Adiponectin treatment improves insulin resistance in mice by regulating the expression of the mitochondrial-derived peptide MOTS-c and its response to exercise via APPL1–SIRT1–PGC-1 α

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Abstract

Aims/hypothesis Adiponectin stimulates mitochondrial biogenesis through peroxisome proliferator-activated receptor- γ coactivator 1 α (PGC-1 α), a major regulator of mitochondrial biogenesis. MOTS-c (mitochondrial open reading frame of the 12S rRNA) is a biologically active mitochondrial-derived peptide encoded by mitochondrial DNA. It influences the mechanisms of obesity and diabetes. We hypothesised that the adiponectin pathway may regulate the production and/or secretion of MOTS-c in skeletal muscle. We aimed to determine whether exercise and adiponectin affect MOTS-c to improve insulin resistance in mice.

Methods To investigate this hypothesis, we used wild-type C57BL/6 mice subjected to high-fat diet, an exercise regimen, and i.p. injection of recombinant mouse adiponectin (Acrp30) or MOTS-c, and adiponectin knockout (*Adipoq*^{−/−}) mice (C57BL/6 background) subjected to i.p. injection of Acrp30. C2C12 myotubes were also treated with sirtuin 1 (SIRT1) inhibitor, PGC-1 α inhibitor, SIRT1 activator, plasmid-expressed active APPL1 (adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper), pcDNA-SIRT1, or siRNA against APPL1, SIRT1 or PGC-1 α .

Results In *Adipoq*^{−/−} mice, MOTS-c levels in the plasma and skeletal muscle were downregulated. In C2C12 myotubes, adiponectin increased the mRNA expression of MOTS-c. APPL1 protein level following adiponectin treatment positively correlated with MOTS-c protein and mRNA levels in C2C12 myotubes. SIRT1 overexpression increased the adiponectin-induced mRNA and protein expression of MOTS-c, SIRT1 and PGC-1 α . Pharmacologic and genetic inhibition of PGC-1 α suppressed the increases in MOTS-c mRNA and protein levels induced by SIRT1 overexpression. In mice, plasma and skeletal muscle MOTS-c levels were significantly downregulated following high-fat-diet. Exercise and i.p. Acrp30 or MOTS-c increased MOTS-c levels and adiponectin mRNA and protein expression in the plasma and skeletal muscle.

Conclusions/interpretation Our findings showed that the APPL1–SIRT1–PGC-1 α pathway regulates the production and/or secretion of skeletal muscle MOTS-c by mediating adiponectin signalling. Our study provides an insight into the cellular and molecular pathways underlying the pathogenesis of diabetes and shows that MOTS-c is a potential novel therapeutic target in the treatment of diabetes.

Keywords Adiponectin · Diabetes · Exercise-mediated signalling · Mitochondrial biogenesis · MOTS-c · Skeletal muscle

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Abbreviations

Acrp30	Recombinant mouse adiponectin
AMPK	AMP-activated protein kinase
APPL1	Adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1
HFD	High-fat diet
KO	Knockout
MOTS-c	Mitochondrial open reading frame of the 12S rRNA-c
mtDNA	Mitochondrial DNA
PGC-1 α	Peroxisome proliferator-activated receptor- γ coactivator 1 α

Research in context

What is already known about this subject?

- Plasma concentrations of adiponectin and MOTS-c are downregulated in people with type 2 diabetes
- MOTS-c prevents diet-induced obesity and insulin resistance
- The protective effects of MOTS-c against the detrimental effects caused by a high-fat diet display many similarities to those of regular exercise

What is the key question?

- Does adiponectin improve insulin resistance by regulating the expression of MOTS-c via APPL1–SIRT1–PGC-1 α ?

What are the new findings?

- This is the first study to indicate that adiponectin is a regulator of MOTS-c in C57BL/6 mice
- Our research preliminarily unravels the signalling mechanism by which exercise increases MOTS-c expression and improves insulin resistance in C57BL/6 mice
- We also found that MOTS-c is autoregulated and is affected by adiponectin

How might this impact on clinical practice in the foreseeable future?

- These findings deepen our understanding of the cellular and molecular pathways underlying the pathogenesis of diabetes, and suggest that MOTS-c has the potential to be a novel therapeutic target in diabetes

sgRNA	Single guide RNA
siRNA	Small interfering RNA
SIRT1	Sirtuin 1

Introduction

Mitochondrial open reading frame of the 12S rRNA-c (MOTS-c) is a biologically active mitochondrial-derived peptide encoded by a short open reading frame in the mitochondrial DNA (mtDNA) [1]. Notably, MOTS-c has been recently demonstrated to regulate insulin sensitivity and metabolic homeostasis [2]. MOTS-c associates positively with insulin resistance, predominantly in lean individuals but not in those with obesity, whereas circulating MOTS-c levels are reduced in male, but not in female, children and adolescents with obesity [3, 4]. In addition, circulating MOTS-c and adiponectin levels are reduced and significantly associated with HbA_{1c} in people with type 2 diabetes [5]. In turn, obesity and type 2 diabetes increase adipose tissue adiponectin but decrease plasma adiponectin [6]. Collectively, these data support the hypothesis that plasma MOTS-c levels may be associated positively with adiponectin levels in individuals with insulin resistance and type 2 diabetes.

