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PAF Contributes to Intestinal Ischemia/Reperfusion-Induced Acute Lung Injury through Neutrophilic Oxidative Stress

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The role of platelet-activating factor (PAF) was investigated in intestinal ischemia/reperfusion (I/R) induced acute lung injury associated with oxidative stress. To induce acute lung injury following intestinal I/R, superior mesenteric arteries were clamped with bulldog clamp for 60 min prior to the 120 min reperfusion in Sprague-Dawley rats. Acute lung injury by intestinal I/R was confirmed by the measurement of lung leak index and protein content in bronchoalveolar lavage (BAL) fluid. Lung leak and protein content in BAL fluid were increased after intestinal I/R, but decreased by WEB 2086, the PAF receptor antagonist. Furthermore, the pulmonary accumulation of neutrophils was evaluated by the measurement of lung myeloperoxidase (MPO) activity and the number of neutrophils in the BAL fluid. Lung MPO activity and the number of neutrophils were increased ($p<0.001$) by intestinal I/R and decreased by WEB 2086 significantly. To confirm the oxidative stress induced by neutrophilic respiratory burst, gamma glutamyl transferase (GGT) activity was measured. Lung GGT activity was significantly elevated after intestinal I/R ($p<0.001$) but decreased to the control level by WEB 2086. On the basis of these experimental results, phospholipase A_2 (PLA_2), lysoPAF acetyltransferase activity and PAF contents were measured to verify whether PAF is the causative humoral factor to cause neutrophilic chemotaxis and oxidative stress in the lung following intestinal I/R. Intestinal I/R greatly elevated PLA_2 activity in the lung as well as intestine ($p<0.001$), whereas WEB 2086 decreased PLA_2 activity significantly ($p<0.001$) in both organs. LysoPAF acetyltransferase activity, the PAF remodelling enzyme, in the lung and intestine was increased significantly ($p<0.05$) also by intestinal I/R. Accordingly, the productions of PAF in the lung and intestine were increased ($p<0.001$) after intestinal I/R compared with sham rats. The level of PAF in plasma was also increased ($p<0.05$) following intestinal I/R. In cytochemical electron microscopy, the generation of hydrogen peroxide was increased after intestinal I/R in the lung and intestine, but decreased by treatment of WEB 2086 in the lung as well as intestine. Collectively, these experimental results indicate that PAF is the humoral mediator to cause acute inflammatory lung injury induced by intestinal I/R.

Key Words: PAF, Intestinal I/R, Neutrophil, Oxidative stress

INTRODUCTION

Adult respiratory distress syndrome (ARDS) is one of the morbid manifestations of multiple organ failure (MOF) derived from various clinical situations (Pittet et al, 1997). One of the etiologies of MOF, acute intestinal ischemia/reperfusion (I/R) has been considered as a serious and enigmatic cause of ARDS (Otamiri et al, 1988). Recently, humoral factors have been suggested as causative factors to understand the pathogenesis of ARDS associated with MOF (Lefer & Lefer, 1993). Xanthine oxidase (XO), one of these humoral factors, is released from endothelial cells of the splanchnic vasculature during intestinal I/R (Adkins et al, 1990). According to Terada et al (1992a), free radical generator XO is the major molecule to generate oxidants from the distant organ through circulation in the body. However, though the inhibition of XO is effective in intestinal I/R induced acute lung

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leak, the protection is not complete. Therefore, some other mechanisms have to be considered to understand the pathogenetic mechanism of acute lung leak following intestinal I/R. Recently, Shen et al (1995) reported the effects of PLA_2 on the sequestration of neutrophils and respiratory burst. Still, it's a controversy, Dana et al (1998) insisted that activation of PLA_2 had a crucial role for the activation of NADPH oxidase in neutrophilic membrane. Moreover, lipid molecules synthesized by PLA_2 have a variety of biological effects including activation of complement system and generation of free radicals from neutrophils (Leslie, 1997). Anderson et al (1991a) suggested PAF, a byproduct of PLA_2, was a strong biological molecule to induce shock like condition. Interestingly, Carter et al (1991) documented that the inhibition of PAF with WEB 2086 decreased acute protein leak in the lung. As the lung is a distant organ from the intestine, the acute pulmonary edema caused by intestinal I/R could be mediated by some humoral factors. As it was confirmed that PLA_2 had a pivotal role to cause acute inflammatory lung injury in the previous study (Lee et al, 1998), the underlying pathogenetic mechanism of acute lung leak by intestinal I/R was investigated associated with the role of PAF in the present study.

