



# Pulmonary exposure to diesel exhaust particles enhances fatty change of the liver in obese diabetic mice

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**Abstract.** In epidemiological studies, exposure to ambient particulate matter (PM) has been reported to be positively associated with mortality in subjects with diabetes mellitus. Diesel exhaust particles (DEP) are major constituents of atmospheric PM. However, there is no experimental evidence for the relation of DEP to diabetes mellitus and its complications. We investigated the effects of DEP inoculated intratracheally on diabetic changes and nonalcoholic fatty liver disease (NAFLD) in diabetic obese and control mice. db/db mice and the corresponding nondiabetic db/+m mice received exposure to vehicle or DEP every two weeks. Animals were examined with biochemistry, histology, and immunohistochemistry for hexanoyl-lysine (HEL) in the liver. In the db/+m mice, pulmonary exposure to DEP did not increase levels of aspartate aminotransferase (AST) or alanine aminotransferase (ALT) compared to that to vehicle. In the db/db mice, however, the exposure to DEP increased the levels of AST and ALT compared to that to vehicle. Only in the db/db mice, DEP enhanced the magnitude of steatosis and formation of HEL, a marker of oxidative stress, in the liver compared to vehicle. These results suggest that pulmonary exposure to DEP, PM, enhances steatosis in the liver of obese diabetic subjects possibly via enhanced oxidative stress.

## Introduction

Previous epidemiological studies have demonstrated that exposure to ambient particulate matter (PM) is positively

associated with increases in the morbidity and daily mortality caused by diseases, including ischemic heart disease (1,2) and chronic obstructive pulmonary disease (3,4), which are closely related to life habits. Diabetes mellitus and its complications are the other typical diseases related to life habits. Over the past several decades, prevalence of type 2 diabetes mellitus has reached epidemic levels in Western countries (5), which is a significant public health interest. The prognosis of patients with diabetes mellitus is worsened generally by a variety of complications including macro- or micro-angiopathy (6), fatty liver (7-10), nephropathy and infection in the presence or absence of obesity. Recent epidemiological studies have reported a positive association between mortality in patients with diabetes mellitus and ambient levels of PM (11,12).

Air pollutants expelled from diesel engine-powered automobiles include diesel exhaust particles (DEP), which are known to be major constituents of atmospheric PM in metropolitan areas. DEP generate reactive oxygen species (ROS) (13) through a nonenzymatic process (14) or enzymatic reactions catalyzed by cytochrome P-450 (Cyp) (15). Furthermore, DEP enhance the gene expression for Cyp enzymes (15). DEP induce a variety of biological damage at least partly through oxidative stress (15).

Oxidative stress resulting from the imbalance between the production of ROS and the activity of antioxidative defense is implicated in the pathogenesis of diabetic complications by the ability to directly oxidize and damage protein, lipid, and DNA. In addition, Cyp enzymes, biological sources of ROS, are typically localized in the liver (16,17). Therefore, it is possible that DEP exposure aggravates diabetic complications or liver diseases possibly through oxidative stress. However, there is little experimental evidence for the association between DEP exposure and the enhancement of fatty liver disease in diabetes mellitus.

The present study was designed to determine the enhancing effects of pulmonary exposure to DEP on nonalcoholic fatty liver disease (NAFLD), a most common chronic liver disease (18) in Western countries, using diabetic mice with obesity. We also examined the role of oxidative stress in the enhancement.

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Table I. Biochemical parameters in the plasma.

	Age (weeks)	db/+m		db/db	
		Vehicle	DEP	Vehicle	DEP
Glucose (mg/dl)	18	261.5±60.0	190.5±10.9	884.2±252.4 <sup>b</sup>	742.4±93.3 <sup>b</sup>
	24	192.3±32.5	189±20.1	1158±51.0 <sup>b</sup>	1030±122.8 <sup>b</sup>
Fructosamine (μmol/l)	18	387.5±32.5	307.0±23.3	403.3±64.8	416.0±29.9 <sup>b</sup>
	24	323.0±14.1	274.0±6.9	470.8±28.2 <sup>b</sup>	429.3±37.5 <sup>b</sup>
Total cholesterol (mg/dl)	18	71.5±2.5	71.0±3.2	273.3±41.2 <sup>b</sup>	318.4±27.0 <sup>b</sup>
	24	83.5±3.8	87.5±2.9	190.5±25.9 <sup>b</sup>	204.5±33.6 <sup>b</sup>
HDL-cholesterol (mg/dl)	18	45.5±7.5	53.8±2.7	110.7±6.8 <sup>a</sup>	131.0±19.4 <sup>b</sup>
	24	38.5±5.6	52.8±3.1	111.8±9.8 <sup>b</sup>	106.8±14.9 <sup>b</sup>
Triglyceride (mg/dl)	18	89.0±20.0	103.0±14.1	116.3±30.3	130.0±19.6
	24	71.2±5.2	88.3±2.7	152.0±10.6 <sup>b</sup>	161.0±27.2 <sup>b</sup>