Adiponectin is a hormone derived from adipocytes and skeletal muscle that regulates energy homeostasis by increasing insulin sensitivity [7, 8]. Ample evidence suggests that adiponectin improves oxidative metabolism in skeletal muscle through its receptors and downstream signals [7, 9–11]. Adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1 (APPL1) is the first identified signalling molecule that mediates

adiponectin function [12]. In mammalian cells, APPL1 interacts with adiponectin receptors and mediates AMP-activated protein kinase (AMPK) and p38-mitogen-activated protein kinase (MAPK) activation, thereby regulating adiponectin signalling and adiponectin-mediated downstream events [12–15]. Notably, studies using mouse primary hepatocytes have confirmed the interaction between APPL1 and sirtuin 1 (SIRT1) [16]. SIRT1 is a member of the sirtuin family of protein deacetylases and plays an important role in metabolic regulation. The deacetylase activity of SIRT1 is controlled by the NAD⁺/NADH ratio; thus, SIRT1 is considered to be a sensor of cellular metabolic status [17].

A recent study has shown that, in skeletal muscle, SIRT1 deacetylates peroxisome proliferator-activated receptor- γ coactivator 1 α (PGC-1 α) [18], a transcriptional coactivator that plays an important role in mitochondrial biogenesis. Moreover, the SIRT1 activator SRT1720 increases PGC-1 α levels [19, 20]. Similarly, adiponectin intervention in primary human myotubes results in increased PGC-1 α , along with mitochondrial biogenesis, as evinced by increased fatty acid oxidation, mtDNA content and citrate synthase levels [21]. Additionally, studies have shown that the adiponectin level is also closely related to mtDNA content in individuals with a family history of type 2 diabetes; these individuals also exhibit skeletal muscle insulin resistance and mitochondrial dysfunction [22]. Collectively, these data suggested a link between adiponectin signalling and MOTS-c. Therefore, we speculated that adiponectin signalling might regulate the production and/or secretion of mitochondrial MOTS-c, at least partially through PGC-1 α [1, 7–21].

Moreover, MOTS-c may serve as a mitochondrial signal that mediates exercise-induced mitohormesis, thereby stimulating physiological adaptation and increasing tolerance to exercise [23–25]. Notably, the protective effects of MOTS-c against the detrimental effects caused by a high-fat diet (HFD) display many similarities to those of regular exercise [26]. Furthermore, MOTS-c increases the cellular level of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), an AMPK agonist, thereby activating AMPK [2], a regulator of gene expression, metabolism and adaptation in response to exercise [27–29]. Consequently, it has been speculated that MOTS-c constitutes an exercise mimetic and may improve exercise capacity [30].

This study aimed to investigate: (1) whether adiponectin regulates the production and/or secretion of MOTS-c in skeletal muscle cells by increasing the levels of APPL1, SIRT1 and PGC-1 α ; and (2) whether exercise can stimulate MOTS-c production by skeletal muscle and result in a parallel increase in the systemic level of MOTS-c.

Methods

Experimental animals All animal experiments in this study were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Research Ethics Committee of China Medical University. A total of 20 male 12-week-old *Adipoq* knockout (*Adipoq*^{−/−}; KO) mice (C57BL/6 background) and their wild-type littermates [31] were obtained from the Shanghai Nanfang Research Center for Model Organisms (Shanghai, China). Five-week-old male C57BL/6 mice (the most widely used genetic background for genetically modified mice used as models of human disease) were purchased from Liaoning Changsheng Biotechnology (Liaoning, China). The C57BL/6 male mice were randomly allocated to two groups, receiving either a standard chow diet (13.9% of digestible energy derived from lipids; $n = 80$) or a HFD (40% of digestible energy derived from lipids; $n = 80$) [32]. Diets were purchased from Jianmin Company (Shenyang, China). After 11 weeks of HFD feeding, the mean weight of the mice in the HFD group was 20% higher than that of the standard chow control group, which indicated successful HFD model establishment [33]. The control and HFD C57BL/6 groups underwent exercise training and received recombinant mouse adiponectin (Acrp30, i.p. 28 $\mu\text{g kg}^{-1} \text{ day}^{-1}$ [34], Novoprotein, China) or vehicle (i.p. 0.9% sodium chloride [saline] injection, 0.5 ml/day) interventions for the last week of exercise training, or MOTS-c (i.p. 0.5 $\text{mg kg}^{-1} \text{ day}^{-1}$ [2], China Peptides, China) or vehicle (i.p. saline 0.5 ml/day) for 8 weeks. The *Adipoq*^{−/−} group and wild-type littermates received Acrp30 (i.p. 28 $\mu\text{g kg}^{-1} \text{ day}^{-1}$) or vehicle intervention alone for 1 week. The experimental set-up is shown in Fig. 1.

The mice were housed in a room under controlled temperature ($23 \pm 1^\circ\text{C}$) with free access to water. Pellets were replaced twice weekly, and weights and food consumption were recorded weekly and daily, respectively. Details of the exercise training were as follows: following 2 days of treadmill running for adaptation, the mice ran for 1 h at a speed of 20 $\text{min}^{-1} \text{ day}^{-1}$ for 5 days each week. Blood glucose was measured from the tail vein after 12 h of fasting, using a blood glucose monitoring system (Sinocare, Hunan, China). The exercise training lasted for 8 weeks. Mice were anaesthetised by i.p. injection of sodium pentobarbital (50 mg/kg body weight; Sinopharm Chemical Reagent, Shanghai, China). Plasma and muscle tissue were collected at 36–48 h after the last exercise session, the samples were frozen in liquid nitrogen, and then stored in a -80°C freezer.