METHODS

Sources of reagents

Dipalmitoyl-(2(9,10(N)-^{3}H)palmitoyl)-phosphatidylcholine, ^{125}I-bovine serum albumin (BSA), and ^{3}H-acetyl CoA were purchased from Dupont NEN research product (Boston, MA, USA). PAF scintillation proximity assay kit was purchased from Amersham International plc (Amersham, UK). WEB 2086, a PAF receptor antagonist, was donated by Boehringer-Ingelheim (Germany). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Procedure for induction of acute lung injury by intestinal I/R

Acute lung injury was induced by intestinal I/R according to the method of Terada et al (1996). Briefly, superior mesenteric arteries were clamped with a bulldog clamp for 60 min prior to the 120 min reperfusion in male Sprague-Dawley rats (B.Wt. 300~350 g). To inhibit PAF in intestinal I/R rats, WEB 2086 (8 mg/kg, i.v.), a PAF receptor antagonist, was given immediately after the clamping of superior mesenteric artery.

Measurement of lung leak

To confirm the acute lung injury following intestinal I/R, protein leak was measured. Rats were anesthetized with ketamine hydrochloride (75 mg/kg). 1.5 h after reperfusion, ^{125}I-BSA (1 μCi) was given intravenously. After 30 min, the trachea was cannulated and the rats were ventilated with respirator. Immediately thereafter, laparotomy and tracheostomy were performed, and heparin was injected into the right ventricle (200 IU, 0.2 ml). Blood was withdrawn (1.0 ml) 10 sec after injection and the lungs were excised after perfusion with phosphate buffered saline to washout the blood. Right lungs and blood samples were counted in the gamma counter. The lung leak index was determined by computing the counts per min of ^{125}I in the lung divided by counters per min of ^{125}I in blood.

Measurement of protein content in BAL fluid

BAL was performed to measure protein content in BAL fluid. Eight ml of normal saline was instilled through the cannula and retrieved three times. Six ml of BAL fluid was obtained from each rat. Before measurement of protein content according to the method of Brown et al (1989), cellular components in the BAL fluid were sedimented by centrifugation at 1,500 rpm for 10 min.

Analysis of lung MPO activity

To confirm the pulmonary infiltration of neutrophils, lung MPO activity was measured according to the method of Goldblum et al (1985). Briefly, left lungs were homogenized in 4.0 ml of potassium phosphate buffer (20 mM, pH 7.4), followed by centrifugation at 30,000×g for 30 min at 4°C. The pellet was resuspended in 4.0 ml of potassium phosphate buffer (50 mM, pH 6.0) with 0.5% hexadecyltrimethyl ammonium bromide. Before incubating for 2 h at 60°C to inactivate MPO inhibitors, samples were sonicated for 90 sec on ice. After incubation, 1.0 ml of lung homogenate was centrifuged again at

12,000 rpm for 3 min and 0.1 ml of supernatant was used as enzyme source. 0.1 ml of supernatant was reacted with 2.9 ml of 0.0168% *o*-dianisidine solution.

Enumeration of neutrophils in BAL fluid

Pulmonary accumulation of neutrophils was confirmed by counting neutrophils in BAL fluid. Briefly, BAL fluid was centrifuged at 1,500 rpm for 10 min at 4°C. Thereafter the pellets were resuspended in 0.5 ml of normal saline and the number of leukocytes was counted. To count neutrophils, 100 μl of resuspended solution was cytospin again on slide glass, Wright stained and differentially counted the number of neutrophils.

