Lard-based high-fat diet increases secretory leukocyte protease inhibitor expression and attenuates the inflammatory response of acute lung injury in endotoxemic rats.

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(Degree)
博士（保健学）

(Date of Degree)
2015-03-25

(Date of Publication)
2016-03-01

(Resource Type)
doctoral thesis

(Report Number)
甲第6307号

(URL)
https://hdl.handle.net/20.500.14094/D1006307

博　士　論　文

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（ラード含有高脂肪食はラットエンドトキシン血症下の
SLPI 発現を増加させ、肺障害を抑制する）

平成　27　年　1　月　15　日提出

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Lard-based high-fat diet increases secretory leukocyte protease inhibitor expression and attenuates the inflammatory response of acute lung injury in endotoxemic rats

Background & aims: Acute lung injury (ALI) is less severe in obese than in nonobese patients, but the mechanism is unclear. Secretory leukocyte protease inhibitor (SLPI) is the key anti-inflammatory protein in various lung diseases. We have previously reported changes of the surgical stress in obese rats using lard-based high-fat diet (HFD). The purpose of this study was to elucidate the effect of lard-based HFD on the pathophysiology of lipopolysaccharide (LPS)-induced ALI, and the role of SLPI expression.

Methods: Male Wistar rats were fed lard-based HFD (60 kcal% fat) or control diet (CD) for either 4 or 12 weeks and were killed after intraperitoneal LPS injection. Analyses included messenger RNA expression of TNF-α, macrophage inflammatory protein (MIP)-2, inducible nitric oxide synthase (iNOS), IL-10 and SLPI in the lung tissue and bronchoalveolar lavage fluid, and histology of the lungs.

Results: Rats fed HFD for 12 weeks showed suppression of the lung injury and oxidative stress after LPS injection, as indicated by reduction of pulmonary TNF-α, MIP-2 and iNOS mRNA expression and 8-hydroxy-2'-deoxyguanosine immunostaining. The increased pulmonary SLPI caused by lard was associated with decreased pro-inflammatory cytokines and oxidative stress, which eventually resulted in the prevention of ALI. Those effects of lard on LPS-induced ALI were greater after 12 weeks than after 4 weeks feeding, as indicated by the reduction of TNF-α, MIP-2 and iNOS levels.

Conclusions: Feeding lard-based HFD for 12 weeks attenuated LPS-induced ALI with increased pulmonary SLPI expression in rats.

Keywords: lard; secretory leukocyte protease inhibitor; acute lung injury; high-fat diet; lipopolysaccharide; sepsis

Introduction

Acute lung injury (ALI) develops in response to other serious medical conditions including sepsis, pneumonia and trauma, and remains a major clinical problem with significant morbidity and mortality [1,2]. ALI is characterized by both alveolar and systemic release of pro-inflammatory cytokines and chemokines that lead to local tissue destruction [1,2] and oxidative stress [3].

Secretory leukocyte protease inhibitor (SLPI) is secreted in neutrophils, macrophages and mucous membrane epithelial cells, and counteracts the activity of several serine proteases in response to acute inflammatory stimuli [4]. SLPI, an antimicrobial and antiviral peptide, is also able to prevent nuclear factor kappa B (NF-κB) activation by preserving the degradation of the NF-κB-inhibitory protein [4,5]. SLPI is highly expressed in the lungs [5] and protects further lung inflammation [6]. SLPI in the bronchoalveolar lavage fluid (BALF) was shown to be elevated as a result of acute respiratory distress syndrome (ARDS) in humans [6].

Recent clinical studies have reported attenuation of ALI in obese patients termed as the “obesity paradox”. Obesity is associated with decreased alveolar epithelial injury and lower odds ratio of death in mechanically ventilated adult ALI patients [7,8] or ARDS patients after surgery [9]. However, the mechanism has not yet been elucidated.

Under-reporting is an important bias in epidemiological studies on diet and obesity in human subjects; therefore animal models have been widely utilized for experiments on dietary obesity [10]. Lionetti et al. reported that lard (40% fat J/J) aggravated liver injury as indicated by increased hepatic TNF-α and infiltration of inflammatory cells compared with fish oil (40% fat J/J) in a 6 week feeding study in rats [11]. In contrast, as for the effect of high-fat diet (HFD) on the lungs in the absence of any acute injury, lard-based HFD (60 kcal% fat) did not influence pro-inflammatory cytokines in the lung tissue after 3 weeks feeding [12], or in BALF after 9 weeks

feeding [13] in mice. According to these results, the effect of lard on the lungs in the absence of any stimulation is fairly well understood, but its effect in the presence of an acute injury, such as in an LPS-induced rat model of ALI, remains unclear.

Based on these studies, we hypothesized that lard-based HFD-induced obesity increases pulmonary SLPI expression and thereby suppresses ALI. Exposure to lipopolysaccharide (LPS) is a well known method to induce pathological ALI [14]. In this study, to evaluate changes in rats with LPS-induced ALI, pulmonary TNF-α, inducible nitric oxide synthase (iNOS) and macrophage inflammatory protein (MIP)-2 levels, myeloperoxidase (MPO) activity and lung histology were assessed.

Materials and methods

Animals and diets

All experiments used 4-week-old male Wistar rats (CLEA Japan, Tokyo, Japan) weighing 70-90 g which were randomly assigned to one of two groups. One group was fed a control diet (CD) in which the fat component consisted of 45% lard and 55% soybean oil (D12450B, 3.8 kcal/g; Research Diets Inc., NJ, USA) and the other was fed a HFD in which the fat component consisted of 90% lard and 10% soybean

Table 1. Fatty acid composition (g/100 g) of diets.

Fatty acid	CD	HFD
C 10:0	0	0.01
C 12:0	0	0.03
C 14:0	0.02	0.36
C 15:0	0	0.03
C 16:0	0.62	6.45
C 16:1 n-7	0.03	0.44
C 17:0	0.01	0.12
C 18:0	0.29	3.48
C 18:1 n-9	1.19	11.19
C 18:2 n-6	1.73	9.45
C 18:3 n-3	0.21	0.67
C 20:0	0	0.05
C 20:1 n-9	0.01	0.19
C 20:2 n-6	0.02	0.26
C 20:3 n-6	0	0.04
C 20:4 n-6	0.01	0.09
C 22:5 n-6	0	0.03
n-6/n-3	**8.36**	**14.19**
SFA (%)	**23.11**	**32.78**
MUFA (%)	**29.75**	**35.97**
n-6 PUFA (%)	**42.11**	**29.21**
n-3 PUFA (%)	**5.03**	**2.04**

CD, control diet; HFD, high-fat diet; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Table 2. Primer sequences used for real-time PCR.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Annealing temperature (℃)
TNF-α	CATCTTCTCAAAACTCGAGTGACAA	TGGGAGTAGATAAGGTACAGCCC	59
SLPI	TCTGATGCTTAACCCTCCCAAT	GCCCTCACAACATTTGTATTTGC	59
IL-10	GGTTGCCAAGCCTTGTCAGAA	GCTCCACTGCCTTGCTTTTATT	61
iNOS	AACCCAAGGTCTACGTTCAAG	AAAGTGGTAGCCACATCCCG	59
MIP-2	GGCACAATCGGTACGATCCAG	ACCCTGCCAAGGTTGACTTC	61
GAPDH	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACACCAGTA	59

SLPI, secretory leukocyte protease inhibitor; iNOS, inducible nitric oxide synthase; MIP-2, macrophage inflammatory protein-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

oil (D12492, 5.2 kcal/g; Research Diets Inc.). Diets were fed for either 4 or 12 weeks (Table 1). The room was maintained at 22℃ on a 12 h light/dark cycle, and diet and water were supplied *ad libitum*. Body weight and food intake were measured daily for each animal. Rats were fasted (i.e., supplied with water only) from the day before LPS injection until they were euthanized. *Escherichia coli* O111:B4 LPS (Sigma-Aldrich, St Louis, MO, USA) was administered by intraperitoneal injection (i.p.) at a dose of 10 mg/kg body weight. Survival rates were monitored for 12 h, and samples collected 0, 1.5 or 6 h, after LPS injection under diethyl ether anesthesia. The lungs were weighed and all samples were stored at -80℃ until analysis. All procedures were approved by the Institutional Animal Care and Use Committee under the Kobe University Animal Experimentation Regulations.