Measurement of MOTS-c-related proteins and plasma hormone levels The levels of insulin, adiponectin and MOTS-c in the plasma along with the levels of MOTS-c-related proteins in muscle and cells were measured using ELISAs according to the manufacturer's instructions (Cloud-Clone Corp., Wuhan, China; full details provided in electronic supplementary material [ESM] Table 1). The intra-batch coefficients of variance were 6.1% (insulin), 6.8% (adiponectin) and 5.5% (MOTS-c); the inter-batch coefficients of variance were 9.2% (insulin), 8.3% (adiponectin) and 10.2% (MOTS-c). MOTS-c protein levels in muscle tissues and cells were normalised to total protein.

Cell culture C2C12 myoblasts were purchased from the Stem Cell Bank of the Chinese Academy of Sciences (Shanghai, China [mycoplasma negative]). The cell line were authenticated by STR (short tandem repeat). The C2C12 cells were routinely cultured in DMEM supplemented with 10% FBS (HyClone, Logan, UT, USA) in a humidity-controlled, 5% CO_2 environment at 37°C . When the cells reached 90% confluence, they were incubated in DMEM supplemented with 2% horse serum (Gibco, Gaithersburg, MD, USA) to induce C2C12 differentiation. The medium was replaced daily. The cells were stimulated with different molecules based on their timing of action as follows: adiponectin (5 $\mu\text{g/ml}$, 24 h), MOTS-c (10 $\mu\text{mol/l}$, 24 h), SR18292 (an inhibitor of PGC-1 α 10 $\mu\text{mol/l}$, 18 h), SRT1720 (an activator of SIRT1 5 $\mu\text{mol/l}$, 24 h), or EX527 (an inhibitor of SIRT1 1 $\mu\text{mol/l}$, 48 h). Multinucleated myotubes were obtained after 4 days of differentiation.

Transfection The plasmids pcDNA-SIRT1 and pcDNA-PGC-1 α were generated by subcloning the respective DNA into the pcDNA3.1 vector (Gene Pharma, Shanghai, China). Plasmid pLDNA-APPL1 was created by subcloning the DNA into a lentiviral vector provided by ABM (Richmond, BC, Canada). Small interfering RNAs (siRNAs) specifically targeting

Fig. 1 Animal experimental scheme. CON, control; EXE, exercise; WT, wild-type

murine SIRT1 and PGC-1 α and scrambled siRNA control were purchased from Gene Pharma. APPL1–single guide RNA (sgRNA) CRISPR and scrambled sgRNA control were designed and synthesised by ABM. The day following differentiation, cells were transfected with pcDNA-SIRT1, pcDNA-PGC-1 α , pLDNA-APPL1, and/or siRNAs and sgRNAs using Lipofectamine3000 (Invitrogen, Carlsbad, CA, USA). After 48 h, SIRT1, PGC-1 α and APPL1 protein and mRNA expression were examined by western blotting and PCR, respectively.

Western blotting Protein lysates from mouse skeletal muscle and C2C12 cells were analysed by western blotting, as previously described [35]. Western blotting [35] was performed at

4°C overnight using primary antibodies against the following proteins: adiponectin, APPL1, PGC-1 α , GAPDH (all from Abcam, Cambridge, UK), SIRT1 (Bioss Antibodies, Shanghai, China), and β -Tubulin (Affinity Biosciences, Cincinnati, OH, USA). Antibodies diluted in tris-buffered saline containing 0.05% Tween (TBS-T). Blots were developed by enhanced chemiluminescence, digitalised with a scanner (MicroChemi4.2, DNR, Neve Yamin, Israel) and analysed by densitometry with ImageJ Software.

RNA extraction and quantitative RT-PCR Total RNA was extracted from mouse skeletal muscle and C2C12 cells using TRIzol reagent (Tiangen, Beijing, China). The total RNA (1 μ g) was reverse transcribed into first-strand cDNA using

Fig. 2 Adiponectin increases the expression of plasma and skeletal muscle MOTS-c. Male wild-type (WT) and *Adipoq*^{-/-} C57BL/6 mice received an i.p. injection of Acrp30 (28 μg kg⁻¹ day⁻¹) or saline for 1 week. (**a**, **b**) MOTS-c levels in (**a**) the circulation and (**b**) skeletal muscle were measured by competition inhibition ELISA. MOTS-c levels

in the muscle were normalised to total protein. (**c**–**f**) Western blot analysis of APPL1, SIRT1 and PGC-1α protein expression in the skeletal muscle. In vivo treatments were repeated in at least three independent experiments. All data are expressed as the means ± SD. Student's *t* test: **p* < 0.05, ***p* < 0.01

PrimeScript RT reagent Kit (TaKaRa, Dalian, China), in a 20 μl reaction mixture. Quantitative RT-PCR was run using TB-Green Premix Ex-Taq II (TaKaRa, Dalian, China), as previously described [36]. Target gene expression was normalised to the amount of β-tubulin or GAPDH and data were analysed based on the 2^{-ΔΔct} method. The specific primers used to amplify the following genes are shown in ESM Table 2: *Adipoq*, *Appl1*, *Sirt1*, *Ppargc1a* (encoding PGC-1α), *Mots-c*, *Tubb3* (encoding β-tubulin) and *Gapdh*.

