Upregulation of Fatty Acyl-CoA Thioesterases in the Heart and Skeletal Muscle of Rats Fed a High-Fat Diet

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In rodent models of diet-induced obesity, prolonged high-fat feeding increases the cellular uptake of fatty acids and causes lipotoxicity in the heart and skeletal muscle, where substrate overload to beta-oxidation generates mitochondrial stress. We examined the hypothesis that, because of its catalytic properties, acyl-CoA thioesterase (ACOT) would counteract these detrimental situations by modulating intracellular acyl-CoA levels. Rats were fed a low- or high-fat diet for up to 20 weeks, and the expressions of ACOT isoforms and fatty acid beta-oxidation enzymes were analyzed by western blotting. The expressions of ACOT1, ACOT2 and ACOT7 proteins in the heart and soleus muscle were significantly increased, by 2.0—7.6-fold, in rats fed the high-fat diet as compared with the low-fat diet group. These effects were accompanied by increases in carnitine palmitoyltransferase and acyl-CoA oxidase expression. However, ACOT was not induced in the extensor digitorum longus muscle or the liver. Subcellular fractionation of heart and soleus muscle homogenates confirmed expression of both the cytosolic and mitochondrial ACOT isoforms. These results underscore the functional relationship between ACOT and fatty acid oxidation, and suggest adaptive upregulation of ACOT to protect against fatty acid oversupply in the heart and skeletal muscle.

Key words acyl-CoA thioesterase; fatty acid oxidation; lipotoxicity; obesity

Over the past few decades, the prevalence of overweight and obesity has increased markedly worldwide, along with the adoption of westernized lifestyles characterized by excessive energy intake and a lack of physical activity. Consequently, obesity and a cluster of obesity-related comorbidities often referred to as the metabolic syndrome have become a serious public health problem and a major risk factor for the development of severe diseases such as cardiovascular disease.[1] Obesity is strongly associated with insulin resistance, in which elevation of circulating fatty acids results in increased fatty acid availability that exceeds the fat disposal capacity of cells, which decreases insulin-stimulated glucose oxidation in muscles and subsequently leads to contractile dysfunction of the heart.[2] The precise mechanisms underlying these "lipotoxic" consequences remain incompletely defined, but it is currently accepted that excessive fatty acid uptake into cells leads to the accumulation of proinflammatory lipid metabolites such as fatty acyl-CoA, diacylglycerol and ceramide. These metabolites stimulate stress-activated kinases, which interfere with insulin signaling.[2,3]

It is also becoming clear that fatty acid beta-oxidation is increased in the insulin-resistant heart and oxidative skeletal muscle, and that mitochondrial overload and incomplete oxidation of fatty acids contribute to the impairment of insulin sensitivity.[2,4] Under these conditions, enhanced beta-oxidation, which is not accompanied by appropriate upregulation of the tricarboxylic acid (TCA) cycle or electron transport chain (ETC) activity, fails to oxidize fatty acids completely to CO_2 and deposits incomplete fat catabolites along with diminished levels of TCA cycle intermediates. These stressful environments created within mitochondria are thought to exacerbate cellular insulin resistance by enhanced oxidative stress, for example.[4] However, acyl-CoA thioesterase (ACOT) exists within the mitochondrial matrix of mammalian cells[5] and its expression is expected to be upregulated in response to fatty acid overload, as demonstrated in diabetic and fasted animals.[6,7]

ACOT comprises a group of enzymes that are localized in multiple compartments in cells and catalyze the hydrolysis of long-chain acyl-CoA thioesters to free fatty acids and CoA-SH. For example, ACOT1 (formerly known as CTE-I or ACH2) and ACOT7a (CTE-II, BACH or ACT) are localized in the cytosol while ACOT2 (MTE-I or ARTISt) and ACOT7b (MTE-II or LACH1) are present in mitochondria.[8—10] These catalytic properties of ACOT mean that it is capable of lowering the increased levels of acyl-CoA imported by carnitine palmitoyltransferase (CPT) across the mitochondrial membranes from the cytosol. Accordingly, ACOT could counteract the enhanced beta-oxidation and reduce the mitochondrial stress caused by the imbalance between beta-oxidation and TCA cycle/ETC activity during fatty acid overload. Moreover, the ACOT isoforms present in the cytosol could scavenge surplus acyl-CoA to prevent mitochondrial overload of fatty acids. In this study, to investigate this hypothesis, we examined the effect of fatty acid oversupply on the ACOT expression level *in vivo*, and confirmed upregulation of the ACOT protein levels in the heart and skeletal muscle of rats fed a high-fat diet.

