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Research Article

Intermittent Fasting Sustainably Improves Glucose Tolerance in Normal Weight Male Mice Through Histone Hyperacetylation

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Abstract

To explore the mechanism by which intermittent fasting (IF) exerts prolonged effects after discontinuation, we examined mice that had been subjected to 4 cycles of fasting for 72 hours and ad libitum feeding for 96 hours per week (72hIF), followed by 4 weeks of ad libitum feeding, focusing on expression of genes for lipid metabolism in the skeletal muscle and histone acetylation in the promoter region. The 72hIF regimen resulted in metabolic remodeling, characterized by enhanced lipid utilization and mitochondrial activation in the muscle. This long-term IF (72hIF) caused stronger metabolic effects than alternate day fasting (24hIF) wherein fasting and refeeding are repeated every 24 hours. Upregulation of lipid oxidation genes and an increase in oxygen utilization were sustained even at 4 weeks after discontinuation of 72hIF, associated with histone hyperacetylation of the promoter region of uncoupling protein 3 (*Ucp3*) and carnitine palmitoyl transferase 1b (*Cpt1b*) genes. An increase in leucine owing to fasting-induced muscle degradation was suggested to lead to the histone acetylation. These findings support the previously unappreciated notion that sustainable promotion of histone acetylation in lipid oxidation genes of the muscle and adipose tissues during and after IF may contribute to sustained metabolic effects of IF.

Key Words: intermittent fasting, glucose tolerance, exercise endurance, histone acetylation, lipid oxidation, mitochondria

Abbreviations: 24hIF, 24-hour fast followed by 24-hour ad lib feeding; 72hIF, 72-hour continuous fast followed by 96-hour ad lib feeding; *Acox1*, acyl-CoA oxidase 1; *Adipoq*, adiponectin; BCAA, branched-chain amino acid; *Cpt1b*, carnitine palmitoyl transferase 1b; ChIP, chromatin immunoprecipitation; CR, calorie restriction; *Dio2*, iodothyronine deiodinase 2; FBS, fetal bovine serum; HAT, histone acetyltransferase; IF, Intermittent fasting; ipGTT, intraperitoneal glucose tolerance test; ITT, insulin tolerance test; *Lipe*, lipase; NAD, nicotinamide adenine dinucleotide; OHB, β-hydroxybutyric acid; PCR, polymerase chain reaction; *Pnpla2*, patatin like phospholipase domain containing 2; *Ppargc1a*, peroxisome proliferator–activated receptor γ coactivator 1α; *Prdm16*, PR domain containing 16.

The number of patients with obesity and diabetes has been increasing rapidly, and the development of sustainable treatment against these metabolic diseases has become an urgent issue. Diet therapy consists of fundamental treatments for lifestyle-related diseases; various approaches, such as calorie restriction (CR), carbohydrate restriction, and appropriate order of eating have been attempted in the clinical situation [1-3]. CR that involves the limiting of daily calorie intake is expected to facilitate weight reduction and glycemic control [4]. Intermittent fasting (IF) has also attracted attention as a diet therapy that can produce clinically significant body weight loss [5].

In animal studies, positive metabolic effects of IF in rodents and the associated mechanisms have been precisely examined in each organ [6]. The white adipose tissue has shown an increase in energy consumption by IF, suggesting a metabolic remodeling, termed as *browning* [7, 8]. In the skeletal muscle, a combination of IF and exercise promotes the use of fatty acids [9]. In addition, reduction in blood pressure [10], neuroprotection [11, 12], relief of inflammation [13], retardation of tumor growth [14], and lifespan extension [15] have been reported as effects of IF in rodents. As a protocol for IF in animals and humans, alternate day fasting, involving fasting for 24 hours and ad libitum feeding for 24 hours every other day, or a schedule of fasting for 16 hours and ad libitum feeding for 8 hours in a day are often used [16]. There is no restriction on food intake other than during the fasting period; therefore, IF can be considered a diet therapy that uses the effect of "fasting stress." Thus, alterations in humoral factors, hormones, and metabolites during fasting are assumed to induce the metabolic remodeling of organs that leads to favorable effects of IF [17].

In human studies, IF consistently produces significant weight loss and improved glycemic control; however, IF is thought to be inappropriate for patients with type 1 diabetes who require insulin therapy or who have type 2 diabetes with poor glycemic control, because it may increase the risk of hypoglycemia or ketosis due to fasting [18]. A randomized controlled trial revealed that IF increased hypoglycemia in

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diabetic patients [19]. Because most human studies of IF have been enrolling individuals who are overweight but not diabetic, evidence for the safety and health effects of IF among diabetic patients is limited [20]. Currently, further evidence is needed to elucidate which type of diabetic patients are suitable for what degree of IF. Particularly, studies that are long enough for obese individuals to show improved insulin resistance are needed to determine the appropriate indication of IF for patients with type 2 diabetes and obesity.

Previous studies have shown prolonged effects of dietary intervention for a certain period after discontinuation [21]. In mice, significant weight loss continued for 12 weeks after discontinuation of IF, when they were subjected to IF for 26 weeks [22]. A single 48-hour fasting in mice induced hypothalamic metabolic adaptations that persisted for a week after discontinuation of fasting [23]. In humans, obese patients who underwent IF or CR showed a prolonged effect of weight loss at least for 12 months after the end of intervention [24, 25]. However, the mechanism by which IF causes the sustainable effects remains unclear. Therefore, we examined prolonged effects of IF by using mice, focusing on gene expression in the skeletal muscle that is related to lipid metabolism. As a mechanism that may be the cause of the prolonged effects on gene expression, histone acetylation of the promoter region of genes related to lipid metabolism was analyzed [26].

In the present study, mice were subjected to 4 cycles of long-term (72 hours) continuous fasting and 96 hours ad libitum feeding period per week (72hIF), which was expected to cause a higher fasting load. They were allowed ad libitum feeding for the subsequent 4 weeks after the completion of a period of 4 weeks of IF, and the prolonged effects of IF were evaluated. The metabolic effects of conventional IF in mice, wherein fasting and refeeding are repeated every 24 hours (24hIF), were also investigated, to examine the hypothesis that the long-term fasting of 72hIF could cause potent and sustainable metabolic effects than 24hIF. Through these experiments, we determined the mechanism of IF-induced metabolic remodeling that leads to sustainable effects.

Materials and Methods

Intermittent Fasting in Mice

Male C57BL/6 mice were obtained from Charles River Laboratories (Tokyo, Japan). All the animal experiments were approved by the local ethics committee (the Laboratory Animal Care and Use Committee of Keio University, permission number #15064) and were conducted as per the domestic law on the protection of laboratory animals that is based on the Declaration of Helsinki. The mice were fed a normal diet (fat 10 kcal%, protein 20 kcal%, carbohydrate 70 kcal%) and subjected to IF from 12 weeks of age. In the 72hIF group, 1 week was set as 1 cycle, and the mice were subjected to fasting during the first 72 hours; the remaining 96 hours were set as ad libitum feeding period. By utilizing 72hIF, a strong fasting stress was expected, and this procedure was performed for 4 continuous cycles. In the 24hIF group, 24 hours fasting and 24 hours refeeding were repeated, setting 1 week as 1 cycle.

After 4 cycles of IF, a high-fat diet (fat 60 kcal%, protein 20 kcal%, carbohydrate 20 kcal%) was fed ad libitum for 4 weeks of the observation period, that is, the mice were followed for 8 weeks. At the 2 time points, just after 4 cycles of IF (16 weeks of age) and after 4 weeks of the subsequent observation period (20 weeks of age), the body weight and rectal temperature of the mice were measured at the age of 12 to 20 weeks. Glucose tolerance tests, respiratory gas analysis, muscular strength examinations, and endurance tests were performed as per standard procedures [27]. The skeletal muscle, white adipose tissue, and brown adipose tissues were collected; the gene expression associated with fat utilization and histone acetylation level of the promoter region were evaluated. We had 10 mice in each group of experiments. That is, for the 72hIF experiments, 40 mice were used of which 20 were subjected to 72hIF and 20 were controls. Ten of the 20 mice were sacrificed at the end of the 4-week fasting period, and the remaining 10 were sacrificed after an additional 4 weeks of observation. Similarly, 40 mice were used for the 24hIF experiments. Thus, there were a total of 80 mice used in the present study. Anesthesia for mice was performed by intraperitoneal administration of a mixture of 3 anesthetics (medetomidine hydrochloride 0.3 mg/kg, midazolam 4 mg/kg, and butorphanol tartrate 5 mg/kg). For sacrifice, cervical dislocation was performed after administration of the above mixture.

Analysis of the Physical Performance, Body Composition, and Metabolic Parameters of the IF Mice

Muscle strength of the forelimbs was measured using a standard dynamometer for mice (MK-380 M, Muromachi Kikai, Tokyo, Japan). Each mouse was placed on a metal mesh and pulled horizontally; the power of traction when the mouse released the mesh was defined as the muscle strength. Running distance was measured on a treadmill for mice (LE8170M, PanLab, Barcelona, Spain) as per a previously described protocol [27]. Briefly, the mice were put on a motor-driven treadmill until they were completely exhausted; exhaustion was defined as the time point at which they remained on the plate giving electrical stimulation for >30 seconds. The oxygen utilization, as an index of energy expenditure, and respiratory quotient, as an index of lipid oxidation, were measured using a respiratory gas analyzer (ARCO-2000 mass spectrometer, ARCO system, Kashiwa, Japan). Respiratory gas analysis was performed at 16 and 20 weeks of age. To perform the intraperitoneal glucose tolerance test (ipGTT), the mice were forced to fast for 16 hours overnight and then intraperitoneally injected with glucose at a dose of 1 g glucose/kg body weight. To perform the insulin tolerance test (ITT), the mice were intraperitoneally injected with insulin at a dose of 1 unit/kg body weight. Their blood glucose levels were measured before and at 15, 30, 60, and 90 minutes after the glucose injection. The ITT was tandemly performed at 48 to 72 hours after ipGTT using the same mice. Blood collection for ipGTT and ITT was performed by capillary aspiration using heparin tubes through tail vein cutting. We measured the weight of the muscle (gastrocnemius), white adipose tissue (epididymal fat), and brown adipose tissue. The electron microscopic analysis of the mitochondria of quadriceps muscle was performed using the standard method. A part of these tissues was used for quantitative polymerase chain reaction (PCR) analysis and chromatin immunoprecipitation (ChIP) assay.

