Enhancement of acute lung injury related to bacterial endotoxin by components of diesel exhaust particles

R Yanagisawa, H Takano, K Inoue, T Ichinose, K Sadakane, S Yoshino, K Yamaki, Y Kumagai, K Uchiyama, T Yoshikawa, M Morita

Background: Diesel exhaust particles (DEP) synergistically aggravate acute lung injury related to lipopolysaccharide (LPS) in mice, but the components in DEP responsible for this have not been identified. A study was undertaken to examine the effects of the organic chemicals (DEP-OC) and residual carbonaceous nuclei (washed DEP) derived from DEP on LPS related lung injury.

Methods: ICR mice were divided into experimental groups and vehicle, LPS, washed DEP, DEP-OC, washed DEP+LPS, and DEP-OC+LPS were administered intratracheally. The cellular profile of the bronchoalveolar lavage (BAL) fluid, pulmonary oedema, lung histology, and expression of proinflammatory molecules and Toll-like receptors in the lung were evaluated.

Results: Both DEP-OC and washed DEP enhanced the infiltration of neutrophils into BAL fluid in the presence of LPS. Washed DEP combined with LPS synergistically exacerbated pulmonary oedema and induced alveolar haemorrhage, which was concomitant with the enhanced lung expression of interleukin-1β, macrophage inflammatory protein-1α, macrophage chemoattractant protein-1, and keratinocyte chemoattractant, whereas DEP-OC combined with LPS did not. Gene expression of Toll-like receptors 2 and 4 was increased by combined treatment with washed DEP and LPS. The enhancement effects of washed DEP on LPS related changes were comparable to those of whole DEP.

Conclusions: These results suggest that the residual carbonaceous nuclei of DEP rather than the extracted organic chemicals predominantly contribute to the aggravation of LPS related lung injury. This may be mediated through the expression of proinflammatory cytokines, chemokines, and Toll-like receptors.

METHODS

Animals
Male ICR mice (6 weeks old, 29–33 g) were purchased from Japan Clea Co (Tokyo, Japan) and fed a commercial diet (Japan Clea Co) and water ad libitum. The mice were housed in an animal facility that was maintained at 24–26°C with 55–75% humidity and a 14/10 hours light/dark cycle.

Preparation of particle samples
A 4JB1 type, light duty, four cylinder, 2.74 litre Isuzu diesel engine (Isuzu Automobile Co, Tokyo, Japan) under computer control was connected to a dynamometer (Mülden-nya, Tokyo, Japan). The details of the condition of the engine has been described previously.19 DEP were raked up from a stainless steel particle collector and stored at −20°C until use.

Preparation of LPS
Lipopolysaccharide (LPS), a major proinflammatory component of Gram negative bacteria, is associated with aggravation of lung diseases. Exposure of rodents to LPS recruits neutrophils and increases the expression of proinflammatory molecules.18–20 We have recently reported that intratracheal instillation with DEP enhances neutrophilic lung inflammation related to LPS inoculated intratracheally.18 In the previous study, however, we did not identify the components of DEP which were responsible for the enhancing effects. The aim of this study was to determine the components of DEP which are critical for the aggravation of acute lung injury related to LPS. Organic chemicals were extracted with dichloromethane leaving the residual carbonaceous nuclei of DEP; the effects of each component on LPS related lung injury in mice were examined.

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Preparation of LPS

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steel tunnel connected to a four cylinder diesel engine. The mass median diameter of the particles recovered was approximately 0.4 µm, and most were globular in shape. Activated charcoal (Norit; Sigma Chemical Co, St Louis, MO) had a diameter of 0.1–0.6 µm.

**Preparation of organic chemicals in DEP (DEP-OC) and washed DEP**

DEP were extracted with dichloromethane (CH2Cl2). Briefly, DEP were suspended in dichloromethane and sonicated for 5 minutes (UD-201; Tomy Seiko, Tokyo, Japan). The suspension was centrifuged at 200g for 20 minutes, the supernatants were transferred to another tube, and the residue washed with dichloromethane. This procedure was repeated three times. The residual particles of DEP were prepared as washed DEP. The extracts were combined, evaporated, dissolved in 100% dimethyl sulfoxide (DMSO, Nacalai Tesque, Kyoto, Japan) and prepared as DEP-OC. They were stored at –80°C before use.

