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Time-restricted feeding prevents high-fat and high-cholesterol diet-induced obesity but fails to ameliorate atherosclerosis in apolipoprotein E-knockout mice

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Abstract: One of the leading risk factors for atherosclerosis is obesity, which is commonly caused by a nutrient-rich Western-style diet, sedentary behaviors, and shift work. Time-restricted (TR) feeding and intermittent fasting are both known to prevent overweight and adiposity, improve glucose tolerance, and decrease plasma cholesterol in high-fat diet-induced obese mice. Here we examined the overall effects of TR feeding of a Western diet (fat, 40.5 Kcal%; cholesterol, 0.21 g%) using 8-week-old *Apoe*$^{-/-}$ mice. Mice were assigned into three groups: (1) an *ad libitum* (AL) group fed an AL Western diet, (2) a TR group with restricted access to a Western diet (15 h/day, 12:00 to 3:00 Zeitgeber time [ZT]); and (3) an Ex/TR group fed a TR Western diet and subjected to physical exercise at 12:00 ZT. Mice in the AL group gained body weight rapidly during the 14-week observation period. With TR feeding, excessive weight gain, liver adiposity, visceral fat, and brown adipose tissue volume were effectively suppressed. Although TR feeding failed to decrease Oil Red O-stained aortic plaques in *Apoe*$^{-/-}$ mice, physical exercise significantly decreased them. Neither TR feeding with exercise nor that without exercise decreased the mean area under the curve of the plasma cholesterol level or the fasting plasma glucose. Collectively, TR feeding of a Western diet prevented the development of obesity but failed to ameliorate atherosclerosis in *Apoe*$^{-/-}$ mice.

Key words: apolipoprotein E-knockout mice, atherosclerosis, obesity, time-restricted feeding, Western-style diet

Introduction

Obesity is associated with many comorbidities, including an elevated risk for diabetes and atherosclerosis [1–6]. Among the causal factors for obesity are a nutrient-rich Western-style diet, sedentary behaviors, and shift work [7–12]. Obesity has a physiological influence on organs such as the liver, as well as visceral fat, and/or the circulatory system, making the atherosclerotic disease modeling and extremely complex endeavor. Apolipoprotein E-knockout (*Apoe*$^{-/-}$) mice represent a classical experimental model by which to mimic elevated plasma cholesterol and development of aortic atherosclerotic plaques [13–15]. The effects of pharmacological or nutritional interventions have been studied using this model.

Time-restricted (TR) feeding is a novel dietary regimen; it entails restricting access to food during a resting

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Supplementary Figures and Table: refer to J-STAGE: https://www.jstage.jst.go.jp/browse/expanim period without altering diet quality or quantity during an active period [16–19]. Compared with conventional *ad libitum* (AL) feeding, TR feeding limits weight gain and adiposity, improves glucose tolerance, and decreases plasma cholesterol in a mouse model of high-fat diet-induced obesity [20–22]. However, the dietary effects of TR feeding in *Apoe*$^{-/-}$ mice have not been elucidated. In this study, we examined whether TR feeding of a Western diet (high in fat and cholesterol) prevents atherosclerosis in *Apoe*$^{-/-}$ mice.

Materials and Methods

Animals

The care and use of all mice were in accordance with the guidelines for the proper conduct of animal experiments (Science Council of Japan). All animal experiments were approved by the Animal Care and Use Committee at Dokkyo Medical University. Six-week-old male C57BL/6J (*Mus musculus musculus*) apolipoprotein E-knockout (*Apoe*$^{-/-}$) mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). They were housed at a constant temperature (23 ± 2°C) and acclimated to a 12:12-h light-dark cycle with AL access to a normal chow diet (CLEA Japan, Inc., Tokyo, Japan) until they were eight weeks old and used for this study. Mice were randomly assigned to each experimental group, and the individual differences in plasma cholesterol and fasting glucose are shown in Supplementary Table 1.

Reproducibility of the TR feeding regimen in a pilot experiment

First, the effectiveness of a TR feeding regimen was confirmed in C57BL/6J *Apoe*$^{-/-}$ mice. We followed the original protocol established by Panda and colleagues [20, 21]; we adopted a TR feeding regimen with 15 h of AL access to a high-fat diet (HFD) food source (Supplementary Fig. 1A). As described in the results section, we confirmed the overall reproducibility of the TR regimen. Therefore, we adopted an experimental protocol to enable us to examine the effects of TR feeding in atherosclerosis-prone *Apoe*$^{-/-}$ mice.