Statistical analysis All data shown are representative of at least three independent experiments and sample replication of in vivo data was obtained from individual mice. Randomisation and blinding to group assignment and outcome assessment were not carried out in these studies. No results were intentionally removed; data were excluded if they were outside the standard curve range, the sample volume was insufficient or the sample was lost during the experiment. Statistical analyses were performed using GraphPad Prism 8 (GraphPad, San Diego, CA, USA). All results were expressed as mean ± SEM. Student's *t* test was used to assess differences between two groups, and ANOVA was used for multigroup difference analysis. All tests were two-sided and *p* < 0.05 was considered statistically significant.

Results

Adiponectin increases MOTS-c expression in the plasma and skeletal muscle Ramanjaneya et al [5] reported that the serum

concentrations of adiponectin and MOTS-c were downregulated in patients with type 2 diabetes. However, the relationship between adiponectin and MOTS-c remains unclear. We employed adiponectin KO (*Adipoq*^{-/-}) mice to investigate whether changes in adiponectin were related to MOTS-c levels. Consistent with findings in a previous report [37], the mean weight and the plasma insulin level of *Adipoq*^{-/-} mice were not significantly different from those of control mice (ESM Fig. 1a,b). Notably, plasma and skeletal muscle MOTS-c protein (Fig. 2a,b) and mRNA (ESM Fig. 1c) were downregulated in *Adipoq*^{-/-} mice. Adiponectin intervention with Acrp30 restored the protein or mRNA expression of plasma and skeletal muscle MOTS-c (Fig. 2a,b and ESM Fig. 1c) in *Adipoq*^{-/-} mice. Moreover, adiponectin treatment reversed the decrease of protein expression of APPL1, SIRT1 and PGC-1α in skeletal muscle of *Adipoq*^{-/-} mice (Fig. 2c–f). These findings clearly indicated that plasma MOTS-c depends on adiponectin levels, and this may be related to the expression of APPL1, SIRT1 and PGC-1α.

Adiponectin regulates MOTS-c expression in C2C12 myotubes by increasing APPL1, SIRT1, and PGC-1α As plasma and skeletal muscle MOTS-c protein levels were downregulated in *Adipoq*^{-/-} mice, we next conducted a detailed investigation of the mechanism by which the adiponectin pathway might regulate the production and/or secretion of skeletal muscle MOTS-c. First, C2C12 myotubes were treated with adiponectin at different concentrations (2.5, 5 or 10 μg/ml) for 24 h, or with 5 μg/ml of adiponectin for different periods (12, 24 or 48 h). The results show that the protein and mRNA

expression of MOTS-c increased significantly following 5 $\mu\text{g/ml}$ Acrp30 treatment (Fig. 3a); the mRNA expression levels of *Appl1*, *Sirt1* and *Ppargc1a* were also increased with 5 $\mu\text{g/ml}$ of adiponectin for 24 h (Fig. 3b). These findings show that adiponectin increases the mRNA expression of *Mots-c* in C2C12 myotubes, with parallel increases in the mRNA expression levels of *Appl1*, *Sirt1* and *Ppargc1a*.

Next, we sought to determine whether adiponectin-regulated skeletal muscle MOTS-c expression was mediated by APPL1. We transfected C2C12 myotubes with an APPL1 overexpression plasmid and then treated them with 5 $\mu\text{g/ml}$ of adiponectin for 24 h. Compared with the control treatment, adiponectin treatment significantly increased the protein and mRNA expression of MOTS-c, as did APPL1 overexpression (Fig. 3g and ESM Fig. 2a). Moreover, APPL1 overexpression further increased

the protein and mRNA expression of APPL1, SIRT1 and PGC-1 α induced by adiponectin (Fig. 3c–f and ESM Fig. 2a). In contrast, after knockdown of APPL1, the adiponectin-induced protein and mRNA expression of MOTS-c, APPL1, SIRT1 and PGC-1 α was suppressed (Fig. 3h–l and ESM Fig. 2b). These data indicate that APPL1 mediates adiponectin-regulated expression of MOTS-c and proteins of the adiponectin pathway.

We next evaluated whether adiponectin regulates PGC-1 α expression in skeletal muscle by increasing SIRT1. C2C12 myotubes treated with a SIRT1 inhibitor (EX527) showed reduced protein and mRNA expression of PGC-1 α compared with that of the control group (Fig. 4a–c and ESM Fig. 3a). Adiponectin reversed the downregulated PGC-1 α protein expression in EX527-treated C2C12 myotubes (Fig. 4a–c and ESM Fig. 3a). Notably, adiponectin upregulated the

