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2-Aminoadipic acid protects against obesity and diabetes

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Abstract

Obesity and type 2 diabetes (T2D) are both complicated endocrine disorders resulting from an interaction between multiple predisposing genes and environmental triggers, while diet and exercise have key influence on metabolic disorders. Previous reports demonstrated that 2-aminoadipic acid (2-AAA), an intermediate metabolite of lysine metabolism, could modulate insulin secretion and predict T2D, suggesting the role of 2-AAA in glycolipid metabolism. Here, we showed that treatment of diet-induced obesity (DIO) mice with 2-AAA significantly reduced body weight, decreased fat accumulation and lowered fasting glucose. Furthermore, *Dhtkd1*^{-/-} mice, in which the substrate of DHTKD1 2-AAA increased to a significant high level, were resistant to DIO and obesity-related insulin resistance. Further study showed that 2-AAA induced higher energy expenditure due to increased adipocyte thermogenesis via upregulating PGC1 α and UCP1 mediated by β 3AR activation, and stimulated lipolysis depending on enhanced expression of hormone-sensitive lipase (HSL) through activating β 3AR signaling. Moreover, 2-AAA could alleviate the diabetic symptoms of *db/db* mice. Our data showed that 2-AAA played an important role in regulating glycolipid metabolism independent of diet and exercise, implying that improving the level of 2-AAA *in vivo* could be developed as a strategy in the treatment of obesity or diabetes.

Key Words

- ▶ 2-aminoadipic acid
- ▶ obesity
- ▶ diabetes
- ▶ β 3 adrenergic receptor
- ▶ thermogenesis
- ▶ lipolysis

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Introduction

Obesity is a severe public health issue worldwide featured by irregular glucose, lipid and hormone metabolism and imbalanced energy expenditure. Obesity is usually present combined with detrimental comorbidities including T2D, insulin resistance, nonalcoholic fatty liver disease (NAFLD), inflammation, and even cancer risk (Flegal *et al.* 2013, Gonzalez-Muniesa *et al.* 2017),

threatening human lives and influencing the quality of life (Calle *et al.* 2003, Jensen *et al.* 2014). T2D is a complicated disorder characterized by hyperglycemia in the setting of insulin resistance (Ashcroft & Rorsman 2012). Causes of T2D are not separated from chronic persistent glucotoxicity (hyperglycemia) and lipotoxicity (elevated lipid levels) (Alejandro *et al.* 2015).

Reducing obesity and associated T2D through diet, exercise and pharmaceuticals brings about health benefits (Colberg & Sigal 2011, Fildes *et al.* 2015).

In humans, adipose tissues are divided into white adipose tissue (WAT) and brown adipose tissue (BAT) according to their functions (Klaus 1997). The main function of WAT is storing energy as a triglyceride when the energy intake is excessive, and meanwhile, hydrolyzing triglyceride and releasing free fatty acid when the energy intake is inadequate. The main function of BAT is thermogenesis, that is, providing heat for life activities (Kajimura *et al.* 2010). BAT contains a large number of mitochondria and a large amount of mitochondrial uncoupling protein 1 (UCP1) is expressed in the inner membrane of mitochondria, participating in the transmission of electron transfer chains to producing heat through cellular respiration and mediating energy expenditure (Argyropoulos & Harper 2002, Ricquier 2005). Previous evidence demonstrated that obesity-resistant mice had a higher expression of UCP1 in WAT relative to obese controls (Xue *et al.* 2007). It has also been known that the activation of UCP1 in beige adipocyte could increase energy expenditure and resist DIO and insulin insensitivity (Guerra *et al.* 1998). The functional mechanism of UCP1-mediated thermogenesis was boosted in recent years by the discovery of UCP1-expressed adipocyte, which could be induced in some WATs (beige fat) of mice to induce fat burning in response to stimuli (Wu *et al.* 2012, Shabalina *et al.* 2013). Chronic stimulation of $\beta 3$ adrenergic receptor ($\beta 3$ AR) could convert WAT into a tissue resembling BAT, which was called 'browning' of white fat (Seale *et al.* 2011, Lee *et al.* 2012, 2014). Despite thermogenesis, $\beta 3$ AR expression and $\beta 3$ AR-mediated lipolysis have been documented in human visceral adipocytes (Lonnqvist *et al.* 1993, 1995, De Matteis *et al.* 2002, Lafontan & Berlan 2003, Yehuda-Shnaidman *et al.* 2010). In 'browning' adipocytes, the free fatty acids produced by $\beta 3$ AR-mediated lipolysis are used up for thermogenesis via UCP1 (Ricquier *et al.* 1986, Yehuda-Shnaidman *et al.* 2010). The activation of $\beta 3$ AR pathway in the white adipocytes makes $\beta 3$ AR-based lipolysis and thermogenesis become a new therapeutic strategy against obesity and diabetes.

2-AAA is a potential regulator of glucose homeostasis, which is higher in T2D patients and considered to be a biomarker for T2D mellitus (Wang *et al.* 2013). But, dramatically, 2-AAA is able to promote insulin secretion and decrease blood glucose in mice (Wang *et al.* 2013, Xu *et al.* 2018). Dehydrogenase E1 and transketolase domain-containing 1 (DHTKD1) protein is a component

of α -ketoacidic acid dehydrogenase complex, which is involved in the metabolism of lysine, hydroxylysine and tryptophan (Hagen *et al.* 2015). DHTKD1 deficiency or mutation affects mitochondrial oxidative phosphorylation and energy metabolism (Xu *et al.* 2012, 2013). Loss of function of DHTKD1 causes accumulation of intermediate metabolite 2-AAA in humans and mice (Danhauser *et al.* 2012, Xu *et al.* 2018).