<sup>a</sup>p<0.05 versus db/+m mice receiving identical exposure. <sup>b</sup>p<0.01 versus db/+m mice receiving identical exposure. Values are the mean ± SEM in each group.

## Materials and methods

**Animals.** Five-week old female C57BL/KsJ-db/db Jcl (db/db) mice (n=24) weighing 23.6-29.1 g and C57BL/KsJ-db/+m Jcl (db/+m) mice (n=16) weighing 15.5-20.4 g as normal controls were supplied by Japan Clea Co. (Tokyo, Japan). The mice were housed in an animal facility that was maintained at 24-26°C with 55-75% humidity and a 14:10-h light:dark cycle; they were fed (Meiji Seika Kaisha Ltd., Saitama, Japan) and given water *ad libitum*. The studies followed the National Institutes of Health guidelines for the experimental use of animals. The Institutional Review Board approved all animal studies.

**Collection of DEP.** A 4JB1-type, light-duty, four-cylinder, 2.74-L, Isuzu diesel engine (Isuzu Automobile Co., Tokyo, Japan) under computer control was connected to a dynamometer (Meiden-sha, Tokyo Japan). The engine was operated on standard diesel fuel at 1500 rpm under a load of 10 torque (kg/m). DEP were collected as previously described (19). The mass median aerodynamic diameter of DEP was 0.4 μm by an Anderson Air Sampler (Shibata Science Technology, Tokyo, Japan). Most of the particles were globular in shape.

**Study protocol.** Db/db mice and db/+m mice were randomly divided into four experimental groups: the db/db-vehicle group, the db/db-DEP group, the db/+m-vehicle group, and the db/+m-DEP group. The vehicle groups intratracheally received 100 μl of phosphate-buffered saline at pH 7.4 containing 0.05% Tween-80 every two weeks. The DEP groups intratracheally received 100 μg DEP (20) in the same vehicle every two weeks. The DEP suspension was sonicated for 3 min with an ultrasonic disrupter (UD-201, Tomy Seiko, Tokyo, Japan). The mice were anesthetized with 4% halothane (Takeda Chemical Industries Ltd., Osaka, Japan) and intratracheally administered DEP or vehicle for 12 or 18 weeks (7 or 9 times). Body weights were continuously measured every week.

At 18 or 24 weeks of age, the animals were exsanguinated under deep anesthesia with diethyl ether, body weights and the weights of the liver, kidney, and heart were measured. Routine laboratory examinations including total protein, albumin, blood urea nitrogen, uric acid, creatinine, glucose, fructosamine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, HDL cholesterol, and triglyceride were conducted on plasma. The livers were fixed in 10% neutral phosphate-buffered formalin for histological examinations.

**Histology and quantitative analysis.** The livers were embedded in paraffin. Three-micrometer sections were affixed to slides, deparaffinized, and stained with hematoxylin and eosin. Tissue sections of the liver from the mice at 24 weeks of age were quantitatively examined by A.S. and M.T., and the magnitude of fatty change was graded in a blinded fashion. The degree of fatty change was performed according to modified criteria based on Brunt *et al* (scored from 0 to 5: 0, none; 1, <20%; 2, 20-40%; 3, 40-60%; 4, 60-80%; 5, >80%) (21).

**Immunohistochemistry.** The generation of hexanoyl-lysine (HEL) adduct in the liver from mice at 18 or 24 weeks of age was detected by immunohistochemistry using anti-HEL monoclonal antibody (supplied by University of Hyogo, Hyogo, Japan). In brief, deparaffinized slides were blocked with 10% goat serum for 1 h. After blocking, anti-HEL antibody (1:500) was incubated with sections for 1 h at room temperature, followed by incubation of a biotinylated secondary antibody and streptavidin-peroxidase conjugate. Then, the slides were incubated with 3-amino, 9-ethyl-carbazole chromogen, and counterstained with hematoxylin using an AutoProbe III kit (Biomedica, Foster City, CA, USA).