Fig. 3 Adiponectin regulates MOTS-c expression in C2C12 myotubes by increasing APPL1, SIRT1 and PGC-1 α . **(a)** C2C12 myotubes were treated with Acrp30 at the indicated concentrations for 24 h. **(b)** Differentiated C2C12 cells were treated with Acrp30 (5 $\mu\text{g/ml}$) for the indicated periods. The mRNA expression levels of *Appl1*, *Sirt1*, *Ppargc1a* and *Mots-c* in the treated cells were measured by RT-PCR. All PCRs were performed in at least three independent experiments, in triplicate. **(c–g)** C2C12 myotubes were transfected with a lentiviral plasmid encoding murine APPL1 (PL-APPL1). At 24 h after transfection, control cells were transfected with a plasmid encoding GFP (PL-Blank). Acrp30 (5 $\mu\text{g/ml}$) or ultrapure water was used to stimulate the

C2C12 myotubes for 24 h, and protein expression was evaluated by western blotting (**c–f**) and ELISA (MOTS-c) (**g**). **(h–l)** Cells were transfected with APPL1-specific sgRNA (sg-APPL1) or scrambled sgRNA (scr-sgRNA) control. The inhibitory effect of sgRNA on APPL1 protein expression was evaluated by western blotting (**h–k**) and ELISA (MOTS-c) (**l**). At 24 h after transfection, the cells were stimulated with Acrp30 (5 $\mu\text{g/ml}$) or ultrapure water for 24 h. The cellular levels of MOTS-c were normalised to total protein. All experiments were performed at least three times independently and each group contained three batches of individual samples. All data are expressed as the means $\pm$ SD. Student's *t* test: **p* < 0.05, ***p* < 0.01

Fig. 4 Adiponectin regulates PGC-1 α expression in skeletal muscle by increasing SIRT1. (**a–c**) C2C12 myotubes were treated with Acrp30 (5 μ g/ml; 24 h), the SIRT1 inhibitor EX527 (1 μ mol/l; 48 h), the SIRT1 activator SRT1720 (5 μ mol/l; 24 h), EX527 (1 μ mol/l; 48 h) + Acrp30 (5 μ g/ml; 24 h), SRT1720 (5 μ mol/l; 24 h) + Acrp30 (5 μ g/ml; 24 h), or vehicle (ultrapure water). Subsequently, SIRT1 and PGC-1 α protein levels were analysed by western blotting. (**d–f**) C2C12 myotubes were transfected with pcDNA-SIRT1 (PC-SIRT1) or PC-Blank control and subsequently treated with EX527 (1 μ mol/l; 48 h), Acrp30 (5 μ g/ml;

24 h), or vehicle (ultrapure water). SIRT1 and PGC-1 α expression was measured by western blotting 48 h after transfection. (**g–i**) C2C12 myotubes were transfected with siRNA targeting *Sirt1* (siSIRT1) or scrambled siRNA (scr-siRNA) control and subsequently treated with Acrp30 (5 μ g/ml; 24 h) or ultrapure water. SIRT1 and PGC-1 α expression was measured by western blotting 48 h after transfection. All experiments were performed at least three times independently and each group contained three batches of individual samples. All data are expressed as the means $\pm$ SD. Student's *t* test: **p* < 0.05, ***p* < 0.01

protein and mRNA expression of PGC-1 α in C2C12 myotubes treated with a SIRT1 activator (SRT1720) (Fig. 4a–c and ESM Fig. 3a). In addition, we transfected C2C12 myotubes with a SIRT1 overexpression plasmid. SIRT1 overexpression increased the protein and mRNA expression of PGC-1 α to similar levels as observed following adiponectin treatment (Fig. 4d–f and ESM Fig. 3b). However, EX527 treatment reduced PGC-1 α protein and mRNA levels, and this downregulation was reversed by SIRT1 overexpression (Fig. 4d–f and ESM Fig. 3b). SIRT1 knockdown reduced PGC-1 α protein and mRNA levels in C2C12 myotubes, whereas adiponectin treatment after SIRT1 knockdown reversed the PGC-1 α protein and mRNA levels, albeit to lower levels than

those in adiponectin-treated C2C12 myotubes without SIRT1 knockdown (Fig. 4g–i and ESM Fig. 3c). These results indicated that adiponectin promotes SIRT1 and in turn regulates PGC-1 α expression.

Finally, we analysed whether SIRT1 regulated skeletal muscle MOTS-c expression through a PGC-1 α -dependent mechanism. We transfected C2C12 myotubes with a SIRT1 overexpression plasmid and found that SIRT1, PGC-1 α and MOTS-c protein and mRNA were increased to levels similar to those observed following adiponectin treatment (Fig. 5a–d and ESM Fig. 4a). Notably, SIRT1 overexpression enhanced the adiponectin-induced increases in SIRT1, PGC-1 α and MOTS-c protein and mRNA levels (Fig. 5a–d and ESM Fig.