In the current study, we evaluated the effect of 2-AAA on lipid metabolism and found that it activated $\beta 3$ AR signaling to protect against obesity and diabetes. Furthermore, 2-AAA obviously ameliorated metabolic status of *db/db* mice. This provides a novel insight on lipid metabolism and reveals the potential pharmaceutical value of 2-AAA.

Materials and methods

Mice

The whole-body knockout *Dhtkd1*^{-/-} mice had been reported previously (Xu *et al.* 2018) and the *db/db* mice were purchased from Nanjing Biomedical Research Institute of Nanjing University. All mice were maintained in a standard specific-pathogen-free condition and free access to diet and water unless otherwise specified. Only male mice were chosen to participate in this research. High-fat diet (HFD) (diet with 60kcal% fat, Research Diets) and 2-AAA (Sangon Biotech) drinking water were executed since the mice were 1 month old. All procedures were approved by the Animal Ethics Committee of Rui-Jin Hospital.

Body composition and indirect calorimetry

Body composition (fat and lean mass) was assessed via quantitative nuclear magnetic resonance relaxometry using an EchoMRI whole-body composition analyzer. Mice were housed individually in LabMaster system (TSE Systems), and after 2 days for the adaptation, indirect calorimetry was measured for 5 consecutive days.

Histopathological analysis

Mouse tissues were isolated and fixed in 10% formalin and sectioned. The staining procedures were performed routinely, including hematoxylin and eosin (H&E) staining, oil-red staining and PAS staining. Besides, mouse liver and BAT were dissected and fixed in

2.5% glutaraldehyde and semithin sections were stained with toluidine blue. Then, the transmission electron microscopy (TEM) was carried out using Philips CM120 instrument at Shanghai Jiao Tong University School of Medicine.

Serum biochemical analysis

The blood samples of mouse taken from retrobulbar vein were centrifuged and the sera were analyzed with an automatic biochemical analyzer. Blood leptin and insulin contents were determined using ELISA method with mouse leptin ELISA kit (Crystal Chem) and insulin ELISA kit (Mercodia), respectively.

GTT and ITT

Mice fasted overnight for 16h were injected intraperitoneally with 2g of glucose per kilogram body weight. Blood samples accessed from the tail vein were collected at the specific time points after injection, and glucose levels were measured using a glucometer (Sinocare). On the other hand, mice fasted for 6h were intraperitoneally injected with 0.75U/kg human insulin (Humulin; Lilly). Tail blood samples were collected and measured with a glucometer (Sinocare) at the specific time points after injection.

Cell culture and treatment

The 3T3-L1 cells (ATCC® CL173™) were purchased from American Type Culture Collection and were cultured in Dulbecco's modified Eagle's medium (DMEM) complete medium containing 10% heat-inactivated bovine calf serum, 100µg/mL streptomycin and 100units/mL penicillin at 37°C in a 5% humidified CO₂ atmosphere. For the experimental group, 20µM 2-AAA (Sigma-Aldrich) was added to the medium.

Quantitative reverse transcription-PCR (qRT-PCR)

Total RNAs were extracted from mouse adipose tissues or cultured cells using TriPure reagent (Roche) and were reverse transcribed into cDNAs using a reverse transcriptase reagent kit with genomic DNA eraser (TaKaRa) according to the manufacturers' instructions. Quantitation was carried out using a SYBR green PCR

kit (TaKaRa) in Mastercycler *ep* realplex instrument (Eppendorf). Relative transcript quantities were calculated using the $\Delta\Delta C_T$ (threshold cycle) method with β -actin as an endogenous reference gene. Each sample was analyzed in triplicate and primers listed in Supplementary Table 1 (see section on [supplementary data](#) given at the end of this article) were accessed from the PrimerBank database (Spandidos *et al.* 2010).

Western blot analysis

Adipose tissues or cells were lysed using lysis buffer, which contained 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS in PBS supplemented freshly with protease and phosphatase inhibitor cocktails (Roche). Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with specific primary antibodies as follows, anti-UCP1 (Santa Cruz), anti-PGC1 α (Santa Cruz), anti-PPAR γ (Santa Cruz), anti-ATGL (Cell Signaling Technology), anti-perilipin A (Sigma-Aldrich), anti-HSL (Cell Signaling Technology), anti-pHSL (Cell Signaling Technology), anti- β 3AR (Abcam), anti-PKA (Cell Signaling Technology), anti-pPKA (Cell Signaling Technology), anti-p38 (Cell Signaling Technology), anti-pp38 (Cell Signaling Technology), anti-Actin (Cell Signaling Technology), anti-Tubulin (Sigma-Aldrich), and anti-GAPDH (Sangon Biotech).

Molecular simulation

The structural data of 2-AAA were from PubChem and the structure of β 3AR were obtained through homologous modeling referring to the structure of β 2AR (PDB code, 5X7D) in Protein Data Bank using SWISS-MODEL. Then, the binding pocket of ARs was calculated and identified according to the location of the natural small molecules and the shape and volume of 2-AAA. Finally, molecular docking of ARs and 2-AAA was carried out by ArgusLab software.

