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Resveratrol attenuates high-fat diet–induced insulin resistance by influencing skeletal muscle lipid transport and subsarcolemmal mitochondrial ß-oxidation

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ABSTRACT

Although resveratrol (RES) is implicated in the regulation of insulin sensitivity in rodents, the exact mechanism underlying this effect remains unclear. Therefore, we sought to investigate how RES affects skeletal muscle lipid transportation and lipid oxidation of subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondrial populations in high-fat diet (HFD)–induced insulin resistance (IR) rats. Systemic and skeletal muscle insulin sensitivity together with expressions of several genes related to mitochondrial biogenesis and skeletal muscle lipid transportation was studied in rats fed a normal diet, an HFD, and an HFD with intervention of RES for 8 weeks. Citrate synthase (CS), electron transport chain (ETC) activities, and several enzymes for mitochondrial β-oxidation were assessed in SS and IMF mitochondria from tibialis anterior muscle. The HFD-fed rats exhibited obvious systemic and skeletal muscle IR as well as intramuscular lipid accumulation. SIRT1 activity and expression of genes related to mitochondrial biogenesis were greatly declined, whereas the gene for lipid transportation, FAT/CD36, was upregulated ($P < .05$). Subsarcolemmal but not IMF mitochondria displayed lower CS, ETC, and β-oxidation activities. By contrast, RES treatment protected rats against diet-induced intramuscular lipid accumulation and IR, increased SIRT1 activity and mitochondrial biogenesis, and reverted the decline in SS mitochondrial CS and ETC activities. Importantly, although expression of FAT/CD36 was increased (11%, $P < .05$), activities of SS mitochondrial β-oxidation enzymes were largely enhanced (41%~67%, $P < .05$). This study suggests that RES ameliorates insulin sensitivity consistent with an improved balance between skeletal muscle lipid transportation and SS mitochondrial β-oxidation in HFD rats.

1. Introduction

Many studies report that consumption of high-fat diet (HFD) leads to obesity, insulin resistance (IR), and ultimately type 2 diabetes mellitus [1-4]. At the cellular level, it seems that IR develops secondary to impaired mitochondrial biogenesis and function [5,6]. Yet there are inconsistencies and contradictions concerning the role of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), a critical regulator of mitochondrial biogenesis. There is evidence that modest overexpression of PGC-1α is sufficient to improve mitochondrial fatty acid oxidation, with a concomitant slight increase in fatty acid transport protein, FAT/CD36, in tibialis anterior (TA) muscle and improved insulin sensitivity [7]. However, PGC-1α transgenic

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mice showed increased mitochondrial density, which was associated with greatly upregulated expression of FAT/CD36. Surprisingly, increased muscle PGC-1α expression did not have any effect on (improve) fat-induced IR [8]. Thus, the role of different increase in PGC-1α levels in regulating insulin sensitivity is controversial; and mechanisms remain unresolved.

Resveratrol (RES), a SIRT1 agonist, is a natural polyphenolic compound mainly found in the skin of grapes [9]. More importantly, it is reported that RES could activate PGC-1α and ameliorate metabolic homeostasis in HFD mice [10,11]. However, the precise molecular mechanisms by which RES improves HFD-induced IR are not fully understood. Moreover, in these studies, the effects of RES on lipid transport were not determined. Thus, the main aim of our study was to observe whether RES influences the key molecules involved in skeletal muscle fatty acid delivery and mitochondrial fatty acid oxidation, therefore benefiting insulin sensitivity.

On the other hand, we can hypothesize that mitochondrial subpopulation may be an important consideration when examining IR and the underlying mechanisms because subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria exhibit different metabolic capacities and are not equally susceptible to developing IR [6]. Although various studies on RES and other SIRT1 agonists have revealed its modulatory effects on mitochondrial capacity [10,12,13], it has not been demonstrated yet whether these changes relate to alterations in skeletal muscle SS or IMF mitochondria. Therefore, another purpose of this study was to assess the effect of RES on SS and IMF mitochondrial tricarboxylic acid (TCA) cycle and electron transport chain (ETC) function as well as β-oxidation capacity in HFD-induced IR rats.

2. Materials and methods

2.1. Animals and diets

Male SD rats aged 8 weeks and housed individually in a controlled environment (22°C ± 3°C, 50%-60% relative humidity) with a 12:12-hour light-dark cycle were initially fed with normal chow and allowed to adapt to their environment for 1 week. After acclimatization, the rats were randomly divided into 3 groups (n =8) as follows: NC8 group (animals were fed ad libitum with normal chow for 8 weeks), HF8 group (animals were fed ad libitum with HFD for 8 weeks), and HF8E group (animals were treated with HFD and administered with RES for 8 weeks). Animals and diets were purchased from the Experimental and Animal Centre of Tongji Medical College affiliated to the Huazhong University of Science Technology (Wuhan, Hubei, China). The normal chow (13.68%, 64.44%, and 21.88% of calories derived, respectively, from fat, carbohydrate, and protein) was provided by the Laboratory Animal Center mentioned above. The HFD (containing, in terms of calories derived, 59% fat, 20% carbohydrate, and 21% protein) was made as described previously by our laboratory [14]. The rats in the HF8E group received an intragastric administration of RES (Sigma, St Louis, MO; 100 mg/kg body weight per day), which was mixed with physiological saline, whereas rats in the NC8 and HF8 groups were treated with physiological saline only. Body weight was analyzed at 0, 2, 4, 6, and 8 weeks after the beginning of diet and RES treatment. All the experimental procedures performed were approved by the Animal Ethics Committee in our university and carried out in accordance with the Hubei Province Laboratory Animal Care Guidelines for the use of animals in research.

2.2. Hyperinsulinemic-euglycemic clamp in conscious rats and measurement of 2-deoxy-D-glucose uptake in TA muscle

The hyperinsulinemic-euglycemic clamp in conscious rats and the rate of 2-deoxy-D-glucose (2-DG) uptake in TA muscle were determined as described previously by our laboratory [15]. In brief, 2-DG (Sigma-Aldrich, St Louis, MO) was administered as a bolus (2 mmol/kg in saline) at 45 minutes before the end of the 2-hour hyperinsulinemic (0.25 U/[kg h])-euglycemic clamp. Fasting blood samples were collected for plasma analyses. The average glucose infusion rate between the 60th and 120th minute ($GIR_{60\text{-}120}$) was used to evaluate insulin sensitivity. During the clamp procedure, plasma glucose was determined every 10 minutes. After 2-hour clamp, rats were killed by a lethal dose (150 mg/kg) of intraperitoneal pentobarbitone sodium. Anterior tibial muscles were rapidly dissected, and 2-DG uptake was then detected on an F-4500 fluorescence spectrophotometer (Hitachi Ltd, Tokyo, Japan) at emission of 615 nm with excitation of 530 nm.