**Study protocol**

Mice were divided into experimental groups and treated as follows: (1) the vehicle group received phosphate buffered saline (PBS) at pH 7.4 (Gibco BRL, Life Technology, Grand Island, NY) containing 0.025% Tween 80 (Nacalai Tesque, Tokyo, Japan) and 0.25% DMSO; (2) the washed DEP or DEP-OC groups received 125 µg washed DEP or DEP-OC, respectively, in the same vehicle; (3) the washed DEP or DEP-OC groups received 125 µg DEP or DEP-OC, respectively, in the same vehicle; (4) the LPS group received 75 µg LPS (E coli B5505, Difco Lab, Detroit, MI, USA) dissolved in the vehicle; and (5) the washed DEP+LPS or (6) the washed DEP+DEP-OC groups received a combination of washed DEP or DEP-OC, respectively, with LPS in the same vehicle.

In another series of experiments the effects of activated charcoal (Norit) were examined in the following groups: (1) vehicle group; (2) Norit group (125 µg/animal); (3) LPS group (75 µg LPS); and (4) Norit+LPS group. The suspension was prepared and inoculated in 100 µl aliquots as previously described.

**Histological evaluation, lung water content, bronchoalveolar lavage**

The lungs were fixed and stained with haematoxylin and eosin as previously described. Lung water content was evaluated as described by Ichinose et al and bronchoalveolar lavage (BAL) and cell counts in BAL fluid were conducted as described by Takano et al.

**Measurement of IL-1β and chemokines in lung tissue supernatants**

The lungs were homogenised and centrifuged as described by Takano et al. ELISA for interleukin-1β (IL-1β; Endogen, Cambridge, MA, USA), macrophage inflammatory protein-1α (MIP-1α; R&D Systems, Minneapolis, MN, USA), macrophage chemotactic protein-1 (MCP-1; R&D Systems), and keratinocyte chemotactic protein (KC; R&D Systems) in the lung tissue supernatants was conducted according to the manufacturer’s instruction.

**Semi-quantitative RT-PCR**

Total RNAs in the lung tissues were extracted in Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. cDNA synthesis and polymerase chain reactions (PCRs) were conducted according to the manufacturer’s protocol (Perkin-Elmer, Foster City, CA, USA). The conditions for PCRs are shown in box 1. PCR products were separated and quantitated as previously described.

**Statistical analysis**

Data are reported as mean (SE) values. Differences between groups were determined using ANOVA with post hoc test as previously described (Statview version 5.0; Abacus Concepts, Inc, Berkeley, CA, USA).

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**RESULTS**

**Effects of DEP-OC and washed DEP on LPS related neutrophil infiltration into BAL fluid**

To estimate the magnitude of neutrophilic lung inflammation we examined the cellular profile of BAL fluid 24 hours after intratracheal instillation. Neither washed DEP nor DEP-OC alone significantly increased the infiltration of neutrophils, but LPS treatment showed a marked increase in the number of neutrophils compared with vehicle alone (fig 1A and B; p<0.01). Administration of LPS combined with either washed DEP or DEP-OC significantly increased the infiltration of neutrophils compared with LPS administered alone (p<0.01). The number of macrophages in BAL fluid was not significantly different between any of the experimental groups (fig 1C and D).

**Effect of DEP-OC and washed DEP on pulmonary oedema related to LPS**

To measure pulmonary oedema we evaluated the lung water content 24 hours after intratracheal treatment. There was a significant increase in lung water content in the LPS and washed DEP groups compared with the vehicle group (fig 2A and B; p<0.01), but there was no significant difference between the DEP-OC and vehicle groups (fig 2B). The combined administration of washed DEP and LPS resulted in a further significant increase in the lung water content compared with LPS or washed DEP administered alone (fig

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**Box 1 Conditions for PCR**

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All PCRs were initiated by denaturation for 10 minutes at 94°C and extended for 7 minutes at 72°C after the last cycle amplification. In each case β-actin was amplified by the same protocol.
Effect of diesel exhaust particles on acute lung injury

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Figure 1 Effects of washed diesel exhaust particles (washed DEP) and organic chemicals in DEP (DEP-OC) on lipopolysaccharide (LPS) related neutrophils (A, B) and macrophages (C, D) in bronchoalveolar lavage (BAL) fluid. *p<0.01 vs vehicle; †p<0.01 vs LPS. Values are mean (SE) of 6 or 8 animals.