**Statistical analysis.** Data were reported as mean ± SEM. Differences among groups were determined using analysis of variance (Stat view version 4.0; Abacus Concepts, Inc., Berkeley, CA). If differences among groups were significant

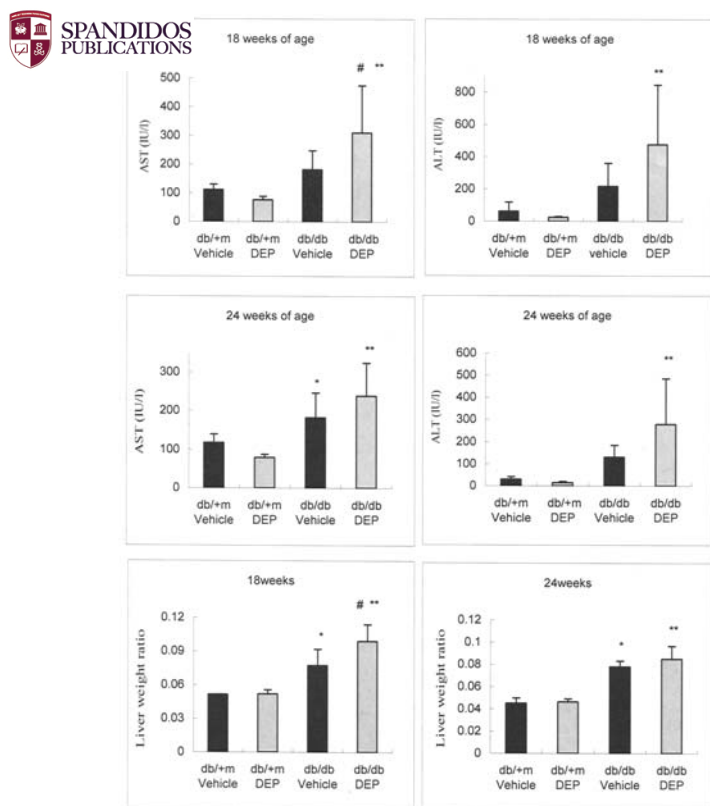


Figure 1. Aspartate aminotransferase, alanine aminotransferase, and liver weight under pulmonary exposure to vehicle or DEP. Mice were intratracheally administered DEP or vehicle every two weeks. Blood samples were collected after exsanguination, the plasma levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured in diabetic (db/db) mice and nondiabetic (db/+m) mice at 18 or 24 weeks of age. Body weights and the weights of the liver were measured. A, plasma levels of AST at 18 weeks of age; B, plasma levels of ALT at 18 weeks of age; C, plasma levels of AST at 24 weeks of age; D, plasma levels of ALT at 24 weeks of age; E, the ratio of liver weights to body weights at 18 weeks of age; F, the ratio of liver weights to body weights at 24 weeks of age. \* $p<0.05$  versus db/+m mice receiving identical exposure. \*\* $p<0.01$  versus db/+m mice receiving identical exposure. # $p<0.05$  versus vehicle exposure in the identical strains. Values are the mean  $\pm$  SEM in each group.

( $p<0.05$  or 0.01), Fisher's protected least significant difference test was used to distinguish between pairs of groups.

## Results

**Effects of pulmonary exposure to DEP on aminotransferase levels.** The levels of AST and ALT (Fig. 1A-D) were greater in the db/db mice than in the db/+m mice under exposure to either DEP or vehicle ( $p<0.01$ : db/+m-DEP versus db/db-DEP for AST and ALT at 18 and 24 weeks of age,  $p<0.05$ : db/+m-vehicle versus db/db-vehicle for AST at 24 weeks of age). In the db/+m mice, the levels of AST and ALT were not significantly different between vehicle exposure and DEP exposure. In the db/db mice, however, DEP exposure enhanced the levels of AST ( $p<0.05$  at 18 weeks of age) and ALT (N.S.) as compared with vehicle exposure.