Western diet regimen and activity schedule

Since Panda and colleagues reported that TR feeding is an effective preventative and therapeutic intervention against various nutritional challenges, such as a high-fructose diet [21], for this study, we selected a Western diet (D12079B, Research Diets Inc., New Brunswick, NJ, USA) that contained high levels of fat and cholesterol and is preferred for atherosclerosis research [23–25]. The energy compositions of this and the other diets used in the study are shown in Table 1. Eight-weeks-old *Apoe*$^{-/-}$ mice were divided into three groups according to dietary regimen (Fig. 1A): (1) an AL group (n=8) with AL access to a Western diet; (2) a TR group (n=8) with TR access to a Western diet for 15 h (12:00 to 3:00 Zeitgeber time [ZT], with ZT 0:00 set as the beginning of the light period); and (3) an Ex/TR group (n=11) fed a TR Western diet and subjected to 20 min of physical exercise at 12:00 ZT, serving as a positive control for the rescue of the phenotype [26–28]. All mice drank water *ad libitum* and the specified feeding regimens were followed for 14 weeks. Food consumption was measured every day by subtracting the weight of residual chow from that of the served chow. Body weight gain and total food consumption were monitored every week during the dietary interventions (Figs. 1B and C).

Forced swimming

Mice assigned to the Ex/TR group were forced to swim for 20 min 5 days per week before their feeding period (from 12:00 ZT, Fig. 1A). Five mice swam simultaneously in a tank containing a 10-cm circulated stream

Table 1. The composition of the diets

Diet type	Normal diet		High-fat diet		Western diet	
Company ID	Clea CE-2		Oriental Yeast HFD-60		Research Diet D12079B	
	g%	Kcal%	g%	Kcal%	g%	Kcal%
Protein	25	25.3	23	18.2	20	17
Carbohydrate	50	62.8	25.3	19.6	50	42.5
Fat	4.8	11.9	35	62.2	21	40.5
Cholesterol	-	-	-	-	0.21	-
Total	-	100	-	100	-	100
Kcal/g	3.499	-	5.062	-	4.686	-

The western diet was purchased from Research Diets Inc. (New Brunswick, NJ, USA), HFD-60 was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan), and the normal diet was purchased from CLEA Japan, Inc. (Tokyo, Japan).

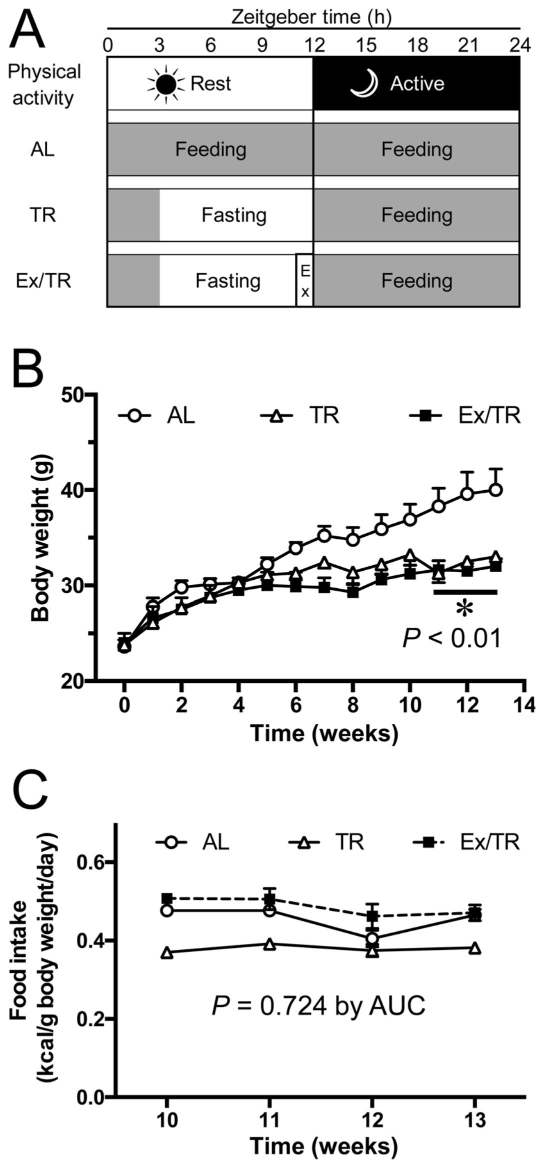

Fig. 1. A TR Western diet prevented weight gain in *Apoe*$^{-/-}$ mice. A. Western diet feeding (nutritional composition shown in Table 1) and activity schedules. Zeitgeber time 0:00 was set as the beginning of the light period. B. Body weight of *Apoe*$^{-/-}$ mice over time. Mice in the AL group gained body weight rapidly when fed a Western diet, whereas weight gain was suppressed in the TR and the Ex/TR groups. C. Daily food intake adjusted by body weight. Data are mean group values, and error bars represent SE. AL, *ad libitum* feeding; TR, time-restricted feeding; Ex/TR, physical exercise plus TR feeding; Ex, 20-min forced swimming imposed after fasting; AUC, area under the curve. *Mean values were statistically different.

of tepid water at a thermo-neutral temperature for mice (30°C). After each swimming session, the mice were towel dried and allowed access to food.