Fig. 5 SIRT1 regulates skeletal muscle MOTS-c expression through a PGC-1 α -dependent mechanism. (**a–d**) C2C12 myotubes were transfected with pcDNA-SIRT1 (PC-SIRT1) or PC-Blank control and subsequently treated with Acip30 (5 μ g/ml; 24 h) or vehicle (ultrapure water). (**e–h**) C2C12 myotubes were transfected with PC-SIRT1 or siRNA targeting *Pparg1a* (siPGC-1 α), or with PC-SIRT1 in the presence of siPGC-1 α . Transfection with scrambled siRNA (scr-siRNA) and PC-Blank were used as controls for siPGC-1 α and PC-SIRT1, respectively. (**i–l**) C2C12 myotubes were treated with the PGC-1 α inhibitor

SR18292 (10 μ mol/l; 18 h) or ultrapure water, and subsequently transfected with PC-SIRT1 or PC-Blank control. All cells were harvested 48 h after transfection. SIRT1 and PGC-1 α protein levels were measured by western blotting, and MOTS-c protein levels were determined by ELISA. Proteins levels were normalised to total protein. All experiments were performed at least three times independently and each group contained three batches of individual samples. All data are expressed as the means $\pm$ SD. Student's *t* test: **p* < 0.05, ***p* < 0.01

4a), corroborating the involvement of the adiponectin pathway in the regulation of MOTS-c expression. In addition, we treated SIRT1-overexpressing C2C12 myotubes with siRNA to interfere with PGC-1 α expression (Fig. 5c–h) or with a PGC-1 α inhibitor (SR18292) (Fig. 5i–l). Both treatments suppressed the MOTS-c expression induced by SIRT1 overexpression and regulated by the SIRT1/PGC-1 α axis (Fig. 5h,l). Notably, PGC-1 α inhibition blocked the mRNA expression of MOTS-c induced by SIRT1 overexpression (ESM Fig. 4b,c). These data indicate that PGC-1 α is also involved in the induction of MOTS-c by SIRT1.

Effects of adiponectin on MOTS-c autoregulation in C2C12 myotubes Mitochondria receive signals in response to stress and metabolic changes (anterograde signals) [38], but emerging data suggest that mitochondria also actively send signals

to the cytoplasm and nucleus (retrograde signals) [39]. It is unclear to date whether MOTS-c, a mitochondrial hormone, transmits signals to the cytoplasm for autoregulation. Treatment of C2C12 myotubes with MOTS-c at different concentrations (5, 10 and 30 μ mol/l) revealed that MOTS-c protein and mRNA expression were significantly increased upon 10 μ mol/l MOTS-c treatment (Fig. 6a and ESM Fig. 5a), with parallel increase in the protein levels of adiponectin, APPL1, SIRT1 and PGC-1 α (Fig. 6b–e). Interestingly, similar to adiponectin treatment, MOTS-c treatment increased MOTS-c protein and mRNA levels in C2C12 myotubes (Fig. 6f and ESM Fig. 5b), indicating that MOTS-c is autoregulated. To confirm whether adiponectin affects the autoregulation of MOTS-c, we utilised combined MOTS-c and adiponectin treatment. The combination treatment had an additive effect in increasing the protein and mRNA

Fig. 6 Adiponectin enhances MOTS-c autoregulation in C2C12 myotubes. (**a–e**) C2C12 myotubes were treated with MOTS-c at the indicated concentrations (shown on the *x*-axis) or ultrapure water (vehicle) for 24 h. (**f–j**) C2C12 myotubes were treated with MOTS-c (10 μmol/l; 24 h), Acip30 (5 μg/ml; 24 h), or ultrapure water, or with MOTS-c (10 μmol/l; 24 h) in the presence of Acip30 (5 μg/ml; 24 h). Protein levels of

adiponectin, APPL1, SIRT1 and PGC-1α were determined by western blotting. MOTS-c protein levels were determined by ELISA and normalised to total protein. All experiments were performed at least three times independently and each group contained three batches of individual samples. All data are expressed as the means ± SD. Student's *t* test. **p* < 0.05, ***p* < 0.01

expression of MOTS-c, APPL1, SIRT1, PGC-1α and adiponectin (Fig. 6f–j and ESM Fig. 5b). These results preliminarily indicate that MOTS-c is autoregulated and is affected by adiponectin, and reveal that the adiponectin pathway regulates MOTS-c expression [38, 39].

Exercise stimulates the production and/or secretion of skeletal muscle MOTS-c MOTS-c reportedly is an exercise mimetic and can improve insulin sensitivity in mice with ageing- and diet-induced obesity [40]; however, there is no direct evidence that exercise regulates MOTS-c production and/or secretion [30]. Therefore, we aimed to investigate the response of MOTS-c to exercise. In this experiment, wild-type mice underwent exercise intervention of 60 min/day for 5 days/

week. After 8 weeks, we found that exercise significantly increased the protein and mRNA levels of plasma and skeletal muscle MOTS-c when compared with those in mice that did not undergo exercise intervention (Fig. 7a,b and ESM Fig. 6a). The experiment also revealed that exercise increased the protein expression of APPL1, SIRT1, and PGC-1α in skeletal muscle (Fig. 7c–f).

On the basis of these results, we next asked whether exercise and adiponectin would have a synergistic effect on the regulation of MOTS-c expression. We found that exercise and adiponectin interventions had additive effects on the mRNA levels of SIRT1, PGC-1α and MOTS-c in skeletal muscle (ESM Fig. 6a). These findings preliminarily indicate that exercise promotes the production and/or secretion of skeletal muscle MOTS-c; thus,

Fig. 7 Effects of exercise (EXE) and Acip30 treatment in C57BL/6 mice. (a–f) Wild-type C57BL/6 male mice were randomly allocated to the control group or exercise group. The exercise group received an i.p. injection of Acip30 (28 $\mu\text{g kg}^{-1} \text{ day}^{-1}$) or saline for the last week of exercise training. (a, b) MOTS-c protein levels in the circulation (a) and the muscle (b) were measured by ELISA. MOTS-c levels in the

muscle were normalised to total protein. (c–f) Western blot analysis of APPL1, SIRT1 and PGC-1 α in mouse muscle. All experiments were performed at least three times independently and each group contained three batches of individual samples. All data are expressed as the means $\pm$ SD. Student's *t* test: **p* < 0.05, ***p* < 0.01

exercise may synergise with the adiponectin pathway to induce skeletal muscle MOTS-c expression [30, 40].

