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Lecithinized superoxide dismutase attenuates phorbol myristate acetate-induced injury in isolated dog lung

Takashige Miyahara [a], Toshishige Shibamoto [a,*], Hong-Gang Wang [a], Tomonobu Koizumi [b], Takayuki Honda [c], Keishi Kubo [b], Morie Sekiguchi [b], Shozo Koyama [a]

[a] *Department of Physiology, Division 2, Shinshu University School of Medicine, Matsumoto 390, Japan*
[b] *First Department of Medicine, Shinshu University School of Medicine, Matsumoto 390, Japan*
[c] *Laboratory Medicine, Shinshu University School of Medicine, Matsumoto 390, Japan*

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Abstract

Lecithinized superoxide dismutase, a lecithin derivative bound to recombinant human CuZn superoxide dismutase, has a higher affinity for cells such as polymorphonuclear leukocytes and endothelial cells than recombinant human CuZn superoxide dismutase has. We determined the protective effects of lecithinized superoxide dismutase on the increased microvascular permeability induced by phorbol myristate acetate (PMA) in isolated dog lungs. Microvascular permeability was assessed by the capillary filtration coefficient ($K_{f,c}$) and solvent drag reflection coefficient (σ_f). PMA (13.3 μg) increased microvascular permeability, as evidenced by an increase in $K_{f,c}$ and the small σ_f value. Lecithinized superoxide dismutase at both low (4800 U) and high doses (48 000 U) inhibited the PMA-induced increase in $K_{f,c}$, but only the high dose of lecithinized superoxide dismutase attenuated the decrease in σ_f. Recombinant human CuZn superoxide dismutase did not affect the PMA-induced increase in vascular permeability at either a low (4800 U) or a high dose (48 000 U). These findings suggest that lecithinized superoxide dismutase has a protective effect against oxygen radical-induced lung injury in isolated dog lungs. © 1998 Elsevier Science B.V.

Keywords: Lecithinized superoxide dismutase; Phorbol myristate acetate; Capillary filtration coefficient; Solvent drag reflection coefficient; Lung injury

1. Introduction

Phorbol myristate acetate (PMA) has been used to induce acute lung injury as a model of the adult respiratory distress syndrome in both intact animals (Loyd et al., 1983; Shasby et al., 1982) and isolated perfused lungs (Allison et al., 1986; Shasby et al., 1982). Reactive oxygen metabolites have been implicated in the PMA-induced lung injury, based on the evidence that PMA activates the oxygen metabolic cycle in polymorphonuclear leukocytes, resulting in the production of substantial amounts of reduction products of oxygen including the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Jackson et al., 1986; Tinsley et al., 1996). Dimethylthiourea, a scavenger of hydroxyl radical ($^{\cdot}OH$), prevents the acute pulmonary edema in in vivo rabbits given PMA, as well as isolated rabbit lungs perfused with polymorphonuclear leukocyte and PMA (Jackson et al., 1986). In addition, catalase, a scavenger of H_2O_2, significantly protects against the acute lung injury induced by PMA. However, the effects of superoxide dismutase, a scavenger of O_2^-, on the PMA-induced injury are variable in the isolated blood-perfused dog lungs (Allison et al., 1988). The evidence that catalase or dimethylthiourea provides better protection against lung injury than does superoxide dismutase (Allison et al., 1988; Jackson et al., 1986; Johnson and Ward, 1982) seems unreasonable because $^{\cdot}OH$ and H_2O_2 are derived from O_2^-. One of the possible reasons why superoxide dismutase does not prevent the PMA-induced injury may be due to its low cell membrane affinity (Igarashi et al., 1992), which might make it difficult for superoxide dismutase molecules to gain access to the plasma membrane or to the intracellular site where O_2^- is generated and exerts its cytotoxic effects.

One way to overcome this disadvantage is to incorporate superoxide dismutase into a drug delivery system. Therefore, lecithinized superoxide dismutase, in which 4

* Corresponding author. Tel.: +81-263-37-2599; fax: +81-263-36-3644; e-mail: shibamo@gipac.shinshu-u.ac.jp

molecules of a phosphatidylcholine derivative are covalently bound to each dimer of recombinant human CuZn superoxide dismutase, has been synthesized (Igarashi et al., 1992). Lecithinized superoxide dismutase has more O_2^- scavenging ability, higher cellular affinity for several kinds of cells, such as polymorphonuclear leukocytes and endothelial cells, and delayed plasma disappearance than unmodified recombinant human CuZn superoxide dismutase (Igarashi et al., 1992, 1994).