2.3. Plasma analyses

Blood was transferred into tubes containing EDTA and centrifuged, and then the plasma was stored at –80°C for later analysis. Basal insulin was quantified by radioimmunoassay with a rat insulin enzyme-linked immunosorbent assay kit (Linco RIA rat insulin kit, St Charles, MO) as described by the manufacturer. Serum free fatty acid (FFA) and triglyceride (TG) measurements were performed by colorimetric enzymatic method (Wako nonesterified fatty acid C kit, Richmond, VA, and GPO-PAP, Roche Diagnostics, Indianapolis, IN, respectively).

2.4. Intramuscular lipid analysis

Muscle FFA determined calorimetrically (Wako nonesterified fatty acid C kit, Richmond, VA) and muscle TG content was measured as previously described [16]. Briefly, 50 mg of frozen TA muscle was extracted with saturated chloroform-methanol (2:1 vol/vol) and homogenized. After 2 hours of lipid extraction, ice-cold phosphate-buffered saline was added to each sample tube before centrifugation at 3000*g* for 15 minutes. The isolated lipids were then resuspended in ethanol, and the TG concentration was determined spectrophotometrically at 490 nm by use of an enzymatic method.

2.5. Preparation of TA muscle SS and IMF mitochondria and assay of citrate synthase activity

Tibialis anterior muscle homogenate, as well as SS and IMF mitochondria, was prepared as described previously [6]. Citrate synthase (CS) activity was assessed in duplicate or triplicate, and measurements were taken every 10 seconds over a 3-minute period at 412 nm at 30°C [6]. Citrate synthase activity, measured in the TA homogenate, was expressed as micromoles per

minute per milligram tissue protein. To determine CS specific activity, measurements were made in isolated SS and IMF mitochondria; and the results were expressed as micromoles per minute per milligram mitochondrial protein and also as micromoles per minute per milligram tissue protein. To exclude the possibility that changes in mitochondrial yield could result from loss of mitochondria during the isolation procedure, we also assessed the recovery of CS activity in the various fractions during the isolation procedure of SS and IMF mitochondria.

2.6. Measurement of individual complexes of the ETC activities in TA muscle mitochondria

Tibialis anterior muscle mitochondrial ETC activities of complexes I to IV were measured in mitochondrial preparations using commercial kits (Genmed Scientifics, Wilmington, DE, USA) and detected by WFJ7200 Spectrophotometer (Unico, Shanghai Instrument Co, Ltd, Shanghai, China). The oxidation of NADH by complex I (NADH dehydrogenase) was recorded using the ubiquinone as electron acceptor. The decrease in absorption was measured at 340 nm at 30°C in both the absence and presence of 5 μg/mL rotenone. The enzyme activity was measured for 3 minutes, with values recorded every 10 seconds after the initiation of the reaction. The complex II (succinate dehydrogenase) activity was determined by measuring the reduction of 2,6-dichlorophenolindophenol in absorbance at 600 nm at 30°C. The oxidation of CoQH2 by complex III (cytochrome *c* reductase) was determined using cytochrome *c* as an electron acceptor. The reaction was started with 10 μg of mitochondrial protein, and the enzyme activity was measured at 550 nm at 30°C. Finally, complex IV activity was determined by measuring the oxidation of cytochrome *c* at 550 nm at room temperature. The assay mixture for complex IV consisted of 10 mmol/L phosphate buffer (pH 7.4) and 20 mmol/L reduced cytochrome *c*. The results of complexes I to IV activities were expressed as nanomoles per minute per milligram tissue protein and nanomoles per minute per milligram mitochondrial protein.

2.7. Carnitine palmitoyltransferase–1, medium-chain acyl-CoA dehydrogenase, and long-chain acyl-CoA dehydrogenase activities

Specific activities of the different enzymes on 96-well plates were measured spectrophotometrically using commercially available kits (Genmed Scientifics). Carnitine palmitoyltransferase–1 (CPT-1) was measured at 412 nm, and medium-chain acyl-CoA dehydrogenase (MCAD) and long-chain acyl-CoA dehydrogenase (LCAD) were measured at 450 nm in isolated SS and IMF mitochondria. All assays were performed at 30°C. The results were expressed as nanomoles per minute per milligram tissue protein and nanomoles per minute per milligram mitochondrial protein.

2.8. TA muscle SIRT1 Activity

To determine if overexpressed SIRT1 by RES had biological activity, enzyme activity assay of SIRT1 was carried out using a commercial kit (Genmed Scientifics). The peptide substrate, consisting of amino acids 379 to 382 of human p53 conjugated to *p*-nitroaniline, was deacetylated and incubated for 60 minutes at 37°C. After release of *p*-nitroaniline using endopeptidase developer, optical absorption was measured at 405 nm. SIRT1 activity was estimated by the difference between tissue homogenate either in the presence or absence of NAD and expressed as micromoles *p*-nitroaniline per minute per milligram tissue protein.

2.9. Real-time polymerase chain reaction

To study progressive alterations in the expression of genes, real-time reverse transcription polymerase chain reaction (RT-PCR) analysis was performed. Total RNA was extracted using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. A 2-step RT-PCR method was used to synthesize single-stranded complementary DNA (Prime Script RT Reagent Kit; Perfect Real Time; TaKaRa Code RR037A, Otsu, Japan). Target genes were analyzed by real-time PCR using Applied Biosystems (Foster City, CA) 7500 Fast Real-Time PCR System with SYBR Green I dye (catalog no. 4309155). Temperature cycles were as follows: 95°C for 30 seconds followed by 42 cycles of 95°C for 5 seconds and 60°C for 30 seconds. The SYBR Green fluorescence was detected at the end of each cycle to monitor the amount of PCR product formed during that cycle. Oligonucleotide sequences of sense and antisense primers are shown in Table 1.

2.10. Statistical analysis

The statistical analysis was performed with SPSS 15.0 software (SPSS, Chicago, IL). All numeric variables are expressed as mean ± SE. Group statistical comparisons were assessed by 1-way analysis of variance; and individual comparisons, by Student-Newman-Keuls test. A P value < .05 was considered statistically significant.