MATERIALS AND METHODS

Animals and Treatments All animals were treated according to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health

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(NIH Publication No. 85-23, revised 1996). The protocol of this study was approved by the Committee of Animal Use and Welfare of Tokyo University of Pharmacy and Life Sciences. Male Wistar rats (9 weeks old) were obtained from Tokyo Laboratory Animals Science (Tokyo, Japan) and acclimatized for 1 week on a standard laboratory chow (CE-2; Clea Japan, Tokyo, Japan). The animals were subsequently fed a low-fat diet (D12450B; Research Diets, New Brunswick, NJ, U.S.A.) or a high-fat diet (D12492; Research Diets) for 4 or 20 weeks *ad libitum*. The low-fat diet contained 3.85 kcal/g, to which the contributions of carbohydrate, fat and protein were 70%, 10% and 20%, respectively. The corresponding values for the high-fat diet were 5.24 kcal/g and 20%, 60% and 20%, respectively. The source of the carbohydrate was a combination of cornstarch, maltodextrin and sucrose, while the source of the fat was soybean oil and lard. The CE-2 diet (3.43 kcal/g) was similar to the low-fat diet in terms of the contributions of carbohydrate, fat and protein to the total energy available (58%, 13%, 29%, respectively). After an overnight fast, the rats were anesthetized with pentobarbital (50 mg/kg, intraperitoneally (i.p.)) and killed by decapitation. The serum was collected, and tissues were excised, snap-frozen and stored at −80 °C until analysis. Serum nutritional and biochemical parameters were analyzed by a clinical testing laboratory (SRL, Tokyo, Japan).

Sample Preparation and Subcellular Fractionation Tissue homogenates were prepared in SETP buffer (0.25 M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Tris–HCl pH 7.5 and Complete protease inhibitor cocktail (Roche, Mannheim, Germany)) using a Polytron tissue processor (Kinematica, Lucerne, Switzerland). For subcellular fractionation, heart and soleus muscle tissues were homogenized in SETP buffer using a Potter–Elvehjem glass homogenizer with a Teflon pestle. The homogenates were centrifuged at 400×***g*** for 10 min, and the pellets were washed once with SETP buffer. The wash solution and supernatant were combined and centrifuged at 25000×***g*** for 10 min, and the resulting supernatant was centrifuged at 105000×***g*** for 60 min. The 400×***g*** (nuclear fraction), 25000×***g*** (particulate fraction) and 105000×***g*** (microsomal fraction) pellets, and the 105000×***g*** supernatant (cytosolic fraction) were suspended in SETP buffer. The protein concentrations were determined using a DC protein assay kit (Bio-Rad, Hercules, CA, U.S.A.) with bovine serum albumin as the standard.

Western Blotting Proteins were resolved in 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels, and transferred onto Immun-blot polyvinylidene difluoride (PVDF) membranes (Bio-Rad). After blocking with 5% skimmed milk, the membranes were incubated with a primary antibody, followed by horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) or anti-mouse IgG secondary antibodies (Invitrogen, Carlsbad, CA, U.S.A.). The enzyme activity was visualized using ECL Advance Western Blotting Detection Reagents (GE Healthcare, Little Chalfont, U.K.) and analyzed using an LAS 3000 Lumino Imaging Analyzer (Fuji Photo Film, Tokyo, Japan). Rabbit polyclonal antibodies against ACOT1,[11] ACOT7,[12] CPT2[13,14] and acyl-CoA oxidase 1 (ACOX1)[14,15] were raised in our previous studies. Mouse monoclonal anti-heat shock protein 60 (HSP60) (Stressgen, Ann Arbor, MI, U.S.A.) and anti-alpha-tubulin (Sigma-Aldrich, St. Louis, MO, U.S.A.) antibodies were obtained from commercial sources.