The purpose of the present study was to determine the effects of the lecithinized superoxide dismutase on the increased pulmonary microvascular permeability induced by PMA, in comparison with the effect of unmodified recombinant human CuZn superoxide dismutase. We assessed vascular permeability changes after PMA by determining the capillary filtration coefficient ($K_{f,c}$) and the solvent drag reflection coefficient (σ_f) in isolated blood-perfused dog lungs. $K_{f,c}$ is considered as an indicator of the microvascular permeability of fluid, although it is really a measure of water conductance. σ_f is an index of microvascular permeability to proteins: if the membrane (endothelium) is freely permeable to protein, $\sigma_f = 0$. By contrast, if the membrane is impermeable to protein, $\sigma_f = 1$. A decrease in σ_f indicates an increase in vascular permeability to proteins. σ_f for protein is estimated by measuring the changes in hematocrit and plasma protein concentration after fluid filtration (Maron et al., 1987; Wolf et al., 1987). Thus, this model permits an accurate and extensive assessment of the PMA-induced increase in microvascular permeability by measuring both the $K_{f,c}$ and σ_f. The present study definitely determined whether lecithinized superoxide dismutase attenuates PMA-induced lung injury.

2. Methods

2.1. Isolated lung preparation

Forty-four mongrel dogs (6–12 kg) of either sex were anesthetized with pentobarbital sodium (25 mg/kg i.v.). They were intubated and mechanically ventilated at a tidal volume of 15–20 ml/kg with room air. The isolated lung preparation and perfusion system have been previously described (Shibamoto et al., 1990). Catheters were placed in the left jugular vein and in the left carotid artery. In the fifth left intercostal space, a thoracotomy was performed, and the left upper lobe was ligated and removed. A ligature was placed around the left pulmonary artery but not tied. The dog was then anticoagulated with heparin (500 U/kg i.v.). Ten minutes later, blood was removed from the carotid artery catheter and placed in the perfusion system. The ligature around the left pulmonary artery was tied to prevent air from entering the artery. The left lower lobe was then excised at the hilum and weighed. Plastic cannulas were secured in the pulmonary artery and vein and the lobar bronchus. Thereafter, perfusion was begun within 5 min after excision of the lung.

2.2. Lung perfusion system

The cannulated lobe was suspended from an electric balance (LF-600, Murakami Koki) and perfused at constant flow with 200 ml of shed blood that was pumped from the venous reservoir through a heat exchanger (37°C) into the pulmonary artery. Airway pressure was maintained at a constant level of 3 cmH_2O. The pump speed and the height of the venous reservoir could be adjusted to maintain the pulmonary arterial and venous pressures at any steady level. The perfused blood was oxygenated in the venous reservoir by continuous bubbling with 95% O_2–5% CO_2.

Pulmonary arterial and venous pressures were measured by using pressure transducers that were placed on the reference points at the level of the hilum of the lung. Blood flow (Q) was measured with an electromagnetic flowmeter (MFV 1200; Nihon Kohden, Tokyo, Japan) and the flow probe was positioned in the venous outflow line. Lung weight (W) was continuously monitored and displayed on the physiograph. Pulmonary arterial pressure and pulmonary venous pressure were initially adjusted to a level within the normal perfusion range in zone 3 (pulmonary arterial pressure > pulmonary venous pressure > airway pressure) and to obtain an isogravimetric state (no weight gain or loss).

2.3. Measurements of pulmonary vascular permeability

2.3.1. Capillary filtration coefficient

The capillary filtration coefficient ($K_{f,c}$) was used as an index of microvascular permeability to fluid (Drake et al., 1978) and was assessed by simultaneously increasing pulmonary arterial and pulmonary venous pressures by 6–8 mm Hg from an isogravimetric state and observing the increase in weight of the lung. The sudden increase in vascular pressure caused a rapid weight gain in the lobe due to an increase in blood volume of the lung. This was followed by a gradual and prolonged weight gain that was attributed to transcapillary fluid filtration (Drake et al., 1978). The weight gain rate ($\Delta W/\Delta t$) each minute following the increase in pressure ($t = 0$) was plotted as a semilogarithmic function with time, and the slow phase of the weight transient was extrapolated to time 0. When the lung was not isogravimetric but was gaining weight, this extrapolated rate was subtracted from the rate of weight gain in the last 1 min just before $K_{f,c}$ determination. The extrapolated rate of weight gain ($(\Delta W/\Delta t)_{t=0}$) was then divided by the increase in pulmonary capillary pressure (ΔP_c). Capillary pressure was measured before and after the increase in vascular pressure, using the double vascular occlusion technique (Townsley et al., 1986). $K_{f,c}$ was

normalized to the initial lung weight of 100 g to yield $K_{f,c}$ (in ml/min per cmH_2O per 100 g wet lung weight).