2A; p<0.01). However, combined treatment with DEP-OC and LPS had no significant effect compared with LPS alone (fig 2B).

Effect of DEP-OC and washed DEP on histological changes in the lung

To determine the effects of DEP components on lung histology we evaluated lung specimens stained with haematoxylin and eosin 24 hours after intratracheal instillation. No pathological changes were seen in lung specimens in the vehicle group (fig 3A). Infiltration of neutrophils was slight in lung tissue of animals treated with washed DEP and DEP-OC (fig 3B and C) and moderate in those treated with LPS (fig 3D). Combined treatment with washed DEP and LPS markedly enhanced neutrophil sequestration, interstitial oedema, and alveolar haemorrhage compared with LPS alone (fig 3E), while treatment with DEP-OC and LPS combined resulted in neutrophil inflammation without alveolar haemorrhage (fig 3F) which was less prominent than in the group treated with washed DEP+LPS.

Effect of DEP-OC and washed DEP on protein expression of proinflammatory molecules related to LPS

Expression of proinflammatory molecules was studied by measuring protein levels of IL-1β, MIP-1α, MCP-1, and KC in lung tissue supernatants 24 hours after intratracheal instillation. The concentrations of these proinflammatory molecules were below the detection limits in those treated with washed DEP (fig 4) and DEP-OC (fig 5). LPS treatment significantly increased the protein levels of IL-1β, MIP-1α, MCP-1, and KC compared with the vehicle (figs 4A and 5; p<0.01). The combined instillation of washed DEP and LPS resulted in further significant increases compared with LPS alone (fig 4A–D; p<0.05 for IL-1β, MIP-1α and KC; p<0.01 for MCP-1). These results were consistent with those for neutrophil inflammation and pulmonary oedema. In contrast, in the DEP-OC+LPS group the concentrations of these proinflammatory molecules decreased compared with the LPS group, particularly MIP-1α (fig 5B; p<0.05) and KC (fig 5D; p<0.01).

Effect of DEP-OC and washed DEP on mRNA expression of proinflammatory molecules related to LPS

The magnitude of mRNA expression in the lung was measured by semi-quantitative RT-PCR 4 hours after the intratracheal treatments. Treatment with DEP-OC slightly increased mRNA expression of IL-1β and MIP-1α (fig 6A and B) compared with the vehicle, whereas treatment with washed DEP did not. Treatment with LPS significantly increased mRNA expression of IL-1β (p<0.01) and MIP-1α (p<0.01) compared with the vehicle. The mRNA expression of these proinflammatory molecules was increased in the washed DEP+LPS group compared with the LPS group, especially for IL-1β (p<0.05), while mRNA expression in the DEP-OC+LPS group did not increase.

Effect of DEP-OC and washed DEP on mRNA expression of TLRs related to LPS

Lung expression of TLRs, important receptors for bacterial endotoxin, 4 hours after intratracheal administration was also investigated. Instillation with DEP-OC slightly increased the expression of TLR2 compared with vehicle instillation, whereas instillation with washed DEP did not (fig 7A). The mRNA expression of TLR2 was higher in the LPS group than in those treated with vehicle only (p<0.01), and was more intense in the DEP-OC+LPS and the washed DEP+LPS groups than in the LPS group. The expression was more prominent in the washed DEP+LPS group (p<0.01 vs LPS) than in the DEP-OC+LPS group. Treatment with DEP-OC, washed DEP, and DEP-OC+LPS slightly increased TLR4 expression compared with vehicle only (fig 7B). A greater increase in TLR4 expression was seen in the washed DEP+LPS group than in the LPS group (p<0.05).
lung is increased by the combined administration of washed DEP. In addition, the mRNA expression of TLR2 and TLR4 in the presence of LPS (fig 3E), which is concomitant with the infiltration of neutrophils in BAL fluid in the presence of LPS (fig 1). Washed DEP synergistically exacerbate pulmonary oedema, alveolar haemorrhage (fig 3F), and decrease the expression of proinflammatory molecules in the presence of LPS (fig 3A). Combined treatment with washed DEP and LPS (E) marked enhanced accumulation of neutrophils, interstitial oedema, and alveolar haemorrhage. Histological changes in lung specimens treated with DEP-OC and LPS (F) were less prominent than those treated with washed DEP and LPS, n=3 per group. Original magnification ×25.