**Effects of pulmonary exposure to DEP on biochemical parameters.** In overall trends, the levels of glucose ( $p<0.01$ : db/+m mice versus db/db mice receiving identical exposure),

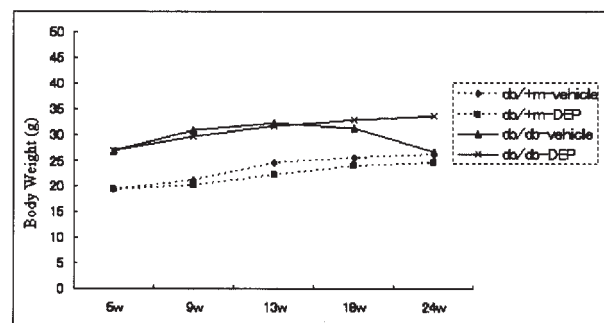


Figure 2. Effects of pulmonary exposure to DEP on body weight. Mice were intratracheally administered DEP or vehicle every two weeks for 12 or 18 weeks. Body weights were continuously measured every week.

fructosamine ( $p<0.01$ : db/+m-DEP versus db/db-DEP,  $p<0.01$ : db/+m-vehicle versus db/db-vehicle at 24 weeks of age), total cholesterol ( $p<0.01$ : db/+m mice versus db/db mice receiving identical exposure), HDL cholesterol ( $p<0.01$ : db/+m-DEP versus db/db-DEP and db/+m-vehicle versus db/db-vehicle at 24 weeks of age,  $p<0.05$ : db/+m-vehicle versus db/db-vehicle at 18 weeks of age), and triglyceride ( $p<0.01$ : db/+m mice versus db/db mice receiving identical exposure at 24 weeks of age) were greater in the db/db mice than in the db/+m mice (Table I). DEP exposure did not enhance the levels of glucose, fructosamine, total cholesterol, HDL cholesterol, and triglyceride as compared to vehicle exposure in both types of mice.

The levels of total protein, albumin, blood urea nitrogen, uric acid, and creatine were not significantly different between the experimental groups (data not shown).

**Effects of pulmonary exposure to DEP on body weight and liver weight.** Overall, db/db mice eventually developed more severe obesity than db/+m mice under exposure either to DEP or vehicle (Fig. 2). However, DEP exposure did not significantly enhance body weight as compared to vehicle exposure in both types of mice.

The ratio of liver weight to body weight (Fig. 1E and F) was significantly greater in the db/db mice than in the db/+m mice under exposure to either DEP ( $p<0.01$  at 18 and 24 weeks of age) or vehicle ( $p<0.05$  at 18 and 24 weeks of age). In the db/+m mice, the ratio was not significantly different between vehicle exposure and DEP exposure. In the db/db mice, however, DEP exposure significantly enhanced the ratio as compared with vehicle exposure ( $p<0.05$  at 18 weeks).

**Effects of DEP on fatty change in the liver.** The magnitude of fatty change in the liver was greater in the db/db mice (Fig. 3C, D, G, H) than in the db/+m mice (Fig. 3A, B, E, F) under exposure to either DEP or vehicle at 18 and 24 weeks of age. In the db/+m mice, the magnitude of fatty change in the liver was not different between vehicle exposure (Fig. 3A and E) and DEP exposure (Fig. 3B and F) at 18 (Fig. 3A and B) and 24 weeks (Fig. 3E and F) of age. In the db/db mice, however, DEP exposure (Fig. 3D and H) enhanced the magnitude of fatty change as compared with vehicle exposure (Fig. 3C and G) at 18 (Fig. 3C and D) and 24 (Fig. 3G and H) weeks of age. The magnitude of fatty change in the liver was scored



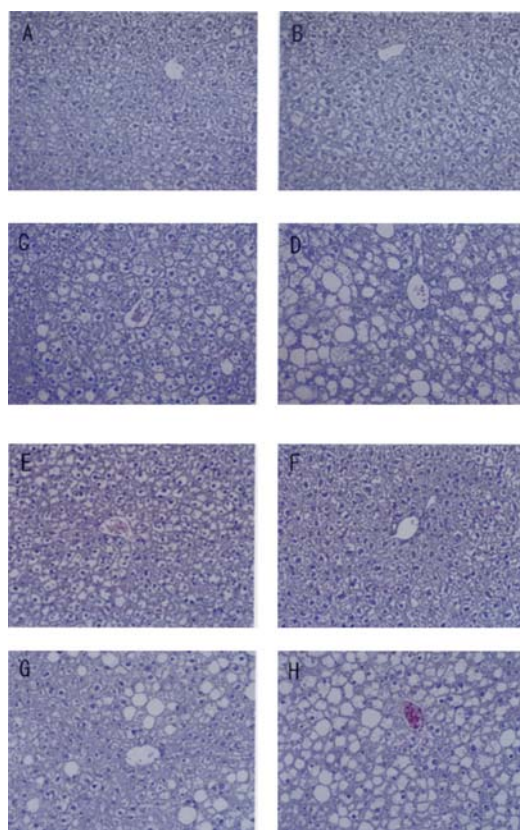


Figure 3. Effects of pulmonary exposure to DEP on fatty change in the liver histology. Mice were intratracheally administered DEP or vehicle every two weeks for 12 or 18 weeks. The livers were fixed and embedded in paraffin. The sections were stained with hematoxylin and eosin. A, db/+m-vehicle group; B, db/+m-DEP group; C, db/db-vehicle group; D, db/db-DEP group at 18 weeks of age. E, db/+m-vehicle group; F, db/+m-DEP group; G, db/db-vehicle group; H, db/db-DEP group at 24 weeks of age. Original magnification x400.