Estimation of Gene Expression by Quantitative PCR Analysis and Histone Acetylation Levels Using ChIP Assays

Histone acetylation is an epigenetic modification that causes prolonged promotion of gene expression and has been

implicated in the development of obesity and diabetes. Estimation of gene expression and histone acetylation levels was performed using quantitative PCR analysis and ChIP assay, respectively, as per the standard protocols. Total RNA was extracted using RNeasy Mini Kit (Qiagen), and reverse transcription was performed with ExScript RT reagent kit (Takara Bio, Otsu, Japan). The gene expression levels were determined with quantitative PCR reactions (ABI 7500, Applied Biosystems, Foster City, CA) in the presence of TB Green Premix ExTaq (Takara Bio) fluorescent dye. The genes examined with PCR analysis were as follows: the lipid utilization-related genes, uncoupling protein 3 (*Ucp3*), iodothyronine deiodinase 2 (*Dio2*), carnitine palmitoyl transferase 1b (*Cpt1b*), acyl-CoA oxidase 1 (*Acox1*), uncoupling protein 1 (*Ucp1*), peroxisome proliferator–activated receptor γ coactivator 1α (*Ppargc1a*), lipase, hormone sensitive (*Lipe*), and patatin like phospholipase domain containing 2 (*Pnpla2*). Expression levels of a transcriptional factor involved in brown adipose tissue maturation, PR domain containing 16 (*Prdm16*) and representative adipokines, adiponectin (*Adipoq*) and leptin (*Lep*) were also examined. The relative quantity of mRNA was normalized to that of the internal control, ribosomal protein S18. The ChIP assay was performed by using the Simple ChIP Plus Kit as per the manufacturer's instructions (#9005, Cell Signaling Technology Japan, Tokyo, Japan). The DNA–protein complexes were immunoprecipitated using ChIP-grade antibodies against pan histone H3 acetylation (H3Ac, #39139, RRID: AB_2614978) and pan H4 acetylation (H4Ac, #39243, RRID: AB_2793550) (Active motif, Carlsbad, CA). The DNA levels in the immunoprecipitants were analyzed with quantitative PCR and normalized to the inputs. PCR primers were designed to amplify the promoter region of *Ucp3* and *Cpt1b*. Target sequences were within 500 base pairs upstream of the transcription start site where augmentation of histone acetylation has been reported. The sequences of PCR primers are shown in Table 1.

Table 1. The sequences of PCR primers used in the present study

Quantitative PCR analysis
Ucp3
F: CTCTGCACTGTATGCTGAAGATG
R: CACGTTCCAAGCTCCCAGA
Dio2
F: CTGGTGTGCGAATGATAACTACTGA
R: GCACCATGACCCAAATGTTCC
Cpt1b
F: ATGTGCTCCTACCAGATGGAGA
R: GAAGCGACCTTTGTGGTAGACAG
Acox1
F: GGCATTGGCATCGTGAGAAC
R: GCAAATCTGATGGCTTTGACTTGA
Ucp1
F: CACTCAGGATTGGCCTCTACGAC
R:GCTCTGGGCTTGCATTCTGAC
Ppargc1a
F: CCGTAAATCTGCGGGATGATG
R: CAGTTTCGTTCGACCTGCGTAA
Prdm16
F: GCATATCCACAGCACGGTGGA
R: GTGCTGAACATCTGCCCACA
Lipe
F: TCCTGGAACTAAGTGGACGCAAG
R: CAGACACACTCCTGCGCATAGAC
Pnpla2
F: AACACCAGCATCCAGTTCAA
R: GGTTCAGTAGGCCATTCCTC
Adipoq
F: AGACTAATGAGACCTGGCCACTTTC
R: GGCATGACTGGGCAGGATTA
Lep
F: CCAGGATCAATGACATTTCACACAC
R: GAAGCCCAGGAATGAAGTCCAA
ChIP
Ucp3 promoter region
F: TGACTCCTCTGCTGGGATCT
R: TGCCCTCATCATCTCTACCC
Cpt1b promoter region
F: CAACCTTGTGGGAAAACACTCTAC
R: GAGAAAACAGATAGGGGTGGAAAC

Abbreviations: F: forward primer, R: reverse primer.

Metabolome Analysis of the Quadriceps in the Fasting Mice

To quantify the metabolites in the quadriceps muscle after 24 or 72 hours fasting, capillary electrophoresis–mass spectrometry (aCE-MS)-based metabolome analysis was conducted in the core facility of Keio university. The quadriceps weighed approximately 150 mg wet weight and were ground; 1 mL methanol containing internal standards (200μM each of l-methionine sulfone and 2-morpholinoethanesulfonic acid) was added. After the addition of 0.5 mL of deionized water and 0.8 mL of chloroform, the mixture was centrifuged at 20 000*g* for 15 minutes at 4 °C. The upper aqueous region of the sample was filtered through a 5-kDa cutoff filter (Merck Millipore, Tokyo, Japan) via centrifugation to remove the protein components. The filtrate was lyophilized and dissolved in 200 μL of deionized water containing reference compounds (200μM each of 3-aminopyrrolidine and trimesate). The amounts of metabolites were normalized with the original tissue samples and quantified as nmol/g of tissue. We quantified 123 representative metabolites in the glycolysis pathway, tricarboxylic acid (TCA) cycle, adenine nucleotides, nicotinate derivatives, and amino acids in the quadriceps of IF-treated mice.

Experiments Using C2C12 Cultured Myocytes

C2C12 (RRID: CVCL_0188) cultured myocytes obtained from RIKEN BioResource Center (Tsukuba, Japan) were grown to a nearly confluent number in Dulbecco's Modified Eagle's Medium (DMEM #11995; Gibco, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) and differentiated into myocytes via incubation with 0.1% FBS before the experiments. The cells were treated with either normal medium (FBS 10%, glucose 1.0 g/L) or low-serum low glucose starvation medium (FBS 1%, glucose

0.5 g/L). The C2C12 cells were examined for the oxygen consumption rate, gene expression, and histone acetylation. The oxygen consumption rate of the cultured myocytes was measured using an extracellular flux analyzer (Seahorse XF-24, Agilent, Santa Clara, CA). The gene expression levels were determined using quantitative PCR reactions (ABI 7500) in the presence of TB Green Premix ExTaq (Takara Bio) fluorescent dye. The histone acetylation levels were estimated by using ChIP assay using antibodies against H3Ac and H4Ac (Active motif).

Statistical Analyses

All the data are expressed as mean ± standard error values. Comparison of the mean values of the 2 groups was performed using Student *t* tests. When more than 2 groups were compared, analysis of variance was used to evaluate the significance of the differences among the groups. If significant differences were confirmed, each difference was further examined with Fisher's protected least significant difference method. A value of $P < .05$ was defined to be statistically significant.

Study Approval

The animal study was approved by the Ethics Committee of the laboratory animal center, Keio University School of Medicine (approval number 15064). All experiments were conducted in accordance with domestic law on the protection of laboratory animals, which is based on the NIH Guide for the Care and Use of Laboratory Animals.

Results

72hIF Upregulates Expression of Lipid Oxidation Genes in Skeletal Muscle and Adipose Tissues, and Improves Glucose Tolerance and Exercise Endurance

Four cycles of 72hIF (4 weeks) were performed to examine the effects of IF with long-term fasting on body weight, glucose tolerance, and exercise endurance (Fig. 1A). All mice survived after 4 cycles of 72 hours of fasting in our breeding condition. Body weight decreased sharply for 24 hours after the start of fasting and decreased gradually thereafter. The body weight increased significantly within 24 hours of refeeding and increased slowly thereafter. At 96 hours after refeeding, the body weight returned to a level similar to that before the fasting. Repeated fasting reduced the weight loss associated with fasting. The body weight at the end of 4 cycles of 72hIF was not significantly different from that in the control ad libitum feeding group (Fig. 1B). Regarding the food intake during this period, there was no significant difference between the IF group and the control group. The intraperitoneal glucose tolerance test (ipGTT) and insulin tolerance test (ITT) at the end of 4 cycles of 72hIF showed that the blood glucose level was significantly lower in the IF group than in the ad libitum feeding group (Fig. 1C and 1D). The rectal temperature decreased significantly at 24 hours after the start of fasting. The decrease was mild between 24 and 48 hours after the start of fasting and further decreased between 48 and 72 hours. In the subsequent ad libitum feeding period, the rectal temperature increased considerably in 24 hours after refeeding and then gradually increased. Then, 96 hours after refeeding, the rectal temperature was restored to the same level as that before the start. Repeated fasting reduced the decrease in rectal temperature during fasting. At the end of 4 cycles of IF, there was no significant difference compared with the control ad libitum feeding group (Fig. 1E). Respiratory gas analysis showed that oxygen consumption significantly increased while the respiratory quotient significantly decreased in the 72hIF group (Fig. 1F and 1G). There is concern regarding the progression of sarcopenia associated with IF; therefore, we measured the grip strength and exercise endurance after 72hIF. Grip strength was not significantly different; however, exercise endurance was significantly increased in the IF group (Fig. 1H and 1I). When the weights of the collected tissues were compared, the gastrocnemius muscle weight was not significantly different; however, the weight of the brown adipose tissue was significantly increased in the 72hIF group, while that of the white adipose tissue was significantly decreased in the 72hIF group (Fig. 1J). The expression of genes involved in mitochondrial activation and lipid oxidation in the skeletal muscle, white adipose tissue, and brown adipose tissue was examined using the quantitative PCR method. In the 72hIF group, mitochondrial uncoupling protein 3 (*Ucp3*), type II iodothyronine deiodinase (*Dio2*), carnitine O-palmitoyltransferase 1 (*Cpt1b*), acyl-CoA oxidase (*Acox1*) in the skeletal muscle; *Ucp1*, *Dio2*, and peroxisome proliferator–activated receptor gamma coactivator 1-alpha (PGC1α or *Ppargc1a*) in the brown adipose tissue; and *Ucp1*, *Dio2*, hormone-sensitive lipase (HSL or *Lipe*), and adipose triglyceride lipase (ATGL or *Pnpla2*) in the white adipose tissue were significantly upregulated (Fig. 1K-1M). In addition, we found that the expression level of PR domain containing 16 (*Prdm16*) was markedly enhanced in the brown adipose tissue after 72hIF, that of adiponectin (*Adipoq*) increased, and that of leptin (*Lep*) decreased in the white adipose tissue after 72hIF (Fig. 1L and M). Electron microscopic observation revealed mitochondrial hypertrophy in the skeletal muscle at the end of the 4 weeks of IF (Fig. 1N). These results indicate that 72hIF causes metabolic remodeling that promotes lipid utilization in the skeletal muscle and adipose tissue and improves glucose tolerance and exercise endurance without reducing calorie intake; these findings are compatible with previous reports.