3. Results

3.1. RES restored systemic and skeletal muscle insulin sensitivity

Although fasting blood glucose levels were not altered by HFD (Fig. 1A), fasting insulin (FINS) levels were significantly

Table 1 – Primer design for genes analyzed by real-time PCR

Gene	Primer sequence (5′-3′; forward, reverse)	GenBank accession no.
PGC-1α	GAGAACAAGACTATTGAGCGAAC GTGGAGTGGCTGCCTTGGGT	AF106698
NRF-1	GCTCTTTGAGACCCTGCTTTC GTGGAGTTGAGTATGTCCGAGT	XM011548
mTFA	CATTGGGATTGGGCACAAGAAG CTACTGTCAAGGCTCAAAAGGTC	M62810
FAT/CD36	CATTACTGGAGCCGTTATTGGT TGCTGCTATTCTTTGCCACTTC	NM031561
β-actin	CACCCGCGAGTACAACCTTC CCCATACCCACCATCACACC	NM012675

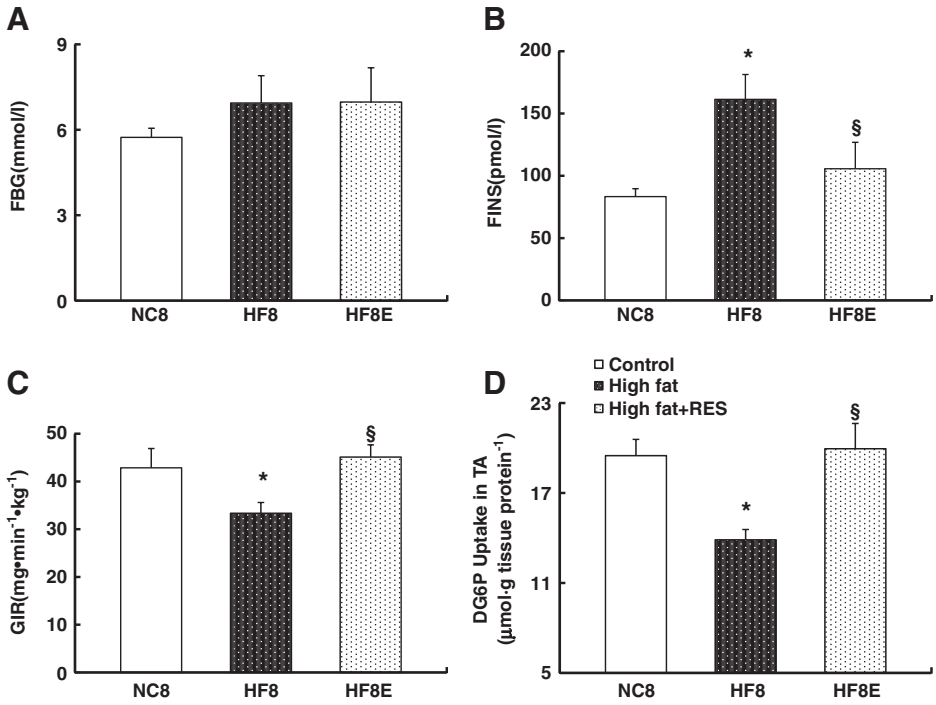

Fig. 1 – Fasting blood glucose (A), FINS (B), $GIR_{60\text{-}120}$ levels (C), and TA muscle 2-DG uptake (D) in the NC8, HF8, and HF8E groups. Values are mean ± SE (n =8). *P < .05 vs NC8; §P < .05 vs HF8.

increased ($P < .05$) (Fig. 1B); and the average $GIR_{60\text{-}120}$ at hyperinsulinemic-euglycemic clamp was decreased (~20%, $P < .05$; Fig. 1C), suggesting systemic IR. In addition, HFD rats showed a reduction in insulin-stimulated glucose uptake in TA muscles (~30%, $P < .05$; Fig. 1D), indicating skeletal muscle IR. Resveratrol treatment resolved both systemic and skeletal muscle IR as shown by a decrease in FINS levels and increased $GIR_{60\text{-}120}$ and TA muscle glucose uptake as compared with HFD rats ($P < .05$) (Fig. 1B-D).

3.2. *Resveratrol decreased TA muscle lipid deposition with a nonsignificant decrease in body weight but without affecting blood lipids*

Average body weight during the course of the experiment is given in Fig. 2A. Animals fed HFD for 8 weeks exhibited 11% increased body weight compared with normal controls ($P < .05$). Resveratrol treatment did induce a decrease in body weight in HFD rats, although the difference was statistically nonsignificant ($P = .057$; Fig. 2A). Blood lipids such as FFA (Fig. 2B) and TG (Fig. 2C) in the fasting state were much higher in the HF8 than in the NC8 group ($P < .05$). We also observed marked accumulation of FFA (Fig. 2D) and TG (Fig. 2E) in TA muscle in HF8 rats ($P < .05$). However, RES treatment had no major impact on blood lipids levels (Fig. 2B, C), but decreased intramuscular FFA and TG in HFD rats (15%~44%, $P < .05$; Fig. 2D, E).

3.3. *RES increased SS mitochondrial TCA and ECT functions*

Subsarcolemmal and IMF mitochondrial TCA and ETC functions of TA muscles at the end of the treatment were determined by using mitochondrial CS and complex I to IV activities, respectively. In the HF8 group, CS activity of homogenate per gram tissue protein was found to be lower than that in the NC8 group ($P < .05$) (Fig. 3A). Furthermore, activities of CS (Fig. 3B, C) and complexes I to IV (Fig. 4A, B) per gram tissue protein in isolated SS and IMF mitochondria declined. This difference was statistically significant for SS mitochondria but failed to reach statistical significance for IMF mitochondria. By contrast, after 8 weeks RES treatment, homogenate CS activity (Fig. 3A) and activities of SS mitochondrial CS (Fig. 3B, C) and complexes I to IV (Fig. 4A, B) per gram tissue protein reverted to normal state ($P < .05$). In addition, recovery of SS and IMF mitochondrial CS activity was quite consistent and comparable in all the experimental groups (Fig. 3D), indicating that the change in mitochondrial yield could not result from loss of mitochondria during the isolation procedure. Interestingly, CS and complexes I to IV specific activities per milligram mitochondrial protein in SS and IMF mitochondria were unchanged in all the groups (data not shown).

3.4. *RES enhanced SS mitochondrial ß-oxidation and TA muscle FAT/CD36 messenger RNA expression*

To explore possible mechanisms underlying the destroyed balance between skeletal muscle lipid transportation and mitochondrial β-oxidation of HFD rats, we measured the activity of CPT-1, MCAD, and LCAD, which are closely related to mitochondrial fatty acid β-oxidation [1,17], and the messenger RNA (mRNA) expression of FAT/CD36, a putative fatty acid transporter [18]. The CPT-1, MCAD, and LCAD activities per gram tissue protein were found to be lower in the HF8 group in isolated SS ($P < .05$; Fig. 5A) and IMF