Blood collection and serum analysis

Every 4 weeks, blood samples were collected from mouse tail veins for plasma cholesterol and glucose assessments after a 5-h fast (at 07:00 ZT). Total plasma cholesterol was measured with a LabAssay Cholesterol kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's instructions. An oral glucose tolerance test was performed during week 14. Briefly, after a 5-h fast, mice were orally gavaged with a 2 g/kg glucose solution. Blood samples were collected before dosing and at 30, 60, and 120 min after dosing. Plasma glucose was determined using a LabAssay Glucose kit (Wako). Colorimetric signals were detected and quantified using a plate reader (Infinite F200 Pro, Tecan, Zurich, Switzerland).

Quantification of atherosclerotic plaques

After the 14-week interventions, mice were euthanized, and blood and tissue samples were harvested. Whole aortas, including the aortic sinus, were perfused with PBS containing 4% paraformaldehyde, dissected, and post-fixed in 4% paraformaldehyde for 16 h at 4°C for the analysis of atherosclerotic plaques. Fixed aortic sinuses were embedded in an optimal cutting temperature compound (Tissue-Tek, Sakura Finetechnical Co., Tokyo, Japan) and frozen at −20°C. Subsequently, 10-μm-thick cross sections at 50-μm intervals were prepared. Plaques were assessed using three serial aortic sinus sections, which included 150-μm lengths of the aortic valve, that were stained with Oil Red O. Digital images of the plaque in sections were acquired (BX53, Olympus, Tokyo, Japan) and quantified using ImageJ 1.48 (NIH, Bethesda, MD, USA). The plaque areas of three sections were averaged. The whole aortic lumen was exposed under a dissection microscope (YS02Z2, Micronet Inc., Saitama, Japan), and any plaque was stained with Oil Red O. Digital images of whole-mount aortic plaque were captured using a BZ-X700 All-in-One Fluorescence Microscope (Keyence, Osaka, Japan). Plaque areas were analyzed using Photoshop (Adobe KK, Tokyo, Japan) and expressed as a percentage of the whole aortic area.

Histological analysis

After euthanizing the mice, their liver, visceral white adipose tissues (vWAT) in their retroperitoneal cavity, and interscapular subcutaneous brown adipose tissues (BAT) were harvested and immediately fixed in 10% formalin for histological analysis. Paraffin-embedded tissues were cut into 5-μm-thick sections, deparaffinized, and stained with hematoxylin and eosin. Cell images of vWAT were acquired using a 530–550 nm band-pass filter for rhodamine to enhance cell membrane visualization.

Statistical analyses

For multiple comparisons of the mean values, Fisher's least significant difference procedure was adopted. Non-Gaussian data (e.g., those in Fig. 3B) required a non-parametric test. Following and analysis of variance using the Kruskal-Wallis test, Mann-Whitney U tests were performed for paired comparisons by groups. Correlations between two variables were assessed using Pearson's correlation coefficient (r). IBM SPSS Statistics version 26 (IBM Corp., Armonk, NY, USA) was used for statistical calculations. Results for numerical data are presented as the mean ± SD. A *P*-value ≤0.05 was considered as statistically significant.

Results

Reproducibility of the TR feeding regimen in the pilot experiment

We first confirmed the effectiveness of a TR feeding regimen in C57BL/6J *Apoe*$^{-/-}$ mice, following the original protocol established by Panda and colleagues [20, 21]. While AL access to HFD (H/AL) rapidly increased body weight during the observation period, TR feeding of HFD (H/TR) prevented excessive weight gain, almost to the similar level of normal chow-fed (N/AL) mice (*P*<0.01 after week 8, Supplementary Fig. 1B). Of note, the overall food intake was comparable between H/AL and H/TR (Supplementary Fig. 1C), suggesting that the weight loss was not a consequence of caloric restriction. The discrepancy could be explained by an altered metabolism and circadian rhythm [20, 21], prompting us to investigate circadian gene expression in liver and brown adipose tissue. Wet tissue weights of the liver, vWAT, and BAT were remarkably increased by the HFD, whereas TR feeding significantly decreased them (Supplementary Fig. 2). Panda and colleagues reported that an HFD causes dysregulation of circadian gene expression for fat metabolism and thermogenesis and that the TR feeding regimen successfully normalizes them [20, 21]. We confirmed that the gene expression for fat metabolism and thermogenesis inversely fluctuated between the active and the resting periods (Supplementary Fig. 3), suggesting that the weight loss was a consequence of altered metabolism and thermogenesis. Therefore, we adopted the protocol to examine the effects of TR feeding in atherosclerosis-prone *Apoe*$^{-/-}$ mice.