24hIF Has Limited Impact on the Improvement in Glucose Tolerance and Exercise Endurance as Compared With 72hIF

Four cycles of 24hIF (4 weeks) were performed to examine the effects of IF with frequent fasting on the body weight, glucose tolerance, and exercise endurance (Fig. 2A). Body weight decreased significantly after a single 24-hours fast, and the weight was restored to a level similar to that before the fasting with 24 hours of refeeding. At the end of the 4 cycles of 24hIF, there was no significant difference in the body weight as compared to the control ad libitum feeding group (Fig. 2B). Regarding the food intake during this period, there was no significant difference between the IF group and the control ad libitum feeding group. The ipGTT and ITT at the end of 4 cycles of 24hIF showed lower blood glucose levels in the IF group as compared with that in the ad libitum feeding group; however, the impact of the change was limited when compared with that of the 72hIF (Fig. 1C and 1D, Fig. 2C and 2D). Regarding the rectal temperature, the largest decrease was observed in the first fasting; the temperature reduction became smaller with each fasting session (Fig. 2E). Respiratory gas analysis showed a significant increase in the oxygen

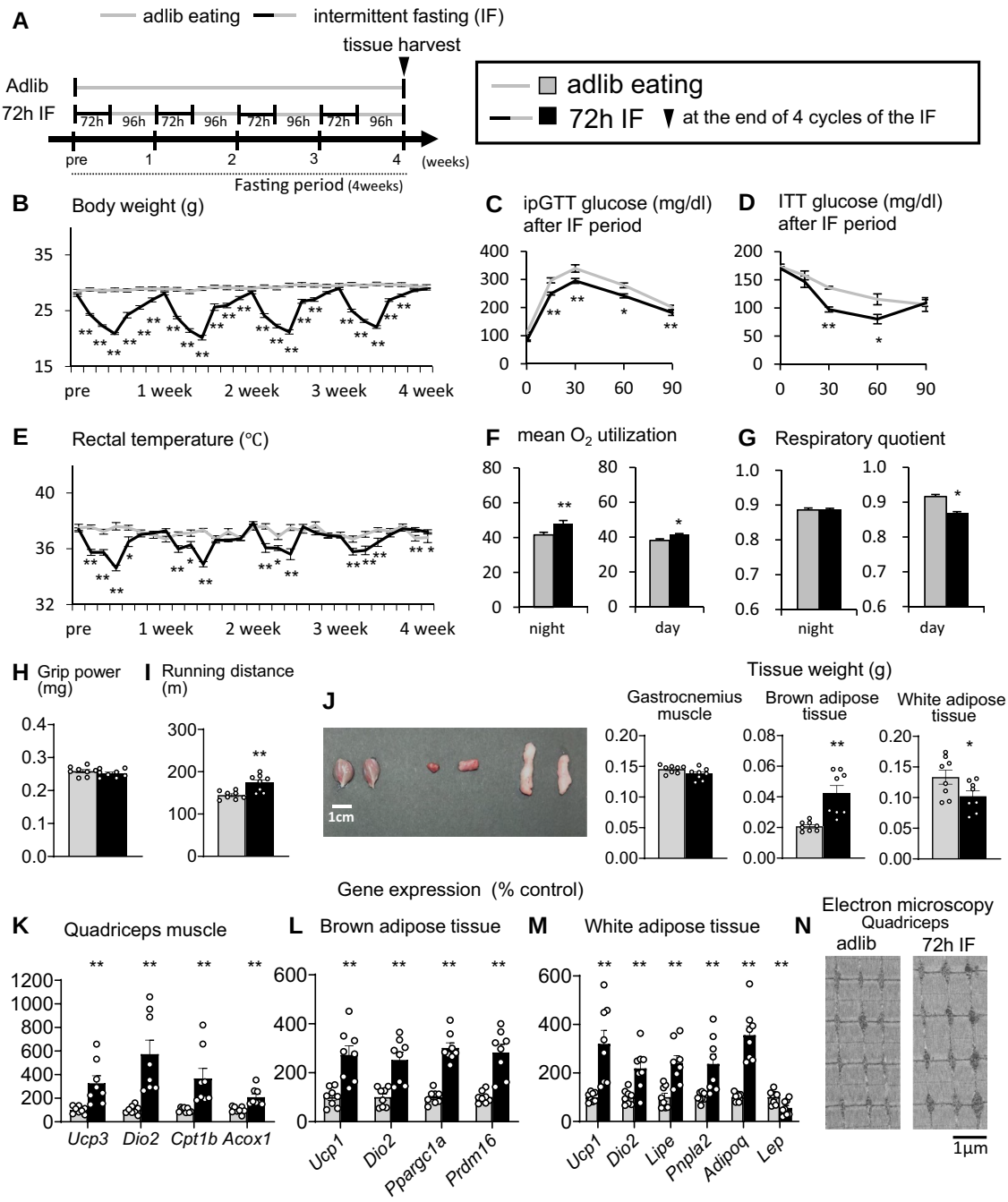

Figure 1. 72hIF upregulates the expression of lipid oxidation genes in the skeletal muscle and adipose tissues and improves glucose tolerance and exercise endurance. To examine the effect of 72hIF on body weight, glucose tolerance, and physical performance, 12-week-old C57BL6 mice were subjected to 4 cycles (4 weeks) of 72hIF and examined just after the completion of the IF period. (A) Schematic presentation of the 72hIF protocol. (B) Body weight during the 4 weeks of IF. (C, D) The blood glucose levels under intraperitoneal glucose tolerance test (ipGTT, C) and insulin tolerance test (ITT, D). Black lines represent 72hIF mice, and gray lines represent the control ad libitum feeding mice. (E) Rectal temperature during the IF period. (F) Oxygen utilization and (G) respiratory quotient measured using a respiratory gas analyzer (N = 4 in each group). (H) Grip power as an index of muscle strength and (I) running distance as an index of exercise endurance. (J) A macroscopic image and tissue weight of the gastrocnemius muscle, brown adipose tissue, and white adipose tissue just after the 4 weeks of IF. Scale bar = 1 cm. Expression of lipid oxidation genes estimated by quantitative PCR analysis in (K) quadriceps, (L) brown adipose tissue, and (M) white adipose tissue. (N) Electron microscopic analysis of the quadriceps. Scale bar = 1 μm. N = 10 in each group unless otherwise indicated. Differences in mean values were statistically analyzed by Student 2-tailed *t* tests. $^{*}P < .05$, $^{**}P < .01$ vs control ad libitum feeding mice. Abbreviations: *Acox1*, acyl-CoA oxidase 1; *Adipoq*, adiponectin; *Cpt1b*, carnitine palmitoyl transferase 1b; *DiO2*, iodothyronine deiodinase 2; *Lep*, leptin; *Lipe*, lipase, hormone sensitive; *Pnpla2*, patatin like phospholipase domain containing 2; *Ppargc1a*, peroxisome proliferator–activated receptor γ coactivator 1α; *Prdm16*, PR domain containing 16; *Ucp1*, uncoupling protein 1; *Ucp3*, uncoupling protein 3.

consumption and a decrease in the respiratory quotient in the 24 hours IF group during daytime. However, this change was smaller when compared to that in the 72hIF group (Fig. 2F and 2G). There was no significant difference in grip strength and exercise endurance between the 24hIF group and the control ad libitum feeding group (Fig. 2H and 2I). With respect to the weight of each tissue, brown fat weight was significantly increased in the 24hIF group although there was no significant