**Figure 2** Effects of (A) washed diesel exhaust particles (washed DEP) and (B) organic chemicals in DEP (DEP-OC) on pulmonary oedema related to LPS. *p<0.01 v vehicle; ††p<0.01 v LPS; ‡p<0.01 v washed DEP. Values are mean (SE) of 7 animals.

**Effect of activated charcoal on pulmonary oedema related to LPS**
To investigate the effects of complete carbonaceous nuclei we evaluated the lung water content 24 hours after intratracheal instillation of Norit. The LPS group showed a significant increase in lung water content compared with the vehicle group (fig 8, p<0.01), and the group treated with Norit showed no change compared with the vehicle group. There was no significant difference between the Norit+LPS and LPS groups.

**DISCUSSION**
We have previously shown that DEP synergistically enhanced acute lung injury related to LPS. The lung injury consisted of pulmonary oedema, alveolar haemorrhage, and infiltration of neutrophils. The present study has shown that both the organic chemicals in DEP extracted with dichloromethane (DEP-OC) and the residual carbonaceous nuclei of DEP after extraction with dichloromethane (washed DEP) enhance the infiltration of neutrophils in BAL fluid in the presence of LPS (fig 1). Washed DEP synergistically exacerbate pulmonary oedema (fig 2A) and induce alveolar haemorrhage in the presence of LPS (fig 3E), which is concomitant with the expression of IL-1ß, MIP-1ß, MCP-1, and KC (figs 4 and 6). In contrast, DEP-OC do not affect pulmonary oedema (fig 2B) or alveolar haemorrhage (fig 3F), and decrease the expression of these proinflammatory molecules in the presence of LPS (fig 5). In addition, the mRNA expression of TLR2 and TLR4 in the lung is increased by the combined administration of washed DEP and LPS (fig 7). The enhancement by washed DEP of the LPS related changes are comparable to those of whole DEP.

Previous reports have shown the potent activities of whole DEP on the respiratory and immune systems in vivo and in vitro. Intratracheal instillation of mice with DEP induces the infiltration of neutrophils and pulmonary oedema, and exposure of mice to DEP increases the antigen specific IgE and IgG responses. Our previous studies have shown that the intratracheal inoculation of DEP enhances antigen specific IgG, production, eosinophilic airway inflammation, and the expression of cytokines in mice. In vitro studies have shown that DEP induce the expression of proinflammatory cytokines and chemokines such as IL-6, IL-8, GM-CSF, and RANTES from human bronchial epithelial cells. Exposure to DEP with LPS or preincubation with LPS before DEP treatment increases IL-1ß secretion in peripheral blood mononuclear cells.

DEP consist of carbonaceous nuclei and a vast number of organic compounds such as polyaromatic hydrocarbons, aliphatic hydrocarbons, and heterocyclics. Previous studies have suggested that organic chemicals in DEP enhance IgE production from peripheral blood mononuclear cells and IgE secreting B cells. Co-exposure of mice to antigen and organic chemicals in DEP in vivo contributes to the enhancement of antigen specific IgE and IgG production. In addition, organic chemicals in DEP induce the proinflammatory molecules in vitro. Exposure of human epithelial cells to organic chemicals in DEP increases the mRNA expression of IL-8, RANTES, and GM-CSF and modulates NF-κB activation and p38 phosphorylation. Organic chemicals in DEP generate reactive oxygen species and subsequently induce apoptosis in alveolar macrophages. These results suggest that the organic chemicals in DEP play a role in DEP related...
Figure 4  Effects of washed diesel exhaust particles (washed DEP) on the expression of proinflammatory molecules related to lipopolysaccharide (LPS) in lung tissue supernatants 24 hours after intratracheal instillation: (A) interleukin 1β (IL-1β), (B) macrophage inflammatory protein 1α (MIP-1α), (C) macrophage chemoattractant protein 1 (MCP-1), and (D) keratinocyte chemoattractant (KC). *p<0.01 v vehicle; †p<0.05 v LPS; ††p<0.01 v LPS. Values are mean (SE) of 5 animals.