TR feeding prevented Western diet-induced weight gain in *Apoe*$^{-/-}$ mice

Ad libitum feeding of a Western diet increased body weight during the 14-week observation period in the AL group (Fig. 1B). In contrast, body weight gain was suppressed in the TR and Ex/TR groups (*P*<0.01 after week 11, Fig. 1B). The mean area under the curve (AUC) values for food intake were as follows: 1.338 ± 0.123, 1.148 ± 0.113, and 1.447 ± 0.239 for the AL, TR, and Ex/TR groups, respectively (*P*=0.724, Fig. 1C). The Ex/TR group consumed slightly more food compared with the AL group (Fig. 1C), but the difference was not statistically significant. Thus, the TR dietary regimen prevented Western diet-induced weight gain in *Apoe*$^{-/-}$ mice.

TR feeding failed to ameliorate aortic atherosclerotic plaques in *Apoe*$^{-/-}$ mice

Plaques in the aortic sinus (Fig. 2A) and the whole aorta were visualized (Fig. 2B) and quantified (Figs. 2C and D). The mean areas of Oil Red O staining (mm^2) in the aortic sinus were as follows: 0.50 ± 0.11, 0.57 ± 0.08, and 0.39 ± 0.13 for the AL, TR, and Ex/TR groups, respectively (Fig. 2C). Plaque areas were significantly different between the TR and Ex/TR groups (*P*=0.012, Fig. 2C). The total areas (%) of Oil Red O staining in the whole aortic lumen were as follows: 8.05 ± 3.20, 10.98 ± 2.43, and 5.88 ± 2.11 for the AL, TR, and Ex/TR groups, respectively (Fig. 2D). A statistically significant difference in the total area of staining was only observed between the TR and Ex/TR groups (*P*=0.002, Fig. 2D).

TR feeding failed to decrease the plasma cholesterol level in *Apoe*$^{-/-}$ mice

The mean values for plasma cholesterol (mg/dl) in the AL, TR, and Ex/TR groups, respectively, at each time point were as follows: at week 0 (before nutritional challenges), 403.50 ± 64.84, 349.93 ± 90.99, and 359.99 ± 49.16 (*P*=0.411, Supplementary Table 1); at week 4, 548.55 ± 361.03, 797.08 ± 312.29, and 783.03 ± 276.08 (*P*=0.336); at week 8, 475.62 ± 254.02, 663.88 ± 364.88, and 808.24 ± 399.87 (*P*=0.377); at week 12, 925.32 ± 351.63, 881.68 ± 347.39, and 522.93 ± 186.19 (*P*=0.06); and at week 14, 956.64 ± 448.28, 936.55 ± 274.04, and 603.69 ± 212.48 (*P*=0.091, Fig. 3A). Although cholesterol tended to be lower in the Ex/TR group at week 12, the mean AUC for plasma cholesterol was not significantly different among groups (*P*=0.551). When plotted, there was no significant correlation between the AUC values for plasma cholesterol and the areas of Oil Red O staining in the aortic sinus (Pearson's r=0.241, *P*=0.267, Fig. 3B).

Neither TR feeding nor physical exercise mitigated abnormal glucose metabolism in *Apoe*$^{-/-}$ mice

The mean values for fasting plasma glucose (mg/dl)