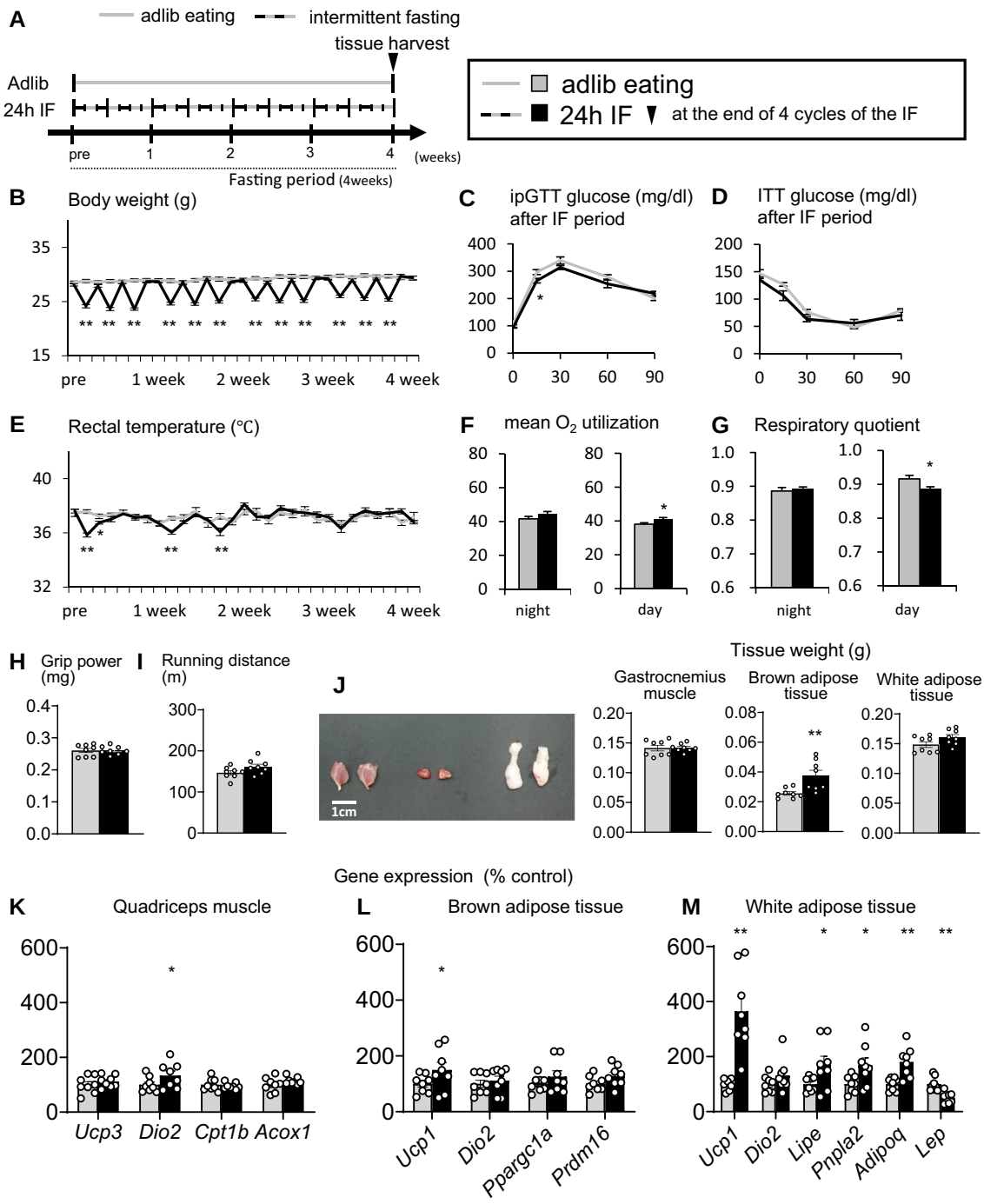

Figure 2. 24hIF has limited impact on improvement in glucose tolerance and exercise endurance. The metabolic effect of frequent IF (24hIF) was examined by using mice subjected to 4 cycles of 24hIF. (A) Schematic presentation of the 24hIF protocol. (B) Body weight during the 4 weeks of IF. (C, D) The blood glucose levels under intraperitoneal glucose tolerance test (ipGTT, C) and insulin tolerance test (ITT, D). Black lines represent 24hIF mice and gray lines represent control ad libitum feeding mice. (E) Rectal temperature during the IF period. (F) Oxygen utilization and (G) respiratory quotient measured using a respiratory gas analyzer (N = 4 in each group). (H) Grip power as an index of muscle strength and (I) running distance as an index of exercise endurance. (J) A macroscopic image and tissue weight of gastrocnemius muscle, brown adipose tissue, and white adipose tissue just after the 4 weeks of IF. Scale bar = 1 cm. Expression of lipid oxidation genes estimated using quantitative PCR analysis in the (K) quadriceps, (L) brown adipose tissue, and (M) white adipose tissue. N = 10 in each group unless otherwise indicated. Differences in mean values were statistically analyzed by Student 2-tailed *t* tests. $*P < .05$, $**P < .01$ vs control mice. Abbreviations: *Acox1*, acyl-CoA oxidase 1; *Adipoq*, adiponectin; *Cpt1b*, carnitine palmitoyl transferase 1b; *DiO2*, iodothyronine deiodinase 2; *Lep*, leptin; *Lipe*, lipase, hormone sensitive; *Pnpla2*, patatin like phospholipase domain containing 2; *Ppargc1a*, peroxisome proliferator–activated receptor γ coactivator 1α; *Prdm16*, PR domain containing 16; *Ucp1*, uncoupling protein 1; *Ucp3*, uncoupling protein 3.

difference in the weight of the gastrocnemius muscle and white adipose tissue between the 24hIF group and the control group (Fig. 2J). When the same examination of gene expression as that for 72hIF was performed using the quantitative PCR method, the upregulation of *Dio2* in the skeletal muscle; *Ucp1* in the brown adipose tissue; and *Ucp1*, *Lipe*, *Pnpla2*, and *Adipoq* in the white adipose tissue, as well as the down-regulation of *Lep*, were significant in the 24hIF group

(Fig. 2K-2M). These results suggest that 24hIF as well as 72hIF causes metabolic remodeling that promotes lipid utilization, although the effect is limited in the present experimental condition. The impact of frequent fasting, 24hIF, on the improvement in glucose tolerance and exercise endurance was considered weaker than that of long-term fasting, 72hIF.

The Effects of 72hIF on Body Weight, Rectal Temperature, and Exercise Endurance Were Sustained for the Subsequent 4 Weeks of Observation

To elucidate whether 72hIF exerts sustainable effects that persist even after the completion of the IF period, the mice were examined for metabolic remodeling after 4 weeks of ad libitum feeding of a high-fat diet (Fig. 3A-3M). During this 4-week observation period after 72hIF, weight gain of the mice in the 72hIF group was significantly suppressed (Fig. 3B). By ipGTT and ITT at the end of the 4 weeks of observation period, the blood glucose level in the 72hIF group continued to be significantly lower than those in the control group (Fig. 3C and 3D). The rectal temperature sustained a significant increase in the 72hIF group (Fig. 3E). Respiratory gas analysis at the end of the observation period continued to show a significant increase in oxygen consumption and a significant decrease in the respiratory quotient in the 72hIF group (Fig. 3F and 3G). The grip strength remained unchanged; however, exercise endurance showed a prolonged increase in the 72hIF group (Fig. 3H and 3I). Regarding the weight of each tissue collected at the end of the observation period, the weight of the gastrocnemius muscle was not significantly different. In contrast, the weight of the brown adipose tissue remained significantly increased in the 72hIF group, while that of the white adipose tissue was significantly decreased in the 72hIF group (Fig. 3J). At the end of the 4 weeks of observation, the gene expression of each tissue was examined using the quantitative PCR method. *Ucp3*, *Dio2*, *Cpt1b*, and *Acox1* in the skeletal muscle; *UCP1*, *Dio2*, *Ppargc1a, and Prdm16* in the brown adipose tissue; and *Ucp1*, *Dio2*, *Lipe*, *Pnpla2, and Adipoq* in the white adipose tissue were still significantly upregulated (Fig. 3K-3M). Thus, the metabolic effects of 72hIF persisted even after 4 weeks of observation. During the 4 weeks of observation in the 24hIF group (Fig. 4A-4M), there was a tendency to suppress weight gain in the IF group; however, there was no significant difference in the body weight (Fig. 4B). At the end of the 4 weeks of observation, ipGTT and ITT showed a decrease in the blood glucose level; however, the change was smaller than that in the 72hIF group (Fig. 3C and 3D, Fig. 4C and 4D). The rectal temperature was also significantly higher in the 24hIF group than in the control group at 1 week after the start of the observation period; however, thereafter, no significant difference was observed (Fig. 4E). Respiratory gas analysis at the end of the observation period showed an increase in the oxygen consumption and a decrease in the respiratory quotient as compared to that in the control; however, the change was not significant (Fig. 4F and 4G). With respect to grip strength and running distance, there was no significant difference between the 24hIF group and the control group (Fig. 4H and 4I). The weight of the gastrocnemius muscle and brown/white adipose tissue showed no significant difference (Fig. 4J). In the quantitative PCR analysis in each tissue, the 24hIF group had a tendency for upregulation of lipid oxidation genes; however, the change in gene expression was smaller than 72hIF group (Fig. 4K-4M).

Enhanced Expression of Lipid Oxidation Genes in the Skeletal Muscle After 72hIF Is Associated With Histone Acetylation

We hypothesized that the change in gene expression associated with IF was caused by histone acetylation and examined the acetylation level of histone H3 (H3Ac) and H4 (H4Ac) in the promoter region of lipid oxidation genes, *Cpt1b* and *UCP3*, in the quadriceps muscle after 72hIF (Fig. 5A). The H3Ac and H4Ac were examined after a single session of 72 hours fasting, at the end of 4 cycles of 72hIF, and after the 4-week observation period. When the ratio of histone acetylation was examined using the ChIP assay; both the genes showed enhanced acetylation after a single 72 hours fasting session and at the end of 4 cycles of 72hIF. These changes in histone acetylation were sustainable even at the end of the 4 weeks of observation (Fig. 5B). Further, assuming that alterations in the metabolites in the skeletal muscle associated with fasting can participate in the regulation of histone acetylation due to IF, a metabolome analysis of the quadriceps muscle after a fasting session was performed; the metabolites in the glycolysis pathway, TCA cycle, adenine nucleotides, nicotinate derivatives, and amino acids were quantified (Fig. 5C). Among the 123 metabolites that were examined, the tissue level of 24 metabolites significantly changed after 24 hours of fasting, and 41 metabolites changed after 72 hours fasting (Table 2). In the glycolysis pathway, metabolites, such as glucose-6-phosphate (G6P) and lactate were significantly reduced after 72 hours of fasting although they showed no significant change after 24 hours of fasting. Although the pyruvate level was stable during fasting, acetyl CoA increased significantly after 24 or 72 hours of fasting, suggesting fasting-induced generation of acetyl CoA via the degradation of proteins, amino acids, and lipids. In the TCA cycle, levels of fumarate and malate were increased while citrate was decreased upon fasting. Regarding adenine nucleotides, ATP remained unchanged even after 72 hours of fasting; however, the ATP degradation products adenosine diphosphate (ADP) and inosine increased. Reduced nicotinamide adenine dinucleotide (NADH) and carnitine did not change after the 24 hours of fasting; however, NADH significantly decreased and carnitine increased between 24 and 72 hours of fasting. Branched-chain amino acids (BCAAs), including valine, leucine, and isoleucine as well as tryptophan, proline, and asparagine steadily increased during 24 to 72 hours of fasting, suggesting substantial muscle degradation. β-Hydroxybutyric acid (OHB) increased significantly after 24 hours of fasting; however, the level decreased between 24 and 72 hours of fasting. These results are consistent with the promotion of muscle degradation and reduction of glucose utilization after a fasting session that increases the tissue level of amino acids. Compared to the changeable amino acids level, the metabolite levels in the glycolysis pathway, TCA cycles, and adenine nucleotides were relatively stable during a fasting session.