Bronchoalveolar lavage fluid (BALF)

One milliliter of iced 0.1% ethylene diamine tetra-acetic acid (EDTA) in phosphate-buffered saline (PBS) was injected into the right lung to wash pulmonary alveoli. This step was carried out three times, and BALF thus collected was then centrifuged at 300×g for 5 min. After removal of the supernatant, the pellet was resuspended in 250 μl of PBS and 750 μl of ISOGEN reagent

(Invitrogen, Carlsbad, CA, USA) and stored at -80℃.

Determination of the wet/dry ratio

To determine pulmonary edema, the wet/dry ratio of the lung was measured. After the rats were euthanized, representative tissue samples were taken from the upper lobe of the left lung and weighed. The samples were weighed again after 24 h of drying at 65℃ and the baseline lung dry mass determined.

Histopathologic analysis

Six hours after LPS injection, lung tissue samples were collected for histological examination. After fixing in 4% (w/v) paraformaldehyde in PBS for 24 h, tissue samples (the sagittal section of the right lobe) were embedded in paraffin. Sections of 3 μm thickness were cut and stained with hematoxylin and eosin (HE), and images taken using a Nikon Digital Sight DS-L2 imaging controller (Nikon Instruments Inc., Tokyo, Japan). The average alveolar septal thickness was quantified by a researcher blinded to the treatment groups by measuring the thickness of all septa along a crosshair placed on each image obtained at ×400 magnification (50 septa per animal), using Image J software (National Institutes of Health, Bethesda, MD).

8-hydroxy-2'-deoxyguanosine (8-OHdG) immunohistochemistry

Damage to lung DNA due to oxidative stress was assessed in samples collected at 6 h by staining tissue sections with a monoclonal antibody against 8-OHdG (N45.1, diluted 1:2000; Japan Institute for the Control of Aging, Shizuoka, Japan) using a previously described immunohistochemical method [15]. The cells were counted in 5 randomly-selected areas per slide under a bright-field microscope, and the results expressed as the ratio of immunoreactive-positive cells to total cells.

RNA extraction and real-time quantitative polymerase chain reaction (PCR) analysis

For RNA extraction from lung tissue and BALF, TRIzol reagent (Invitrogen) and ISOGEN reagent were used, respectively, according to the manufacturers' instructions. RNA concentration and purity were determined based on the absorbance at 260 and 280 nm. Complementary DNA synthesis and real-time PCR analysis were performed as previously described [15,16]. RNA (1 μg) extracted from each lung tissue sample and from each BALF sample was reverse transcribed to complementary DNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. All PCR analysis was performed in duplicate using SYBR Green Real time Master Mix (TOYOBO, Osaka, Japan) with the primers listed in Table 2, and reactions were analyzed using the MyiQ Real-Time PCR system (Bio-Rad). All expression values were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control gene. For each sample, the expression was calculated based on the ΔΔCt method [15,16]. Briefly, the ΔCt values in each sample were calculated as follows: threshold cycle (Ct) (target gene) − Ct (internal control gene). The relative expression of each target gene (ΔΔCt) was obtained by subtracting the ΔCt of each sample of the treated groups from the mean ΔCt of the CD group. Finally, the relative expression value, normalized to an endogenous reference, was given by $2^{-\Delta\Delta Ct}$. The ratio was based on the amount of mRNA in each of the CD-fed rats at 0 h.

Western blot analysis

Lung tissue samples were collected 6 h after LPS injection and homogenized in 600 μl PRO-PREP solution (iNtRON Biotechnology Inc., Seoul, Korea) containing a protease inhibitor mixture (EDTA, leupeptin, pepstatin, aprotinin and phenylmethanesulfonyl fluoride). Western blot analysis, including protein

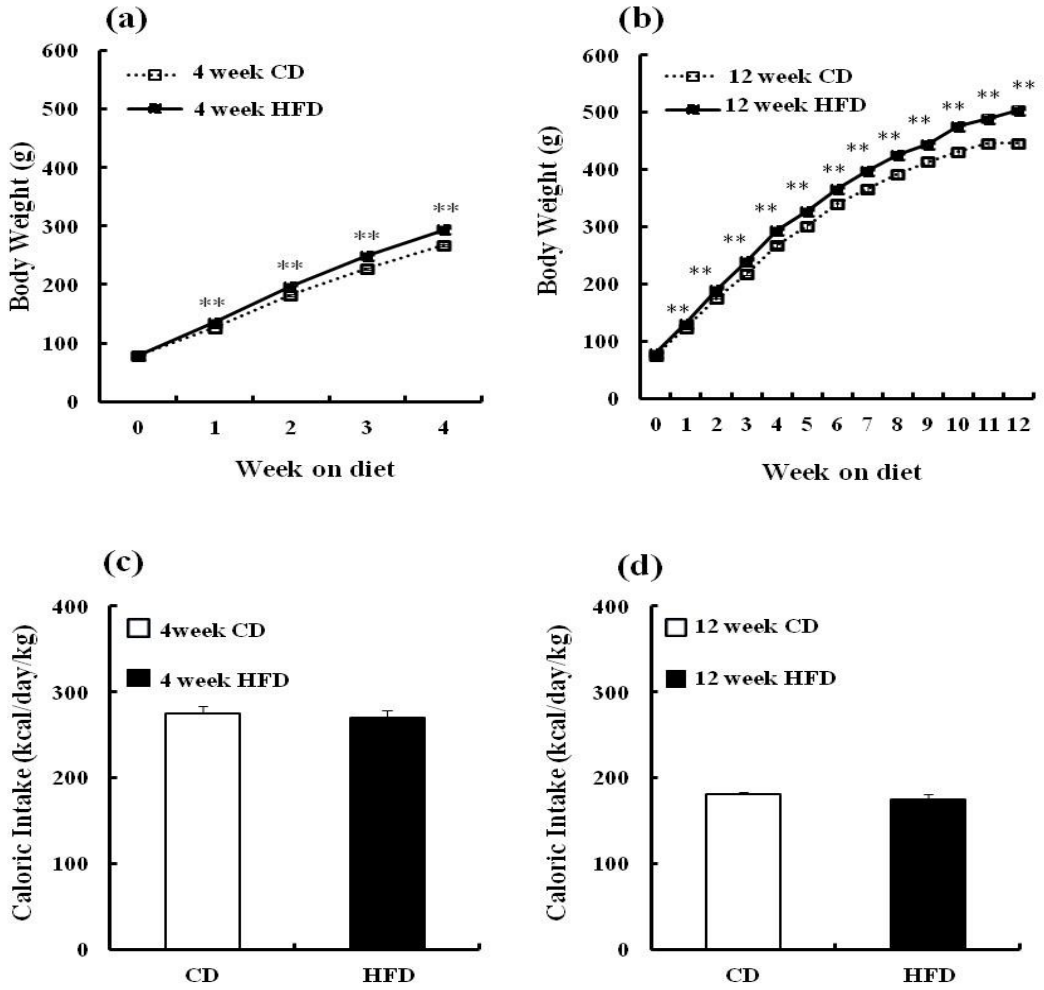

Fig. 1. Effect of HFD on body weight and energy consumption.

Weekly body weight gain of rats fed CD or HFD for (a) 4 weeks and (b) 12 weeks, and daily caloric intake of the same rats after (c) 4 weeks and (d) 12 weeks. Open squares with a dashed line indicate CD-fed rats, and closed squares with a solid line indicate HFD-fed rats. Values represent the mean ± SEM of 27-30 animals per group. $^{**}p < 0.01$ *vs*. CD.

concentration measurement, was performed as described previously [17]. Briefly, the Lowry method was used to determine the protein concentration of the supernatant after centrifugation at 16,200×g for 5 min (4℃). Extracted proteins (20 μg) were separated using 18% acrylamide gels and transferred to polyvinylidene difluoride membranes (GE Healthcare, Amersham, Bucks, UK).

After blocking with Tris-buffered saline/Tween 20 containing 50 g/l skimmed milk, membranes were probed with a polyclonal rabbit anti-SLPI antibody (diluted 1:1000; Cell Signaling Technology, Beverley, MA, USA) or β-actin (diluted 1:5000; Sigma-Aldrich). For development of blots, the ECL-plus Western Blotting Detection System (GE Healthcare) was used before exposing membranes to Hyperfilm (GE Healthcare).