GGT assay in the lung

GGT activity in the lung was measured according to the method of Meister et al (1981). Briefly, right lungs were homogenized with 4.0 ml of homogenizing buffer (0.15 M KCl, 10 mM Tris base (pH 7.4), 10 mM EDTA, 50 μg/ml PMSF, 3 μg/ml leupeptin), and sonicated for 90 sec at maximum power. Glycylglycine (0.2 ml, 0.1 M, pH 8.0) and L-gamma-glutamyl-p-nitroanilide (0.2 ml, 5 mM, pH 8.0) were mixed with 0.6 ml of Tris-HCl buffer (0.1 M, pH 8.0) and 0.1 ml of lung homogenate. The mixture was incubated at 37°C for 60 min and the reaction was stopped by adding 0.5 ml of 50 mM serine-borate solution. Spectrophotometry was carried out at 410 nm. The production of 1 μmol per min of p-nitroaniline was defined as 1 unit.

Assay of intestinal and pulmonary PLA_2

Intestinal and pulmonary PLA_2 activity was measured by the method of Katsumata et al (1986). Briefly, the blood-free excised lung and small intestine were homogenized with polytron homogenator in 2.0 ml of phosphate buffer (20 mM, pH 7.4), then sonicated for 90 sec on ice. 2 μCi of L-α-dipalmitoyl-2(9,10(N)^{3}H-palmitoyl)-phosphatidylcholine (30 Ci/mmol) was incubated with 100 μl of the intestine and lung homogenate in 880 μl of 100 mM glycine buffer at pH 9.0, containing 10 g/L of BSA, 2.5 mM sodium deoxycholate, 0.1 mM dipalmitoylphosphatidylcholine, 2.0 mM $CaCl_2$ and 1.75 M absolute ethanol containing 200 mM EDTA. The reaction mixture was incubated for 60 min at 37°C and then the reaction was stopped by adding 200 μl of 5% Triton X-100. The fatty acids released by hydrolysis were extracted by 5.0 ml of hexane containing 0.1% acetic acid and 2.5 g of Na_2SO_4. After vortexing, the hexane layer was separated and counted in the liquid scintillation counter. Snake venom PLA_2 (*Crotalus adamanteus*) control samples (0.01 U/ml) were assayed with all the other samples to confirm the reproducibility. One unit was defined as an activity to hydrolyze 1 μmol of substrate per min.

PAF scintillation proximity assay

PAF content was measured in the lung, intestine and plasma by the method of PAF scintillation proximity assay. Briefly, left lung and a small portion of intestine were homogenized in 0.2 M citric acid-normal saline solution. For PAF assay in plasma, blood was withdrawn after 10 min of reperfusion. The withdrawn blood was mixed with 2.0 ml of 0.2 M citric acid in normal saline. These samples were mixed with 3.0 ml of chloroform-methanol mixture (1 : 2, v/v) and vigorously vortexed. Again, 1.0 ml of normal saline was added, vortexed and centrifuged at 10,000 × g for 10 min. Organic layer was separated and dried under nitrogen. These samples were resuspended in assay buffer (0.05 M Tris-HCl buffer, pH 7.4 containing 0.9% NaCl, 0.01% Triton X-100, 0.1% gelatin and 0.0057% thimerosal). Resuspended samples were mixed with anti PAF antibody, SPA protein A reagent and tracer. Before measuring the radioactivity with β-scintillation spectroscopy, the mixture was incubated for 18 h at room temperature with shaking.

Measurement of lysoPAF acetyltransferase

The PAF remodelling enzyme, lysoPAF acetyltransferase activity was measured in the lung and intestine according to the method of Bussolino et al (1986). Briefly, left lung and intestinal tissue were immersed in 1.0 ml of 0.25 M sucrose solution containing 1 mM DTT and homogenized before sonication for 90 sec. Thereafter 100 μl of sample solution was mixed well with 0.5 ml of 0.1 M Tris-HCl buffer (pH 6.9) containing 40 μM lysoPAF, 200 μM ^{3}H-acetyl CoA (0.5 μCi), 40 μg of BSA. Reactions were stopped with 2.0 ml of chloroform-methanol mixture (1 : 2, v/v). Lipids were extracted

according to the method of Bligh & Dyer (1959). The lipid in chloroform layer were dried under nitrogen, and radioactivity was measured with beta-scintillation spectroscopy.