Reverse Transcription (RT)-Polymerase Chain Reaction (PCR) After oligo$(dT)_{18}$-primed RT of total RNA extracted from the heart and soleus muscle of rats, PCR was performed for ACOT7 transcript variants with 5′-TCCGGTCCCAC-CACCGACACG-3′ (*Acot7_v1*), 5′-GATGAGGGCTGTCA-GAACCAG-3′ (*Acot7_v2*) or 5′-CTCCCAGGCTCATTCA-TTCG-3′ (*Acot7_v7*) as a sense primer and 5′-CCACGTTG-GCATCATCCGGAC-3′ as an antisense primer. RT reactions were incubated at 42 °C for 90 min with Superscript III RNase H^- reverse transcriptase (Invitrogen) and PCR was performed with Taq DNA polymerase (New England BioLabs, Beverly, MA, U.S.A.). The cycling conditions were 94 °C for 2 min, followed by 40 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. Aliquots of the reactions were run on an agarose gel, stained with SYBR green (BioWhittaker Molecular Applications, Rockland, ME, U.S.A.), and analyzed by the LAS 3000 Lumino Imaging Analyzer.

Statistical Analysis The statistical significance of differences among values was examined by one-way factorial analysis of variance followed by Bonferroni's multiple comparison test. Values of $p<0.05$ were considered to indicate statistical significance.

RESULTS

Adult male Wistar rats were fed a low-fat or high-fat diet for 4 or 20 weeks. Weight gain was 1.3-fold greater in the rats fed the high-fat diet than in those fed the low-fat diet after 20 weeks. The weight of the white adipose tissue (WAT), the relative WAT weight to body weight, and the liver weight tended to increase for the high-fat diet compared with the low-fat diet (Table 1). Regarding the serum parameters, there were no significant differences in the aminotransferase levels among all of the groups or glucose levels between the high-fat and low-fat diet groups at each time-point. However, the triglyceride level was 3.2-fold higher in the low-fat diet group than in the high-fat diet group after 4 weeks. This increase in the low-fat diet diminished and became insignificant after 20 weeks, although it still remained 1.8-fold higher than that in the high-fat diet group. Similar trends were seen for free fatty acid and total cholesterol.

Under these conditions, the expression levels of ACOT1, ACOT2 and ACOT7 proteins in the heart and soleus muscle were significantly increased by 1.8—7.6-fold in the high-fat diet group after 4 and 20 weeks, as compared with the baseline values and the low-fat diet group for the same feeding period. However, the ACOT2 expression in the heart was not significantly affected by 4 weeks of the high-fat diet compared with the baseline value (Figs. 1A, B, E—J). High-fat feeding for 20 weeks significantly increased heart ACOT1 and ACOT2 and soleus muscle ACOT2 expression compared with that at 4 weeks ($p<0.001$). The low-fat diet did not affect the expression of these ACOT proteins.

In the heart and soleus muscle, the expressions of the mitochondrial beta-oxidation enzyme CPT2 and the peroxisomal beta-oxidation enzyme ACOX1 were also significantly increased by the high-fat diet ($p<0.05$). In the heart, CPT2

Table 1. Body Weight Gains, Tissue Weights, and Serum Nutritional and Biochemical Parameters

	Baseline	4 weeks		20 weeks	
		Low-fat diet	High-fat diet	Low-fat diet	High-fat diet
Body weight gain (g)	—	192±5	203±3	423±30	534±19†
WAT weight (g)	4.2±0.2	17.2±0.8	19.7±1.1	31.2±3.5*	43.0±7.8*
Relative WAT weight (%)	1.3±0.1	3.2±0.2	3.6±0.2*	4.1±0.4*	5.0±0.9*
Liver weight (g)	9.9±0.2	14.8±0.9*	14.0±0.2*	16.5±0.5*	18.4±1.3*
Serum level of					
AST (IU/l)	140±6	155±32	146±12	143±11	160±16
ALT (IU/l)	37±1	34±5	40±1	45±9	54±6
Glucose (mg/dl)	101±5	135±16	143±4*	132±4	146±9*
Triglyceride (mg/dl)	60±10	230±59*	73±9†	132±20	74±6
Free fatty acid (mEq/dl)	0.43±0.03	0.69±0.05*	0.47±0.07	0.56±0.05	0.43±0.03
Total cholesterol (mg/dl)	50±3	90±7*	65±3†	91±5*	103±7*

White adipose tissue (WAT) was excised from the retroperitoneal region of rats, and the weight was used to estimate the degree of obesity, together with the relative WAT weight [WAT weight (g)/body weight (g)×100]. Data are expressed as means±S.E.M. of 4 rats. AST, aspartate aminotransferase; ALT, alanine aminotransferase. *† $p<0.05$, significantly different to baseline and the low-fat diet group for the same feeding period, respectively.