Neutrophil accumulation in the lungs

MPO activity in tissue was measured as neutrophil accumulation using an MPO Assay Kit (Hycult Biotechnology b.v, UDEN, The Netherlands), according to the manufacturer's instructions. Briefly, lung tissue samples were homogenized in 20 ml lysis buffer/g tissue weight. Lysis buffer consisted of 200 mM NaCl, 5 mM EDTA, 10 mM tris hydroxymethyl aminomethane (Tris), 10% (w/v) glycerine, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin (pH 7.4), and homogenization was done on ice. The 3, 3', 5, 5'-tetra-methylbenzidine solution was added to the supernatant and the activity was measured by absorbance at 450 nm. The results were calculated as MPO activity per gram of tissue.

Statistical analysis

Statistical analyses were performed using Statcel2 software (OMS Publishing Inc. Saitama, Japan). The Tukey-Kramer test was performed for comparisons with each basal level. Differences between CD- and HFD-fed rats at each time point were assessing using a t-test. A log-rank test was used to assess differences in the survival rate between CD- and HFD-fed rats. All results are expressed as mean ± standard error of the mean (SEM), and a p value < 0.05 was considered to demonstrate a significant difference.

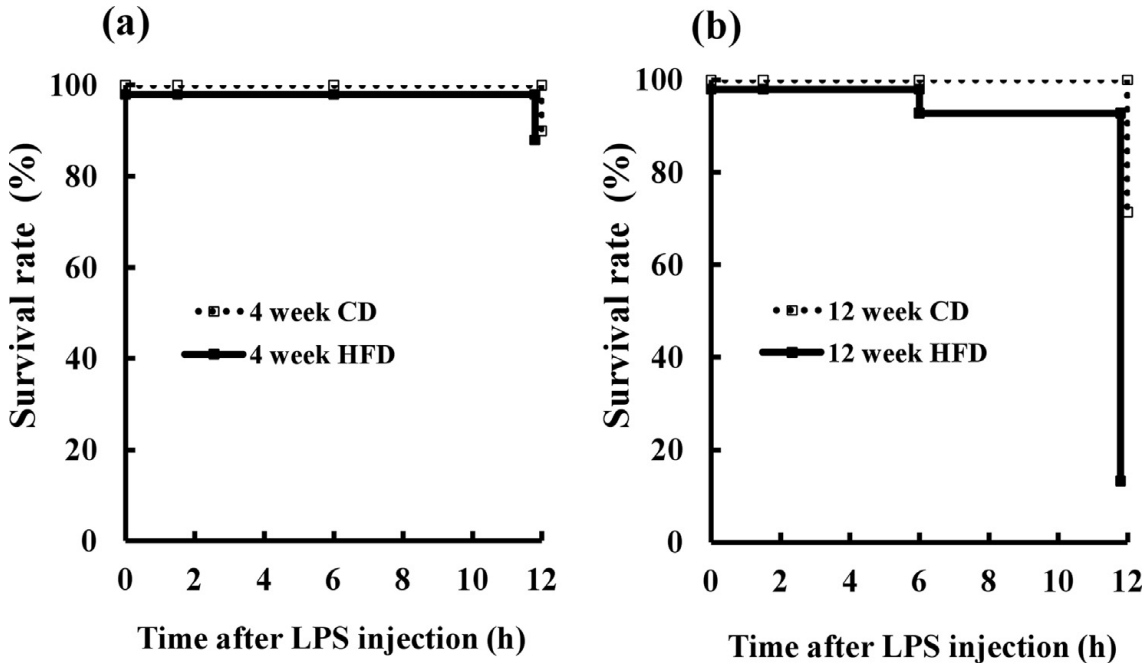

Fig. 2. Effect of HFD on survival rate after LPS injection.

The survival rate of rats fed CD or HFD diet for (a) 4 weeks or (b) 12 weeks, for up to 12 h after LPS injection. Open squares with a dashed line indicate CD-fed rats, and closed squares with a solid line indicate HFD-fed rats. Values represent the means ± SEM of 7-10 animals for each time point. LPS, lipopolysaccharide.

Results

Body weight and energy consumption

HFD-fed rats showed a significant weight gain compared with CD-fed rats after 1 week of the feeding period (Fig. 1). Body weight increased from 79.3 ± 0.4 to 294.0 ± 2.8 g after 4 weeks feeding and from 78.5 ± 0.7 to 504.4 ± 7.3 g after 12 weeks feeding HFD (Fig. 1(a and b); $p < 0.01$). The caloric intake per day was not different between CD- and HFD-fed rats at the end of either the 4 week or 12 week feeding period (Fig. 1(c and d)).

Survival rate after LPS injection

There was no difference in survival rate between CD- and HFD-fed rats in either feeding period 6 h after LPS injection. After 12 h, the survival rate was 90% for both CD- (9/10) and HFD-fed rats (9/

10) at 4 weeks (Fig. 2(a)). After 12 weeks feeding, the survival rate 12 h after LPS injection in CD- and HFD-fed rats was 71.4% (5/7) and 14.3% (1/7), respectively (Fig. 2(b)). Thus, the survival rate in HFD-fed rats after 12 weeks feeding was lower than that in CD-fed rats after LPS injection.

Lung injury

Several histopathological changes including pulmonary congestion, inflammatory cell infiltration, and alveolar septal thickening were caused by LPS (Fig. 3(a-h)). To quantify these histological differences, alveolar septal thickness was measured. The morphometric analysis showed statistical differences between CD and HFD-fed rats (Fig. 3(i and j)). At the baseline for each feeding term, the thickness in HFD-fed rats was significantly higher than that in CD-fed rats (4 weeks CD: 3.35 ± 0.17 μm *vs.* HFD: 5.96 ± 0.86 μm, and 12 weeks CD: 5.23 ± 0.63 μm *vs.* HFD: 11.37 ± 0.57 μm; Fig. 3(i), $p < 0.01$; Fig. 3(j), $p < 0.05$). Compared with rats fed HFD for 4 weeks, septal thickness was significantly increased in rats fed HFD for 12 weeks in the absence of LPS stimulation ($p < 0.01$). After LPS injection, the thickness in rats fed CD for 4 weeks was significantly lower than that in HFD-fed rats after 6 h (CD: 6.31 ± 0.18 μm *vs*. HFD: 9.10 ± 0.48 μm; Fig. 3(i), $p < 0.01$). In rats fed for 12 weeks, there was no difference between CD- and HFD-fed rats after LPS injection (CD: 13.33 ± 0.93 μm *vs*. HFD: 10.99 ± 1.50 μm; Fig. 3(j)). Six hours after LPS injection, the thickness in rats fed either diet for 12 weeks showed a significant increase compared with rats fed the same diet for 4 weeks ($p < 0.05$).

Wet/dry ratio

Ratios between the weight of wet and dry lungs in rats induced by LPS were 6.89 ± 1.03 in CD-fed rats and 4.68 ± 0.50 in HFD-fed rats after 4 weeks feeding (Fig. 4(a)).