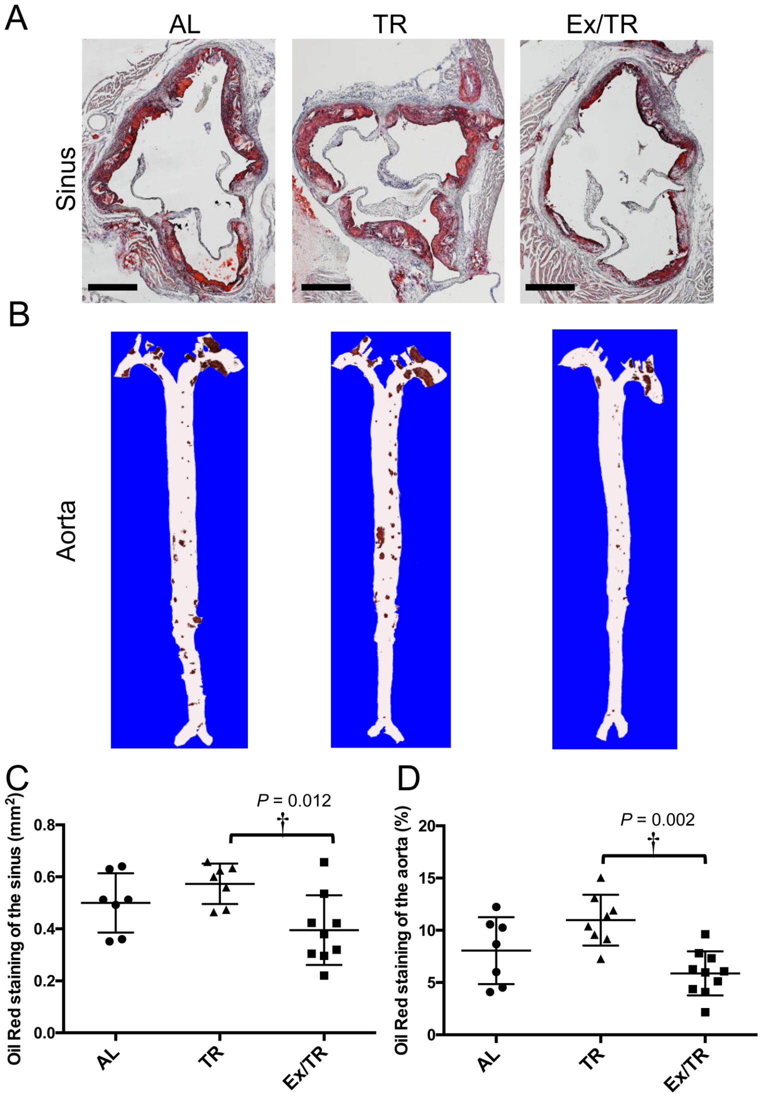

Fig. 2. A TR Western diet failed to ameliorate atherosclerotic plaques in *Apoe*$^{-/-}$ mice. A. Histology of Oil Red O-stained coronal sections of aortic sinuses at the level of the aortic valve. Atherosclerotic plaques were visualized by red color. Scale bars: 300 μm. B. Macroscopic images of Oil Red O-stained whole aortic lumens. Plaques tended to accumulate in the aortic arch. C. Areas of atherosclerotic plaquing in the sinus showed that the difference between the TR and Ex/TR groups was statistically significant (as shown), although there was no significant difference between the AL and TR groups or between the Ex/TR and AL groups (P=0.325 and P=0.146, respectively). D. Total area (%) of plaquing in the aorta revealed that there was a statistically significant difference between the TR and Ex/TR groups (as shown) and that there was no significant difference between the AL and TR groups or between the Ex/TR and AL groups (P=0.104 and P=0.188, respectively). Scatter plots show individual data. Bars represent mean values ± SD. †Mean values were statistically different. AL, *ad libitum* feeding; TR, time-restricted feeding; Ex/TR, physical exercise plus TR feeding.

in the AL, TR, and Ex/TR groups, respectively, at each time point were as follows: at week 0 (before nutritional challenges), 125.20 ± 34.19, 129.43 ± 35.00, and 115.07 ± 31.16 (P=0.53, Supplementary Table 1); at week 4, 171.46 ± 29.44, 183.19 ± 45.63, and 212.12 ± 60.21 (P=0.443); at week 8, 165.21 ± 27.70, 181.50 ± 32.91, and 214.85 ± 48.41 (P=0.105); at week 12, 241.16 ± 51.90, 181.99 ± 36.92, and 201.91 ± 68.29 (P=0.062); and at week 14, 206.76 ± 49.26, 155.98 ± 36.15, and 149.66 ± 54.61 (P=0.077, Fig. 4A). Although fasting plasma glucose tended to be lower in the TR and Ex/TR groups at week 14, the mean AUC for fasting plasma glucose was not significantly different among groups (P=0.585). Based on oral glucose tolerance testing, the