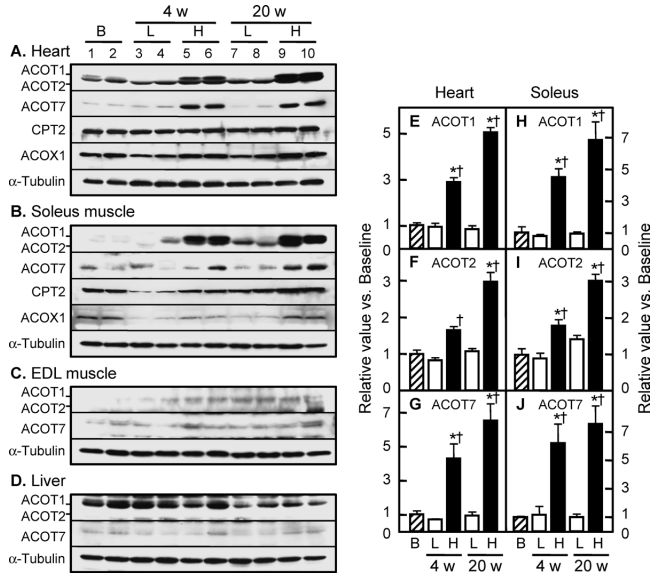

Fig. 1. Changes in the Expression Levels of ACOT Isoforms in the Heart and Skeletal Muscle of Rats Fed a High-Fat Diet

After 1 week of acclimatization [baseline (B)], the rats were fed a low-fat (L) or a high-fat (H) diet for 4 or 20 weeks (w). The protein expression levels of the indicated enzymes were analyzed by Western blotting in the heart, soleus muscle, extensor digitorum longus (EDL) muscle and liver, as shown in panels A—D, respectively. The blots for 2 rats are presented for each group (20 μg protein/lane). The antibody against ACOT1 (46 kDa) cross-reacted with ACOT2 (45 kDa). The signal intensities of the bands corresponding to ACOT1, ACOT2 and ACOT7 (43 kDa) in the heart and soleus muscle were measured and are expressed relative (mean±S.E.M. of 4 rats) to the baseline values set as 1 (E—J). * $p<0.05$ *versus* baseline; † $p<0.05$ *versus* the low-fat diet group at the same feeding time. CPT2, carnitine palmitoyltransferase 2; ACOX1, acyl-CoA oxidase 1 (52 kDa subunit). Alpha-tubulin was used as a loading control.

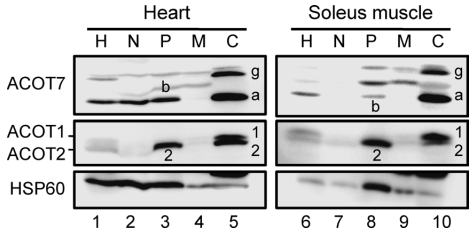

Fig. 2. Subcellular Fractionation

Homogenates prepared from the heart and soleus muscle of rats fed a high-fat diet for 4 weeks were fractionated by sequential centrifugation, and analyzed by Western blotting with antibodies against ACOT1, ACOT7 and heat shock protein 60 (HSP60) as a mitochondrial marker. The detected bands corresponding to ACOT1, ACOT2 and ACOT7 isoforms 7a, 7b and 7g are indicated on the blots by 1, 2, a, b and g, respectively. Lanes 1—5: whole homogenate (H) and nuclear (N), particulate (P), microsomal (M) and cytosolic (C) fractions, respectively (20 μg protein/lane). Data are presented for one of two independent experiments. It should be noted that, in lane 2 of the middle panel (ACOT1 and ACOT2), no bands are detected in the region around 45 kDa because the antibodies were blocked by a large amount of protein present there, most probably actin.

was increased by 1.7- and 1.4-fold and ACOX1 expression was increased by 2.6- and 2.4-fold by high-fat feeding for 4 and 20 weeks, respectively. Similarly, in the soleus muscle, CPT2 expression was increased by 2.3-(although not significant) and 1.9-fold and ACOX1 was increased by 2.0- and 1.9-fold. In contrast, in the extensor digitorum longus (EDL) muscle, the expression levels of ACOT1, ACOT2 and ACOT7 remained very low in all of the groups, and no significant changes were observed in the high-fat diet group compared with the low-fat diet group at both time-points (Fig. 1C). In addition, there were no significant changes in the expressions of these ACOT proteins in the liver (Fig. 1D).