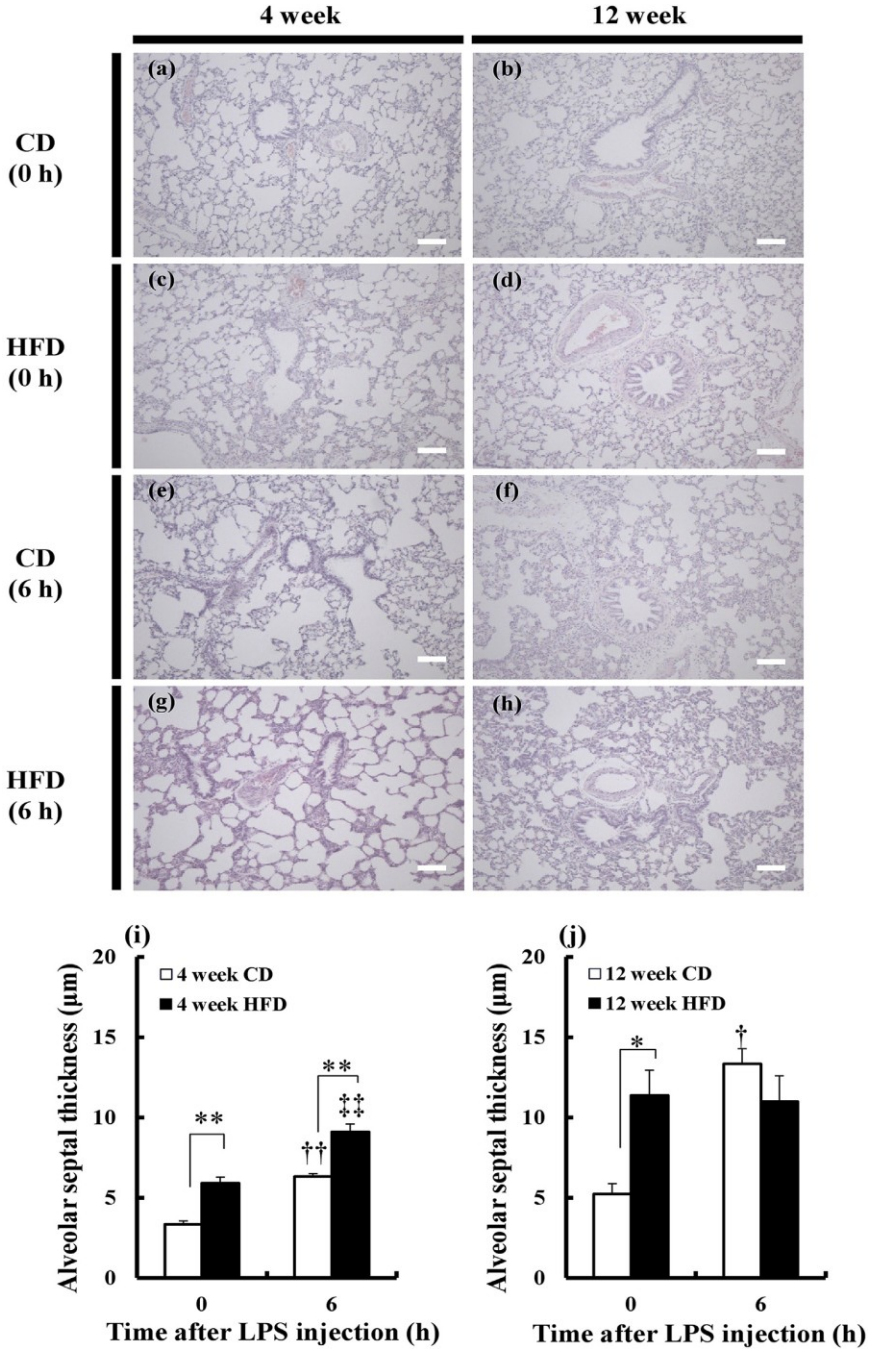

Fig. 3. Effect of HFD on pathological changes.

(a) 4 weeks CD, 0 h after LPS injection; (b) 12 weeks CD, 0 h; (c) 4 weeks HFD, 0 h; (d) 12 weeks HFD, 0 h; (e) 4 weeks CD, 6 h; (f) 12 weeks CD, 6 h; (g) 4 weeks HFD, 6 h; (h) 12 weeks HFD, 6 h (hematoxylin and eosin staining, 200× magnification, internal scale bars indicate 100 μm). Alveolar septal thickness after (i) 4 weeks and (j) 12 weeks feeding was evaluated using Image J software. Open squares indicate CD-fed rats, and closed squares indicate HFD-fed rats. Values represent the mean ± SEM of 4-5 animals per group at each time point. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ *vs*. CD, 0 h, $^{\ddagger\ddagger}p < 0.01$ *vs*. HFD, 0 h, $*p < 0.05$, $**p < 0.01$ *vs*. CD at the same time point. LPS, lipopolysaccharide.

After 12 weeks feeding, the ratios were 4.95 ± 0.67 in CD-fed rats and 3.93 ± 0.21 in HFD-fed rats (Fig. 4(b)). These results show that there was no difference in the degree of edema between CD- and HFD-fed rats.

Expression of TNF-a in the lungs

Prior to LPS injection for each feeding period, TNF-α expression in the lung tissue was not different between CD- and HFD-fed rats (Fig. 5). After 4 weeks feeding, TNF-α expression in the lung tissue increased approximately 10-fold 1.5 h after LPS injection (Fig. 5(a)). Six hours after LPS injection, there was no difference in TNF-α expression in either lung tissue or BALF between CD- and HFD-fed rats. In the 12 weeks feeding group, TNF-α expression in the lung tissue was increased approximately 25 times 1.5 h after LPS injection, compared with the expression prior to injection (Fig. 5(b)).

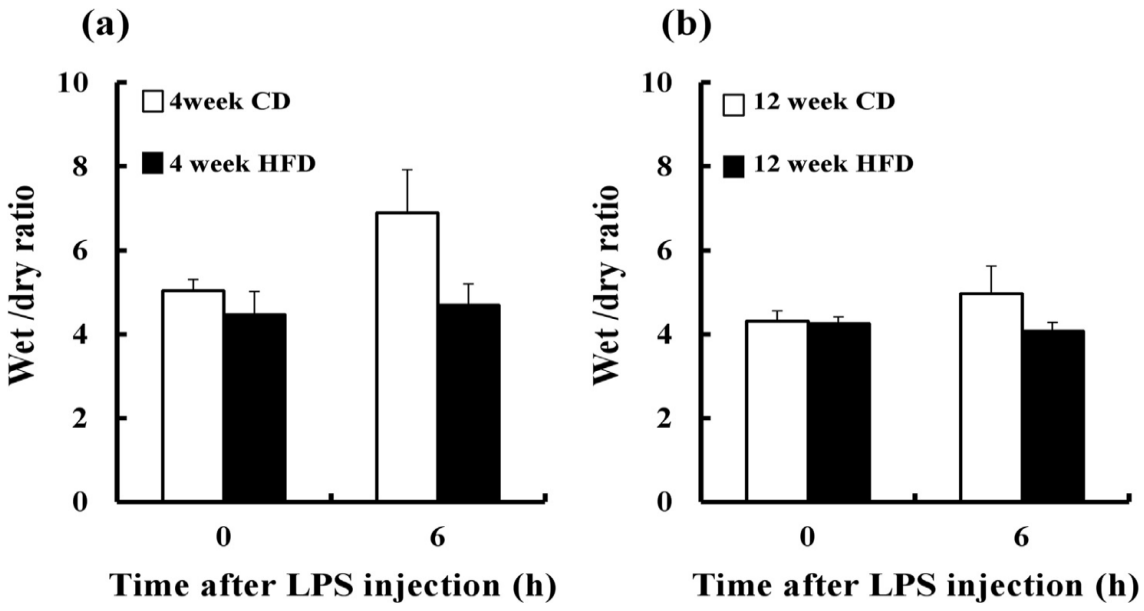

Fig. 4. Wet/dry ratio.

Tissue damage was evaluated using the wet/dry ratio. Wet/dry ratio after LPS injection in rats fed the experimental diets for (a) 4 weeks or (b) 12 weeks. Open squares indicate CD-fed rats, and closed squares indicate HFD-fed rats. Values represent the mean ± SEM of 4-5 animals per group at each time point. LPS, lipopolysaccharide.

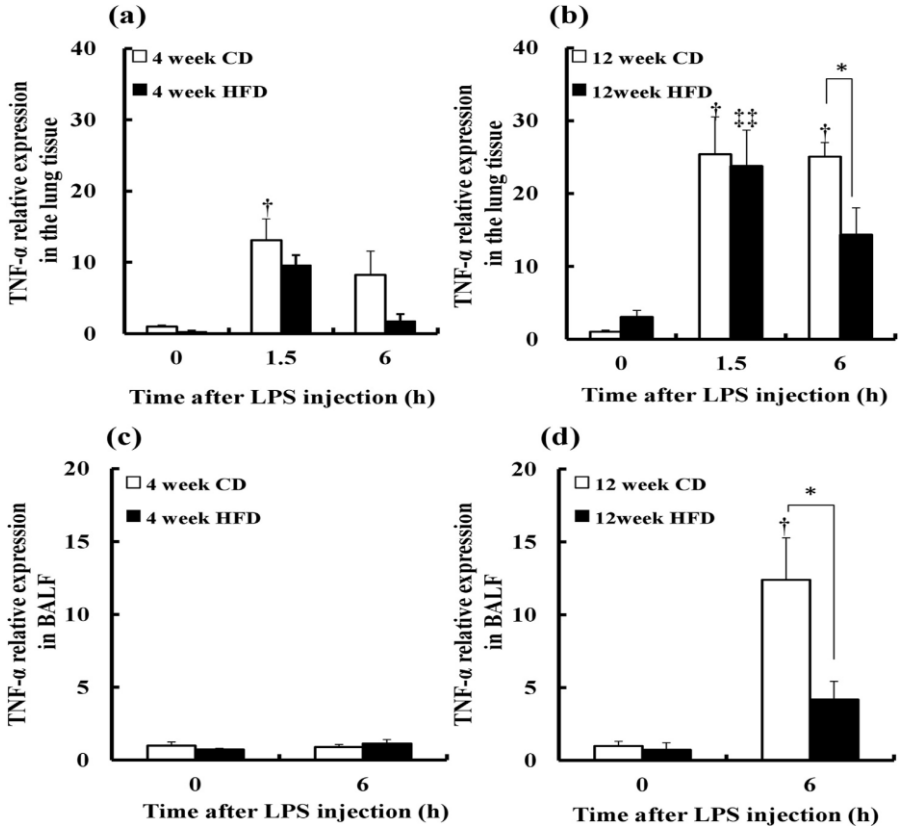

Fig. 5. Effect of HFD on TNF-α mRNA.