$$K_{f,c} = \frac{(\Delta W/\Delta t)_{t=0}}{\Delta P_c} \quad (1)$$

2.3.2. Solvent drag reflection coefficient

The solvent drag reflection coefficient for total proteins (σ_f) was another index of permeability to proteins and was estimated by measuring the changes in hematocrit and plasma protein concentration after fluid filtration (Wolf et al., 1987; Ehrhart and Hofman, 1992). The initial and final blood samples (1 ml) were obtained before and after fluid filtration, respectively, that was induced by raising the venous reservoir during measurement of $K_{f,c}$. σ_f was calculated using

$$\sigma_f = 1 - \frac{C_{h1}(1-H_1) + C_{h2}(1/H_2 - 1)(V_r - H_1)}{C_h * \left[(1-H_1/H_2) + V_r/H_2\right]} \quad (2)$$

where H_1 and H_2 are the initial (before filtration) and final (after filtration) hematocrits, respectively, C_{h1} and C_{h2} are the initial and final plasma protein concentrations, respectively, corrected for the effects of hemolysis by subtracting the initial and final plasma hemoglobin concentrations, $C_h *$ is the log mean of plasma protein concentrations during filtration $[C_h * = (C_{h1} - C_{h2})/\ln(C_{h1}/C_{h2})]$, and V_r is the volume of erythrocytes hemolyzed as a fraction of the initial (before filtration) perfusate volume calculated as

$$V_r = \frac{[Hb]_2 H_1(1/H_2 - 1) - [Hb]_1(1-H_1)}{[Hb]_2(1/H_2 - 1) + 33} \quad (3)$$

where $[Hb]_1$ and $[Hb]_2$ are the initial and final plasma hemoglobin concentrations, respectively. Plasma protein concentration was measured by refractometry, and plasma hemoglobin concentration was measured by Spectrophotometer (DU 640, Beckman, USA) (Shinowara, 1954).

The total vascular resistance (R_t) was calculated as follows

$$R_t = (P_{pa} - P_{pv})/Q \quad (4)$$

where P_{pa} and P_{pv} are pulmonary arterial and venous pressures, respectively.

2.4. Experimental protocol

Hemodynamic variables of the perfused lung were monitored for at least 20 min after the start of perfusion to reach an isogravimetric state at pulmonary arterial pressure of 9–17 mmHg, pulmonary venous pressure of 2.5–4 mm Hg, and a flow > 0.7 l/min per 100 g wet lung weight. All lobes with a baseline flow < 0.7 l/min per 100 g wet lung weight were excluded from the present study. Each lung in this study was challenged with PMA (13.3 μg), which was injected into the pulmonary artery. Initially, 5 mg PMA (Sigma) was dissolved in 1 ml dimethylsulfoxide (DMSO), divided into aliquots of 50 μg/10 μl each, and frozen at -40°C. Just before its use, 1 aliquot was thawed, and diluted with saline (50 μg/200 μl). Papaverine (2×10^{-4} M) was added to the perfusate of all lungs studied to prevent strong pulmonary vasoconstriction after PMA administration. This dose of papaverine was lower than that reported to increase vascular permeability (Maron and Pilati, 1988). Fifteen minutes after papaverine, baseline $K_{f,c}$ was measured. After hemodynamic stability was reestablished, either 5% mannitol (scavenger vehicle, 0.4 ml, PMA group, $n = 5$), low-dose (4800 U) lecithinized superoxide dismutase ($n = 7$), high-dose (48 000 U) lecithinized superoxide dismutase ($n = 7$), low-dose (4800 U) recombinant human CuZn superoxide dismutase ($n =$

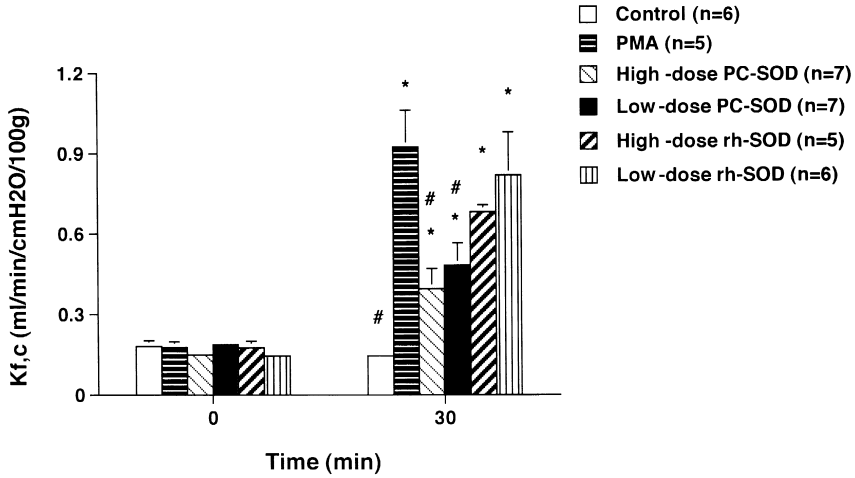

Fig. 1. $K_{f,c}$ in the control ($n = 6$), PMA ($n = 5$), high-dose PC-SOD ($n = 7$), low-dose PC-SOD ($n = 7$), high-dose rh-SOD ($n = 5$), and low-dose rh-SOD ($n = 6$) groups (mean ± S.E.M). n = no. of lungs; PC-SOD = lecithinized superoxide dismutase; rh-SOD = recombinant human CuZn superoxide dismutase. $* P < 0.05$ vs. time 0. $^{\#}P < 0.05$ vs. the PMA group.