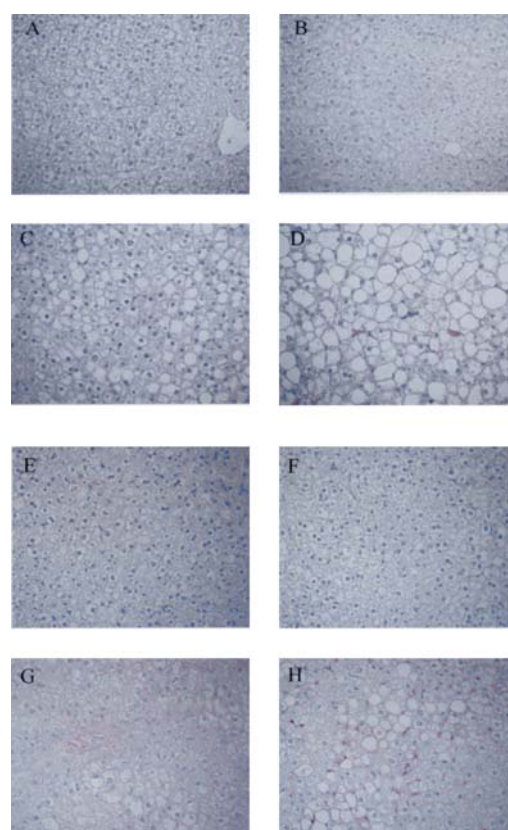


Figure 4. Effects of pulmonary exposure to DEP on the oxidative stress in the liver. Mice were intratracheally administered DEP or vehicle every two weeks for 12 or 18 weeks. The livers were fixed and embedded in paraffin. In the sections, the generation of hexanoyl-lysine (HEL) adduct was detected by immunohistochemistry using anti-HEL antibody. A, db/+m-vehicle group; B, db/+m-DEP group; C, db/db-vehicle group; D, db/db-DEP group at 18 weeks of age; E, db/+m-vehicle group; F, db/+m-DEP group; G, db/db-vehicle group; H, db/db-DEP group at 24 weeks of age. Original magnification x400.

Table II. Quantitative analysis for fatty change.

Strain	Exposure	Score of fatty change
db/+m	vehicle	0.5±0.289
db/+m	DEP	1.0±0.577
db/db	vehicle	2.6±0.927 <sup>a</sup>
db/db	DEP	4.0±0.577 <sup>a</sup>

<sup>a</sup>p<0.05 versus db/+m mice receiving identical exposure. Values are the mean ± SEM in each group.

in each specimen at 24 weeks of age. The scores of fatty change were significantly greater in the db/db mice than in the db/+m mice receiving exposure to vehicle or DEP (p<0.05; Table II). In the db/db mice, pulmonary exposure to DEP enhanced the magnitude of fatty change as compared to that to vehicle, although it didn't reach statistical significance.

*Effects of pulmonary exposure to DEP on HEL formation.* The staining for HEL was not apparent under exposure to either

vehicle or DEP in the db/+m mice (Fig. 4A and B) at 18 or 24 weeks (Fig. 4E and F) of age. Positive staining for HEL was slightly observed in the livers of db/db mice exposed to DEP at 18 weeks of age (Fig. 4D), but not in those exposed to vehicle (Fig. 4C). At 24 weeks of age, however, positive staining for HEL was apparent in the livers of the db/db mice (Fig. 4G and H), which was far more prominent under exposure to DEP (Fig. 4H)

## Discussion

The present study has shown that pulmonary exposure to DEP, particulate air pollutants, enhances fatty change in the livers of diabetic obese mice. The enhancement is concomitant with oxidative stress in the liver.