Cytochemical electron microscopy of hydrogen peroxide in the lung and intestine

In order to estimate production of hydrogen peroxide in the lung, cytochemical electron microscopy was performed by the modified method of Hobson et al (1991). Briefly, lung samples (1 mm^3) were incubated in cerium chloride media (2.0 mM $CeCl_3$, 10 mM 3-amino-1,2,4-triazol, 0.1 M Tris-maleate buffer, 7% sucrose, 0.0002% Triton X-100) for 60 min. Then, the samples were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, post fixed, dehydrated, and embedded in Epoxy resin. Counter staining was not performed to increase the contrast between the reaction product and tissue.

RESULTS

Effect of WEB 2086 on intestinal I/R induced lung leak and protein contents in BAL fluid

The effect of PAF inhibition was evaluated by the measurement of lung leak and protein contents in BAL fluid. Intestinal I/R increased ($p<0.001$) lung leak index and protein contents in BAL fluid compared with a sham group. Treatment of WEB 2086 attenuated lung leak and protein content in BAL fluid compared with an intestinal I/R group ($p<0.001$, Table 1).

Effect of WEB 2086 on intestinal I/R induced pulmonary accumulation of neutrophils

Intestinal I/R increased ($p<0.001$) lung MPO activity and the number of neutrophils in BAL fluid. In contrast, WEB 2086 decreased ($p<0.001$) lung MPO activity and the number of neutrophils in the BAL fluid (Table 2). These results suggest the involvement of neutrophils in oxidative stress induced by respiratory burst.

Table 1. Changes of lung leak index and protein contents in bronchoalveolar lavage (BAL) fluid following intestinal ischemia/reperfusion (I/R) and treatment of WEB 2086

	Sham	I/R	I/R+WEB 2086
Lung leak index	0.071±0.008 (n=7)	0.229±0.018*** (n=9)	0.127±0.006***,### (n=7)
Protein contents (mg/ml of BAL fluid)	3.649±0.215 (n=7)	5.406±0.156*** (n=7)	3.391±0.161### (n=9)

Values are mean±SE, n indicates number of rats.
***$p<0.001$ compared with Sham values. ###$p<0.001$ compared with I/R values.

Table 2. Changes of lung myeloperoxidase (MPO) activity and the number of neutrophils following intestinal ischemia/reperfusion (I/R) and treatment of WEB 2086

	Sham	I/R	I/R+WEB 2086
Lung MPO activity (U/g of wet lung)	2.836±0.625 (n=7)	38.256±2.878*** (n=8)	13.401±2.430**,### (n=8)
Number of neutrophils (millions /two lungs)	0.045±0.013 (n=7)	4.272±0.378*** (n=9)	2.537±0.361***,## (n=9)

Values are mean±SE, n indicates number of rats.
**$p<0.01$, ***$p<0.001$ compared with Sham values. ##$p<0.01$, ###$p<0.001$ compared with I/R values.

Effect of WEB 2086 on the lung GGT activity

As an index of oxidative stress in the lung, the GGT activity was measured in control, I/R and I/R with WEB 2086 treated rats. Intestinal I/R increased ($p<0.001$) GGT activity in the lung compared to sham rats. In contrast, WEB 2086 decreased ($p<0.001$) GGT activity to the control level (Table 3).

Effect of WEB 2086 on lung and intestinal PLA_2 activity

Intestinal I/R increased ($p<0.001$) pulmonary and intestinal PLA_2 activity. In contrast, WEB 2086 decreased ($p<0.001$) pulmonary and intestinal PLA_2 compared to those of intestinal I/R rats (Table 4). The increased PLA_2 activity following I/R signifies that there is some humoral factor which links the intestinal and lung injury.