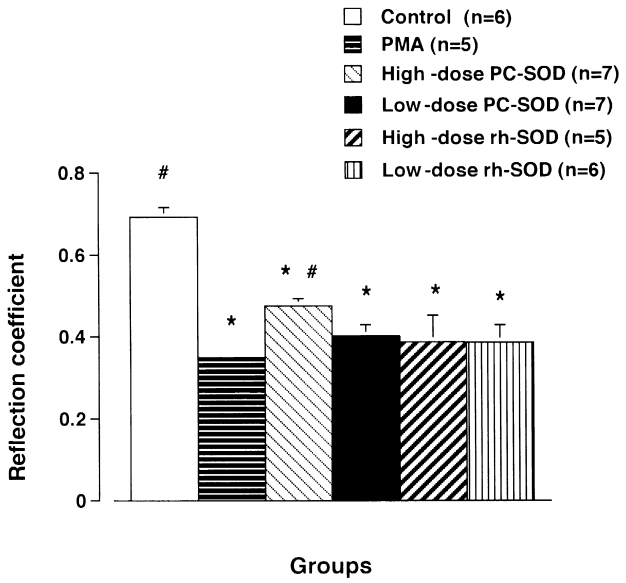

Fig. 2. Estimation of solvent drag reflection coefficient (σ_f) in the control ($n = 6$), PMA ($n = 5$), high-dose PC-SOD ($n = 7$), low-dose PC-SOD ($n = 7$), high-dose rh-SOD ($n = 5$), and low-dose rh-SOD ($n = 6$) groups (mean ± S.E.M.). $* P < 0.05$ vs. the control group. $^{\#}P < 0.05$ vs. the PMA group.

6), or high-dose (48,000 U) recombinant human CuZn superoxide dismutase ($n = 5$) was added to the perfusate. Ten minutes later, PMA (13.3 μg) was injected into the pulmonary artery. Thirty minutes after PMA injection, the venous reservoir was elevated for measurement of post-PMA $K_{f,c}$ and σ_f. For calculation of σ_f, the initial blood sample was obtained just after PMA injection and the final ones were obtained at 10-min intervals after the start of post-PMA $K_{f,c}$ measurement until filtration was sufficient to increase the hematocrit by $> 10\%$ from the initial value obtained immediately after PMA injection. In the lecithinized superoxide dismutase alone ($n = 4$) and recombinant human CuZn superoxide dismutase alone ($n = 4$) groups, these experiments were carried out in the same manner as for the high-dose lecithinized superoxide dismutase group and the high-dose recombinant human CuZn superoxide dismutase group, respectively, except an injection of DMSO (dimethylsulfoxide) (3 μl) plus saline (53.3 μl) was given instead of PMA. In the control group (control group, $n = 6$), isolated lungs were perfused in the same manner as the PMA group except an injection of DMSO (3 μl) plus saline (53.3 μl) was given instead of PMA.

For determination of blood leukocyte counts in the PMA group, blood samples were drawn from the left carotid artery before thoracotomy and after exsanguination, and from the venous line of the isolated perfusion system at baseline and 30 min after PMA administration. Leukocyte counts were performed by using a microcell counter (F-520; Toa, Kobe, Japan) and differential leukocyte counts were determined manually (May-Giemsa stain).

The lungs in the PMA ($n = 4$), high-dose lecithinized superoxide dismutase ($n = 3$), low-dose lecithinized superoxide dismutase ($n = 2$) and control groups ($n = 5$) were prepared for determination of the polymorphonuclear leukocyte number in the lung tissue by fixation in 10% neutral-buffered formalin at the end of protocol. The left upper lobe excised before exsanguination ($n = 4$) was also prepared. Samples were embedded in paraffin, sectioned into 6-μm pieces, and stained with hematoxylin–eosin. The numbers of alveoli and polymorphonuclear leukocytes were counted at a magnification of $\times 400$, averaging a total 100 alveoli for each specimen.

$K_{f,c}$ was measured at baseline and 30 min after PMA injection in each group. In some lungs, we reduced the perfusate flow rate to 50% of the baseline flow rate to