Exercise, adiponectin and MOTS-c increase plasma and skeletal muscle MOTS-c or adiponectin and improve insulin resistance Our results show that adiponectin treatment increased MOTS-c expression in C2C12 myotubes (Fig. 3a) and exercise increased plasma and skeletal muscle expression of MOTS-c (Fig. 7a,b and ESM Fig. 6a). To investigate the regulation of MOTS-c production and/or secretion by the adiponectin pathway under conditions of insulin resistance, we subjected mice that had received 11 weeks of HFD to 8 weeks of exercise and 1 week of adiponectin treatment. The body weights, insulin levels and fasting glucose levels of HFD mice were significantly higher than those in the control group (ESM Fig. 7a,b,g). Notably, plasma and skeletal muscle MOTS-c levels were significantly downregulated in HFD mice (Fig. 8a,b and ESM Fig. 7c). After 8 weeks of exercise, the body weights, insulin levels and fasting glucose levels of HFD mice were reduced to levels similar to those following 1 week of adiponectin treatment (ESM Fig. 7a,b,g). The protein and mRNA expression of plasma (Fig. 8a) and skeletal muscle (Fig. 8b and ESM Fig. 7c) MOTS-c was increased. Notably, the combination of exercise and adiponectin interventions reduced plasma insulin levels (ESM Fig. 7b), increased plasma MOTS-c levels (Fig. 8a) and increased the mRNA expression of skeletal muscle *Mots-c*, *Appl1*, *Sirt1* and *Ppargc1a* (ESM Fig. 7c), and these

effects were synergistic. These data suggest that exercise and adiponectin, or both synergistically, modulate MOTS-c production and/or secretion through the adiponectin pathway, thereby improving insulin resistance.

Next, we sought to determine whether MOTS-c regulates adiponectin expression in plasma and skeletal muscle. Following exercise and MOTS-c treatment for 8 weeks, the body weights and insulin levels of HFD mice were decreased (ESM Fig. 7d and e). Simultaneously, it reversed the plasma and muscle downregulation of adiponectin caused by obesity (Fig. 8f,g and ESM Fig. 7f). The combination of exercise and MOTS-c increased the mRNA and protein expression of skeletal muscle APPL1, SIRT1 and PGC-1 α (Fig. 8g and ESM Fig. 7f).

Discussion

The data presented herein demonstrate that adiponectin regulates the production and/or secretion of MOTS-c in skeletal muscle cells, and that exercise intervention increases MOTS-c production and improves metabolism in mice. Adiponectin signalling is believed to be an important regulator of skeletal muscle MOTS-c based on the following findings: (1) adiponectin treatment in *Adipoq*^{−/−} mice reversed the plasma and skeletal muscle levels of MOTS-c and increased the skeletal muscle expression of APPL1, SIRT1 and PGC-1 α ; (2) adiponectin increased MOTS-c expression in C2C12 myotubes, and this response was increased by APPL1 overexpression and decreased by

Fig. 8 Effects of exercise (EXE), Acpr30 and MOTs-c intervention in HFD mice. (a–e) C57BL/6 male mice that received HFD were randomly allocated to the HFD exercise group and the HFD control (CON) group. The mice received i.p. injection of Acpr30 ($28 \mu\text{g kg}^{-1} \text{ day}^{-1}$) or saline for the last week of exercise training. (f–g) HFD mice received i.p. injection of MOTs-c ($0.5 \text{ mg kg}^{-1} \text{ day}^{-1}$) or saline for 8 weeks. (a–b) Competition inhibition ELISA was used to measure MOTs-c in the circulation and the muscle. MOTs-c levels in the muscle were normalised to total protein.

APPL1 inhibition; knockdown or pharmacologic inhibition of PGC-1α and simultaneous SIRT1 overexpression suppressed MOTs-c expression induced by the SIRT1–PGC-1α axis; and (3) exercise increased the plasma and skeletal muscle levels of MOTs-c in mice, increased skeletal muscle expression of APPL1, SIRT1 and PGC-1α, and reduced body weight and plasma insulin levels. This study thus provides evidence to support the hypothesis that adiponectin increases plasma and skeletal muscle MOTs-c levels by increasing APPL1, SIRT1 and PGC-1α expression.