Figure 5  Effects of organic chemicals in diesel exhaust particles (DEP-OC) on the expression of proinflammatory molecules related to lipopolysaccharide (LPS) in lung tissue supernatants 24 hours after intratracheal instillation: (A) interleukin 1β (IL-1β), (B) macrophage inflammatory protein 1α (MIP-1α), (C) macrophage chemoattractant protein 1 (MCP-1), and (D) keratinocyte chemoattractant (KC). *p<0.01 v vehicle; †p<0.05 v LPS; ††p<0.01 v LPS. Values are mean (SE) of 6 animals.
inflammatory responses in vitro. However, there is no evidence that organic chemicals in DEP aggravate non-allergic inflammation in vivo. On the other hand, it remains unclear whether the residual particles in DEP after extraction can affect the expression of proinflammatory molecules and the subsequent inflammatory responses in vivo and in vitro.

We have recently reported that treatment with whole DEP synergistically aggravates LPS related acute lung injury including neutrophilic inflammation, pulmonary oedema, and alveolar haemorrhage in mice. The present study shows that washed DEP and DEP-OC enhance the infiltration of neutrophils into BAL fluid in the presence of LPS. Washed DEP synergistically exacerbated pulmonary oedema and induced alveolar haemorrhage in the presence of LPS. The magnitude of lung injury in mice treated with LPS and washed DEP at a dose of 125 µg in the present study was comparable to that in mice treated with LPS and whole DEP at a dose of 250 µg in the previous study. In contrast, DEP-OC at a dose of 125 µg did not affect pulmonary oedema and alveolar haemorrhage in the presence or absence of LPS. These results indicate that the residual carbonaceous nucel of DEP after extraction with dichloromethane have a more critical role in aggravating LPS related lung injury than the extracted organic chemicals. Since the organic fraction of DEP constitutes around 50% of total particle mass, we administered washed DEP and DEP-OC intratracheally at a dose of 125 µg—that is, 50% of the 250 µg dose of whole DEP used in our previous study. IL-1ß is a proinflammatory cytokine involved in the recruitment and activation of neutrophils. It also triggers recruitment of chemokines which play an important role in the process of inflammation. Anti-IL-8 treatment significantly suppresses LPS induced lung permeability in rabbits. The intratracheal instillation of LPS in rats results in acute

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**Figure 6** Effects of washed diesel exhaust particles (washed DEP) and organic chemicals in DEP (DEP-OC) on mRNA expression of the lipopolysaccharide (LPS) related proinflammatory molecules (A) interleukin 1ß (IL-1ß) and (B) macrophage inflammatory protein 1α (MIP-1α) 4 hours after intratracheal instillation. The amplification cycles were 33 and 35 for IL-1ß and 30 and 32 for MIP-1α; β-actin was amplified by the same protocol. The top panel in each case shows the RT-PCR gel photographs and the bottom panel shows band density ratios of arbitrary density units for (A) IL-1ß and (B) MIP-1α to β-actin. *p<0.01 v vehicle; †p<0.05 v LPS group. Each ratio represents the mean (SE) of at least 3 animals per group.

**Figure 7** Effects of washed diesel exhaust particles (washed DEP) and organic chemicals in DEP (DEP-OC) on mRNA expression of (A) Toll-like receptor 2 (TLR2) and (B) Toll-like receptor 4 (TLR4). The amplification cycles were 30 and 32 for TLR2, and 28 and 30 for TLR4. In each case β-actin was amplified by the same protocol. The top panel in each case shows the RT-PCR gel photographs of TLR2 and TLR4 with β-actin, and the bottom panel shows band density ratios of arbitrary density units for TLR2 and TLR4 to β-actin. *p<0.01 v vehicle; †p<0.05 v LPS; ††p<0.01 v LPS. Each ratio represents the mean (SE) of at least 3 animals per group.