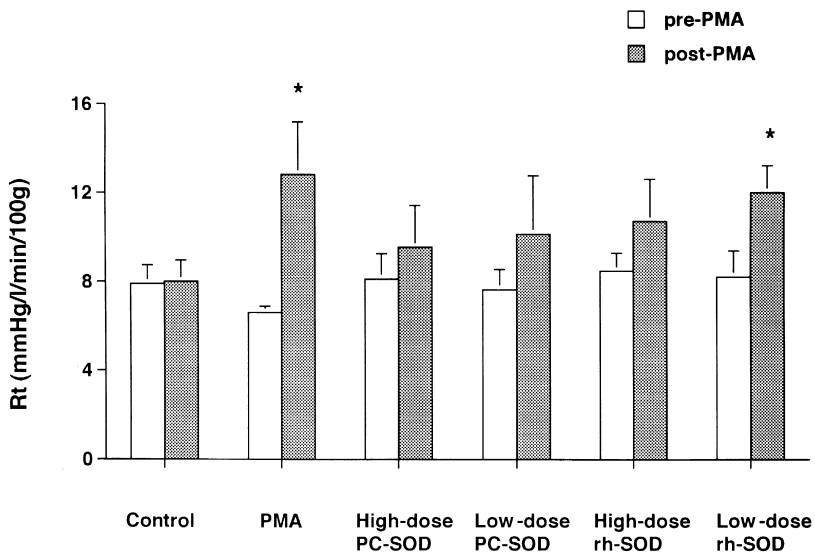

Fig. 3. Total pulmonary vascular resistance (R_t) in the control ($n = 6$), PMA ($n = 5$), high-dose PC-SOD ($n = 7$), low-dose PC-SOD ($n = 7$), high-dose rh-SOD ($n = 5$), and low-dose rh-SOD ($n = 6$) groups (mean ± S.E.M.). $* P < 0.05$ vs. pre-PMA.

Table 1
Total blood leukocyte and polymorphonuclear leukocyte (PMN) counts before and during isolated perfusion in the PMA group

	Pre-thoracotomy	Post-exsanguination	Baseline	30 min
Leukocyte (mm^{-3})	8323 ± 670	4718 ± 561[a]	1945 ± 352[a]	675 ± 191[a]
PMN (mm^{-3})	6032 ± 570	2527 ± 435[a]	451 ± 136[a]	201 ± 160[a]

Values are means ± S.E.M.
[a] $P < 0.05$ vs. pre-thoracotomy.

keep pulmonary arterial pressure within 5 mmHg of the baseline values to prevent excessive edema formation. The reduction of perfusate flow caused a decrease in pulmonary venous pressure below airway pressure, resulting in induction of zone 2 (pulmonary arterial pressure > airway pressure > pulmonary venous pressure). In such lungs, $K_{f,c}$ at 30 min was measured at zone 3 following an increase in pulmonary venous pressure by 3 mm Hg for 3 min. This was achieved by raising the height of the reservoir because $K_{f,c}$ depends on vascular surface area (Shibamoto et al., 1990). This last 1-min weight gain was subtracted from the extrapolated rate of weight gain for calculation of $K_{f,c}$.

2.5. Drugs

Lecithinized superoxide dismutase (120 000 U/ml), recombinant human CuZn superoxide dismutase (540 000 U/ml), and 5% mannitol were provided by Seikagaku Kogyo, (Tokyo, Japan). Lecithinized superoxide dismutase and recombinant human CuZn superoxide dismutase were dissolved in 5% mannitol (12 000 U/ml and 54 000 U/ml, respectively).

2.6. Statistical analysis

All values are expressed as means ± S.E.M. Among the different groups, differences were analyzed by one-way analysis of variance (ANOVA) followed by Fisher's procedure, and changes within a group were analyzed by the Student t test with a Bonferroni correction. P values less than 0.05 were considered significant.

3. Results

Fig. 1 shows the changes in $K_{f,c}$ in all groups except the lecithinized superoxide dismutase alone and recombinant human CuZn superoxide dismutase alone groups. There were no significant differences in the baseline $K_{f,c}$ among any of the groups studied. In the PMA group, $K_{f,c}$ increased dramatically from 0.18 ± 0.02 before PMA to 0.92 ± 0.14 ml/min per cmH_2O per 100 g after PMA. The pretreatment with the low or high dose of lecithinized superoxide dismutase attenuated significantly the increase in $K_{f,c}$ after PMA and there were no significant differences between the low- and high-dose lecithinized superoxide dismutase groups. However, in both lecithinized superoxide dismutase groups, the $K_{f,c}$ after PMA was significantly greater than that of each baseline value, indicating that lecithinized superoxide dismutase did not completely block the PMA-induced increased permeability. The pretreatment with recombinant human CuZn superoxide dismutase at either a low or a high dose did not attenuate the PMA-induced increase in $K_{f,c}$.

Fig. 2 shows the estimation of σ_f in all groups except the lecithinized superoxide dismutase alone and recombinant human CuZn superoxide dismutase alone groups. In the PMA group, the σ_f value, 0.35 ± 0.01, was significantly smaller than that of the control group (0.69 ± 0.06). This finding suggests that PMA increased microvascular permeability, since a decrease in σ_f indicates an increase in permeability. Only in the high-dose lecithinized superoxide dismutase group was the σ_f value, 0.48 ± 0.02, significantly greater than that of the PMA group, whereas the σ_f in the high-dose lecithinized superoxide dismutase group was significantly smaller than that of the control group.