MOTS-c is a novel type of endocrine signalling molecule derived from mitochondria. Thus, mtDNA dysfunction may lead to a decline in mitochondrial function and a progressive decrease in MOTs-c expression [2]. Several lines of evidence suggest that

(c–e) Western blot analysis of APPL1, SIRT1 and PGC-1α in the mouse muscle was conducted. (f) ELISA was used to measure adiponectin in circulation. (g) mRNA expression levels of *Appl1*, *Sirt1*, *Pparg1a* and *Adipoq* in the muscle were measured by RT-PCR. All experiments were performed at least three times independently and each group contained three batches of individual samples. All data are expressed as the means $\pm$ SD. Student's *t* test: * $p < 0.05$, ** $p < 0.01$

MOTS-c may be linked to adiponectin levels in insulin resistance. To test this, we employed adiponectin KO mice and found that, although the plasma and skeletal muscle MOTs-c levels were decreased in these mice, the plasma MOTs-c level was much higher than that reported by other groups [2, 5]. This discrepancy will be further explored by comparing different detection methods or optimising the test process in future research. Adiponectin treatment restored the mRNA levels of MOTs-c, demonstrating that adiponectin might regulate MOTs-c levels. Notably, adiponectin treatment in KO mice also reversed the downregulation of APPL1, SIRT1 and PGC-1α in skeletal muscle [2, 4, 5, 22].

As an adiponectin receptor-binding protein, APPL1 mediates adiponectin signalling in various cells [12, 14, 41, 42].

Adiponectin treatment promotes the interaction of APPL1 with SIRT1, which is associated with PGC-1 α . Although PGC-1 α is a transcriptional coactivator that plays an important role in mitochondrial biogenesis [43], to our knowledge, the role of PGC-1 α in the regulation of MOTS-c expression has not been reported. The present study reveals that adiponectin-treated C2C12 myotubes exhibit increased mRNA levels of MOTS-c. The APPL1 protein level in adiponectin-treated C2C12 myotubes was positively correlated with the protein and mRNA expression of skeletal muscle MOTS-c. Notably, knockdown or pharmacologic inhibition of PGC-1 α and simultaneous SIRT1 overexpression suppressed the MOTS-c expression induced by SIRT1. Hence, adiponectin signalling may regulate MOTS-c expression in C2C12 myotubes through PGC-1 α . Moreover, MOTS-c and adiponectin treatments showed an additive effect toward increasing MOTS-c expression in C2C12 myotubes, further illustrating that the adiponectin pathway may regulate MOTS-c expression through PGC-1 α . However, PGC-1 α is a transcriptional coactivator that does not directly bind DNA but regulates gene expression by interacting with transcription factors [44]. Therefore, the direct upstream factors that regulate MOTS-c expression remain to be elucidated [12, 14, 16, 18–20, 41–44].

Mitochondria constitute the major cellular source of bioenergy and fuel skeletal muscle with energy during exercise. They also transmit exercise-induced signals to other organs [23, 45–48]. Notably, the protective effects of MOTS-c against the adverse effects caused by an HFD display many similarities to those of regular exercise [26]. In addition, a key factor for the skeletal muscle reprogramming induced by regular exercise is the activation of the PGC-1 α axis, which enhances mitochondrial biogenesis and increases mitochondrial content [1]. From this perspective, exercise may improve the endocrine and paracrine functions of MOTS-c in skeletal muscle [1]. Our study revealed that exercise increased the plasma and skeletal muscle expression of MOTS-c, along with skeletal muscle expression of APPL1, SIRT1, and PGC-1 α in normal mice. As exercise simultaneously increased skeletal muscle MOTS-c expression and activated the adiponectin pathway, we performed combined exercise and adiponectin interventions in mice, and found a significant additive effect. These results suggest that the regulatory effects of exercise on MOTS-c may have a synergistic effect with the adiponectin pathway. However, the effects of exercise are broad and are achieved through a complex network of signalling factors that coordinate physiological functions [30]. Therefore, the signalling mechanism by which exercise regulates MOTS-c expression requires further investigation.

Exercise intervention reduced plasma insulin levels in obese mice while simultaneously increasing plasma MOTS-c levels. It also increased the mRNA expression of *Appl1*, *Sirt1*, *Pparg1a*

and *Mots-c* in skeletal muscle to levels similar to those following adiponectin treatments. The increase in MOTS-c expression and improvement in insulin resistance by exercise and/or adiponectin in our study were in line with results reported by Lee et al [2], who found that i.p. injection of MOTS-c improved insulin sensitivity in HFD-induced obese mice. In contrast to our findings, in their study, i.p. injection of MOTS-c improved insulin resistance through AMPK activation and increased GLUT4 expression in skeletal muscle. We found that MOTS-c treatment in HFD mice can improve the levels of adiponectin in plasma and muscle in mice. In summary, this is the first study to show that adiponectin is a regulator of MOTS-c, and that it regulates the production and/or secretion of skeletal muscle MOTS-c through the APPL1–SIRT1–PGC-1 α pathway in *Adipoq*^{−/−} mice and C2C12 myotubes. Furthermore, this study preliminarily demonstrates that MOTS-c responds to exercise, and unravels the signalling mechanism by which exercise increases MOTS-c expression and improves insulin resistance in C57BL/6 mice. These findings show promise in yielding a better understanding of the cellular and molecular pathways underlying the pathogenesis of diabetes.

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Data availability All data generated or analysed during this study are included in this published article (and its additional files). Requests for material should be made to the corresponding authors.

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Contribution statement QG conceived and designed the research, performed the experiments, analysed the data, and wrote the manuscript. BC, QLY and STX performed experiments and analysed the data. SCC conceived and designed the research. XJY contributed to drafting the manuscript. All authors interpreted the results, contributed to the discussion, edited, and revised the manuscript and read and approved the final version of the manuscript. SCC is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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