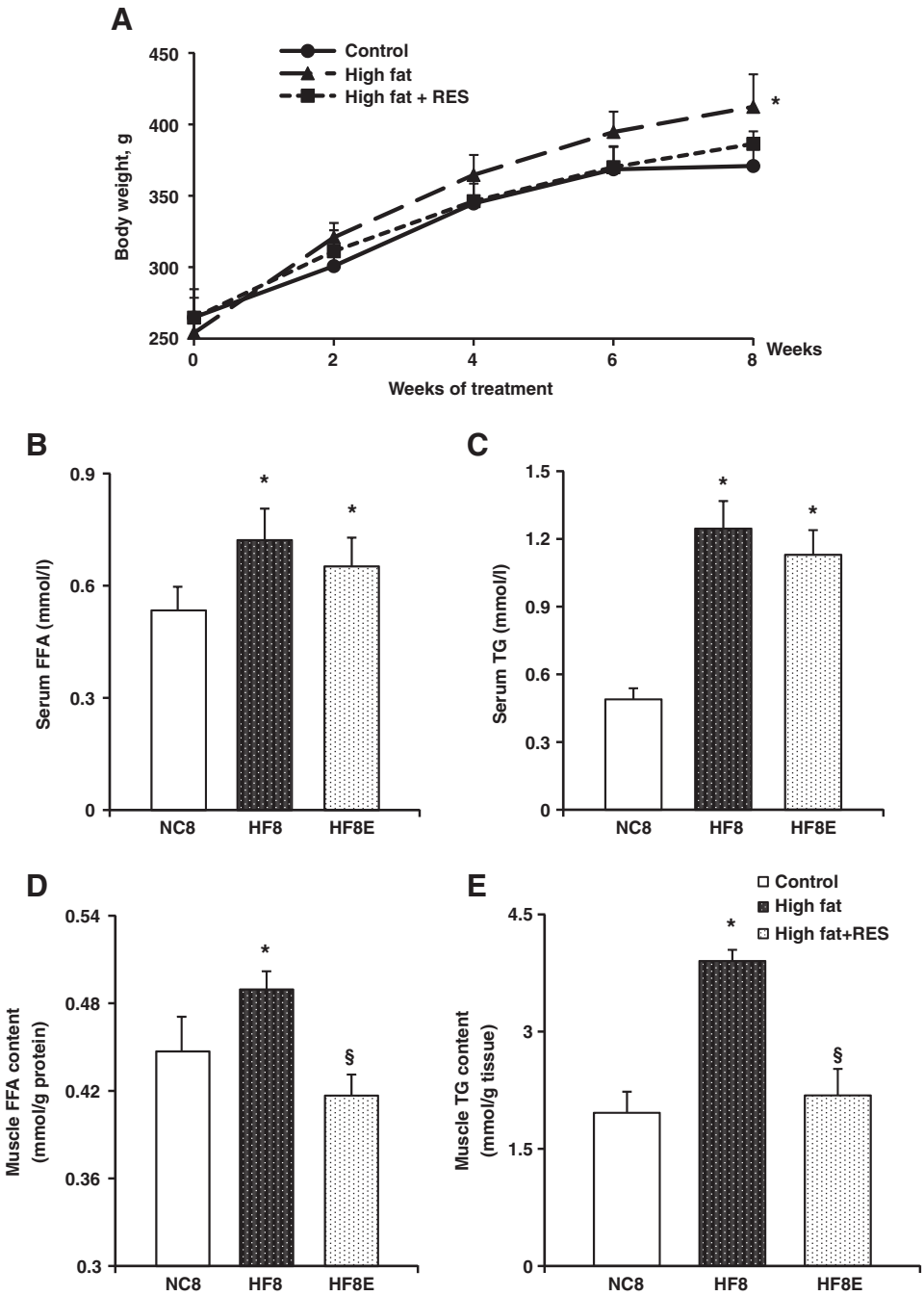

Fig. 2 – Body weight growth curves (A) in the NC8, HF8, and HF8E groups. Each point represents mean ± SE (n =8). Serum FFA (B) and TG (C) levels and TA muscle FFA (D) and TG (E) content in the NC8, HF8, and HF8E groups. Values are mean ± SE (n =8). *P < .05 vs NC8; §P < .05 vs HF8.

mitochondria ($P > .05$; Fig. 5B). However, after 8 weeks of RES treatment, CPT-1, MCAD, and LCAD activities per gram tissue protein of SS mitochondria were found to be much higher in the HF8E than in the HF8 group (41%~67%; $P < .05$) (Fig. 5A). In IMF mitochondria, although CPT-1, MCAD, and LCAD activities per gram tissue protein tended to be elevated by RES treatment, the increase was not statistically significant (Fig. 5B). In addition, CPT-1, MCAD, and LCAD specific activities per milligram mitochondrial protein in SS and IMF mitochondria were unchanged in the HF8 group. Notably, the specific activities per milligram mitochondrial protein of CPT-1, MCAD, and LCAD in the HF8E group were markedly increased (16%~36%; $P < .05$) (Fig. 5C) in SS mitochondria, whereas no differences were found in IMF mitochondria (data not shown). On the other hand, FAT/CD36 mRNA levels of TA muscle were increased by 25% in HF8 rats ($P < .05$) (Fig. 6A), with an additional increase after RES treatment (11%; $P < .05$) (Fig. 6A).

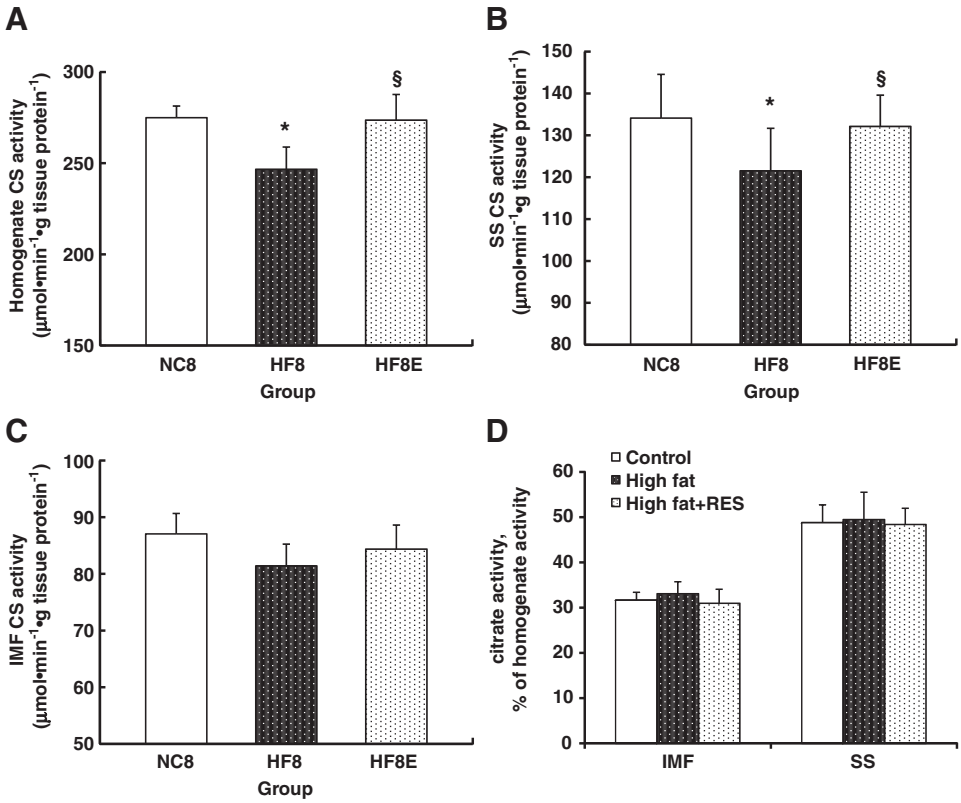

Fig. 3 – Homogenate (A), isolated SS (B), and IMF (C) mitochondrial CS activities in the NC8, HF8, and HF8E groups. D, Percentage recovery of CS activity during mitochondrial isolation procedure in IMF and SS mitochondria. Values are mean ± SE (n =8). *P < .05 vs NC8; §P < .05 vs HF8.