Effect of intestinal I/R on the content of PAF in the lung, intestine and plasma

Intestinal I/R significantly increased PAF content in the lung ($p<0.05$), intestine ($p<0.001$) and plasma ($p<0.001$) compared to those of sham rats (Table 5). The increase of PAF content in the lung could cause the accumulation of neutrophils and oxidative stress

Table 3. Change of lung gamma glutamyl transferase (GGT) activity following intestinal ischemia/reperfusion (I/R) and treatment of WEB 2086

	Sham	I/R	I/R+WEB 2086
Lung GGT activity (U/g of wet lung)	2.541±0.163 (n=6)	4.191±0.193*** (n=7)	2.898±0.122### (n=10)

Values are mean±SE, n indicates number of rats.
***p<0.001 compared with Sham values. ###p<0.001 compared with I/R values.

Table 4. Changes of intestinal and pulmonary PLA_2 activity following intestinal ischemia/reperfusion (I/R) and treatment of WEB 2086

	Sham	I/R	I/R+WEB 2086
Intestinal PLA_2 (mU/g of wet intestine)	33.823±9.644 (n=6)	546.534±56.221*** (n=10)	98.144±21.277### (n=12)
Pulmonary PLA_2 (mU/g of wet lung)	33.604±5.228 (n=9)	414.375±58.869*** (n=8)	55.293±19.721### (n=8)

Values are mean±SE, n indicates number of rats.
***p<0.001 compared with Sham values. ###p<0.001 compared with I/R values.

Table 5. Changes of PAF contents in plasma, intestine, and lung following intestinal ischemia/reperfusion (I/R)

PAF	Sham	I/R
Plasma (ng/ml of blood)	0.434±0.104 (n=9)	2.220±0.293*** (n=9)
Intestine (ng/g of intestine)	2.300±0.448 (n=5)	14.056±1.118*** (n=9)
Lung (ng/g of lung)	19.332±2.943 (n=5)	32.322±2.939* (n=9)

Values are mean±SE, n indicates number of rats.
*p<0.05, ***p<0.001 compared with Sham values.

in the lung. The increased level of the PAF in the intestine might be the source of plasma PAF.

Table 6. Changes of lysoPAF acetyltransferase activity (PAF, nmol/g/min) in intestine and lung following intestinal ischemia/reperfusion (I/R)

	Sham	I/R
Intestine	0.341 ± 0.059 (n=5)	$1.312 \pm 0.331^{*}$ (n=8)
Lung	0.294 ± 0.024 (n=5)	$0.461 \pm 0.057^{*}$ (n=8)

Values are mean ± SE, n indicates number of rats.
*$p < 0.05$ compared with Sham values.

Effect of intestinal I/R on the lysoPAF acetyltransferase activity in the lung and intestine

Intestinal I/R increased ($p < 0.05$) lysoPAF acetyltransferase activity in the lung and intestine compared to those of sham rats (Table 6). The increase of lysoPAF acetyltransferase activity implicates the increased remodelling of lysoPAF to PAF.

Cytochemical electron microscopy for detection of hydrogen peroxide in the lung and intestine

Intestinal I/R increased the deposits of cerrous perhydroxide in intestine compared to sham rats (Fig.