Statistical analysis

All data were presented as means $\pm$ s.d. and a two-tailed Student's *t* test was used to compare the differences between two groups. A *P* value of less than 0.05 was used to define statistical significance (**P*<0.05, ***P*<0.01, and ****P*<0.001).

Results

2-AAA protected against DIO via increasing energy expenditure in mice

To elucidate the role of 2-AAA on metabolism, we conducted daily treatment of C57 mice with 2.5 g/L of 2-AAA on either a standard chow diet (SCD) or HFD for 24 weeks. Interestingly, the body weight of 2-AAA-treated mice was significantly lighter than that of controls, especially when they were fed with HFD (Fig. 1A). From the photographs of mice, we found that 2-AAA-treated mice were slenderer than controls whether they were fed with SCD or HFD (Fig. 1B). Besides, 2-AAA-treated mice accumulated less fat in the abdomen compared to controls (Fig. 1B). Body composition analyses showed that the percentage of fat mass and lean mass both increased pronouncedly under HFD (Fig. 1C). However, 2-AAA-fed mice displayed a significant reduction in lean body mass without an alteration in fat mass on SCD. On the HFD, a significant decrease in body weight in the 2-AAA-treated mice was mainly due to a marked reduction in the percentage of fat body mass (Fig. 1C). Both the sizes of BAT and epididymal (visceral) and subcutaneous WAT of 2-AAA-treated mice

on either SCD or HFD were smaller compared to those of controls (Fig. 1D). Next, organ weight showed that the weights of adipose tissues of 2-AAA-treated mice were less than those of controls fed with HFD, but with no alteration in liver and kidney (Fig. 1E). Histological analysis showed the adipocyte sizes of 2-AAA-treated mice were smaller than those of controls when they were fed with HFD (Fig. 1F). Chronic exposure to HFD could cause fatty liver containing large lipid-containing vacuoles. However, less lipid droplets accumulated in the liver of 2-AAA-treated mice were observed (Fig. 1F). The serum leptin was significantly decreased in 2-AAA-treated mice when they were fed with HFD (Fig. 1G). Plasma lipid-profile testing showed that the total cholesterol (TC), triglyceride (TG) and low-density lipoprotein cholesterol (LDL-C) levels of 2-AAA-treated mice were significantly lower than those of controls, while the other blood biochemical indexes were comparable (Supplementary Fig. 1). This is a rather interesting result that 2-AAA could protect mice against DIO. The lower body weight of 2-AAA-treated mice was closely related to a large reduction in fat mass. Food intake was of no significant difference between the groups (Supplementary Fig. 2A), suggesting that the obesity-resistant effect is primarily

Figure 1

2-AAA intake protects mice against obesity. (A) Body weights of mice ($n = 12$ mice/group). (B) Photographs of mouse morphology and anatomical abdomen. (C) MRI analysis shows the contents of fat mass and lean mass ($n = 5$ mice/group). (D) Photographs of isolated adipose tissues, liver and kidney. (E) Isolated organ weights of mice ($n = 5$ mice/group). (F) HE staining of adipose tissues and oil-red staining of liver. Scale bar, 100 μ m. (G) Serum leptin levels of mice ($n = 8$ mice/group). A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0157>.

associated with altered metabolism, such as enhanced energy expenditure and heat production. Indirect calorimetry studies of 2-AAA-treated mice revealed an increase in the heat production during both day and night (Supplementary Fig. 2B), while the locomotor activity of 2-AAA-treated mice in the metabolic cage was comparable with controls (Supplementary Fig. 2C). The respiratory exchange ratio (RER) was elevated in 2-AAA-treated mice (Supplementary Fig. 2B), suggesting a markedly increased use of glucose as an energy source. Additionally, the increases in O_2 consumption and CO_2 exhalation were detected in 2-AAA-treated mice compared with those of controls (Supplementary Fig. 2B). These reveal that the emaciation of 2-AAA-treated mice is mainly due to their high energy expenditure.

Dhtkd1^{-/-} mice were resistant to DIO due to enhanced energy expenditure

Now that 2-AAA could protect mice from predisposing to obesity, we consider how to increase the level of 2-AAA *in vivo*. Previously, we proved that *Dhtkd1*-knockout (*Dhtkd1*^{-/-}) mice whose urine 2-AAA levels were increased by about 120 times due to *Dhtkd1* deficiency (Xu *et al.* 2018). We speculate that the deletion of *Dhtkd1*

could cause the accumulation of its substrate, 2-AAA. Otherwise, we found the expression of DHTKD1 protein was regulated by diet and cold stress. It increased when the mice were fasted and recovered after refed. And it decreased when the mice were exposed into cold environment (Supplementary Fig. 3). All above implied the role of DHTKD1 in energy metabolism. Besides, in our daily observations, we found *Dhtkd1*^{-/-} mice were slightly thinner and smaller than wt mice, which was much more obvious when they were fed with HFD (Supplementary Fig. 4A). *Dhtkd1*^{-/-} mice appeared normal at birth, but showed decreased body weight compared with age-matched littermates after weaning. These differences were more obvious when given an HFD diet, because wt mice became obese rapidly. In contrast, *Dhtkd1*^{-/-} mice maintained approximately the same body weight on HFD as on SCD (Fig. 2A). The photographs of anatomical abdomen showed that *Dhtkd1*^{-/-} mice accumulated less fat than wt mice whether they were fed with SCD or HFD (Fig. 2B). Body composition analyses showed that HFD-fed *Dhtkd1*^{-/-} mice had marked less fat as a percentage of total body weight compared with wt mice (Fig. 2C), resembling the effects of 2-AAA treatment. The percentage of lean mass was comparable between *Dhtkd1*^{-/-} mice and wt mice (Fig. 2C). Further anatomy showed that the weights