3.5. *RES treatment upregulated TA muscle SIRT1 activity and mitochondrial biogenesis*

Because RES is associated with upregulation of SIRT1 level and mitochondrial biogenesis in skeletal muscle [11], we therefore assessed the TA muscle SIRT1 activity and expressions of PGC-1α, nuclear respiratory factor–1(NRF-1), and mitochondria transcription factor A (mtTFA), which have been identified as key regulators of mitochondrial biogenesis [19]. The HF8 rats showed about 40% reduction in TA muscle SIRT1 activity (Fig. 6B) and significantly decreased PGC-1α, NRF-1, and mtTFA (Fig. 6C-E) expressions compared with the NC8 rats (18%~25%, $P < .05$). Conversely, oral administration of RES to HFD rats significantly normalized the altered SIRT1 activity as well as PGC-1α, NRF-1, and mtTFA expressions (Fig. 6B-E) ($P < .05$).

4. Discussion

Our study highlights an important role of RES in modulating the transporters and enzymes involved in skeletal muscle lipid transportation and mitochondrial lipid oxidation. Our data suggest that a physiologically relevant increase in skeletal muscle SIRT1 activity by RES is sufficient to prevent HFD-induced increased skeletal muscle lipid deposition, and systemic and peripheral (skeletal muscle) IR. The increase in SIRT1 activity is consistent with an improved balance between skeletal muscle lipid transportation and SS mitochondrial β-oxidation, and increased SS mitochondrial TCA and ETC functions.

It has been previously demonstrated that RES stimulates SIRT1-mediated deacetylation of PGC-1α, which improves systemic IR [11]. In this article, we showed that RES intervention resulted in increased levels of SIRT1 and PGC-1α; and there was an improvement not only in systemic IR but also in local IR. The effect of RES on peripheral IR is not unexpected, as supplementation of SRT1720, a small molecule but a more potent activator of SIRT1, to Zucker *fa/fa* rats for 4 weeks results in about 55% higher glucose disposal into skeletal muscle [12]. Thus, SIRT1 activation can be used as a potential therapeutic approach to combat both systemic and peripheral IR.

It is to be noted that the improvement in IR with RES was accompanied by a decrease in body weight that did not reach statistical significance. This may be possibly related to dosage and sample size. It has been demonstrated that the effect of RES on body weight is dose dependent [20]. Baur et al [10] demonstrated that 22.4 mg/(kg d) RES treatment for 1 year did not result in weight loss in HFD mice, whereas the 10- to 20-fold higher dose of RES for only 3 months used in the study of Lagouge et al [11] was sufficient to decrease body weight. Thus, it is suggested that weight loss is more relevant to dose rather than treatment course by RES. On the other hand, because the difference is very near to statistical significance, it is possible that had our sample size been larger, the decrease

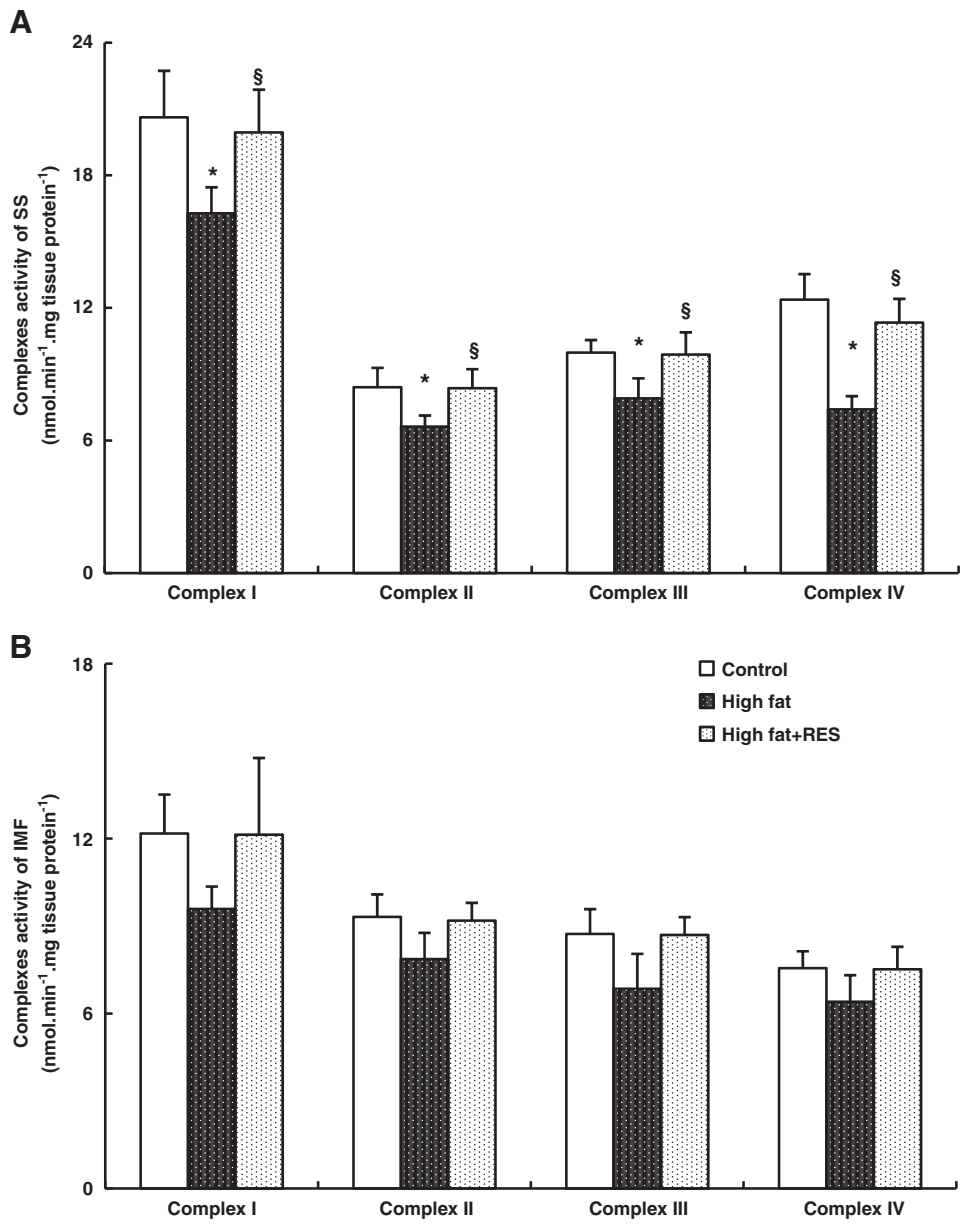

Fig. 4 – Analyses of TA muscle ETC complexes I to IV activities in isolated SS (A) and IMF (B) mitochondria in the NC8, HF8, and HF8E groups. Values are mean ± SE (n =8). $^{*}P < .05$ vs NC8; $^{\S}P < .05$ vs HF8.