In the above Western blot analyses, we used whole tissue homogenates. Therefore, the different ACOT7 isoforms, which are derived from a single gene and are localized in the mitochondria and cytosol, and possibly the nucleus, could not be distinguished because they have similar molecular masses.[16—18] To address this issue, the heart and soleus muscle homogenates were fractionated by centrifugation and the fractions were subjected to Western blotting analyses (Fig. 2). In both cases, the ACOT7 protein (43 kDa) was mainly recovered in the particulate and cytosolic fractions, with an additional isoform (50 kDa) detected in the latter fraction (Fig. 2, top panels). Small amounts of these two proteins were also found in the nuclear fractions, which were likely, at least in part, to be derived from cells that had escaped disruption by homogenization and been centrifuged into the pellets at low gravity. As indicated by the distribution patterns of the cytosolic ACOT1 and mitochondrial ACOT2, as well as HSP60 as a mitochondrial marker, the subcellular fractionations of the muscle tissue homogenates were successful and the mitochondria were concentrated in the particulate fraction. However, ACOT2 and HSP60 that leaked from damaged mitochondria were also found in the cytosolic fraction, particularly in the heart homogenates. These analyses

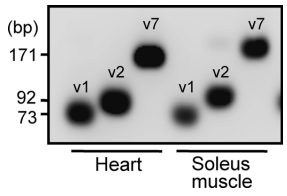

Fig. 3. RT-PCR of ACOT7 Transcript Variants

Total RNA was extracted from the heart and soleus muscle of rats fed a high-fat diet for 4 weeks and subjected to RT-PCR using primer pairs specific to ACOT7 transcript variants. Aliquots of the reactions were run on an agarose gel and stained with SYBR green. The detected bands corresponding to the variants *Acot7_v1*, *Acot7_v2* and *Acot7_v7* are indicated by v1, v2 and v7, respectively.

revealed that the mitochondrial and cytosolic ACOT7 isoforms were expressed in the heart and soleus muscle of rats fed the high-fat diet. It is likely that the former isoform was ACOT7b (43 kDa) and the latter isoforms were ACOT7a (43 kDa) and ACOT7g (50 kDa). The mRNA expressions of ACOT7a, ACOT7b and ACOT7g (transcript variants *Acot7_v1*, *Acot7_v2* and *Acot7_v7*, respectively[9]) were confirmed by RT-PCR in both tissues from the high-fat diet-fed rats (Fig. 3). The expression levels of ACOT7g protein were relatively low in the heart and soleus muscle, and were not affected by high-fat feeding for 4 and 20 weeks (data not shown).

DISCUSSION

In this study, we demonstrated upregulation of the ACOT protein levels in the heart and soleus muscle of rats chronically fed a high-fat diet. Both the cytosolic (ACOT1 and ACOT7a) and mitochondrial (ACOT2 and ACOT7b) isoforms were markedly induced and were accompanied by increased levels of CPT2 and ACOX1, indicating upregulation of fatty acid beta-oxidation. However, these changes in ACOT expression did not occur in the EDL muscle. The EDL muscle comprises white/fast-twitch fibers (type 2) that have a low oxidative, high glycolytic capacity favoring anaerobic energy production. In contrast, the soleus muscle comprises red/slow-twitch fibers (type 1) that have a high oxidative, low glycolytic capacity favoring aerobic energy production, similar to the highly oxidative heart muscle.[19] Therefore, the present findings underscore the functional relationship between ACOT and fatty acid oxidation, which also accounts for the lack of induction of ACOT in the liver where fat oxidation is reduced in response to prolonged high-fat exposure, in contrast to the increased mitochondrial fatty acid catabolism in muscle.[4] Previous studies using RT-PCR suggested increased expression of ACOT in diet-induced obese rats. For example, Durgan *et al.*[20] reported that high-fat feeding for 4 weeks increased heart ACOT1 mRNA by 5.5-fold compared with low-fat feeding, while Wilson *et al.*[21] reported that ACOT1 and ACOT2 mRNA expressions were increased by 5.5- and 1.5-fold in the heart and 5.3- and 2.9-fold in the soleus muscle, respectively, after high-fat feeding for up to 24 weeks. Thus, our finding of ACOT protein upregulation in the present study is compatible with these previous findings.[20,21]