Figure 2

Dhtkd1 deficiency protects mice against obesity. (A) Body weights of mice ($n = 10$ mice/group). (B) Photographs of anatomical abdomen of mice. (C) MRI analysis of the contents of fat mass and lean mass ($n = 5$ mice/group). (D) Photographs of isolated adipose tissues, liver and kidney. (E) Isolated organ weights of mice ($n = 5$ mice/group). (F) HE staining of adipose tissues and oil-red staining of liver. Scale bar, 100 μ m. (G) Serum leptin levels of mice ($n = 8$ mice/group). A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0157>.

of subcutaneous, epididymal fat and BAT of *Dhtkd1*^{-/-} mice were smaller than those of wt mice on either SCD or HFD (Fig. 2D). Except heart, almost all weighed tissues or organs were lighter in *Dhtkd1*^{-/-} mice compared with wt mice when they were fed with HFD (Fig. 2E). Wt mice fed with HFD and SCD for 6 months showed severe lipid droplet accumulation in the liver while relatively smaller adipocytes were found in *Dhtkd1*^{-/-} mice compared to control mice (Fig. 2F). The serum leptin was significantly decreased in *Dhtkd1*^{-/-} mice when they were fed with HFD, but it was comparable when fed with SCD (Fig. 2G). Plasma lipid-profile testing also demonstrated that the TC and triglyceride (TG) levels of *Dhtkd1*^{-/-} mice fed with SCD or HFD were obviously lower than those of wt littermates. In addition, the level of HDL-C was higher in *Dhtkd1*^{-/-} mice. Furthermore, some biochemical indexes, such as TP, ALB, GLOB, UA and CRE, were abnormal in SCD, while ALT, AST, LDH, BUN and CRE were abnormal in HFD (Supplementary Fig. 4B). Taken together, these results suggested that like 2-AAA-treated mice, *Dhtkd1*^{-/-} mice were also resistant to obesity. Besides, we aimed to detect whether the decreased weight in *Dhtkd1*^{-/-} mice is due to reduced energy intake or increased energy expenditure. We found that *Dhtkd1*^{-/-} mice consumed more food and less exercise than controls (Supplementary Fig. 5A). Increased energy expenditure rather than decreased food intake contributed to the weight loss in *Dhtkd1*^{-/-} mice,

which was further confirmed by increased O₂ consumption, CO₂ production, heat production and RER (Supplementary Fig. 5B), indicating that *Dhtkd1*^{-/-} mice were always keeping in a high energy metabolic state.

2-AAA enhanced thermogenesis in brown adipocytes

The body temperatures of *Dhtkd1*^{-/-} mice were almost able to be maintained in normal range up to 6 h in a cold environment (4°C), while the body temperature of wt mice had fallen, even below 34°C (Fig. 3A). The increase in energy expenditure might reflect a high level of thermogenesis in *Dhtkd1*^{-/-} mice. In fact, the expression of *Ucp1*, PPARγ coactivator-1α (*Pgc1α*), *Hsl*, carnitine palmitoyltransferase 1 B (*Cpt1b*), transcription factor A mitochondrial (*Tfam*), *Adrb3*, phytanoyl-CoA hydroxylase (*Phyh*), and *Ppara*, a number of brown fat-specific genes in BAT thermogenesis or mitochondria biogenesis were highly increased in the BAT of *Dhtkd1*^{-/-} mice after exposed in 4°C for 4 h (Fig. 3B). As shown in Supplementary Fig. 6, the expression of thermogenic genes were upregulated significantly in the BAT of 2-AAA-treated mice after 4 h exposure to 4°C. But the differences were significantly narrowed at 24 h. These indicated that the thermogenic effect of 2-AAA was of short duration. Western blotting confirmed that the protein of UCP1 and PGC1α were elevated in BAT of *Dhtkd1*^{-/-} mice under the cold stress

Figure 3

2-AAA administration promotes thermogenesis in BAT. (A) Body temperature changes of *Dhtkd1*^{-/-} and wt mice upon cold (4°C) stress (*n* = 10 mice/group). (B) RNA expression of thermogenic genes in BAT from mice 4 h after 4°C exposure. (C) Western blot analysis in BAT from mice 4 h after 4°C exposure. (D) TEM pictures of BAT from *Dhtkd1*^{-/-} and wt mice fed with HFD. Black arrows, mitochondria. Scale bar, 2 μm. (E) *Ucp1* and *Pgc1α* RNA expression in BAT. (F) Western blot analysis in BAT from *Dhtkd1*^{-/-} and wt mice fed with HFD. (G) Western blot analysis in BAT from 2-AAA-treated mice fed with HFD.