The Starvation Culture Enhances Histone Acetylation of Lipid Oxidation Genes in the Cultured Myocytes

Using cultured C2C12 myocytes, we examined the changes in energy consumption and gene expression after 72 hours of

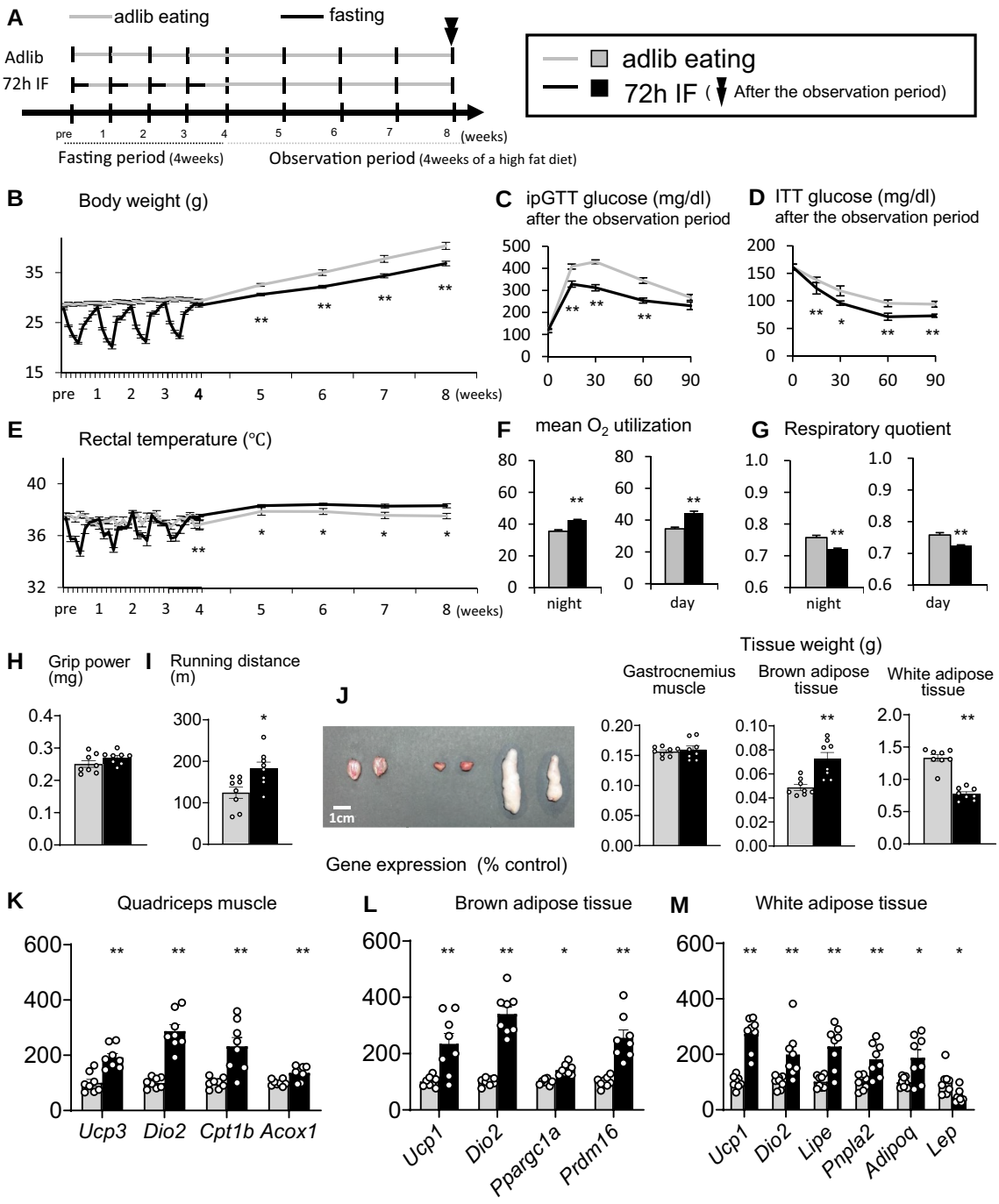

Figure 3. The effects of 72hIF on body weight, rectal temperature, and exercise endurance are sustainable for the subsequent 4 weeks of observation period. To examine the sustainable effects of 72hIF that persist even after the completion of the IF period, (A) 4 weeks of the IF period were followed by 4 weeks of observation. (B) Body weight during and after the IF period. (C, D) The blood glucose levels under intraperitoneal glucose tolerance test (ipGTT, C) and insulin tolerance test after 4 weeks of observation (ITT, D). (E) Rectal temperature during and after the IF period. (F) Oxygen utilization and (G) respiratory quotient measured using a respiratory gas analyzer after the observation period (N = 4 in each group). (H) Grip power as an index of muscle strength and (I) running distance as an index of exercise endurance. (J) A macroscopic image and tissue weight of the gastrocnemius muscle, brown adipose tissue, and white adipose tissue after the 4 weeks of observation. Scale bar = 1 cm. Expression of lipid oxidation genes estimated using quantitative PCR analysis in (K) quadriceps, (L) brown adipose tissue, and (M) white adipose tissue. N = 10 in each group unless otherwise indicated. Differences in mean values were statistically analyzed by Student 2-tailed *t* tests. $*P < .05$, $**P < .01$ vs control mice. Abbreviations: *Acox1*, acyl-CoA oxidase 1; *Adipoq*, adiponectin; *Cpt1b*, carnitine palmitoyl transferase 1b; *DiO2*, iodothyronine deiodinase 2; *Lep*, leptin; *Lipe*, lipase, hormone sensitive; *Pnpla2*, patatin like phospholipase domain containing 2; *Ppargc1a*, peroxisome proliferator–activated receptor γ coactivator 1α; *Prdm16*, PR domain containing 16; *Ucp1*, uncoupling protein 1; *Ucp3*, uncoupling protein 3.

incubation with a low-sugar low-serum starvation culture medium that mimics a fasting environment (Fig. 6A). The starvation culture for 72 hours significantly increased the maximum oxygen consumption as compared to the control with a standard medium (Fig. 6B and 6C). Expression of the lipid oxidation genes, *Ucp3* and *Cpt1b*, was significantly increased with the starvation culture (Fig. 6D). The ChIP assay showed that acetylation of histones H3 and H4 (H3Ac and H4Ac) in