At 0, 1.5 and 6 h after LPS injection, lung tissue and BALF were harvested from rats fed CD or HFD for 4 or 12 weeks. The expression of TNF-α mRNA in lung tissue from rats fed for (a) 4 weeks or (b) 12 weeks, and in BALF from rats fed for (c) 4 weeks or (d) 12 weeks was measured using real-time PCR, and is expressed as a ratio based on the amount of mRNA in CD-fed rats at 0 h. Open squares indicate CD-fed rats, and closed squares indicate HFD-fed rats. Values represent the mean ± SEM of 4-8 animals per group at each time point. $^{\dagger}p < 0.05$ *vs.* CD 0 h, $^{\ddagger\ddagger}p < 0.01$ *vs.* HFD 0 h, $^{*}p < 0.05$ *vs.* CD at the same time point. BALF, bronchoalveolar lavage fluid; LPS, lipopolysaccharide.

Of note, TNF-α expression in HFD-fed rats at 6 h had decreased by approximately two-thirds compared with at 1.5 h (Fig. 5(b); $p < 0.05$ *vs.* CD 6 h). In addition, in BALF, the expression in HFD-fed rats was approximately one-third of that in CD-fed rats 6 h after LPS injection (Fig. 5(d); $p < 0.05$). Collectively, these results show that in the 12 week feeding study, pro-inflammatory cytokines after LPS injection were lower in HFD-fed rats than in CD-fed rats, as shown in both in lung tissue and BALF.

Expression of SLPI and IL-10 in the lungs

Figure 6 shows SLPI and IL-10 expression in the lungs after LPS injection. In the lung tissue, SLPI mRNA expression of HFD-fed rats was the same as that of CD-fed rats at 0 h (Fig. 6(a and b)). After LPS injection, the expression in the lung tissue increased drastically in a time-dependent manner both in CD- and HFD-fed rats. Six hours after LPS injection, SLPI expression in CD-fed rats was increased approximately 20-fold compared with baseline in each feeding group. Of particular note, the SLPI level in rats fed HFD for 12 weeks was 2-fold higher than that in CD-fed rats at 6 h (Fig. 6(b); $p < 0.05$). In BALF, the level of SLPI mRNA in HFD-fed rats was higher than that in CD-fed rats 6 h after LPS injection for both the 4 and 12 week feeding studies (Fig. 6(e and f)). The abundance of SLPI protein in the lung tissue was higher in HFD-fed rats compared with CD-fed rats in the 12 week study (Fig. 7). These data show that LPS induced expression of SLPI in both lung tissue and BALF, especially in HFD-fed rats. There were no significant differences in IL-10 expression in either lung tissue or BALF between the groups after LPS injection (Fig. 6(c, d, g and h)).

Oxidative stress in the lungs

To assess the effect of HFD on oxidative stress in the lungs, we performed 8-OHdG immunostaining and measured iNOS mRNA expression (Figs. 8 and 9).

At baseline, the number of positive cells by oxidative stress in HFD-fed rats was not different compared with CD-fed rats (Fig. 8(i and j)). The lung tissue in CD-fed rats had been damaged in response to LPS-induced oxidative stress. The amount of oxidatively damaged DNA was increased in the alveolar epithelium and interstitial area after LPS injection. The ratio in CD-fed rats was higher than that in HFD-fed rats after LPS injection in the 4 week study (28.0% and 20.2%, respectively; Fig. 8(i), $p < 0.05$).

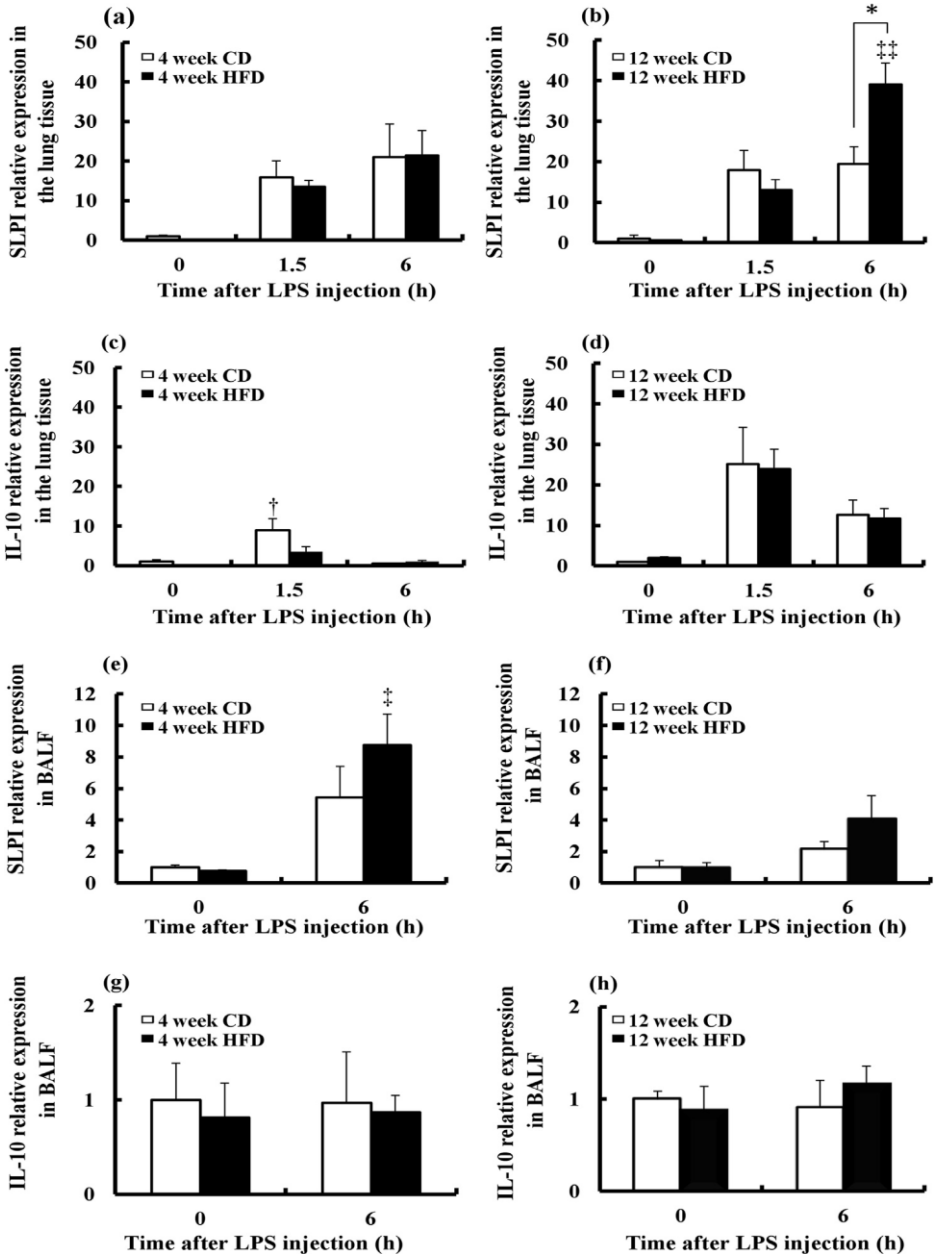

Fig. 6. Effects of HFD on SLPI and IL-10 mRNA.