(Fig. 3C and Supplementary Fig. 7). Consistent with the higher oxygen consumption and enhanced energy expenditure described earlier, these results indicated that *Dhtkd1*^{-/-} mice displayed enhanced thermogenesis. Notably, though a significant alteration in the weight of interscapular BAT was detected in the *Dhtkd1*^{-/-} mice with HFD, we observed a significant increase in the number of mitochondria in BAT of *Dhtkd1*^{-/-} mice by the electron microscope (Fig. 3D). This result indicated that absence of *Dhtkd1* increased thermogenesis, partly because of an increase in mitochondria number. Like cold stimulation, we found the expression levels of UCP1 and PGC1 α were significantly increased in the BAT of *Dhtkd1*-deficient or 2-AAA-treated mice, whether they were fed with SCD or HFD (Fig. 3E, F and G). These observations suggested that enhanced energy dissipation in 2-AAA-treated or *Dhtkd1*^{-/-} mice were dependent on BAT-induced thermogenesis.

2-AAA induced browning of epididymal WAT

To determine whether increased adaptive thermogenesis was due to browning of WAT, we performed transcript and protein analysis and found that 2-AAA could induce

a high expression of a number of brown fat-specific genes in WAT, including *Ucp1*, *Pgc1a*, *Prdm16*, *Dio2* and *Tfam* (Fig. 4A and B). Among them, *Ucp1* gene expression was extremely highly induced in the 2-AAA-treated mice, which was in accordance with the increased expression of *Ucp1* gene in *Dhtkd1*^{-/-} mice (Fig. 4A). Taken together, these data demonstrate that 2-AAA induces browning of WAT. Activation of β 3AR can induce a functional 'brown-like' adipocyte phenotype. In addition, β 3AR stimulation triggers PKA-p38 (MAPK)-PGC1 α kinase cascade which induces UCP1-mediated thermogenesis. Consistently, we observed an increase in PKA and P38 phosphorylation in 2-AAA-treated and *Dhtkd1*^{-/-} mice on HFD (Fig. 4C). Consistent with the *in vivo* data, the gene and protein expression of UCP1 and β 3AR was upregulated in differentiated 3T3-L1 adipocytes treated with 2-AAA (Fig. 4D, E and Supplementary Fig. 7).

Lipolysis was enhanced in WAT of 2-AAA-treated and *Dhtkd1*^{-/-} mice through activating β 3AR signaling

We next explored whether the marked decrease in fat mass in the 2-AAA-treated and *Dhtkd1*^{-/-} mice was due

Figure 4

2-AAA intake enhances lipolysis and browning of WAT via activating β 3AR signaling. (A and B) mRNA expression of brown fat-specific genes in WAT. (C) Western blot analysis of lipolysis- and browning-related proteins in WAT. (D) mRNA expression in 3T3L1 cells upon 2-AAA treatment. (E) Western blot analysis of proteins in 3T3L1 cells upon 2-AAA treatment for 48 h. (F) mRNA expression of lipolysis-related genes in WAT.

to alterations in lipid lipolysis, fat oxidation, lipogenesis, differentiation, mitochondrial biogenesis, thermogenesis or TG synthesis (Supplementary Fig. 8). A significant increase in key lipolytic enzymes at both mRNA and protein levels was detected in the epididymal fat from the 2-AAA-treated and *Dhtkd1*^{-/-} mice fed with HFD (Fig. 4C and F). HSL, a key enzyme controlling the adipocyte lipolytic activity, known to be highly regulated through adrenergic receptors, was found significantly increased in the epididymal fat of 2-AAA-treated and *Dhtkd1*^{-/-} mice fed with HFD (Fig. 4F). Since the enzyme activity and translocation of HSL to lipid droplets need HSL phosphorylation, we conducted Western blot analysis and found a significant increase in the degree of HSL phosphorylation level in the epididymal fat from 2-AAA-treated and *Dhtkd1*^{-/-} mice fed with HFD (Fig. 4C). Perilipin A is a protein covering the lipid droplets and serving as an essential interaction partner for HSL. The protein level of perilipin A showed a marked increase in the epididymal fat from 2-AAA-treated and *Dhtkd1*^{-/-} mice fed with HFD (Fig. 4C). Another main lipase involved in the control of adipocyte lipolysis is desnutrin/ATGL, like HSL, which is regulated by adrenergic receptor activity. Also, 2-AAA-treated and *Dhtkd1*^{-/-} mice had significantly higher levels of ATGL in their adipocytes (Fig. 4C and F). The two adrenergic receptors expressed in the dissected epididymal fat both increased in the 2-AAA-treated and *Dhtkd1*^{-/-} mice. However, the β 3AR, which is more abundantly expressed in adipocytes, displayed a marked enhancement (Fig. 4B and C). Stimulation of β 3AR signaling phosphorylates HSL and increases HSL-mediated lipolysis dependent on protein kinase A (PKA). As shown in Fig. 4C, we found that PKA phosphorylation was increased in the adipocytes in 2-AAA-treated and *Dhtkd1*^{-/-} mice. Our results showed that β 3AR-induced lipolysis via PKA-mediated HSL activation, leading to reduced fat mass in mice. This process produces a lot of fatty acids, but we did not found any pathological change in the liver (Supplementary Fig. 9). The biochemical indicators of liver function also showed no statistical difference (Supplementary Figs 1 and 4). These results suggest that increased lipolysis in adipocytes and reduced adiposity in the 2-AAA-treated or *Dhtkd1*^{-/-} mice are mediated by specific regulation of β 3AR to indirectly enhance HSL enzyme activity and induce UCP1-dependent thermogenesis related to β 3AR signaling.