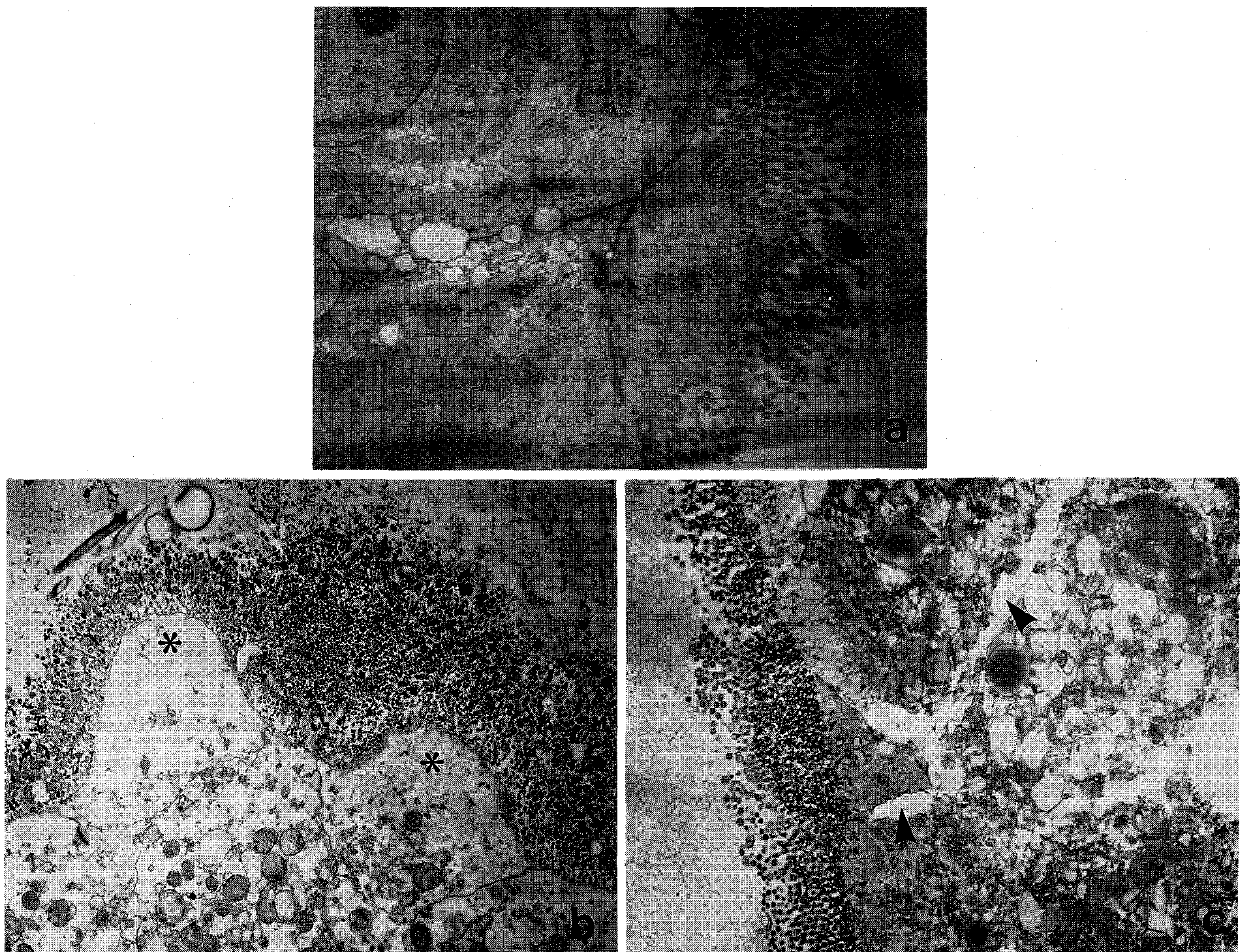

Fig. 1. Cytochemical electron microscopic findings of intestine following intestinal ischemia/reperfusion (I/R). Comparing with sham rats (panel a), I/R treated rat's intestine shows abundant cerrous perhydroxide deposits in the microvilli and severe submucosal injuries (panel b), whereas treatment of WEB 2086 decreases the deposits of cerrous perhydroxide in the microvilli and the tissue injury was diminished (panel c).