**Figure 8** Effects of activated charcoal on pulmonary oedema related to lipopolysaccharide (LPS). *p<0.01 v vehicle. Values are mean (SE) of 6 animals.
neutrophilia and markedly increases the mRNA expression of MIP-1α in BAL fluid cells. Anti-MIP-1α treatment reduces the infiltration of neutrophils into BAL fluid after LPS challenge in rats. Intraperitoneal injection of LPS to mice increases the expression of MCP-1 and leucocyte accumulation in lung tissue and subsequently induces pulmonary oedema. We have previously reported that the enhancement of LPS related lung injury by DEP is concomitant with the enhanced expression of proinflammatory molecules including IL-1β, MIP-1α, MCP-1, and KC. In the present study combined treatment with washed DEP and LPS increased the protein levels of these molecules in the lung compared with LPS alone, in parallel with neutrophilic inflammation and pulmonary oedema. These results and those of previous studies suggest that the expression of these proinflammatory molecules is critical in the enhancement of LPS related lung injury by washed DEP.

TLRs are a family of mammalian proteins homologous to Drosophila toll which mediate the responsiveness to LPS. In our previous study we reported that combined treatment with LPS and whole DEP increased mRNA expression of TLR2 and TLR4. In the present study combined treatment with washed DEP and LPS resulted in enhanced expression of TLR2 and TLR4. The critical effects of washed DEP on LPS related lung injury might be mediated, at least partly, by the increased expression of TLRs. DEP-OC enhanced the infiltration of neutrophils into BAL fluid in the presence of LPS. In contrast to washed DEP, however, treatment with DEP-OC and LPS did not increase lung expression of IL-1β, MIP-1α, MCP-1, and KC compared with LPS alone. These results suggest that the enhancement of neutrophilic inflammation by DEP-OC is independent of the expression of these proinflammatory molecules. Complement activation in the serum was not significantly different between the experimental groups (data not shown). The concentrations of soluble intercellular adhesion molecule-1 in the experimental groups were not associated with the severity of the lung injury (data not shown). The DEP-OC+LPS group had decreased lung expression of IL-10 compared with the LPS group (data not shown). IL-10 is known to inhibit the activation of other proinflammatory molecules, so the enhancement of neutrophilic inflammation related to LPS by DEP-OC might be associated with the decreased local expression of IL-10. In the present study combined treatment with washed DEP and LPS enhanced the infiltration of neutrophils into BAL fluid, increased the production of IL-10. In the present study the expression of both TLR2 and TLR4. The critical effects of washed DEP on LPS related lung injury might be mediated, at least partly, by the increased expression of TLR4. The enhancement of neutrophilic inflammation related to LPS by DEP-OC might be associated with the decreased local expression of TLRs.

In conclusion, this study has shown that washed DEP exaggerates acute lung injury and the expression of proinflammatory molecules in the presence of bacterial endotoxin. Residual carbonaceous nuclei rather than the organic chemicals in DEP are the main contributors to the aggravation of lung injury related to bacterial toxin. The enhancement effects may be mediated through the expression of proinflammatory molecules including cytokines, chemokines, and TLRs.

ACKNOWLEDGMENTS

The authors acknowledge the assistance of Dr Takahiro Kobayashi, National Institute for Environmental Studies, in preparing the DEP extraction and Miho Sakurai for technical assistance.

REFERENCES

LUNG ALERT

Mechanical ventilation may damage organs other than the lungs

In this laboratory study, acute respiratory distress syndrome was induced by acid aspiration in 24 rabbits which were then randomised to injurious (tidal volume 15–17 mL/kg, PEEP 0–3 cm H₂O) or non-injurious ventilation (tidal volume 5–7 mL/kg, PEEP 9–12 cm H₂O). Serum levels of chemokines putatively associated with ventilator induced lung injury and involved in renal apoptosis (monocyte chemotactic protein 1, IL-8 and growth regulated oncogene) together with LDH, AST and creatinine were all significantly higher in the injurious strategy group. The apoptotic index was increased in renal tubules and small intestinal villi from 1.86% and 0.97% (non-injurious) to 10.9% and 6.7% (injurious). Plasma from the rabbits ventilated by the injurious strategy induced more apoptosis in a culture of renal proximal tubule cells. The degree of apoptosis was reduced by binding soluble Fas ligand. Using plasma from human subjects in an earlier study of protective ventilation, soluble Fas ligand was higher in those ventilated by an injurious strategy and changes in its level correlated with changes in creatinine over 72 hours.

This study shows that injurious mechanical ventilation can induce apoptosis in distal organs and may contribute to the development of multiple organ dysfunction. It suggests mediation by circulating pro-apoptotic factors such as soluble Fas ligand.

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