in body weight could have become statistically significant. Although it has been shown that RES possesses antihyperglycemic properties that are independent of its effect on body weight [11], we cannot rule out that, in our study, the improvement in IR was not related to some extent to the decrease in body weight even if it was nonsignificant.

Because the molecular underpinnings of IR are known to be driven by disturbance of lipid metabolism, we investigated whether RES had any effect on circulating lipid levels and ectopic lipid deposition in the skeletal muscle. Although it was evident that, after an HFD, blood lipid and intramuscular lipid (IMCL) would be raised, we observed that RES intervention decreased only IMCL but not plasma lipid levels. Resveratrol has previously been shown to decrease hepatic lipid content in diet-induced obesity model [10], and its concentration in the liver is 8-fold higher than that in skeletal muscle [21]. The fact that RES induced a reduction in IMCL underscores its powerful pharmacological activities despite a low concentration in skeletal muscle. However, the observation that RES did not affect blood lipid levels in the current study is unexpected but consistent with previous reports [10,11] in HFD rats treated with RES and might be due to its low bioavailability in mammals [22]. Therefore, its role to directly reduce blood lipid might be limited, although the pharmacodynamics of the compound are yet to be fully understood.

Given the significant decrease in IMCL in the HFD rats treated with RES, we investigated whether this could be attributed to increased fatty acid oxidation in the skeletal muscle. Carnitine palmitoyltransferase-1 is a rate-limiting

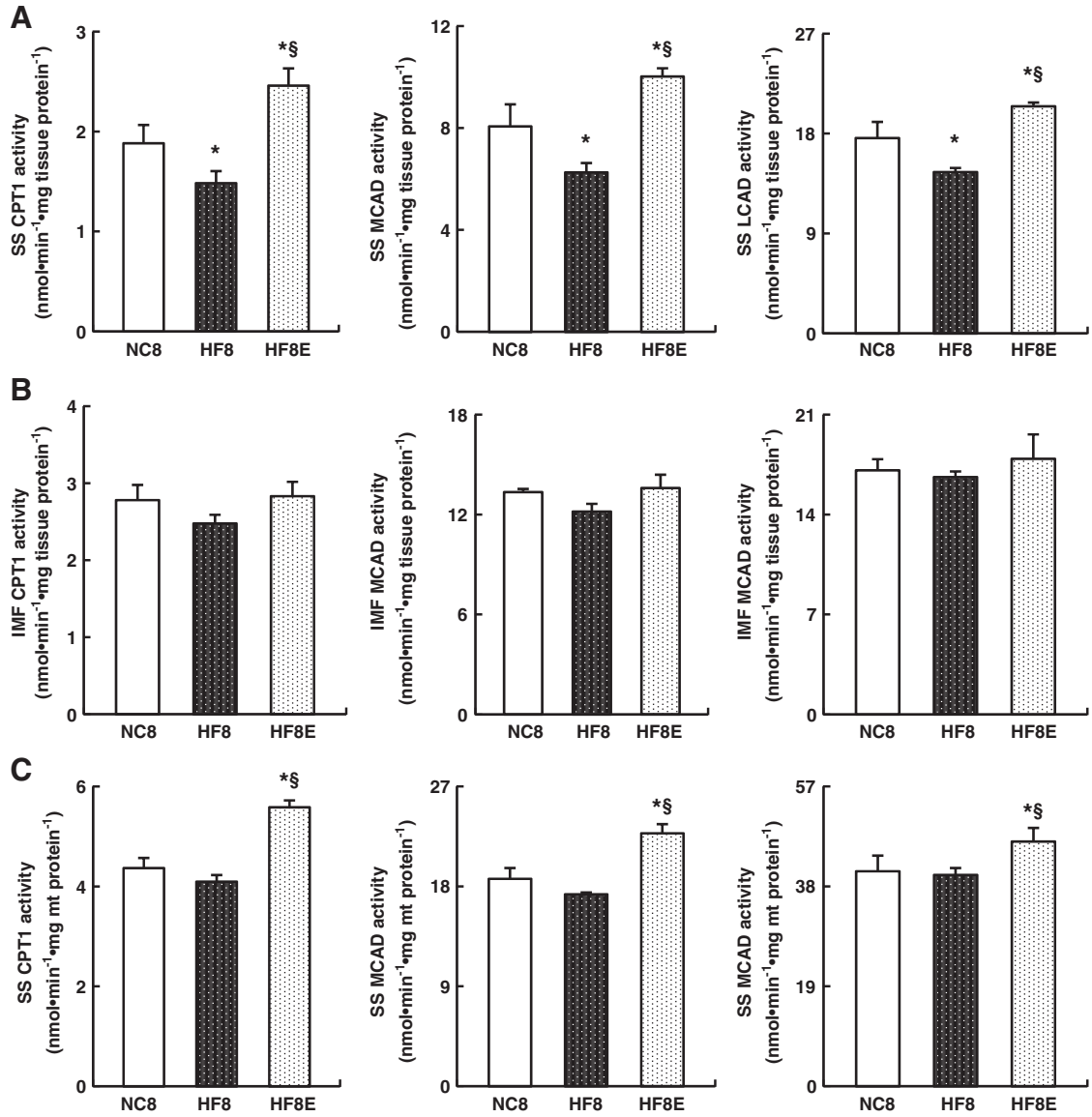

Fig. 5 – The CPT-1, MCAD, and LCAD activities per gram tissue protein in isolated SS (A) and IMF (B) mitochondria as well as the CPT-1, MCAD, and LCAD activities per milligram mitochondrial protein in isolated SS (C) mitochondria in the NC8, HF8, and HF8E groups. Values are mean ± SE (n =8). *P < .05 vs NC8; §P < .05 vs HF8.