2-AAA was simulated to bind to β 3AR

Interestingly, computer simulation showed that 2-AAA binds to β 3AR (Supplementary Fig. 10). The binding sites were located at extracellular domain of β 3AR

(Supplementary Fig. 10A and B), and the optimal binding free energy was -7.35 kcal/mol. To evaluate whether the value was sufficient for their binding, we found a crystal structure of aspartate- β -semialdehyde dehydrogenase from *Streptococcus pneumoniae* (spASADH) with 2',5'-adenosine diphosphate and D-2-AAA (PDB code, 3PWS). The optimal binding free energy of 2-AAA with spASADH was -7.19 kcal/mol, which was less than that of 2-AAA with β 3AR, suggesting that the binding of 2-AAA with β 3AR was stabler than that with spASADH. Besides, the optimal binding free energy of 2-AAA with its enzyme, aminoadipate aminotransferase (AADAT), was -7.71 kcal/mol. The binding free energy obtained by molecular docking is linearly related to the molecular weight of small molecules (Zhao *et al.* 2018). Because the molecular weight of 2-AAA is relatively small, the binding free energy is relatively little. As a metabolic intermediate of lysine metabolism, 2-AAA has similar molecular weight with lysine. Thus, we found the free energy they bound with β 3AR are comparable. Furthermore, the binding of 2-AAA and β 3AR mainly depended on the N atoms and the O atoms with double bonds (Supplementary Fig. 10C), and the sizes of hydrogen bonds were 1.84, 2.86, 2.59 and 2.76 Å, respectively (Supplementary Fig. 10D).

Recently, several clinical trials about β 3AR agonist have failed due to problems of unspecific binding to β 1AR/ β 2AR resulting in tachycardia and tremors. We further examined cardiac parameters using ultrasound and found no significant difference between two groups (Supplementary Fig. 11).

Effects of 2-AAA on glucose metabolism

HFD-induced elevated blood glucose, impaired glycemic response, hyperinsulinemia and enhanced insulin resistance compared with mice fed with SCD. Daily administration of 2-AAA significantly reduced the overall glycemic levels of mice in HFD (Fig. 5A). Furthermore, plasma insulin concentration significantly descended to fasting insulin levels in 2-AAA-treated mice 2h after feeding (Fig. 5B). GTT and ITT indicated that 2-AAA-treated mice showed improved glucose tolerance and insulin sensitivity as evidenced by lower blood glucose levels compared with controls on HFD (Fig. 5C and D). Thus, we supposed that elevated insulin sensitivity might be associated with the body weight of 2-AAA-treated or *Dhtkd1*^{-/-} mice due to their lean phenotype. The PAS staining of liver indicated more glycogen accumulation in 2-AAA-treated mice compared with controls (Fig. 5E). Consistent with the effects of 2-AAA on glucose, *Dhtkd1*

Figure 5

Both 2-AAA-treated and *Dhtkd1*-deficient mice show abnormal glucose metabolism induced by HFD. (A) Serum glucose levels of 2-AAA-treated mice fed with HFD ($n = 10$ mice/group). (B) Serum insulin levels of 2-AAA-treated mice fed with HFD ($n = 10$ mice/group). (C and D) GTT (C) and ITT (D) in 2-AAA-treated mice fed with HFD ($n = 8$ mice/group). (E) PAS staining of liver from 2-AAA-treated mice fed with HFD. Scale bar, 100 μ m. (F) Serum glucose levels of *Dhtkd1*^{-/-} and wt mice fed with HFD ($n = 10$ mice/group). (G) Serum insulin levels of *Dhtkd1*^{-/-} and wt mice fed with HFD ($n = 10$ mice/group). (H and I) GTT (H) and ITT (I) in *Dhtkd1*^{-/-} and wt mice fed with HFD ($n = 8$ mice/group). (J) TEM pictures of liver from *Dhtkd1*^{-/-} and wt mice fed with HFD. Scale bar, 5 μ m. (K) PAS staining of liver and muscle from *Dhtkd1*^{-/-} and wt mice fed with HFD. Scale bar, 100 μ m. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0157>.

deficiency also caused lower serum glucose level and reduced postprandial hyperinsulinemia with HFD in mice (Fig. 5F and G). As expected, *Dhtkd1*^{-/-} mice also showed improved glucose tolerance and insulin sensitivity in GTT and ITT (Fig. 5H and I). The electron microscope analysis showed more glycogen combined with less and smaller lipid droplets in *Dhtkd1*^{-/-} liver than those in wt mice (Fig. 5J) and the PAS staining showed more glycogen accumulation in the liver and skeletal muscle of *Dhtkd1*^{-/-} mice (Fig. 5K). Consistently, the glycogen synthesis-related genes expression was also upregulated in *Dhtkd1*^{-/-} liver (Supplementary Fig. 12). These results suggested that improved HFD-induced glucose homeostasis existed in mice with high 2-AAA concentrations *in vivo*.