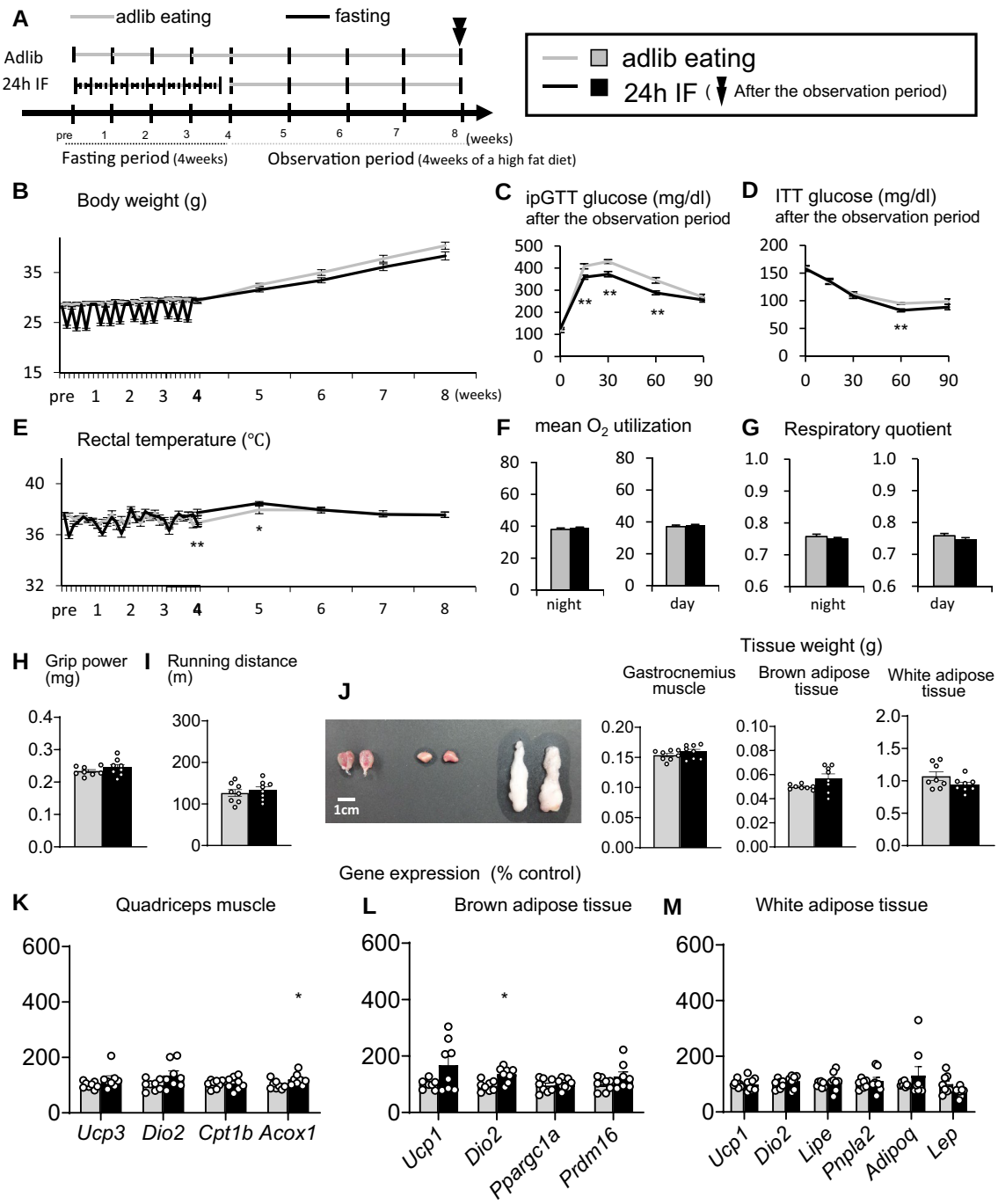

Figure 4. During the 4 weeks observation period of the 24hIF group, the weight gain tends to be suppressed in the IF group. 24hIF mice were subjected to (A) 4 weeks of observation period after 4 weeks of IF. (B) Body weight during and after the IF period. (C, D) The blood glucose levels under intraperitoneal glucose tolerance test (ipGTT, C) and insulin tolerance test after 4 weeks of observation (ITT, D). (E) Rectal temperature during and after the IF period. (F) Oxygen utilization and (G) respiratory quotient measured using a respiratory gas analyzer after the observation period (N = 4 in each group). (H) Grip power and (I) the running distance. (J) A macroscopic image and tissue weight of the gastrocnemius muscle, brown adipose tissue, and white adipose tissue after the 4 weeks of observation. Scale bar = 1 cm. Expression of lipid oxidation genes estimated using quantitative PCR analysis in the (K) quadriceps, (L) brown adipose tissue, and (M) white adipose tissue. N = 10 in each group unless otherwise indicated. Differences in mean values were statistically analyzed by Student 2-tailed *t* tests. * $P < .05$, ** $P < .01$ vs control mice. Abbreviations: *Acox1*, acyl-CoA oxidase 1; *Adipoq*, adiponectin; *Cpt1b*, carnitine palmitoyl transferase 1b; *DiO2*, iodothyronine deiodinase 2; *Lep*, leptin; *Lipe*, lipase, hormone sensitive; *Pnpla2*, patatin like phospholipase domain containing 2; *Ppargc1a*, peroxisome proliferator–activated receptor γ coactivator 1α; *Prdm16*, PR domain containing 16; *Ucp1*, uncoupling protein 1; *Ucp3*, uncoupling protein 3.

the promoter region of *Ucp3* and *Cpt1b* was significantly increased with the starvation culture for 72 hours (Fig. 6E). Furthermore, we investigated the effect of the intermittent starvation culture that simulates IF on the gene expression and histone acetylation of lipid oxidation genes. Similar to in vivo experiments, the cultured myocytes were subjected to the cycles of a starvation for 24 hours and a subsequent standard incubation for 24 hours. Metabolic remodeling of the cells at 1 cycle of starvation culture, 4 cycles of starvation culture, and 1 week after the end of the 4 cycles starvation

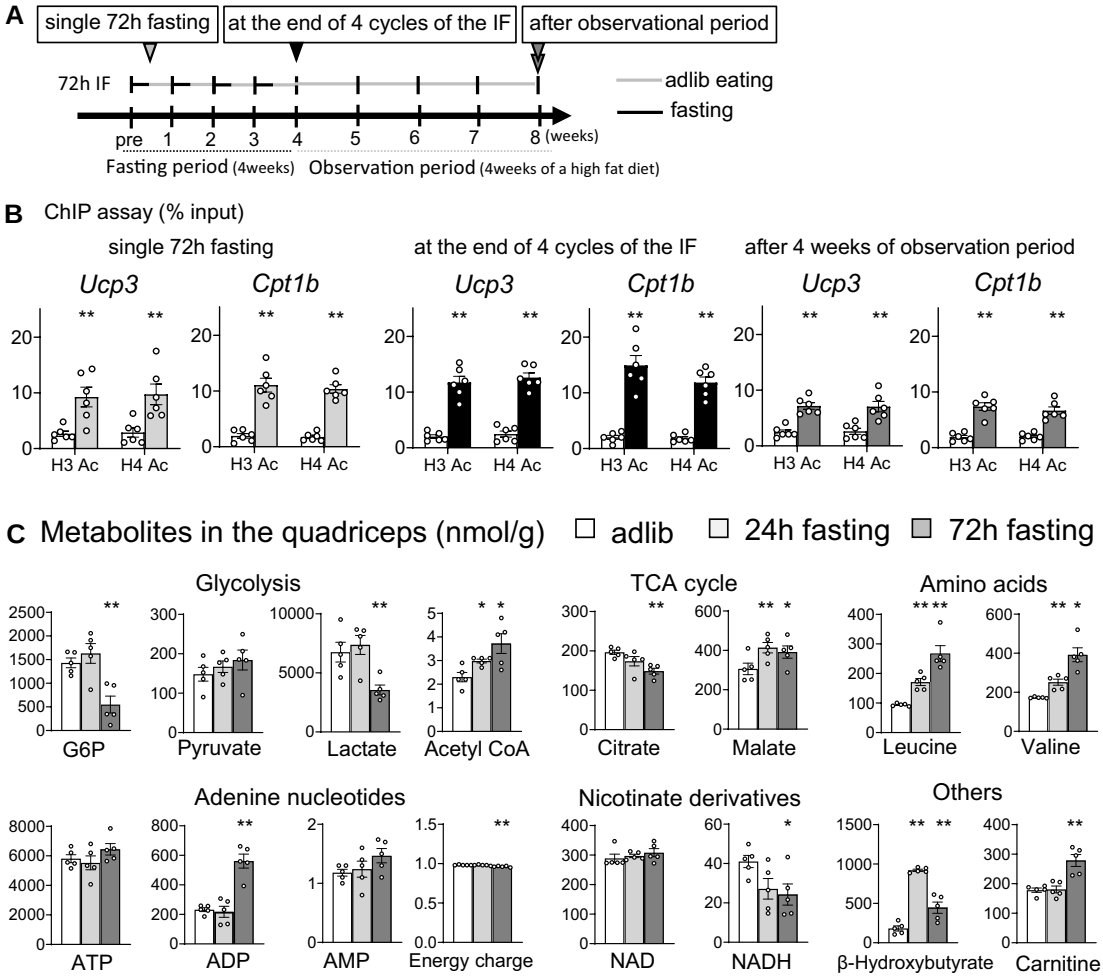

Figure 5. Enhanced expression of lipid oxidation genes in the skeletal muscle after 72hIF is associated with histone acetylation. (A) Schematic presentation of the experimental protocol. (B) ChIP analysis of the acetylation level of histone H3 (H3Ac) and H4 (H4Ac) in the promoter region of *Ucp3* and *Cpt1b* genes. (N = 6 in each group) (C) Metabolome analysis of the metabolites in the quadriceps muscle after 24 hours fasting or 72 hours fasting. (N = 5 in each group) Differences in mean values were statistically analyzed by Student 2-tailed *t* tests. $*P < .05$, $**P < .01$ vs control mice. Abbreviations: *Cpt1b*, carnitine palmitoyl transferase 1b; *Ucp3*, uncoupling protein 3.

culture (after the observation period) was evaluated (Fig. 6F). The maximum oxygen consumption increased after 1 cycle of starvation culture and was significantly enhanced by repeated starvation culture (4 cycles of starvation culture) (Fig. 6G). The expression of both the *Ucp3* and *Cpt1b* genes was significantly increased with the starvation culture, and the upregulation was sustained even 1 week after the discontinuation of starvation culture (after the observation period) (Fig. 6H). When the H3Ac and H4Ac of the promoter region of *Ucp3* and *Cpt1b* were examined using the ChIP assay, the histone acetylation of both genes was significantly increased, and the enhancement was sustained even at 1 week after the discontinuation of the starvation culture (Fig. 6I, after the observation period). The upregulation of *Ucp3* and *Cpt1b* with the starvation culture was suppressed by the treatment with a histone acetyltransferase (HAT) inhibitor, L002 (Fig. 6J). Thus, enhanced histone acetylation of the lipid oxidation genes was reproducible with the in vitro condition of myocytes, implying that an intracellular mechanism that responds to starvation might directly affect the histone acetylation. Based on recent findings of the "metabolism-epigenome link," meaning the regulation of epigenome by intracellular metabolites, we assumed that starvation-responsive metabolites might affect the histone acetylation. Among the metabolites that showed considerable alteration in the metabolome analysis of the quadriceps muscle in mice subjected to fasting, leucine has been noted for the ability to influence gene expression [28], although the precise mechanism is unclear. To examine the hypothesis that leucine is involved in the metabolism-epigenome link that regulates the expression of lipid oxidation genes, leucine-induced histone acetylation was examined using the cultured myocytes treated with 1 mM leucine. The expression of *Ucp3* and *Cpt1b* was enhanced by the leucine treatment, and the upregulation of the lipid oxidation genes by leucine was suppressed by L002 (Fig. 6K). H3Ac and H4Ac in the promoter region of both *Ucp3* and *Cpt1b* genes were enhanced in a time-dependent manner after leucine treatment. (Fig. 6L). These results imply that the fasting-responsive molecule, leucine, may participate in the metabolic regulation of cultured myocytes after fasting.

Discussion

The actual procedure of IF includes various fasting/refeeding periods, and the most effective IF protocol in terms of metabolic benefits has not been established, partially because the precise mechanism of metabolic remodeling remains unclear.