Although the roles of ACOT in lipid metabolism have not been fully established, a model concerning the roles of ACOT1 and ACOT2 is emerging.[7,8,20—23] In this model, fatty acids entering a cell across the plasma membrane are rapidly activated to acyl-CoA and partitioned into the oxidative and nonoxidative metabolic pathways.[20,21] In the heart and soleus muscle, where the expression level of cytosolic ACOT1 is very low, mitochondrial fatty acid oxidation may be the default primary pathway for acyl-CoA metabolism. Thus, an increase in cellular uptake of fatty acids readily results in an elevated level of cytosolic acyl-CoA and thereby enhances beta-oxidation when rats are fed a high-fat diet. In this context, upregulation of ACOT1 would decrease acyl-CoA to alleviate its overload on mitochondrial beta-oxidation. In addition, acyl-CoA synthesized at the expense of ATP utilization would be repeatedly hydrolyzed, leading to the development of a futile energy-wasting cycle.[8,20,21] Moreover, some of the fatty acids released by acyl-CoA hydrolysis *via* ACOT1 may reach and activate a nuclear receptor, peroxisome proliferator-activated receptor alpha (PPAR alpha), which in turn stimulates the expression of genes related to lipid catabolism, including ACOT itself[24—26] and uncoupling protein 3 (UCP3)[27] to form a positive feedback loop.[8]

On the other hand, in the mitochondrial matrix, acyl-CoA transported *via* CPT is subjected to beta-oxidation. However, some of the acyl-CoA is hydrolyzed by ACOT2 and regenerates CoA-SH, which is required for pyruvate dehydrogenase, 2-ketoglutarate dehydrogenase and 3-ketoacyl-CoA thiolase reactions. Thus, upregulation of ACOT2 would constrain the beta-oxidation rate by limiting substrate supply and also maintain the level of coenzymes utilized in the TCA cycle and beta-oxidation to support complete fatty acid oxidation, especially when acyl-CoA is overloaded into mitochondria. Moreover, fatty acids released by ACOT2 are exported from the mitochondria *via* UCP3 to the cytosol where they can be reactivated by acyl-CoA synthetase. Thus, an additional futile cycle mediated by ACOT2 would be promoted.[7,8,21—23] In the futile cycle, AMP produced by acyl-CoA synthetase provides an adenine nucleotide substrate for proton gradient-driven ATP synthesis, which may serve to maintain the expected ETC activity.

Taken together, the upregulation of ACOT expression observed in the present study can be explained by adaptive induction of processes to prevent deranged mitochondrial fatty acid oxidation caused by prolonged high-fat exposure, and thus avoid mitochondrial stress that may cause contractile dysfunction in the heart and skeletal muscle. Increased expressions of ACOT1 and ACOT2 would reduce esterification of fatty acids to proinflammatory lipid metabolites and modulate mitochondrial fatty acid uptake and beta-oxidation to avoid impairing the balance between beta-oxidation and TCA cycle/ETC activity, while consuming ATP. Thus, upregulation of ACOT1 and ACOT2 may alleviate insulin resistance, while maintaining fatty acid oxidation at an adequate rate, even during fatty acid oversupply. Therefore, if ACOT expression can be favorably controlled, this may offer a novel strategy to relieve lipotoxicity and insulin resistance in obesity.

We have corroborated previous findings[20,21] concerning the upregulation of ACOT expression in rats fed a high-at diet, and extended the investigation by demonstrating the coordinated induction of ACOT7 isoforms for the first time. Since ACOT7 has acyl-CoA thioesterase activity that is two-orders of magnitude higher than those of ACOT1 and

ACOT2,[8,11] the increased expressions of these isoforms may have a profound impact on intracellular acyl-CoA disposal. The ACOT7 isoforms may also have similar contributions to those of ACOT1 and ACOT2 to correct acyl-CoA overload.[8,28] However, it is unclear whether ACOT7a with ACOT1 and ACOT7b with ACOT2 share roles in the respective intracellular compartments. Moreover, the mechanisms underlying the induction of ACOT7 in the heart and skeletal muscle are also unclear, while the induction of ACOT1 and ACOT2 seems to be mediated by PPAR alpha.[7,25,26] We have pointed out beneficial aspects of ACOT upregulation with respect to alleviation of lipotoxicity and insulin resistance. However, it has not been determined whether the lipotoxic conditions detrimental to obese animals are associated with maladaptation of ACOT expression, because our diet-induced obesity model fed a high-fat diet, but not a Western diet,[21] showed no apparent metabolic problems. Therefore, further studies are needed to establish the protective roles of ACOT against lipid-induced mitochondrial stress.

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