2-AAA alleviated the diabetic symptoms of *db/db* mice

To assess the benefits of 2-AAA in therapies to ameliorate DIO, *db/db* (*Lepr*^{-/-}) mice were treated with 2-AAA daily for 16 weeks. We found 2-AAA-treated *db/db* mice were thinner than controls (Fig. 6A) combined with the decreased percentages of fat and lean mass in 2-AAA-treated *db/db* mice (Fig. 6B). The weights of liver and WAT were obviously decreased in 2-AAA-treated mice than those of untreated mice (Fig. 6C). Consistent with improved glucose homeostasis in a genetically predisposed diabetes model with 2-AAA treatment, we observed that over-eat, over-drink and polyuria, as typical

symptoms for diabetes, were obviously ameliorated in *db/db* mice after 2-AAA intake (Fig. 6D and Supplementary Fig. 13A). In addition, *db/db* mice developed obvious hyperglycemia, but those *db/db* mice receiving 2-AAA showed a pronounced reduction in blood glucose after 1 week treatment (Fig. 6E). Elevated serum insulin levels after 30 min of refeed were observed in 2-AAA-treated *db/db* mice, suggesting an insulin sensitivity to food (Fig. 6F). Meanwhile, we found hepatic glycogen concentration in 2-AAA-treated *db/db* mice was increased obviously (Fig. 6G), suggesting that more glucose in peripheral blood was stored in liver in the form of glycogen. Intraperitoneal glucose and insulin challenge were performed to evaluate the effects of 2-AAA on significant promotion of glucose homeostasis and insulin resistance (Fig. 6H and I). Transcriptional analysis revealed that the upregulation of multiple key transcription factors involved in thermogenesis, mitochondrial biogenesis, peroxisomal fatty acid oxidation and lipolysis were correlated with ameliorating diabetic symptoms of *db/db* mice under 2-AAA treatment (Supplementary Fig. 13B). Consistent with gene expression, the protein levels of adipose TG lipase (ATGL), the rate-limiting enzyme for lipolysis, perilipin A and pHSL were increased in 2-AAA-treated *db/db* mice (Supplementary Fig. 13C). In summary, our findings revealed that the anti-obesity effect of 2-AAA was attributed to improve insulin resistance and alleviate metabolic disorders in diabetic mice.

Figure 6

2-AAA ameliorates diabetes mellitus symptoms in *db/db* mice. (A) Body weights of 2-AAA-treated *db/db* mice ($n = 9$ mice/group). (B) MRI analysis of the contents of fat mass and lean mass in 2-AAA-treated *db/db* mice ($n = 5$ mice/group). (C) Organ weights of 2-AAA-treated and -untreated *db/db* mice ($n = 5$ mice/group). (D) Food and water consumption of 2-AAA-treated (3 weeks) and untreated *db/db* mice ($n = 5$ mice/group). (E) Serum glucose levels of 2-AAA-treated *db/db* mice ($n = 9$ mice/group). (F) Serum insulin levels of 2-AAA-treated *db/db* mice ($n = 9$ mice/group). (G) Hepatic glycogen of 2-AAA-treated *db/db* mice ($n = 3$ mice/group). (H and I) GTT (H) and ITT (I) in 2-AAA-treated *db/db* mice ($n = 9$ mice/group).

Discussion

The accumulation of 2-AAA in the body is the major cause of 2-aminoacidic and 2-oxoacidic aciduria and Charcot-Marie-Tooth disease 2Q (Danhauser *et al.* 2012, Xu *et al.* 2018). But one thing has two sides. 2-AAA also brings us the gospel. Previous studies showed that after exogenous administration of 2-AAA, high levels of 2-AAA were detected in islets and adipose tissues (Wang *et al.* 2013), indicating that its major action sites were probably in islets and adipocytes. Our previous results also showed that mice with DHTKD1 deficiency caused 2-AAA accumulation to increase insulin production and secretion (Xu *et al.* 2018). In addition, diabetic patients were found to have a significant decrease in 2-AAA levels (Wu *et al.* 2014), indicating the effect of 2-AAA on the development of diabetes. As we observed in this study, 2-AAA is responsible for the improvement of glycolipid metabolism and insulin sensitivity, thus making mice resistant to DIO independent of diet and exercise.

In this study, 2-AAA-fed mice showed weight loss, decreased adipocytes and reduced body fat, which were more pronounced in the HFD. Increased oxygen consumption, carbon dioxide production and heat production no matter on normal diet or HFD, suggesting 2-AAA-treated mice are in a state of hypermetabolism. Thermogenesis in BAT contributed to increased energy metabolism in the 2-AAA-treated mice. BAT has long been considered to be the primary tissue responsible for thermogenesis, in which process, PGC1 α and UCP1 play crucial roles (Puigserver & Spiegelman 2003, Ricquier 2005). PGC1 α can regulate the

expression of mitochondrial protein UCP1 and improve the mitochondrial oxidation capacity. The expression of thermogenesis-related genes, including *Ucp1*, *Pgc1 α* and *Ppara*, was increased significantly in the BAT of 2-AAA-treated mice. The expression level of UCP1 also increased significantly in the WAT of 2-AAA-treated mice, suggesting that 2-AAA could promote the transformation from WAT to BAT, and in addition, the expression of lipogenesis and

Figure 7

Schematic representation of the potential mechanisms of DHTKD1 and 2-AAA in lipolysis. Loss of function of DHTKD1 causes obvious increase of 2-AAA level. Then accumulated 2-AAA overactivates β 3AR signaling, leading to enhanced lipolysis and thermogenesis. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0157>.