At 0, 1.5 and 6 h after LPS injection, lung tissue and BALF were harvested from rats fed CD or HFD for 4 or 12 weeks. The mRNA expression of SLPI at (a) 4 weeks and (b) 12 weeks, and of IL-10 at (c) 4 weeks and (d) 12 weeks in the lung tissue, and of SLPI at (e) 4 weeks and (f) 12 weeks and of IL-10 at (g) 4 weeks and (h) 12 weeks in BALF was measured using real-time PCR, and is expressed as a ratio based on the amount of mRNA in CD-fed rats. Open squares indicate CD-fed rats, and closed squares indicate HFD-fed rats. Values represent the mean ± SEM of 4-8 animals per group. $^{\dagger}p < 0.05$ *vs.* CD 0 h, $^{\ddagger}p < 0.05$, $^{\ddagger\ddagger}p < 0.01$ *vs.* HFD 0 h, $^{*}p < 0.05$ *vs.* CD at the same time point. SLPI, secretory leukocyte protease inhibitor; BALF, bronchoalveolar lavage fluid; LPS, lipopolysaccharide.

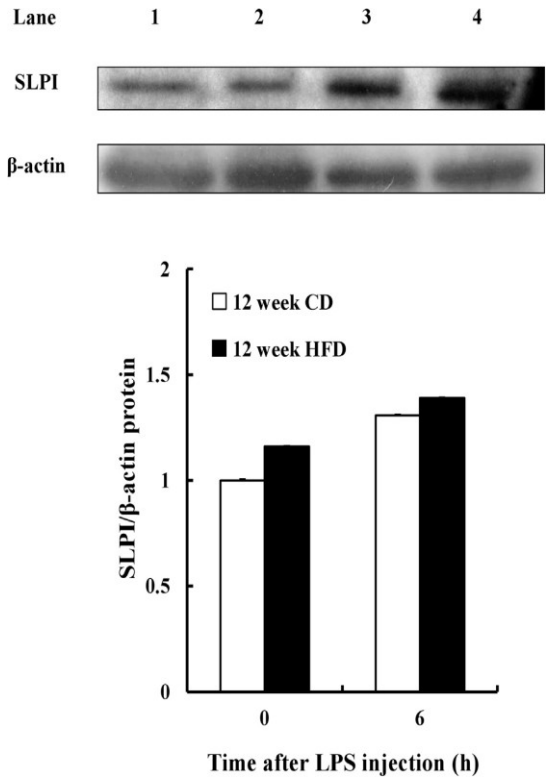

Fig. 7. Effects of HFD on SLPI protein.

At 0 and 6 h after LPS injection, lung tissue protein was harvested from rats fed CD and HFD for 12 weeks. The SLPI and β-actin of CD, 0 h (lane 1), HFD, 0 h (lane 2), CD, 6 h (lane 3) and HFD, 6 h (lane 4) were measured using western blotting, and are expressed as a ratio based on the amount of protein in rats from the CD group. Open squares indicate CD-fed rats, and closed squares indicate HFD-fed rats. Values represent the mean ± SEM of 3 animals per group.
SLPI, secretory leukocyte protease inhibitor;
LPS, lipopolysaccharide.

After 12 weeks feeding, the number of positive cells was further increased and the ratio in CD-fed rats was also higher than that in HFD-fed rats (67.5% and 38.6%, respectively; Fig. 8(j), $p < 0.01$). After LPS injection, the ratio in rats fed for 12 weeks showed a significant increase compared with the respective diet group in the 4 week feeding study ($p < 0.01$).

The expression of iNOS mRNA was measured as a marker of oxidative stress in the lung tissue and BALF at 0 and 6 h after LPS injection (Fig. 9). The expression of iNOS in the lung tissue of endotoxemic rats was approximately 20-fold higher in rats fed CD diet for 4 weeks and 100-fold higher in those fed CD for 12 weeks compared with the respective baseline level (Fig. 9(a and b)). After 12 weeks of HFD, iNOS level in the lung tissue 6 h after LPS injection was decreased to approximately half compared with CD-fed rats (Fig. 9(b), $p < 0.05$). In BALF, the expression in HFD-fed rats was 50% lower than that in CD-fed rats in the 12 week feeding group (Fig. 9(d)).

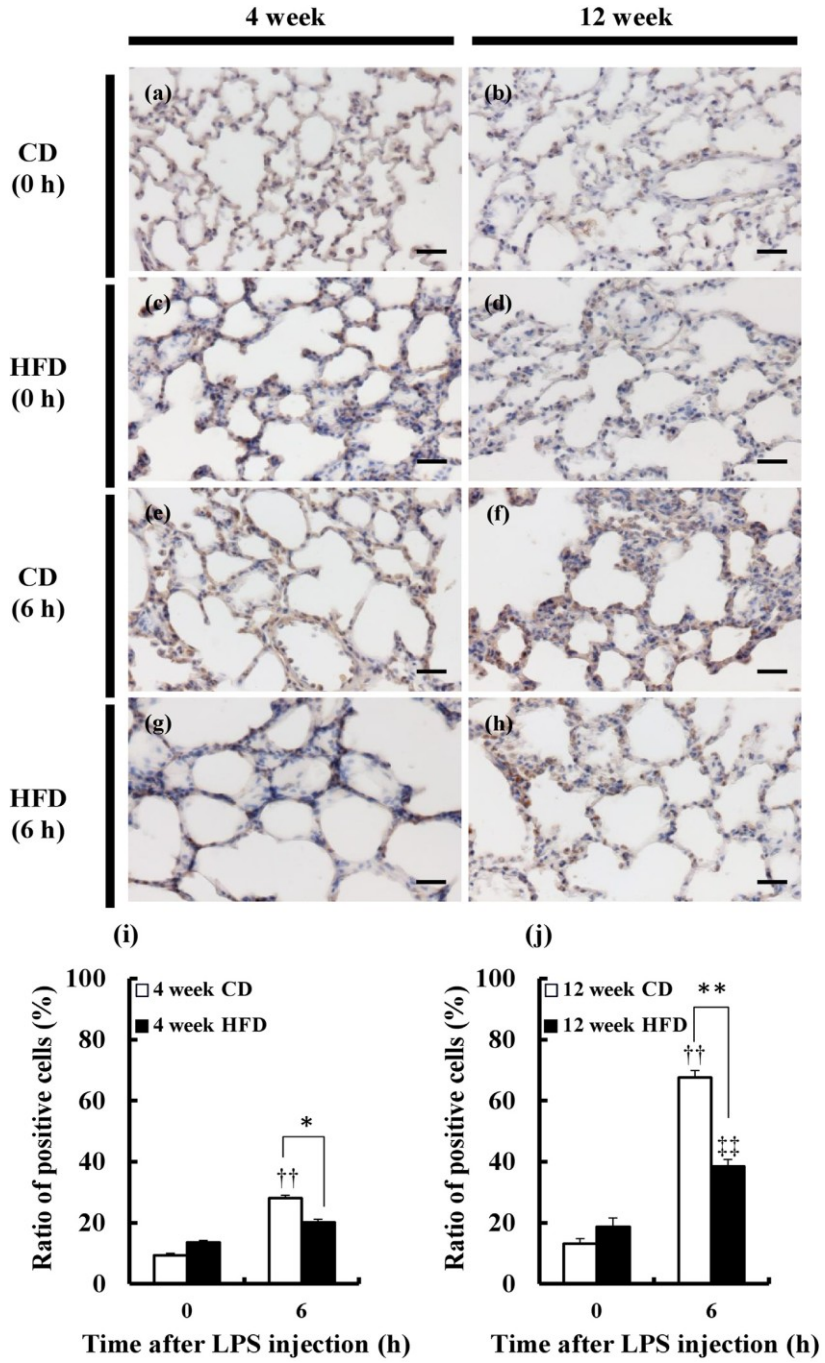

Fig. 8. Effect of HFD on oxidative stress.

(a) 4 week CD, 0 h after LPS injection; (b) 12 week CD, 0 h; (c) 4 week HFD, 0 h; (d) 12 week HFD, 0 h; (e) 4 week CD, 6 h; (f) 12 week CD, 6 h; (g) 4 week HFD, 6 h; (h) 12 week HFD, 6 h (8-OHdG staining, 400× magnification, internal scale bars indicate 50 μm). Positive ratio in rats fed for (i) 4 weeks and (j) 12 weeks was expressed as the mean percentage of immunoreactive-positive cells/total cells. Open squares indicate CD-fed rats, and closed squares indicate HFD-fed rats. Values represent the mean ± SEM of 4-5 animals per group at each time point. $^{\dagger\dagger}p < 0.01$ *vs.* CD 0 h, $^{\ddagger\ddagger}p < 0.01$ *vs.* HFD 0 h, $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* CD at the same time point. LPS, lipopolysaccharide.