enzyme for fatty acid entry into the mitochondria for β-oxidation [1], and MCAD and LCAD catalyze the first step of mitochondrial fatty acid β-oxidation [17]. Moreover, mitochondria in the skeletal muscle are of 2 types, SS and IMF; and each exhibit different metabolic capacities [23]. We found that the activities of SS mitochondrial CPT-1, MCAD, and LCAD, which were decreased by HFD, were not only reversed but also significantly enhanced so as to largely exceed the normal β-oxidation activities. On the other hand, neither HFD nor RES affected the IMF mitochondrial β-oxidation function. Furthermore, we used SS and IMF mitochondrial protein to standardize the β-oxidation activities and found that RES increased SS but not IMF mitochondrial specific β-oxidation function. Hence, our data show that RES targets the SS mitochondria but has no impact on IMF mitochondria, suggesting that the alterations in skeletal muscle SS mitochondria may have special significance for the pathogenesis of IR. This is not surprising because it is recognized that SS mitochondria provide energy for membrane-related processes that include substrate oxidation and insulin action, whereas the IMF mitochondria more directly support muscle contraction [6]. In addition, SS mitochondria may be more susceptible to damage by ectopic lipid deposition because lipid content decreases exponentially from immediately below the sarcolemma toward the central region of the muscle fiber [24]. Moreover, because of their proximity with the sarcolemmal membrane, SS lipids can more easily interfere with key proteins involved in the insulin signaling cascade than IMF lipids [25]. Furthermore, a previous study has demonstrated that mice treated with a powerful SIRT1 activator, SRT1720, displayed obviously increased CPT-1, MCAD, and LCAD expressions even in the

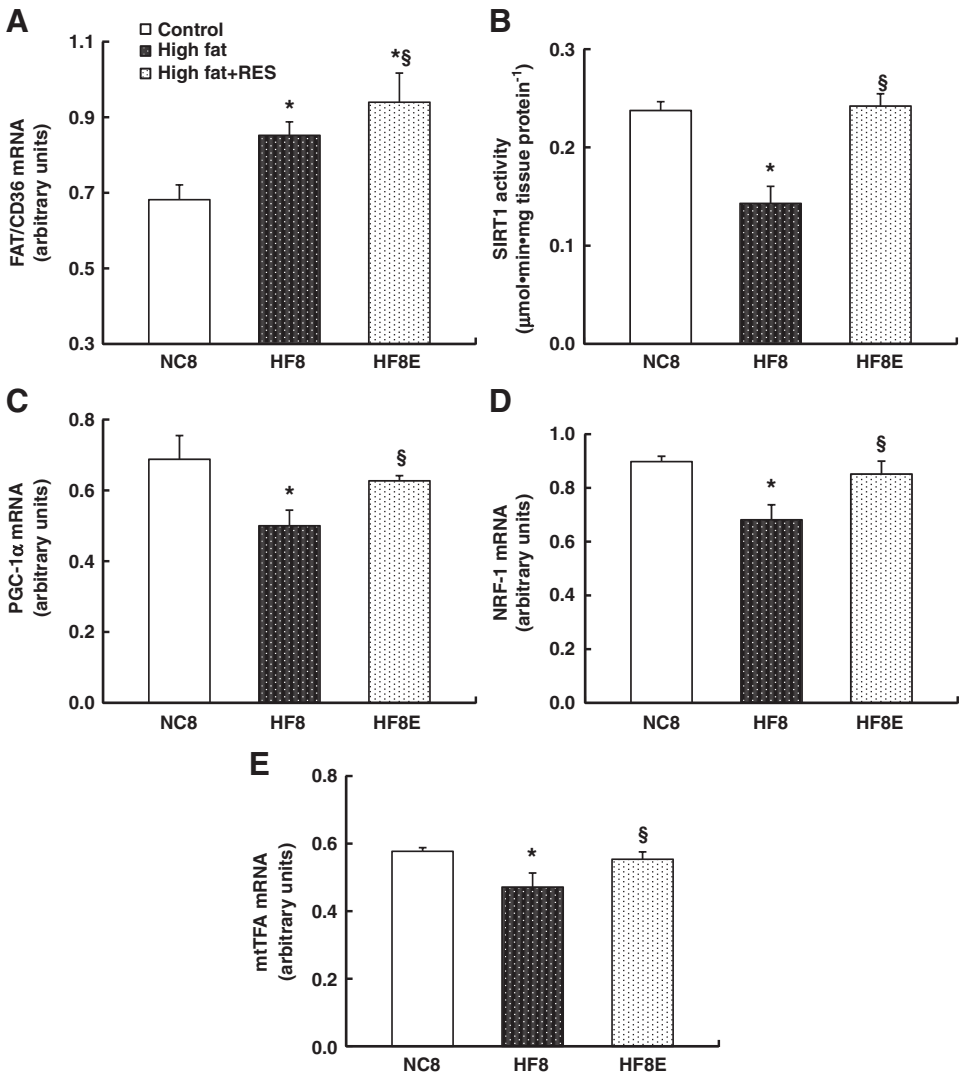

Fig. 6 – Tibialis anterior muscle FAT/CD36 mRNA expression (A); SIRT1 activity (B); and PGC-1α (C), NRF-1 (D), and mtTFA (E) mRNA expressions in the NC8, HF8, and HF8E groups. Values are mean ± SE (n =8). *P < .05 vs NC8; § P < .05 vs HF8.

absence of significant changes in PGC-1α and CS expressions in skeletal muscle [26]. Thus, we tentatively speculate that SIRT1 may directly enhance SS mitochondrial β-oxidation independent of upregulation of mitochondrial biogenesis in skeletal muscle.

Excess lipid uptake [27] and impaired disposal [28] may account, in part, for skeletal muscle lipid accumulation and the development of IR. It is recognized that the transport of FA into skeletal muscle is a highly regulated process mainly involving FA transporters, including plasma membrane-associated fatty acid-binding protein and FAT/CD36 [29]. In their study, Bonen et al [30] pointed out that, at week 6, palmitate transport rates were increased by about 70% in red muscles of ZDF rats, whereas FAT/CD36 protein was elevated by about 30% without any changes in plasma membrane-associated fatty acid-binding protein levels. Thus, the upregulation of FAT/CD36 appears to be a key mechanism contributing to fatty acid transport [31]. Vettor et al [32] previously found elevated muscle FAT/CD36 mRNA expression in lean Zucker rats after 3 hours of lipid infusion, and Koonen et al [27] reported an increase in FAT/CD36 protein levels in the skeletal muscle of mice fed an HFD. Although our study confirmed these previous findings, we also demonstrate that, after RES treatment, the expression of FAT/CD36 was further upregulated. It has been demonstrated that FAT/CD36 promoter contains a PPAR response element sequence, which may be a target of PGC-1α [33]. Therefore, increased expression of PGC-1α by SIRT1 activation in our study can at least partly explain the increased FAT/CD36 expression. Although RES increases fatty acid incorporation into skeletal muscle, it can significantly improve β-oxidation function even in a single SS mitochondrion. Thus, the balance between fatty acid delivery and fatty acid oxidation is maintained, reducing ectopic lipid deposition and finally ameliorating IR.