It has been reported that ambient PM containing elementary carbon, sulfate, heavy metals, and organic compounds can cause and enhance cardiopulmonary diseases (22,23). DEP form a large constituent of ambient urban PM. Inhalation or intratracheal instillation of DEP or the components of DEP has been shown to enhance lung inflammation and asthma (20,24), and to deteriorate biological cardiovascular functions (25). On the other hand, cardiovascular disorders are critical



its in life habit diseases. Diabetes mellitus is another life habit disease and is characterized by complicated cardiovascular risk factors (26-28). In epidemiological studies, individuals with diabetes mellitus have higher risk for death from exposure to polluted ambient air (11,12). However, few experimental studies have elucidated the association between ambient air pollution and diabetes mellitus. We have previously described that nitrogen dioxide air pollution near ambient levels is an atherogenic risk primarily in obese diabetic subjects (29).

db/db mice develop obesity, hyperglycemia, hyperlipemia, and insulin resistance, which is a good animal model for human type 2 diabetes mellitus, as do the corresponding control mice (db/+m). Also in the present study, db/db mice developed obesity, hyperglycemia, hyperlipemia, and fatty change in the liver, whereas the control db/+m mice did not. Thus, db/db mice should be useful for determination of the effects of air pollution on fatty change in the liver of type 2 diabetic obese subjects.

In the present study, pulmonary exposure of obese diabetic mice to DEP enhanced the levels of AST, ALT, the ratio of liver weight, and the magnitude of fatty change of the liver in histology as compared to that to vehicle. To our knowledge, this should be the first report that pulmonary exposure to DEP, air pollutants, can aggravate fatty change in the liver in type 2 diabetic subjects. Interestingly, pulmonary exposure of normal mice to DEP did not affect the liver as compared to vehicle exposure. Thus, the present results can, at least partly, provide experimental evidence to the previous epidemiological reports that diabetic subjects are susceptible populations to PM air pollution.

Our previous researches have shown that pulmonary exposure to DEP induces the expression of Cyp1A1 (30) and ROS (31,32) in the lung, which might play an important role in the present aggravation of fatty change by DEP. DEP contain carbonaceous nuclei which absorb a vast number of organic compounds, such as polycyclic aromatic hydrocarbons, nitropolycyclic aromatic hydrocarbons, heterocyclics, quinones, aldehydes, and aliphatic hydrocarbons (33-35). Particularly, some quinones can generate ROS (36), including superoxide, hydrogen peroxide, and ultimately hydroxyl radical, resulting in biological damage. Cyp enzymes, which can be induced by DEP exposure, are known to produce ROS (37). Cyp1A1 has been reported to be an active Cyp source of superoxide production (38). Also, Cyp2E1 is an enzyme that is closely related to oxidative stress developed in NAFLD patients with steatosis (39,40). In the present study, however, pulmonary exposure to DEP did not enhance the expression of Cyp1A1 and Cyp2E1 in the livers of both types of mice (data not shown).

On the other hand, oxidative stress in mitochondria is considered to play an important role in the pathogenesis of NAFLD. Mitochondria are involved in fatty acid  $\beta$ -oxidation and in oxidative phosphorylation and are an important source of ROS. The increased production of ROS by mitochondria is known to cause lipid peroxidation. Lipid peroxidation products impair the flow of electrons along the respiratory chain, which may cause overreduction of respiratory chain components, and result in further increasing mitochondrial ROS formation and lipid peroxidation (41). It has been reported that DEP decreased the mitochondrial membrane potential

and increased ROS, followed by cytochrome c release and inner mitochondria membrane damage. The DEP-induced mitochondrial damage could be involved in the present enhancing effects on NAFLD, especially in the enhancing effects on oxidative stress. HEL used in this study is a lipid peroxide-modified lysine residue, which is a marker of oxidative stress at an earlier stage compared with malondialdehyde (42). In the present study, extensive HEL staining was observed only in db/db mice, which was far more enhanced by exposure to DEP than to vehicle. These results suggest that lipid peroxidation or oxidative stress is responsible, at least partly, for the enhancement of NAFLD change in db/db mice after DEP exposure.

The present study is the first demonstration that pulmonary exposure to DEP can cause oxidative stress in the liver, a remote organ from the direct site of exposure. It is possible that ROS, lipid peroxides, or inflammatory cytokines originating in the lung reach the liver. It is also possible that DEP or soluble components in DEP, including organic chemicals or metals, can move from the lung to the liver via systemic circulation.

In conclusion, pulmonary exposure to DEP aggravates fatty change in the liver of subjects with type 2 diabetes mellitus with obesity possibly via oxidative stress. Future epidemiological studies should focus on the effects of air pollution on NAFLD in diabetic obese subjects.

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