lipolysis genes was upregulated in WAT. We found that the expression of HSL and ATGL, which controlled lipolysis, increased significantly in WAT of 2-AAA-fed mice. ATGL mainly promotes triglyceride hydrolysis (Villena *et al.* 2004, Zimmermann *et al.* 2004) and phosphorylated HSL mainly promotes the hydrolysis process of diacylglycerol (Vaughan *et al.* 1964, Zechner *et al.* 2009). These results suggest that, on one hand, the lipogenesis increases with the action of insulin; on the other hand, lipolysis also increases. The activation of ATGL and HSL will help to reduce triglyceride storage in various tissues and organs and resist DIO and diacylglycerol accumulation (Osuga *et al.* 2000, Harada *et al.* 2003, Haemmerle *et al.* 2006). Since the activation of β AR is considered to be the major marker of lipolysis (Zechner *et al.* 2009), the upregulation of transcripts, *Adrb2* and *Adrb3*, in the WAT of 2-AAA-fed mice demonstrated that 2-AAA promoted lipolysis. Computer simulation showed that 2-AAA binds to β 3AR at extracellular domain of β 3AR. Pharmacological β 3AR activation leads to increased adipose 'browning' and insulin release. Epinephrine or the β 3AR agonist such as CL 316,243 (CL) are aimed to improve metabolic function to treat obesity. Natural metabolite 2-AAA-mediated DIO resistance is via stimulated adrenergic signaling pathways mainly involving β 3AR. Moreover, presumed as a β 3AR agonist, enhanced 2-AAA contents led to a browning response in inguinal (iWAT), along with reduced WAT and BAT mass regardless of high fat intake.

2-AAA enhances energy metabolism and reduces the accumulation of fat, one of the greatest benefits of which is that it will improve glucose metabolism and increase insulin sensitivity in mice. Actually, we observed that 2-AAA-fed mice showed increased insulin sensitivity, decreased blood glucose and improved glucose tolerance, which was more obvious in HFD-fed mice. The relationship between lipid metabolism and insulin sensitivity mainly reflects in two aspects. First, 2-AAA reduces the size of adipocyte, and smaller adipocytes are more sensitive to insulin stimulation (Foley *et al.* 1980, Bluher *et al.* 2002). Second, besides WAT, increased triglyceride will also accumulate in other tissues or organs, such as liver and muscle, which will weaken the effect of insulin on fat cells and reduce insulin sensitivity (Hulver & Dohm 2004). Given an HFD, the mice showed an increased amount of fat cells, excessive triglycerides stored in the liver and muscle, and weakened effect of insulin on fat cells. But when given 2-AAA simultaneously, the mice showed enhanced hydrolysis of fat in the liver and muscles, and enhanced insulin sensitivity, so they resisted to DIO. Our research demonstrates that the metabolite 2-AAA could

stimulate insulin synthesis and secretion and improve insulin sensitivity. When 2-AAA is used as a treatment tool, we found that 2-AAA could decrease blood glucose of diabetic *db/db* mice and significantly improve their symptoms of diabetes mellitus. Although its effects on reducing weight in *db/db* mice were not as obvious as those in wt mice, it still reduced fat accumulation, enhanced lipolysis and increased insulin sensitivity in *db/db* mice.

DHTKD1 participates in lysine metabolism and catalyzes the conversion of 2-oxoglutarate to succinyl-CoA and regulates 2-AAA levels in both mice and humans (Danhauser *et al.* 2012, Wu *et al.* 2014, Xu *et al.* 2018). DHTKD1/2-AAA axis was highlighted by that reduced in DHTKD1 levels was associated with higher urinary 2-AAA levels in *Dhtkd1*^{-/-} mice (Xu *et al.* 2018). Our studies reveal that *Dhtkd1*^{-/-} mice exhibited weight loss, active energy metabolism, and increased lipid hydrolysis and free fatty acid β -oxidation, resulting in decreased WAT volume and triglyceride content. At the same time, the increase of UCP1 expression in *Dhtkd1*^{-/-} BAT may increase thermogenesis, which enhances the energy metabolic status, eventually leading to increased insulin sensitivity and resistant to age or HFD-induced obesity. We have known that *Dhtkd1* deficiency leads to metabolic substrate 2-AAA accumulation. Therefore, we hypothesized that abnormal metabolic status found in *Dhtkd1*^{-/-} mice was actually closely related to 2-AAA. Then we artificially fed mice with 2-AAA. Consequently, we found similar phenotypes in 2-AAA-fed mice to those found in *Dhtkd1*^{-/-} mice. Our study identifies that DHTKD1 influences lipid metabolism through regulation of 2-AAA levels in both humans and mice. Obesity can cause a lot of health hazards. Among them, cardiovascular disease and diabetes are the major complications with a high concentration of plasma lipid and glucose levels. Nowadays, serum lipid levels can be decreased by statin drugs and diabetes are treated with various hypoglycemic drugs or insulin. Despite the availability of drug and surgical therapeutics, many individuals are intolerant to drugs. Thus, gene therapy brings new directions for treatment, and elevated 2-AAA levels via the inhibition of DHTKD1 may provide a new direction for treating obesity or diabetes.

Overall, the results of our study demonstrated to harness metabolic 2-AAA as a natural regulator in the clinical management of glycolipid metabolism (Fig. 7). It will be a star project that how to reduce the toxicity of 2-AAA to peripheral nerve to the minimum, while using it to treat obesity or diabetes. Anyhow, we hope to extract the essence and discard the dross, making the culprit that causes the human amino acid metabolic and peripheral nervous system diseases benefit the world.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-19-0157>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

W Y X, H Z and Z G W designed experiments and discussed data. W Y X, Y S, H Z, J G, C Z, L T and Y H W performed experiments, and analyzed and interpreted data. L T, S Y L, C L S and H X Z provided reagents and materials. W Y X, H Z, P M and Z L wrote the manuscript. J F participated in discussing data. Z G W supervised the work.

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