Neutrophil recruitment

Because ALI is largely dependent on neutrophil recruitment, we determined MIP-2 expression and MPO activity in the lungs (Fig. 10). In the 4 week feeding groups, MIP-2 levels in HFD-fed rats were increased by approximately 3-fold compared with CD-fed rats in both lung tissue and BALF (Fig. 10(a), $p < 0.05$; Fig. 10(c), p=0.05). Compared with the 4 weeks feeding models, MPO activity was increased in rats fed CD for 12 weeks.

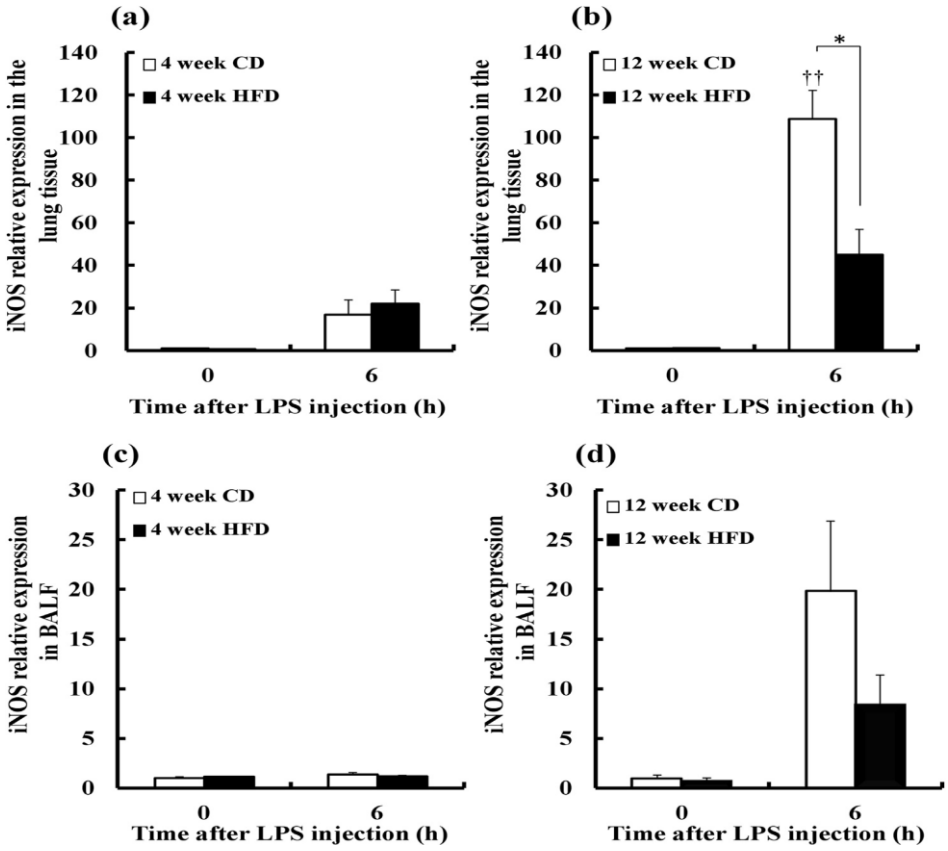

Fig. 9. Effect of HFD on iNOS mRNA.

At 0 and 6 h after LPS injection, lung tissue and BALF were harvested from rats fed CD or HFD for 4 or 12 weeks. The expression of iNOS mRNA in lung tissue from rats fed for (a) 4 weeks or (b) 12 weeks, and in BALF from rats fed for (c) 4 weeks or (d) 12 weeks was measured using real-time PCR, and is expressed as a ratio based on the amount of mRNA in CD-fed rats at 0 h. Values represent the mean ± SEM of 4-8 animals per group at each time point. Open squares indicate CD-fed rats, and closed squares indicate HFD-fed rats. $^{\dagger\dagger}p < 0.01$ *vs.* HFD 0 h, $^{*}p < 0.05$ *vs.* CD at the same time point. iNOS, inducible nitric oxide synthase; BALF, bronchoalveolar lavage fluid; LPS, lipopolysaccharide.

In the 12 week feeding groups, MIP-2 level 6 h after LPS injection was significantly suppressed to 20% in the lung tissue of HFD-fed rats compared with that of CD-fed rats (Fig. 10(b), $p < 0.01$). LPS injection dramatically increased lung MPO activity. The activity 6 h after LPS injection in rats fed HFD for 12 weeks was higher than that in rats fed the same diet for 4 weeks ($p < 0.05$). However, in both 4 and 12 week feeding groups, there was no difference between CD- and HFD-fed rats 6 h after LPS injection (4 weeks CD: 153.0 ± 18.7 μg/ml *vs.* HFD: 125.3 ± 18.1 μg/ml, and 12 weeks CD: 250.6 ± 55.0 μg/ml *vs.* HFD: 220.4 ± 19.3 μg/ml; Fig. 10(e and f)).

Discussion

The present study investigated the effects of lard-based HFD on the pathophysiology of LPS-induced ALI. Increased SLPI expression in the lung tissue and BALF was detected in HFD-fed rats after both 4 and 12 weeks feeding. Both lung injury and oxidative stress were attenuated in rats fed HFD for 12 weeks, as indicated by reduced TNF-α, MIP-2 and iNOS expression and 8-OHdG immunostaining in the lung tissue and BALF. This suggests that the reduction of pro-inflammatory mediators in HFD-fed rats after 12 weeks was caused by SLPI, which increased in the lungs in response to LPS. LPS-induced inflammatory responses including alveolar septal thickness, MPO activity and oxidative stress increased progressively after 12 weeks feeding compared with after 4 weeks feeding. Our results suggest that 12 weeks of lard-based HFD increased pulmonary SLPI expression and attenuated LPS-induced ALI.