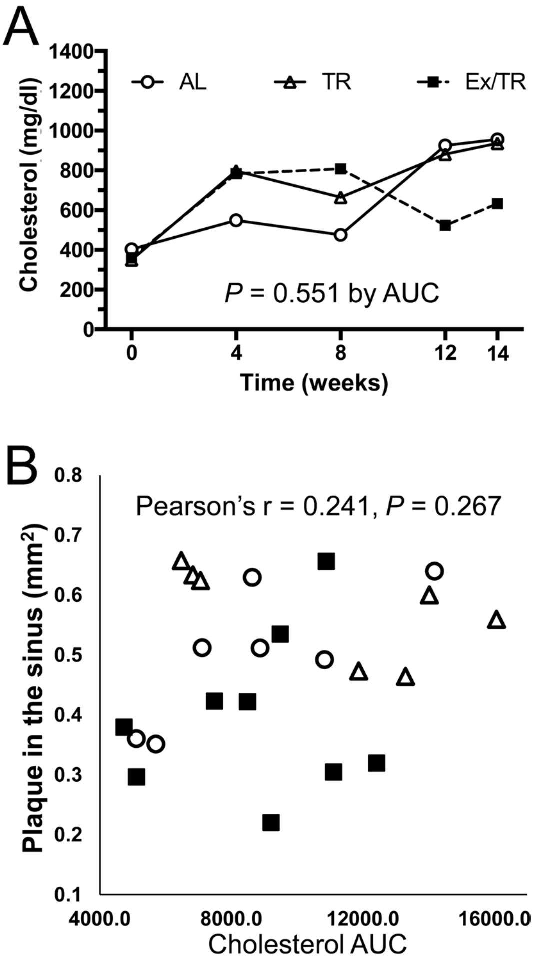

Fig. 3. TR feeding failed to decrease plasma cholesterol in *Apoe*$^{-/-}$ mice. A. Plasma cholesterol from weeks 0 to 14. Data are mean group values. B. The correlation between plaque area and AUC for plasma cholesterol was not statistically significant. Each dot indicates the intersection of the plaque area and AUC for plasma cholesterol for a single *Apoe*$^{-/-}$ mouse. AL, *ad libitum* feeding; TR, time-restricted feeding; Ex/TR, physical exercise plus TR feeding; AUC, area under the curve.

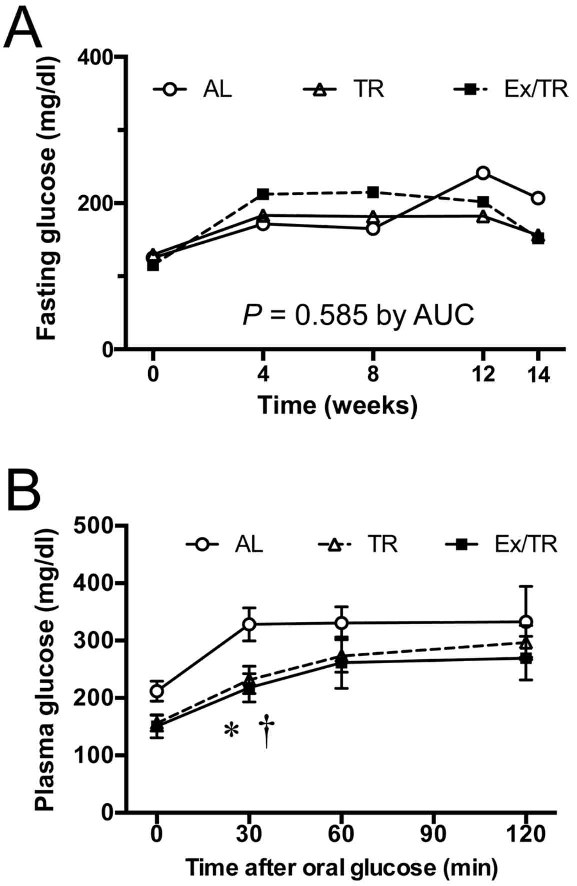

Fig. 4. Neither TR feeding nor physical exercise mitigated abnormal glucose metabolism in *Apoe*$^{-/-}$ mice. A. Fasting plasma glucose from weeks 0 to 14. Data are mean group values. B. An oral glucose tolerance test was performed during week 14. Mean (± SE) plasma glucose by groups over time after oral glucose ingestion. In all groups, glucose concentrations did not drop within 2 h after glucose ingestion, thereby confirming abnormality of glucose metabolism. *AL vs TR: *P*=0.041. †Ex/TR vs. AL: *P*=0.008. AL, *ad libitum* feeding; TR, time-restricted feeding; Ex/TR, physical exercise plus TR feeding; AUC, area under the curve.

plasma glucose levels (mg/dl) in the AL, TR, and Ex/TR groups, respectively, at each time point were as follows: fasting state, 211.67 ± 49.96, 155.97 ± 38.64, and 150.84 ± 67.27 (*P*=0.065); 30 min after glucose ingestion, 328.32 ± 81.37, 231.27 ± 68.00, and 217.74 ± 81.05 (AL vs TR, *P*=0.041 and Ex/TR vs AL, *P*=0.008); 60 min after glucose ingestion, 330.54 ± 79.84, 273.11 ± 80.65, and 269.07 ± 125.70 (*P*=0.318); and 120 min after glucose ingestion, 332.71 ± 163.60, 296.32 ± 83.86, and 269.52 ± 150.13 (*P*=0.483; Fig. 4B). Glucose did not drop in any groups at 60 or 120 min after glucose ingestion (Fig. 4B).