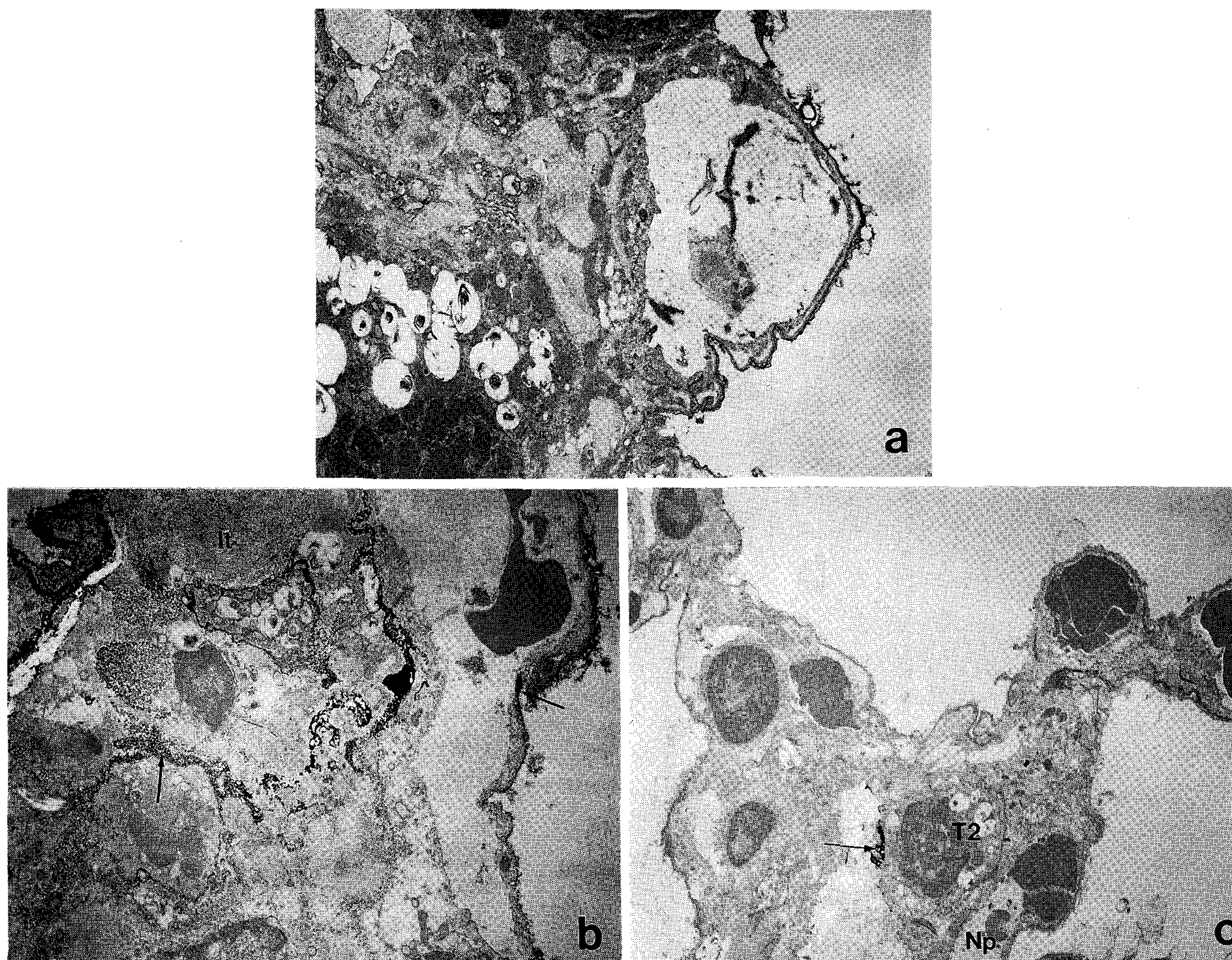

Fig. 2. Cytochemical electron microscopic findings of the lung following intestinal ischemia/reperfusion (I/R). Comparing to sham rats (panel a), cerrous perhydroxide production was evident in the lung tissue especially around neutrophils and apical portion of alveolar type 2 cells (panel b, arrows). These changes were diminished by the treatment of WEB 2086 (panel c), i.e. the production of hydrogen peroxide was decreased and tissue was protected from oxidant injury by WEB 2086. It: interstitium, T2: alveolar type II cell, Np: neutrophil

1, a, b). Treatment of WEB 2086 decreased deposits of cerrous perhydroxide in intestine (Fig. 1, c). This means the decreased production of hydrogen peroxide by the treatment of WEB 2086. Again, in the lung, intestinal I/R increased the cerrous perhydroxide production but WEB 2086 decreased the production of cerrous perhydroxide effectively (Fig. 2, a, b, c).