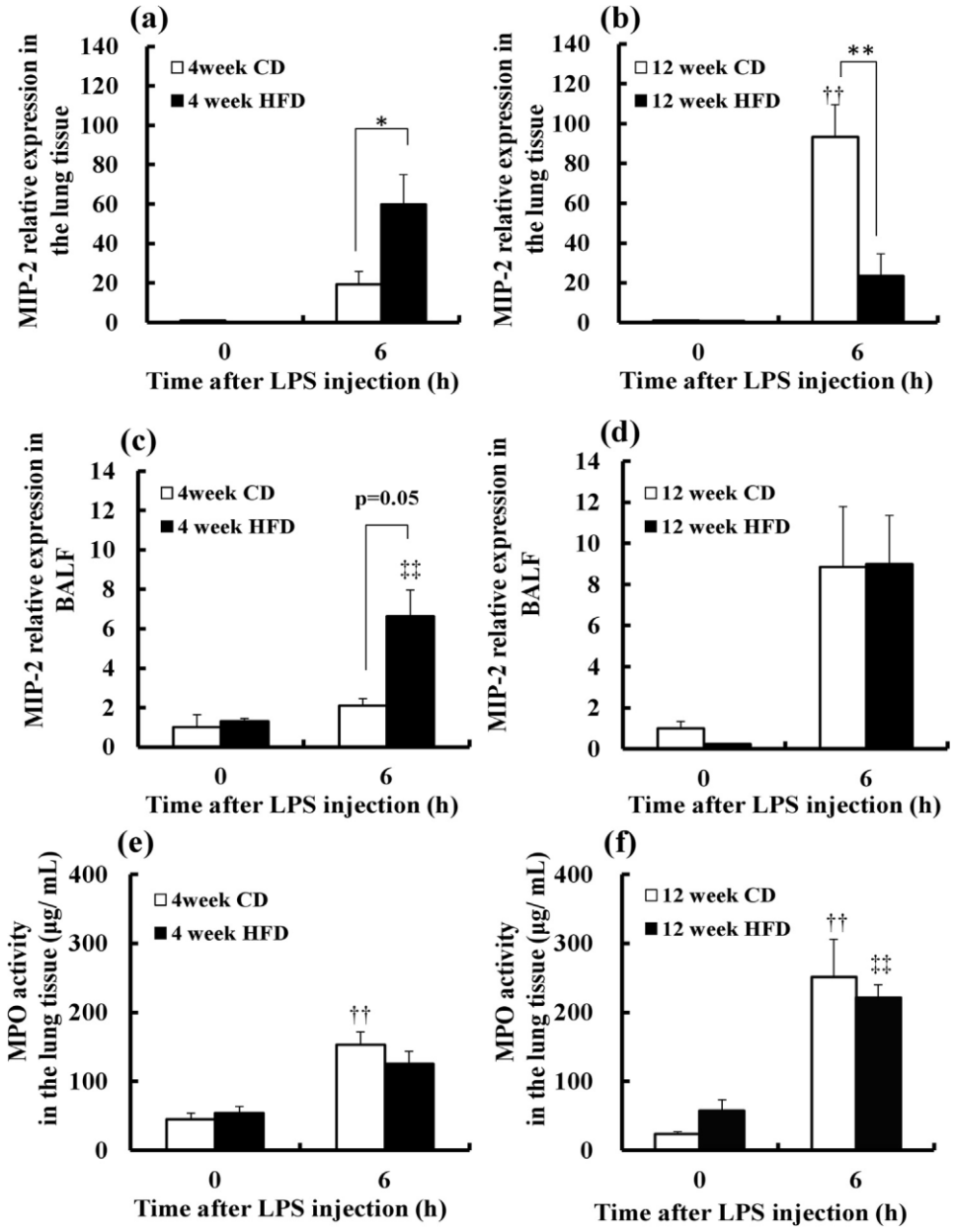

Fig. 10. Effect of HFD on MIP-2 mRNA expression and MPO activity.

At 0 and 6 h after LPS injection, lung tissue and BALF were harvested from rats fed CD or HFD for 4 or 12 weeks. The expression of MIP-2 mRNA in lung tissue from rats fed for (a) 4 weeks or (b) 12 weeks, and in BALF from rats fed for (c) 4 weeks or (d) 12 weeks, was measured using real-time PCR and is expressed as a ratio based on the amount of mRNA in CD-fed rats at 0 h. MPO activity in lung tissue from rats fed for (e) 4 weeks or (f) 12 weeks was measured using an MPO assay ELISA kit. Values represent the mean ± SEM of 4-8 animals per group at each time point. Open squares indicate CD-fed rats, and closed squares indicate HFD-fed rats.

$^{\dagger\dagger}p < 0.01$ *vs.* CD 0 h, $^{\ddagger\ddagger}p < 0.01$ *vs.* HFD 0 h, $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* CD at the same time point.

MIP-2, macrophage inflammatory protein; MPO, myeloperoxidase; BALF, bronchoalveolar lavage fluid; LPS, lipopolysaccharide.

The pulmonary pathological changes including inflammatory cell infiltration and alveolar septal thickening 6 h after i.p. LPS are in agreement with reports using LPS-induced ALI models with intraperitoneal (i.p.), intratracheal and aerosolized administration [14,18]. Jesmin et al. demonstrated that increases of pulmonary TNF-α and iNOS with neutrophil infiltration and alveolar septal thickening were occurring 1 h after i.p. LPS in rats [18], suggesting that the onset of ALI pathogenesis could be detected very early in this model.

The increased SLPI mRNA expression in the lung tissue and BALF in response to lard-based HFD suggests the prevention of further pro-inflammatory cytokine and chemokine production in LPS-induced ALI. Green et al. and Henriksen et al. reported that SLPI could inhibit LPS-induced TNF-α transcription in U937 cells [19] and reduce endothelial chemokine release through NF-κB inhibition [20]. An ALI model produced by intratracheal instillation of IgG immune complex demonstrated that blockade of endogenous SLPI with a neutralizing antibody enhanced pulmonary neutrophil accumulation and increased lung vascular permeability [21]. Taken together, our findings suggest that increased pulmonary SLPI caused by lard could attenuate the development of ALI.

Our observations at the baseline in rats fed HFD for either 4 or 12 weeks showed no change in pulmonary TNF-α or MIP-2 expression in the absence of LPS stimulation, which is in agreement with other lard-based HFD studies [12,13]. These results showed that lard itself had little influence on lung inflammation regardless of the feeding period. The favorable effect of lard-based HFD in LPS-induced ALI in our study is in agreement with several reports. In mouse models of inhaled LPS, lard-based HFD (60 kcal% fat) showed no overt effect on TNF-α or MIP-2 expression in BALF after 9 [13] or 20 weeks feeding [22]. Kordonowy et al. reported a negative correlation between airspace neutrophilia and body weight and suggested 20 weeks

lard-based HFD feeding led to attenuated LPS-induced ALI by altered neutrophil function in mice [22]. Therefore, our results and others' [22] show that the effect of lard is consistent in LPS-induced ALI regardless of the feeding period or the administration method of LPS.

Our observation of worse pulmonary changes with increased alveolar septal thickness, oxidative stress and MPO activity after 12 weeks feeding compared with 4 weeks feeding is in agreement with a study of endotoxemic mice by Bodas et al., which indicated increased lung injury with aging [23]. The observed reduction of survival rate, which is contrary to the suppression of ALI, in rats fed HFD for 12 weeks is due to severe hepatic injury following 80% of LPS i.p. accumulation in the liver [17], reported in our series of experiments using lard-based HFD-fed rats [15,16]. Development of ALI is a secondary outcome due to the overproduction of pro-inflammatory mediators with LPS stimuli [24]. This series of experiments indicated vast changes in both pro-inflammatory and anti-inflammatory lipid mediators in the liver assessed using lipidomics analysis [16]; similar analyses in relation to ALI should be performed to further explain the mechanism by which HFD attenuates the inflammatory response in the lungs.

Our study focused on changes in the lungs, but SLPI is expressed in various tissues such as the salivary gland and genital organs [5]. Evaluation of SLPI in response to lard in these organs may have possibilities. Furthermore, the influence of lard on other pulmonary diseases related to SLPI such as lung cancer and pneumonia may be an interesting focus of future research.

Regarding the mechanism of the “obesity paradox”, the effect of various drugs administered to obese patients has been suggested in clinical studies [25]. In contrast, our results showing the attenuation of ALI in rats fed lard-based HFD suggest that diet itself including lard may be important for controlling the pathophysiology associated with acute

injury. The combination of this study and our previous series of experiment with aggravation of LPS-induced liver injury in rats fed HFD for 12 weeks [16] shows that the effect of lard-based HFD varies according to organ. Our observations are also consistent with some reports without surgical stress indicating that lard-based HFD induced hepatic inflammation [11], but had little influence in lung tissue and BALF [12,13].

In our series of study, even 4 weeks lard-based HFD showed the reduction of hepatic mitochondrial DNA following oxidative stress without excessive weight gain [15]. As for body weight gain, our rats showed an 11% weight gain after 12 weeks HFD feeding [16] which was lower than that of 40% defined as severe obesity in animal models [10]. Taken together, lard itself without severe obese condition was associated with not only the increase in liver injury but also the reduction of lung injury. As for the role of fatty acids constituent in the diet, saturated fatty acids activate inflammatory response via signaling through toll like receptor 4 [26], while oleic acid rich in olive oil reduces the risk of co-morbidities associated with obesity [27]. The lard-based HFD used in this experiment containing 10-fold higher amount of palmitic acid (C16:0), stearic acid (C18:0) and oleic acid (C18:1) than CD indicates that this composition of lard we used is quite different from olive oil having anti-inflammatory effects [27].

The alveolar septal thickness, 3- to 5-μm in 4 and 12 weeks, in the CD-fed rats without LPS was notably lower than that, 10 μm, of the control rats in Norozian et al.'s study [28]. The higher amount of vitamins E and D_3 for preventing oxidative rancidity of fat in both the CD and HFD than that of typical normal chow may contribute to thinner alveolar septal thickness at baseline in this rats [29]. Therefore, our results using lard-based HFD rat models suggested the possibility that the intake of lard leads to the “obesity paradox” in ALI. Further study is required to examine the specific effect of dietary fat on each organ from

the pathophysiologic point of view with chronic and/or acute inflammation.

In summary, feeding rats lard-based HFD for 12 weeks led to a significant reduction of pro-inflammatory cytokines, chemokines and oxidative stress after LPS injection, but this effect was not seen after 4 weeks of feeding the same diet. Furthermore, the elevation of pulmonary SLPI might be associated with the reduction of lung injury in these rats.

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