Resveratrol has previously been shown to increase mitochondrial number and biogenesis [10]. However, the effect of RES on TCA and ETC capacity of mitochondrial

subpopulations has not been considered. In our study, we observed that TCA and ETC activities that were decreased by HFD in SS mitochondria were back to normal levels after RES intervention, which is a novel finding. The activities in IMF mitochondria were not affected by HFD or RES, which is consistent with the results obtained for β-oxidation. However, a certain mismatch is evident for SS mitochondria in which, unlike the TCA and ETC activities, the markers for β-oxidation were greatly enhanced and not simply brought back to baseline. Moreover, standardization of CS and complexes I to IV activities using SS and IMF mitochondrial protein did not reveal any significant differences between each group, indicating that the TCA and ETC functions of individual mitochondrion were not affected by RES. Thus, our study is in support of the suggestion that β-oxidation is not proportionally matched by the TCA cycle [34] and that RES may effectively protect SS mitochondrial function by increasing total SS mitochondrial TCA cycle and ETC function rather than that of single SS mitochondria.

One of the essential regulators of mitochondrial biogenesis is PGC-1α [35]. Benton and colleagues [7] demonstrated that modest PGC-1α overexpression in muscle in vivo increased mitochondrial lipid oxidation rate and ameliorated IR, which is consistent with our study. On the other hand, Choi et al [8] reported that PGC-1α transgenic mice showed deleterious effects on insulin action, which is in contrast with the data reported here. They demonstrated that excessive PGC-1α expression may lead to a relative increase in skeletal muscle fatty acid uptake, which would exceed mitochondrial fatty acid oxidation. This, in turn, overcomes the benefits arising from increased PGC-1α expression. However, it is to be noted that their study demonstrated a 3-fold increase in PGC-1α and a 2-fold upregulation of FAT/CD36. With RES intervention in our study, the overexpression of PGC-1α was approximately 25%; and FAT/CD36 upregulation was only 11%. Taken together, these 2 conflicting conclusions could most likely be attributed to different effects on lipid transport caused by different increases in PGC-1α levels. There seems to be a threshold for PGC-1α upregulation below which insulin sensitivity is preserved and beyond which it worsens it. Our study thus provides evidence that RES treatment is a potential therapeutic way to combat IR by having an advantage over exclusive PGC-1α upregulation.

We have to point out yet that our investigations are indirect assessments of lipid transport and oxidation, and the interpretation of our results should therefore be cautious. Furthermore, RES, which is not a pure agonist of SIRT1, also stimulates AMPK activity; and many of the biomarkers measured in this study (mitochondrial biogenesis and CS activities) are in fact also regulated by AMPK [36]. In addition, RES can target the whole body and may improve insulin sensitivity possibly by acting on other target organs of insulin. Therefore, additional experiments are needed to make a model that specifically activates or blocks SIRT1 in skeletal muscle to confirm the effective power of SIRT1 on the improvement of systemic and skeletal muscle IR in HFD rats.

In summary, we demonstrate RES has a profound anti-IR action in HFD-induced obese animal models consistent with improved balance between skeletal muscle lipid transport and SS rather than IMF mitochondrial β-oxidation. In brief, upregulated PGC-1α expression by SIRT1 activation increases lipid incorporation into skeletal muscle and SS mitochondrial biogenesis that would increase SS mitochondrial TCA, ETC, and fatty acid oxidation functions. In addition, RES and/or SIRT1 may increase skeletal muscle lipid oxidation through other pathways independent of PGC-1α. As a result, increased fatty acid oxidation is sufficient to match the elevated skeletal muscle lipid transport, thereby preventing cellular lipid accumulation. With fewer lipid molecules around, the damage to SS mitochondria induced by HFD was

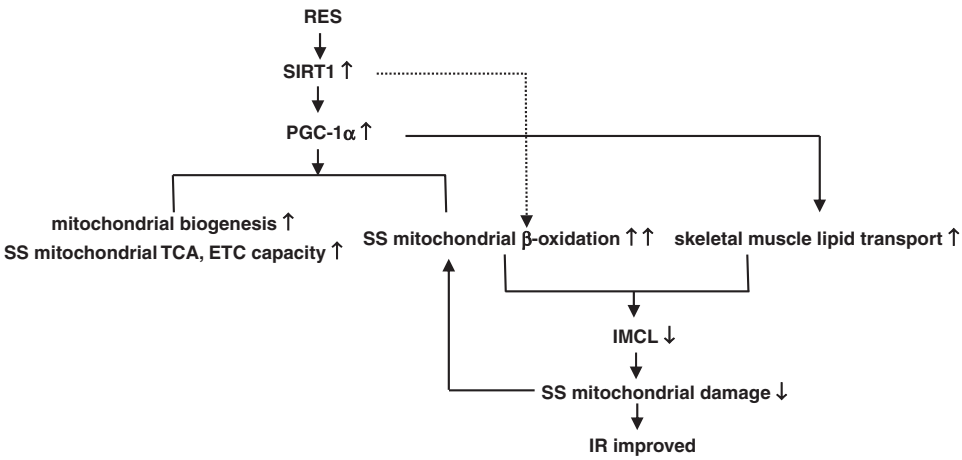

Fig. 7 – Proposed pathway by which RES influences mitochondrial biogenesis, SS mitochondrial fatty acid oxidation, and skeletal muscle lipid transport. Increased SIRT1 activity by RES upregulates PGC-1α, which would increase mitochondrial biogenesis, SS mitochondrial number, and especially SS mitochondrial fatty acid oxidation. Although upregulated PGC-1α also increases lipid incorporation into skeletal muscle, the balance between SS fatty acid oxidation and skeletal muscle lipid transport is ameliorated, thereby preventing cellular lipid accumulation. With fewer lipids around, the damage of SS mitochondria is reduced, leading to increased SS mitochondrial fatty acid β-oxidation. Subsequently, IMCL is further decreased; IR is ultimately improved.

subsequently reduced, leading to increased SS mitochondrial β-oxidation and further decrease in IMCL. In addition, a decline in lipids around SS mitochondria will not interfere with insulin signaling pathway, thereby improving IR (Fig. 7). Collectively, our study is the first to demonstrate that RES could increase not only total SS mitochondrial TCA and ETC capacities but also β-oxidation function of single SS mitochondrion. Importantly, RES probably plays a critical role in keeping the balance between fatty acid flux into skeletal muscle and SS mitochondrial β-oxidation in HFD rats. Our results assist us in further understanding the underlying molecular mechanisms of RES in the improvement of HFD-induced metabolic disorders.

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Conflict of Interest

The authors declare that they have no conflict of interest regarding this manuscript.

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