Fig. 3 shows the changes in R_t in all groups except the lecithinized superoxide dismutase alone and recombinant

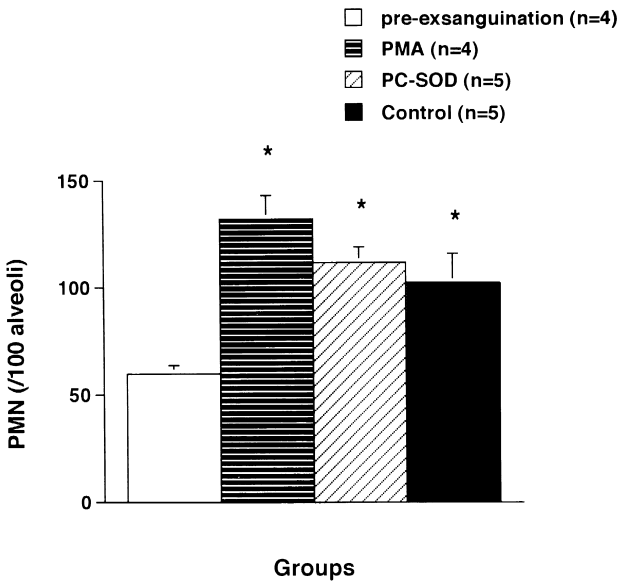

Fig. 4. Polymorphonuclear leukocyte (PMN) counts in the lung in pre-exsanguination ($n = 4$) and the PMA ($n = 4$), PC-SOD ($n = 5$) and control ($n = 5$) groups (mean ± S.E.M.). * $P < 0.05$ vs. pre-exsanguination.

Table 2
Effects of PC-SOD and rh-SOD without PMA on pulmonary vascular resistance and microvascular permeability in isolated perfused dog lungs

Group	n	R_t (mmHg/l per min per 100 g)		$K_{f,c}$ (ml/min per cmH_2O per 100 g)		σ_f
		Baseline	30 min	Baseline	30 min	
PC-SOD alone	4	8.18 ± 1.03	8.26 ± 1.03	0.16 ± 0.02	0.16 ± 0.03	0.68 ± 0.06
rh-SOD alone	4	8.32 ± 0.46	8.34 ± 0.39	0.17 ± 0.01	0.16 ± 0.01	0.71 ± 0.11

Values are means ± S.E.M. PC-SOD = lecithinized superoxide dismutase; rh-SOD = recombinant human CuZn superoxide dismutase; n = no. of lungs.

human CuZn superoxide dismutase alone groups. In this study, in order to assess $K_{f,c}$ accurately and to prevent excessive edema formation, pulmonary arterial pressure was kept at almost baseline level by pretreatment with papaverine and by reducing the perfusate flow rate. Therefore, the magnitude of pulmonary vasoconstriction was evaluated by the changes in R_t. In the PMA and low-dose recombinant human CuZn superoxide dismutase groups, R_t increased significantly after PMA administration despite the papaverine (2.0×10^{-4} M) pretreatment. In the other groups, R_t did not change significantly from the baseline values. There were no significant differences in R_t after PMA administration among all groups.

Table 1 summarizes the changes in the total leukocyte and polymorphonuclear leukocyte counts in the PMA group. The polymorphonuclear leukocyte and leukocyte counts of the blood obtained at the end of exsanguination prior to perfusion were significantly lower than those pre-thoracotomy (6030 ± 570 vs. 2530 ± 440 mm^{-3} for polymorphonuclear leukocytes, 8320 ± 670 vs. 4720 ± 560 mm^{-3} for leukocytes). This indicates that exsanguination produced leukocytopenia, especially neutropenia. Polymorphonuclear leukocyte and leukocyte counts of the perfusate further decreased after PMA administration (200 ± 160 and 680 ± 190, respectively).

Fig. 4 shows the number of polymorphonuclear leukocytes in the lungs in pre-exsanguination and the PMA, high- and low-dose lecithinized superoxide dismutase, and control groups. The number of lung tissue polymorphonuclear leukocytes in the control group (102 ± 14/100 alveoli) was significantly greater than that in the intact lung excised before exsanguination (60 ± 4). Because all the lung tissue samples in the recombinant human CuZn superoxide dismutase groups and some ones in the lecithinized superoxide dismutase groups were accidentally not collected, polymorphonuclear leukocyte numbers were determined only in three of the high-dose lecithinized superoxide dismutase group (115 ± 8) and in two of the low-dose lecithinized superoxide dismutase group (97, 114). The data of these two groups were combined and presented as the lecithinized superoxide dismutase group (112 ± 7) in the Fig. 4. There were no significant differences in the number of polymorphonuclear leukocytes among the PMA, lecithinized superoxide dismutase, and control groups.

Table 2 shows the total vascular resistance and microvascular permeability in the lecithinized superoxide dismutase alone and recombinant human CuZn superoxide dismutase alone groups. None of the measured variables of pulmonary vascular resistance, capillary filtration coefficient and solvent drag reflection coefficient changed significantly after the injection of lecithinized superoxide dismutase or recombinant human CuZn superoxide dismutase.

4. Discussion

In the present study, we used $K_{f,c}$ and σ_f, sensitive indicators of microvascular permeability, to determine the effects of lecithinized superoxide dismutase, in comparison with those of unmodified recombinant human CuZn superoxide dismutase, on the increased microvascular permeability induced by PMA in the isolated blood-perfused dog lung. PMA dramatically increased $K_{f,c}$ and decreased σ_f, indicating an obvious increase in microvascular permeability. This PMA-induced increase in microvascular permeability was significantly inhibited by lecithinized superoxide dismutase but not by recombinant human CuZn superoxide dismutase.