Table 2. Fasting alters the metabolites in the quadriceps muscle of mice

Category (total 123 metabolites)	24 hours fasting		72 hours fasting	
	Increase	Decrease	Increase	Decrease
Glycolysis pathway (11 metabolites)	Acetyl CoA		Acetyl CoA	Fructose 1,6-diphosphate Glucose 6-phosphate Lactate
TCA cycle (10 metabolites)	Fumarate Malate		Fumarate Malate	
Amino acids (20 metabolites)	Valine Isoleucine Leucine Tryptophan Proline Asparagine	Glycine Alanine Serine Lysine Arginine Glutamine	Valine Isoleucine Leucine Threonine Histidine Phenylalanine Tryptophan Methionine Proline Asparagine Aspartic Acid	Glycine Lysine Citrate
Adenine nucleotides (9 metabolites)	Inosine		ADP Inosine	
Nicotinate derivatives (4 metabolites)				NADH
Others (59 metabolites)	β-Hydroxybutyrate S-Adenosyl-L-methionine Spermidine	Hypotaurine ADMA Ornithine Citrulline Hydroxyproline	β-Hydroxybutyrate L-Carnitine Spermidine Taurine Creatine UDP IDP ITP	Hypotaurine ADMA Ornithine Citrulline Hydroxyproline Gluconate 6-Phosphogluconate D-Sedoheptulose 7-phosphate 5-Phosphoribosyl-pyrophosphate β-Alanine

A metabolome analysis of the quadriceps muscle was performed after a fasting session. Among the 123 metabolites that were examined, the tissue level of 24 metabolites had significantly changed after 24 hours of fasting and that of 41 metabolites had changed after 72 hours fasting. N = 5 in each group.

In the case of CR, even if an obese individual exhibited weight loss once, regaining of the lost weight within a short period after discontinuation maybe a problem [29]. Meanwhile, the effect of IF is expected to sustain for a longer time, even after it is stopped [24, 25], although the superiority of IF over CR in the persistence of the effects after discontinuation has not been demonstrated so far. Therefore, we examined the effect of IF at 4 weeks after completion of the IF period by using mice to understand the mechanism that induces sustainable metabolic effects of IF. Mice were subjected to a 72-hour continuous fasting period every week (72hIF), which causes a higher fasting load than conventional 24hIF with a cycle of 24 hours fasting and subsequent 24 hours refeeding. The expression of lipid oxidation genes that affect energy metabolism was examined after IF in association with the chromatin epigenetic modification, a mechanism responsible for the long-term regulation of gene expression.

In animal studies, several mechanisms for metabolic remodeling in each organ associated with IF have been elucidated. The promotion of lipid oxidation in the skeletal muscle has been reported as an effect of IF that is associated with enhanced expression of fatty acid transporters and lipid oxidation genes [30-32]. In the white adipose tissue, IF promotes intestinal microbiome-mediated production of acetate and lactate, both of which cause the conversion of white adipose tissue to the thermogenic beige type [7, 8]. In the brown adipose tissue, thermogenesis promotion after IF that is associated with enhanced *Ucp1* expression has been reported [33]. In the liver, the activation of mitochondrial biogenesis after IF accelerates lipid oxidation [34]. In these ways, IF in rodents reportedly promotes lipid utilization in each organ, changes fat distribution from visceral to subcutaneous, and decreases ectopic fat [35]. These alterations after IF are compatible with improved glucose tolerance [36]. In the present study, using mice, the conventional 24hIF for 4 cycles (4 weeks) caused a slight improvement in the glucose tolerance without significant weight changes. The result supported the concept that IF improves glucose tolerance via metabolic remodeling in each

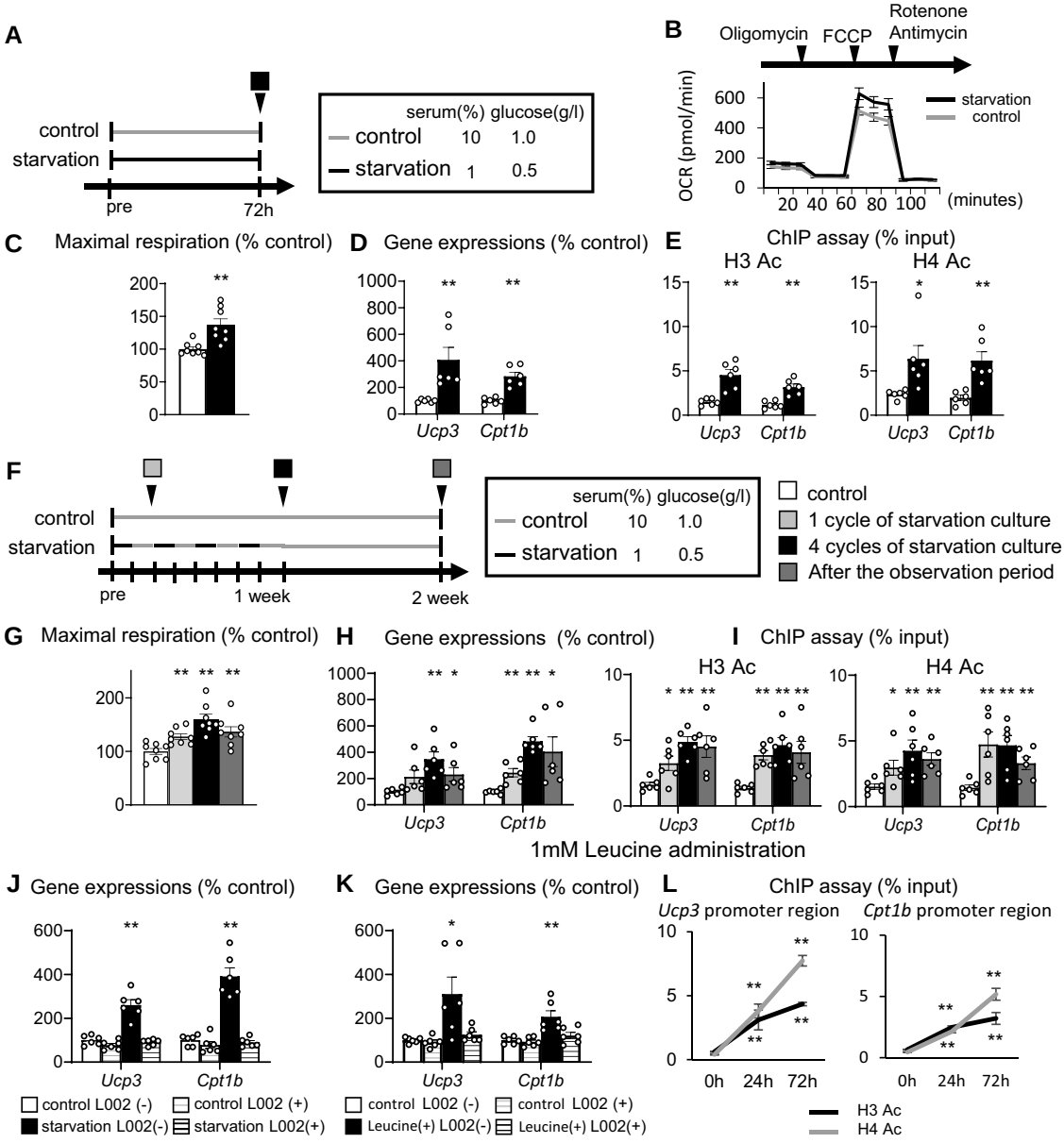

Figure 6. The starvation culture enhances histone acetylation of lipid oxidation genes in the cultured myocytes. (A) Schematic presentation of the 72 hours starvation culture in which low-sugar and low-serum medium was administered to the cultured myocytes, C2C12. (B) Oxygen consumption rate as an index of mitochondrial function. (C) Maximum oxygen consumption. (D) Expression level of *Ucp3* and *Cpt1b* genes estimated using quantitative PCR analysis and (E) histone acetylation in the promoter region of *Ucp3* and *Cpt1b* genes of C2C12 cells after starvation culture estimated using ChIP analysis. (F) Schematic presentation of the intermittent starvation culture for investigating the metabolic remodeling of C2C12 cells by the low-sugar and low-serum starvation medium. Metabolic remodeling of the cells at 1 cycle of starvation culture, 4 cycles of starvation culture, and 1 week after the end of observation period were evaluated. (G) Maximum oxygen consumption. (H) Expression level of *Ucp3* and *Cpt1b* genes and (I) Histone acetylation in the promoter region of *Ucp3* and *Cpt1b* genes. (J) Expression level of *Ucp3* and *Cpt1b* genes after 4 cycles of starvation culture with or without a HAT inhibitor, L002. (K) Expression level of *Ucp3* and *Cpt1b* genes after 72-h treatment of 1 mM leucine with or without L002. (L) Alteration in histone H3 and H4 acetylation of the promoter region of *Ucp3* and *Cpt1b* with treatment of 1 mM leucine to C2C12 cells (N = 6 in each group). N = 12 in each group unless otherwise indicated. Differences in mean values were statistically analyzed by Student 2-tailed *t* tests. $*P < .05$, $**P < .01$ vs control. Abbreviations: *Cpt1b*: carnitine palmitoyl transferase 1b; *Ucp3*: uncoupling protein 3.

organ, characterized by enhanced lipid utilization. When the higher fasting load of 72hIF is applied in mice for 4 weeks, the upregulation of genes for the promotion of lipid utilization was stronger than that with 24hIF; further, there was considerable improvement in glucose tolerance. Thus, the length of single fasting was suggested to be more important than the frequency of fasting for the induction of the metabolic effects of IF. Assuming that the improvement of lipid oxidation was caused by the activation of mitochondrial metabolism, we examined the expression of *Ucp3*, *Dio2*, *Cpt1b*, and *Acox1*, which promote mitochondrial lipid oxidation. These genes were upregulated in the skeletal muscle, and mitochondrial hypertrophy was confirmed on electron microscopy. Heat production in the brown adipose tissue and lipolysis in the white adipose tissue were suggested to be promoted in association with enhanced expression of *Ucp3*, *Dio2*, *Lipe*, and *Pnpla2*. To our knowledge, this is the first report to examine the difference between long-term fasting (72hIF) and frequent

fasting (24hIF) and show the benefits of long-term fasting for efficient metabolic remodeling that ameliorates obesity and diabetes. The enhancement of endurance found in the present study is compatible with the promotion of mitochondrial lipid oxidation in the skeletal muscle. In a previous report using mice, the combined use of exercise and IF increased endurance more effectively than exercise alone [9].