DISCUSSION

Intestinal I/R has been implicated in the pathogenesis of distant organ injury including the lung (Terada et al, 1992a). Therefore, the mechanisms which are responsible for intestinal I/R induced acute lung injury have received increased attention. Some authors (Anderson et al, 1991b; Terada et al, 1992b) have demonstrated that XO inhibitors were effective to decrease the intensity of the tissue injury following I/R, and one of the protective effects was the diminution of neutrophilic production of free radicals. However, although the inhibition of XO significantly reduced the intensity of the injury, it was not fully protective. This fact suggests that other mechanisms are involved in the pathogenesis of acute lung injury following intestinal I/R.

Recently, several experimental and clinical evidences of the contribution of PLA_2 to provocation of

acute lung injury have been issued (Liu et al, 1993; Turnage et al, 1995; Cuzzocrea et al, 1997). For example, PLA_2 appears to play an important role in interleukin-1 induced as well as intestinally mediated acute lung injury (Kurose et al, 1997; Lee et al, 1997). In addition, Koike et al (1995) reported that intestinal PLA_2 activity was increased after intestinal I/R.

There are increasing informations that PLA_2 is intimately involved in the inflammatory processes (Forehand et al, 1993). Among lipid mediators produced by PLA_2, PAF is one of the potent mediators of acute circulatory collapse and MOF (De Kimpe et al, 1995). Ever since Filep et al (1989) reported that PAF was increased in plasma after intestinal I/R, PAF has been regarded as one of the putative molecules to cause circulatory shock. Especially, Mozes et al (1989) insisted that PAF was an endogenous mediator to cause circulatory collapse in the mesenteric-ischemia-reperfusion-induced shock. In the present study, intestinal I/R evoked acute protein leak and pulmonary accumulation of neutrophils which signifies oxidative stress caused by neutrophilic respiratory burst. Furthermore, the production of hydrogen peroxide was increased in cytochemical electron microscopy, which implicated the oxidative stress occurred which might be one of the pathogenetic mechanisms of acute lung injury by intestinal I/R. Another evidence of oxidative stress in the lung is the increased activity of GGT after intestinal I/R. All of these parameters were effectively decreased by the inhibition of PAF with PAF receptor antagonist WEB 2086 in the present study. The effects of WEB 2086 on the decrease of lung leak and pulmonary accumulation of neutrophils suggests that PAF is intimately involved in neutrophilic respiratory burst. Even if the role of PLA_2 has been implicated in the pathogenesis of ARDS by way of pulmonary accumulation of neutrophils, still it has not been elucidated what is the direct cause of pulmonary accumulation of neutrophils and respiratory burst in the lung.

After intestinal I/R, there was increase of lysoPAF acetyltransferase which is crucial to remodel lysoPAF into PAF. The remodelling is the major pathway to synthesize of PAF *in vivo* system (Snyder, 1995). Thus, it is not surprising that there was increase of PAF content in intestine and plasma. Therefore it seems that lysoPAF synthesized by PLA_2 in intestine caused the increase of PAF which was remodelled by lysoPAF acetyltransferase. Moreover, there were increases of lysoPAF acetyltransferase activity and PAF content in the lung also. Recently, the PAF remodelling process is thought to be an autocrine, positive feedback cycle which potentiates inflammatory process in the tissue. In this autocrine process, PAF, PLA_2, cytokines and free radicals are involved. In addition, PAF stimulates lysoPAF acetyltransferase (Doebber et al, 1987) and activates PLA_2 in neutrophils (Sun et al, 1994). Consequently, the activation of PLA_2 increased the production of PAF which in turn activates PLA_2 and lysoPAF acetyltransferase. All of these processes are closely linked to the generation of free radicals. As is shown in the present study, PAF in the plasma appears to be a humoral mediator to cause PLA_2 activation in the lung. And Rao et al (1995) insisted that hydrogen peroxide could activated PLA_2.

Considering the roles of PAF to initiate the respiratory burst in neutrophils, it is quite natural to consider that PAF is a humoral mediator to activate PLA_2 which in return caused to synthesize PAF in the lung. The fact that WEB 2086 decreases PLA_2 activity in the lung and intestine supports the role of PAF in the activation of the PLA_2. These results suggest that PAF is one of the major humoral mediators to induce pulmonary accumulation of neutrophils and PLA_2 activation which result in the oxidative stress induced acute lung injury.

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