It is well established that acute lung injury induced by PMA is oxygen radical dependent (Allison et al., 1988; Jackson et al., 1986; Shasby et al., 1982). PMA can stimulate various cells to release reactive oxygen species. These cells include polymorphonuclear leukocytes, endothelial cells, and alveolar macrophages, all of which are normally present in lung tissue (Kuroda et al., 1987; Selvaraj et al., 1987). Removal of all circulating leukocytes from isolated perfused lungs of dogs (Allison et al., 1986), rats (Perry and Taylor, 1988) or rabbits (Jackson et al., 1986; Shasby et al., 1982), or selective depletion of circulating granulocytes in an intact rabbit by nitrogen mustard (Shasby et al., 1982), protects the lung from PMA-induced injury. In the present study, blood polymorphonuclear leukocyte counts decreased after exsanguination in the PMA group, and tissue polymorphonuclear leukocyte numbers in the intact lung of the control group increased as compared with those in lung excised before exsanguination. These findings strongly suggest that polymorphonuclear leukocytes might be sequestered in the lung during exsanguination. Indeed, hypovolemic shock produced by phlebotomy promotes polymorphonuclear

leukocyte sequestration in lungs (Anderson et al., 1991b). However, the sequestered polymorphonuclear leukocytes alone do not injure the lung unless these polymorphonuclear leukocytes become activated by a second stimulus (Anderson et al., 1991a). Therefore, in the present study, the sequestered polymorphonuclear leukocytes in the lung might be stimulated by PMA and then release reactive oxygen metabolites, resulting in an increase in vascular permeability.

Although many investigators consider oxygen radicals to underlie PMA-induced injury, the beneficial effects of antioxidants on this injury have not necessarily been observed in all reports. Catalase or dimethylthiourea significantly inhibits the PMA-induced lung injury in isolated rat (Okuda et al., 1992; Johnson and Ward, 1982), rabbit (Jackson et al., 1986) and dog (Allison et al., 1988) lungs. In contrast, superoxide dismutase is effective in isolated rat (Okuda et al., 1992) and rabbit (Tinsley et al., 1996) lungs, but not in isolated rat (Johnson and Ward, 1982), and dog (Allison et al., 1988) lungs. Indeed, in the present study, unmodified recombinant human CuZn superoxide dismutase did not prevent the increased microvascular permeability.

The effect of exogenous superoxide dismutase is limited by its low affinity for the cell membrane or tissue and its rapid metabolism (Igarashi et al., 1992, 1994). This explains why unmodified superoxide dismutase was insufficient to inhibit cell injury and to scavenge excess O_2^- at the cell membranes and in the cytoplasm. Therefore, lecithinized superoxide dismutase was synthesized to overcome these problems (Igarashi et al., 1992). Lecithinized superoxide dismutase has 4 to 20 times higher affinity in vitro for polymorphonuclear leukocytes and endothelial cells than that of unmodified superoxide dismutase, and a high cellular uptake by both endothelial cells and polymorphonuclear leukocytes. In addition, lecithinized superoxide dismutase is 100 times more potent than unmodified recombinant human CuZn superoxide dismutase against human vascular endothelial cell damage caused by O_2^- generated by PMA-stimulated polymorphonuclear leukocytes (Igarashi et al., 1992, 1994). Lecithinized superoxide dismutase also has a delayed disappearance from plasma in whole animals, because of the increase in molecular weight due to lecithinization (Igarashi et al., 1992, 1994). Therefore, the pharmacological activities of lecithinized superoxide dismutase seem to be over 200 times greater than those of unmodified recombinant human CuZn superoxide dismutase (Igarashi et al., 1992, 1994).

It has been reported that the molecular oxygen in polymorphonuclear leukocytes is converted to O_2^- by a membrane-bound enzyme such as NADPH oxidase and that the O_2^- releasing site is located on the inner side of the plasma membrane of polymorphonuclear leukocytes (Fujii and Kakinuma, 1990). Polymorphonuclear leukocytes stimulated with PMA produce exclusively O_2^- as the primary oxygen metabolite and release it into the extracellular medium (Makino et al., 1986). Although unmodified superoxide dismutase is not able to gain access to the plasma membranes of polymorphonuclear leukocytes or pulmonary endothelial cells (Fujii and Kakinuma, 1990), lecithinized superoxide dismutase can reach the cell membrane and accumulates in the cytoplasm of these cells (Igarashi et al., 1994). This high membrane affinity of lecithinized superoxide dismutase accounts for the protective effect against the PMA-induced lung injury.