In human studies, IF has been studied mainly for anti-obesity effects; however, the expected effects of IF include influences on a wide range of chronic disorders, including cardiovascular and neuronal diseases [37]. Therefore, IF has been characterized as a dietary regimen that may provide positive constitutional improvement in various individuals, including nonobese and nondiabetic individuals. Elucidation of these effects in humans, clarification of the differences between IF and CR, and identification of suitable indications for IF in the clinical setting are future challenges. In clinical application of IF, it is expected to avoid muscle loss, in other words, sarcopenia [38], because IF does not require a reduction in calorie intake. Sarcopenia in some older adults has been expected to be a good indication for IF and the investigation to examine whether IF can retain lean mass better than CR has been performed. However, when nonobese subjects underwent IF, the lean mass was significantly decreased at 4 weeks of IF as compared with ad libitum feeding [39]. It is now thought to be probable that IF does not help to retain more lean mass than CR during weight loss [5]. Further studies to examine the safety and impact of IF on sarcopenia are needed before IF can be widely recommended to older adults [40].

In humans, the mechanism of metabolic remodeling associated with IF is not as well understood as it is in animals. However, previous studies have shown that the metabolic effects of dietary therapy including IF and CR prolong for a certain period even after discontinuation [24, 25], and consequently, clinical benefits from the persistence of effects of IF is expected. Similarly, in human large-scale clinical trials to investigate the occurrence of cardiovascular diseases, the beneficial effects of drug treatment on blood pressure, blood glucose, and lipid levels can persist even after discontinuation of the treatment [41]. The phenomenon is attracting attention as a "memory phenomenon" of therapeutic intervention [42, 43]. In animal studies, the mechanism of "memory phenomenon" of drug therapy have been reported [44, 45] and have suggested that epigenetic modification is important in the sustained effects of a medication [46]. However, studies focusing on the sustained effects of dietary therapy have been rare, even in animals [21-23]. Therefore, we aimed to investigate the sustainability of the metabolic effects of IF and the possible underlying mechanism. Mice were examined at the end of 4 weeks of ad libitum feeding subsequent to 4 cycles of 72hIF. During the 4 weeks of observation period with ad libitum high-fat feeding, the increase in body weight was significantly suppressed, and the weight of white adipose tissue was reduced. After 4 weeks of ad libitum feeding, the mice subjected to 4 cycles of 72hIF showed sustained improvement in glucose tolerance, exercise endurance, and lipid utilization. The increased expression of genes for lipid utilization in the skeletal muscle and brown/white adipose tissue continued even at 4 weeks after the discontinuation of IF, suggesting sustainability of metabolic remodeling that promotes lipid oxidation.

In the present study using mice, we found that histone acetylation might act as "fasting memory" after IF by which the effect of transient IF persists in each organ even after discontinuation of the treatment. As a molecular basis of such memory phenomenon, the significance of chromatin epigenetic modification that imprints the influence of environmental factors on gene expression, has been reported. Histone acetylation has been shown to be linked to energy metabolism [47], and the relation to lipid oxidation and the development of obesity and diabetes is known in both humans [48] and animals [49, 50]. Various metabolites that increase during fasting, such as nicotinamide adenine dinucleotide (NAD) and ketone bodies, including OHB, exert metabolic effects via the regulation of histone acetylation [51]. In fact, these metabolites serve as essential cofactors for chromatin-modifying enzymes, including histone acetyltransferases (HATs), histone deacetylases (HDACs, Sirtuins), and DNA methyltransferases (DNMTs). The effects of these chromatin-modifying enzymes on gene expression are dependent on the intracellular metabolites, such as NAD, OHB, and acetyl CoA. This relation has been termed as "metabolism-epigenome link" [52]. However, the regulatory mechanism of chromatin epigenetic modification during and after IF and the participation of metabolites have not been sufficiently investigated.

We hypothesized that histone acetylation, a mechanism that opens chromatin and sustainably promotes gene expression, is involved in the prolonged upregulation of lipid oxidation genes after IF and evaluated histone H3 and H4 acetylation in the promoter region of *Ucp3* and *Cpt1b* genes. The ChIP analysis revealed accelerated acetylation of histone H3 and H4 in the promoter region for these genes in the quadriceps muscle of 72hIF mice. Moreover, the enhancement of histone acetylation and gene expression was persistent even at the end of the subsequent 4 weeks of the ad libitum feeding period. Therefore, it was suggested that the histone hyperacetylation for upregulation of lipid oxidation genes would consist the "fasting memory" after IF. We further hypothesized that the enhancement of histone acetylation by 72hIF was caused by the "metabolism-epigenome link," that is, the alteration of intracellular metabolites associated with fasting affected histone acetylation of lipid oxidation genes. Therefore, we performed a metabolome analysis of the quadriceps muscle subjected to a single fasting for 24 or 72 hours and distinguished the fasting-responsive metabolites from the nonresponsive metabolites. It is noteworthy that the majority of metabolites were stable even after 72 hours of fasting. Among the 123 metabolites that were examined, a persistent increase during 24 to 72 hours of fasting was clearly observed in acetyl CoA, carnitine, and BCAAs. Furthermore, among the 41 metabolites that showed a significant increase or decrease after 72 hours of fasting, 13 molecules were amino acids. Therefore, we speculate that amino acids that were generated by muscle degradation during fasting may play significant roles in metabolic remodeling via epigenetic modification. In addition, metabolites that increase between 24 and 72 hours of fasting might account for the upregulation of lipid oxidation genes that caused potent metabolic effects associated with 72hIF.

Previous in vitro studies have shown that acetyl CoA acts as a donor of acetyl group and promotes histone acetylation by HATs [53]. OHB also increases histone acetylation via the suppression of HDACs [54]. In these ways, many fasting-responsive metabolites may participate in the metabolism-epigenome link in the fasting-induced metabolic remodeling. With respect to the amino acids in the skeletal muscle, a fasting-induced increase

was most noteworthy in the case of BCAAs. BCAAs are known to improve exercise endurance via mitochondrial activation and muscle enhancement [27]. One of the BCAAs, leucine, has been noted for the ability to affect gene expressions [28]; however, the precise underlying mechanism remains unclear. In this study, we used cultured myocytes to demonstrate that leucine can enrich histone acetylation of the lipid oxidation genes and upregulate expression. Therefore, BCAAs that are generated by fasting-induced muscle degradation may affect the expression of lipid oxidation genes via histone acetylation. To verify the hypothesis that fasting stress induces histone acetylation that alters the metabolic characteristics in the muscle, C2C12 myocytes were cultured in a low-sugar low-serum medium that is considered to mimic a fasting stimulation. A significant increase in maximum mitochondrial respiration, increased expression of both *Ucp3* and *Cpt1b* genes, and increased histone acetylation in the promoter region were observed. That is, the metabolic remodeling shown in IF mice could be mimicked by the in vitro conditions. As a mechanism of metabolic remodeling with starvation culture, a previous report showed an increase in NAD^+/NADH during starvation culture that enhanced the mitochondrial activity [31]. The in vitro experiment showed that repeated starvation had a stronger effect on the promotion of mitochondrial respiration and the effect persisted even at 1 week after discontinuation of the starvation. The upregulation of *Ucp3* and *Cpt1b* by the starvation culture was suppressed by the treatment with a HAT inhibitor, L002. Accordingly, we concluded that the regulation of gene expression via histone acetylation was involved in the starvation-induced metabolic remodeling.

In these ways, the metabolic remodeling associated with IF was examined by using fasting mice and myocytes with starvation culture. To our knowledge, this is the first report to focus on the sustainable metabolic effects of IF and identify the roles for histone acetylation in the prolonged upregulation of lipid oxidation genes after IF. The findings support the following concepts: (i) as previously reported, IF results in metabolic remodeling of the muscle and adipose tissues in mice, characterized by enhanced lipid utilization; (ii) long-term IF (72hIF) results in stronger metabolic effects that lead to more noticeable improvements in terms of obesity, diabetes, and exercise endurance than frequent IF (24hIF); (iii) metabolic remodeling due to IF sustains for a certain period even after discontinuation of the treatment; (iv) histone acetylation of the promoter region contributes to the upregulation of lipid oxidation genes during and after IF; (v) an increase in BCAAs due to fasting-induced muscle degradation might lead to histone hyperacetylation of the lipid oxidation genes. These findings in animal and cell culture experiments reveal the previously unknown roles for histone acetylation in the sustainable metabolic effects of IF. The main results of this study using mice suggest that the IF-induced changes in gene expression, reduction in body weight, and improvement in insulin resistance may be sustained for a certain period. However, few appropriate human studies have evaluated the sustainable effects of IF long enough for a sufficient reduction in body weight and improvement in insulin resistance. To determine the applicability in humans of the present findings in mice, we believe that longer-term clinical studies of IF should be conducted.

Meanwhile, there are limitations of this study. Obese model mice were not used in this study, despite obesity being a good indication for IF in humans, because the main purpose was to analyze the effects of IF on skeletal muscle and not on body weight. The IF period was as short as 4 weeks and the follow-up period was 4 weeks after a return to ad libitum feeding. As a result, the effects of IF observed in the present study on the enhancement of muscle lipid oxidation genes in mice should not be directly applied to human patients with obesity or diabetes. The average duration of intervention in studies that examined the effects of IF is approximately 2 to 3 months in animal studies using short-lived rodents, and 4 to 6 months in human clinical studies [55]. The majority of IF studies to date have been run for a short duration. Long-term follow-up of IF in human studies has been at most 1 year [24, 25, 56]. Therefore, further studies focusing on the safety and efficacy of IF for longer periods of time and examining the long-term sustainability of the effects after discontinuation are needed. We expect that future study will elucidate precise mechanisms and appropriate indications of IF.

Acknowledgments

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Author Contributions

S.E., A.U., K.M., and M.S. designed the research and performed the experiments; S.E., A.U., and K.M. analyzed the data and wrote the paper. All authors read the manuscript critically and contributed to the refinement; H.I. supervised the research project.

Disclosures

The authors have nothing to disclose.

Data Availability

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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