TR feeding prevented Western diet-induced adiposity in Apoe$^{-/-}$ mice

The results for fat accumulation are shown in Fig. 5. In the AL group, and abundance of lipid droplets was observed in the cytoplasm of hepatocytes, consistent with the typical pathology for a fatty liver (Fig. 5A). In contrast, substantially fewer lipid droplets were observed in the liver in the TR and the Ex/TR groups. Hypertrophy of adipocytes in vWAT and BAT was evident in the AL group compared with the smaller size of adipocytes in the TR and Ex/TR groups. BAT adipocytes in the AL groups resembled hypertrophied white adipocytes. The TR and Ex/TR interventions successfully rescued the mesh-like intracellular structures of BAT adipocytes. The body weights (g) at the end of the observation period were 42.90 ± 6.40, 31.86 ± 2.79, and 32.09 ± 2.18 for the AL, TR, and Ex/TR groups, respectively (Fig. 5B). Other measurements for the AL, TR, and Ex/TR groups were as follows: liver mass (‰ of body weight),

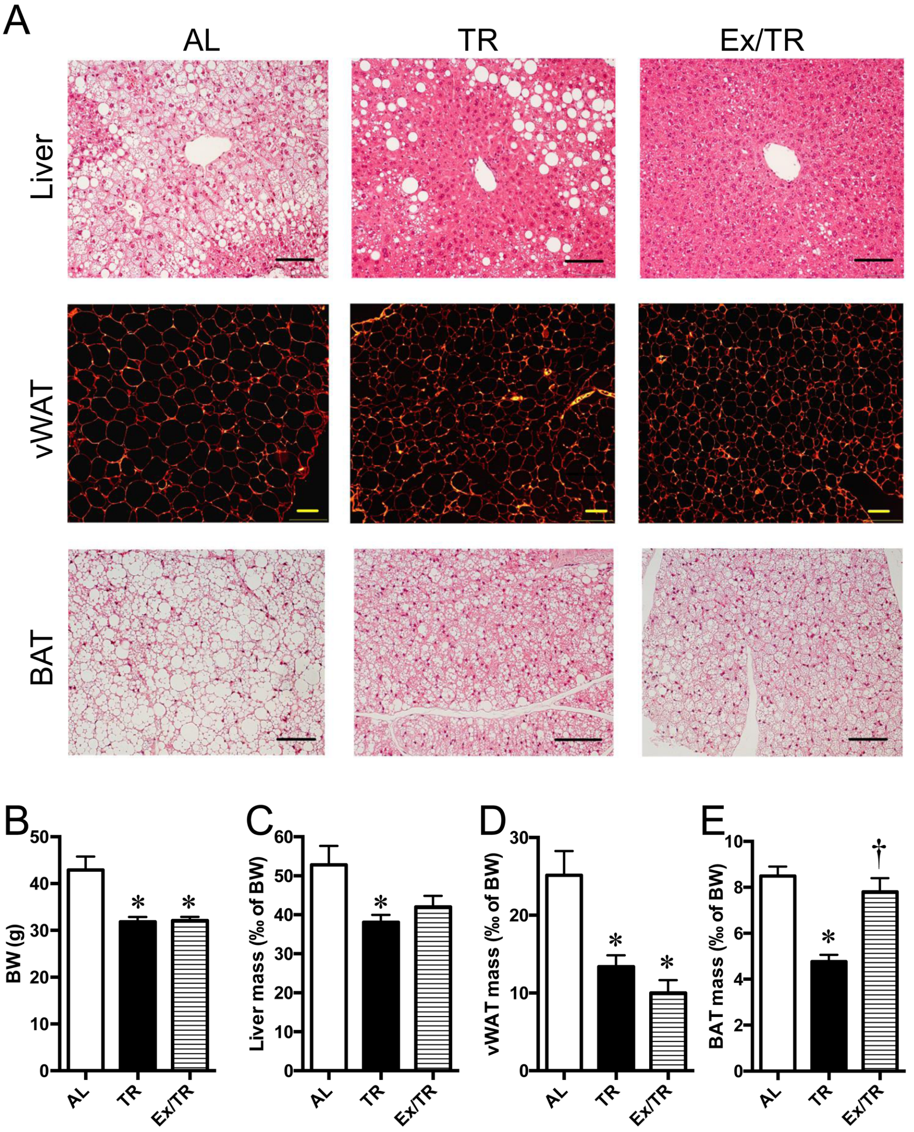

Fig. 5. TR feeding prevented Western diet-induced adiposity in *Apoe*$^{-/-}$ mice. A. Hematoxylin eosin-stained sections of liver, visceral white adipose tissue (vWAT), and brown adipose tissue (BAT). AL: *Ad libitum* feeding of a Western diet (left column). In the liver (upper row), the TR and Ex/TR interventions (middle and right columns, respectively) resulted in remarkably fewer ectopic lipid droplets surrounding a portal vein and mitigated a fatty liver compared with the AL group. In the vWAT (middle row), the adipocytes were smaller in mice in the TR and Ex/TR groups compared with the AL group. In the BAT (lower row), the AL intervention transformed the morphology of brown adipocytes so that they appeared to resemble hypertrophied white adipocytes. The TR and Ex/TR interventions restored typical mesh-like structures (lower middle and right). Scale bars: 200 μm. B. Body weight at week 14. C. Liver mass normalized by each body weight. D. vWAT mass normalized by each body weight. E. BAT mass normalized by each body weight. Bar graphs show mean values + SE. *Statistically different from AL group. †Statistically different from TR group. AL, *ad libitum* feeding; TR, time-restricted feeding; Ex/TR, physical exercise plus TR feeding; BW, body weight.

52.80 ± 10.92, 38.09 ± 5.30, and 42.01 ± 7.47 (Fig. 5C); vWAT mass (‰ of body weight), 25.18 ± 6.95, 13.39 ± 4.14, and 10.03 ± 4.02 (Fig. 5D); and BAT mass (‰ of body weight), 8.50 ± 0.91, 4.77 ± 0.83, and 7.81 ± 1.46 (Fig. 5E). Ex/TR did not decrease the overall tissue weight of BAT.

Discussion

Here we demonstrated that TR feeding of a Western diet, compared with AL feeding, prevented weight gain and limited adiposity but failed to ameliorate atherosclerosis and glucose intolerance in *Apoe*$^{-/-}$ mice.