The high dose of lecithinized superoxide dismutase, however, could not completely block the increased permeability induced by PMA, because the $K_{f,c}$ measured at 30 min was significantly greater than the corresponding baseline value, and the σ_f was significantly smaller than that of the control group. We have four possible explanations why lecithinized superoxide dismutase did not completely block the PMA-induced lung injury. First, more toxic metabolites, including $^{\cdot}OH$, may be intimately related to the pathogenesis of PMA-induced lung injury. O_2^- could be converted to $^{\cdot}OH$, which is made by a metal-catalyzed reaction between O_2^- and H_2O_2 generated by superoxide dismutase (Fenton reaction). However, superoxide dismutase has O_2^- as a substrate and provides the second layer of defense after glutathione peroxidase or catalase against free radical injury. Considered to be a primary antioxidant enzyme, superoxide dismutase prevents free radical chain reactions by decreasing free radicals available to initiate the process (Heffner and Repine, 1989). Although the high dose of lecithinized superoxide dismutase in the present study was sufficient to prevent the biosynthesis of $^{\cdot}OH$, $^{\cdot}OH$ might have been generated and contributed to the PMA injury. Second, the dose of lecithinized superoxide dismutase used in the present study might have been suboptimal. However, this possibility is unlikely, since lecithinized superoxide dismutase at 1000 U/kg body weight attenuates the Forssman antiserum-induced respiratory obstruction in guinea pig (Igarashi et al., 1992) and lecithinized superoxide dismutase at 50 000 U/kg body weight inhibits ischemia–reperfusion paw edema in mice (Igarashi et al., 1994). Therefore, the high dose of lecithinized superoxide dismutase (48 000 U) injected into a perfusing blood volume of 200 ml in the present study, which is equivalent to 21 840 U/kg body weight, seems to be comparable to that of the previous two studies and sufficient to scavenge the oxygen radicals generated by PMA. Third, lecithinized superoxide dismutase might act as a vasodilator or block the vasoconstrictor activity of PMA. If lecithinized superoxide dismutase causes vasodilation, it probably would increase $K_{f,c}$, which might explain why lecithinized superoxide dismutase only attenuated and did not completely block the PMA-induced increase in $K_{f,c}$. However, in the present study, lecithinized superoxide dismutase did not act as a vasodilator or block the vasoconstriction in the lecithinized superoxide dismutase alone group, as shown in Table 2. Finally, other neutrophil constituents, such as neutrophil elastase, might

also participate in PMA-induced lung damage. Indeed, oxygen radicals may inactivate antiproteases and enhance the toxicity of neutrophil elastase (Carp and Janoff, 1979). Therefore, when closely attached to endothelial cells, polymorphonuclear leukocytes might release proteases, resulting in direct cellular injury, although exogenous superoxide dismutase can eliminate extracellular oxidants that might well inactivate antiproteases (McDonald et al., 1989).

In the present study, microvascular permeability was assessed by $K_{f,c}$ and σ_f. $K_{f,c}$ is a specific index of vascular permeability to fluid, provided the microvascular surface area is maintained constant (Townsley et al., 1987). σ_f is an index of microvascular permeability to proteins and is independent of the perfused surface area. In the high-dose lecithinized superoxide dismutase group, both $K_{f,c}$ and σ_f were significantly different from those in the PMA group. In contrast, in the low-dose lecithinized superoxide dismutase group, only $K_{f,c}$, but not σ_f, was significantly different from that of the PMA group. These results indicate that the low dose of lecithinized superoxide dismutase attenuated the increased permeability to water, but not the increased permeability to proteins, while the high dose of lecithinized superoxide dismutase attenuated the increased permeability to both water and proteins. There is a theory that different size pores, small (80 Å) and large pores (200 Å), are responsible for the movement of water and protein across the microvascular barrier of the lung (Taylor and Granger, 1984). According to this theory, the low dose of lecithinized superoxide dismutase might prevent damage to the small pore only, but not damage to the large pore, and the high dose of lecithinized superoxide dismutase might prevent damage to both the small and the large pores.

Many investigators report that PMA causes an increase in pulmonary artery pressure in whole animals (Dyer and Snapper, 1986; Loyd et al., 1983) and isolated lungs (Carpenter and Roth, 1987; Johnson, 1988). It is well known that reactive oxygen radical products generated by PMA stimulate arachidonic acid metabolism in lungs, and that vasoactive products of arachidonate, such as the potent vasoconstrictor thromboxane A_2, might mediate O_2-metabolite-induced pulmonary vasoconstriction (Tate et al., 1984). Moreover, it has been recently reported that O_2^- shortens the life of endothelium-derived vascular relaxing factor (believed to be nitric oxide (NO)) (Gryglewski et al., 1986; Rubanyi and Vanhoutte, 1986). In the present study, although papaverine was injected in the perfusate, PMA increased R_t significantly in the PMA and low-dose recombinant human CuZn superoxide dismutase groups. The high dose of lecithinized superoxide dismutase, which inhibited significantly the increased microvascular permeability, attenuated the increased R_t to a greater extent than was seen in the other groups. Therefore, these findings reinforce the suggestion that reactive oxygen radical products play a crucial role in PMA-induced pulmonary vasoconstriction.

In summary, pulmonary microvascular damage induced by PMA in the isolated dog lung is inhibited by lecithinized superoxide dismutase but not by recombinant human CuZn superoxide dismutase. Accordingly, lecithinized superoxide dismutase has the potential to be used in the treatment of various oxygen radical-related diseases.

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