In humans, obesity is associated with many comorbidities, including an elevated risk for diabetes and atherosclerosis [1–6]. After we reproduced the remarkable effects of TR feeding against obesity (Supplementary Figs.1–3), we expected that TR feeding could also influence the atherosclerosis phenotype in *Apoe*$^{-/-}$ mice. The apparent discrepancy between the anti-obesity effects and the failure of atherosclerosis prevention by TR feeding could be due to a species-related difference in lipid metabolism [29]. Lipoprotein metabolism in rodents is evolutionally distant from that in humans and some researchers prefer to use another species, such as the rabbit [30–32]. The *Apoe*$^{-/-}$ mouse is one of the most frequently used hypercholesterolemia models [33, 34], but the elevation of very-low-density lipoprotein (VLDL) does not precisely mimic the human pathology [35]. Panda and colleagues originally reported that TR feeding decreases plasma cholesterol in a genetically normal background [20, 21]. The lack of reproducibility could be explained by skewed balance between high-density lipoprotein and VLDL in *Apoe*$^{-/-}$ mice [35]. Thus, one has to be careful when interpreting the physiological significance of the apparently negative data.

The limitation of our study is that the experiment was performed according to the least stringent TR feeding regimen reported previously by Panda and colleagues [21] (Fig. 1A). Chaix *et al.* thoroughly compared the most stringent TR diet regimen (9-h access to food during the active period) with those of the least stringent TR regimen (15-h access to food) [21]. We adopted the least stringent regimen because the comparisons by Chaix *et al.* showed that all the TR protocols were sufficient to improve glucose homeostasis and decrease plasma cholesterol [21]. We initially performed our own pilot study and confirmed that the least stringent TR regimen prevented obesity and the alteration of circadian rhythms in terms of gene expression (Supplementary Figs. 1–3). We subsequently replaced the high-fat diet with a Western diet because Chaix *et al.* had already demonstrated that the beneficial effects of a TR feeding were applicable to other nutritional challenges, such as high-fructose diet [21]. To our disappointment, the least stringent TR feeding regimen for a Western diet failed to decrease plasma cholesterol (Fig. 3) and was unsuccessful in ameliorating atherosclerotic plaques in *Apoe*$^{-/-}$ mice (Fig. 2). However, our results did indicate that the least stringent TR feeding regimen is sufficient to prevent overweight and adiposity in *Apoe*$^{-/-}$ mice (Figs. 1 and 5). When a TR regimen is applied in a clinical setting, clinicians may want to consider the adoption of the most stringent protocol to obtain optimal beneficial effects. Results of recent clinical studies based on the most stringent TR protocol suggest that the TR dietary regimen appears promising among patients with prediabetes [19].

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