Vitamin C rescues high-fat diet-induced predisposition to metabolic dysregulation in GLUT10 deficient mouse model

Chung-Lin Jiang  
Academia Sinica

Chang-Yu Tsao  
Academia Sinica

Yi-Ching Lee (✉️ yiching@gate.sinica.edu.tw)  
Academia Sinica

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Abstract

Background

The development of type 2 diabetes mellitus (T2DM) is highly influenced by complex interactions between genetic and environmental (dietary and lifestyle) factors. Vitamin C (ascorbic acid, AA) has been considered as a complementary nutritional treatment for T2DM. However, evidence for the significance and beneficial effects of AA in T2DM is thus far inconclusive. We hypothesize that combined genetic and dietary factors may influence AA effects on metabolism.

Purpose

We aimed to explore whether AA effects on metabolism are influenced by the combination of genetic and dietary factors.

Methods

We investigated the impacts of AA on high-fat diet (HFD)-induced metabolic dysregulation in wild type (WT) and $GLUT10^{G128E}$ mice. We have previously showed that genetic polymorphisms in glucose transporter 10 (GLUT10) gene are associated with a T2DM intermediate phenotype in non-diabetic population, and $GLUT10^{G128E}$ mice (carrying human orthologous $GLUT10^{G128E}$ variant) are highly sensitive to HFD-induced metabolic dysregulation.

Results

Here we show that AA has greater metabolic impacts on HFD-fed $GLUT10^{G128E}$ mice than HFD-fed WT mice. Remarkably, AA rescues defective epididymal white adipose tissue (eWAT) development in $GLUT10^{G128E}$ mice at early developmental stages. Furthermore, AA attenuates the predisposition of $GLUT10^{G128E}$ mice to HFD-triggered eWAT inflammation, adipokine dysregulation, ectopic fatty acid accumulation, metabolic dysregulation and body weight gain, as compared with WT mice.

Conclusions

Taken together, AA has greater metabolic impacts on HFD-fed $GLUT10^{G128E}$ mice than HFD-fed WT mice. AA plays an important role in improving WAT development and HFD-induced metabolic dysregulation in $GLUT10^{G128E}$ mice. Our results suggest that proper WAT development is essential for metabolic regulation later in life. Furthermore, when considering the usage of AA as a complementary nutrition for prevention and treatment of T2DM, an individual’s genetics and dietary patterns should be taken into account.

Background
Type 2 diabetes mellitus (T2DM) is a major medical problem worldwide, and its development is highly affected by complex interactions between genetic and environmental (dietary and lifestyle) factors. While genetic factors have been associated with T2DM in population studies, the effect sizes of identified variants are typically very small. In addition to their marginal effects, many genetic factors are thought to alter susceptibility by environmental factors, such as high-fat diet (HFD), accelerating the development of T2DM.

During oversupply of nutrients, white adipose tissue (WAT) actively regulates whole-body energy homeostasis by storing lipids and secreting adipokines. Obesity is frequently associated with WAT inflammation, which contributes to further ectopic fat accumulation and finally T2DM. Hence, antioxidants, such as vitamin C (ascorbic acid, AA) have been proposed as a means to attenuate WAT inflammation and T2DM. However, clinical trials examining the effects of AA on T2DM have thus far been inconclusive. We hypothesize that beneficial effects of AA on metabolism might be influenced by combinations of genetic and environmental factors. Such complex interactions between various genetic risk factor combinations and specific diet or lifestyle characteristics make clinical studies on the effects of AA on T2DM difficult to design. Therefore, we first used a genetically predisposed mouse model to investigate whether AA effects on metabolism might be influenced by particular genetic and diet.

In this study, we examined AA effects on metabolism in wild-type (WT) mice and GLUT10G128E mice fed with a normal diet (CD) or HFD, as we previously showed that GLUT10G128E mice are highly sensitive to HFD-induced T2DM. The chromosomal region around the SLC2A10 locus (human gene encoding glucose transporter 10; GLUT10) has been associated with T2DM in sib-pair studies, however, the gene was not directly associated with T2DM in genome-wide association studies. Along these lines, we further demonstrated that the SLC2A10 locus is associated with T2DM intermediate phenotypes in non-diabetic human subjects. In addition, loss-of-function mutations in SLC2A10 lead to a rare autosomal recessive connective tissue disorder called arterial tortuosity syndrome (ATS; OMIM 208050). We found that GLUT10 transports the oxidized form of AA (dehydroascorbic acid) and regulates intracellular AA status in fibroblasts, aortic smooth muscle cells (ASMCs), and adipocytes using GLUT10 deficient cells, and these results were then validated in fibroblasts from ATS patients by other groups. Interestingly, our studies on the effects of GLUT10 genetic variants on metabolism using a mouse model that carries a rare human genetic variant of SLC2A10 (GLUT10G128E mice) revealed that GLUT10G128E mice have impaired WAT development and are highly sensitive to HFD-induced obesity and metabolic dysregulation compared with WT mice.

In this study, we utilize the GLUT10G128E mice to demonstrate that AA differentially affects metabolism depending on genetic predisposition and diet, providing strong support for the idea that clinical studies on the effects of AA in T2DM prevention should account for interactions between diet and specific genetic variants.
Results

AA supplementation attenuates HFD-induced metabolic dysregulation in GLUT10G128E mice

To evaluate the effects of AA supplementation on metabolism in WT and GLUT10G128E mice on a CD or HFD, we began supplementing the drinking water with 3.3g/L AA for breeding pairs, nursing females, and mice after weaning. This protocol of AA supplementation was previously demonstrated to maintain optimal physiological AA levels in AA synthesis-deficient mice. Male mice were then placed on a CD or HFD (Fig. 1A). We first analyzed the effect of AA supplementation on serum AA levels in WT and GLUT10G128E mice at 3 and 20 weeks of age. We found that AA supplementation of pregnant and nursing mothers led to significantly increased serum AA levels in both WT and GLUT10G128E pups at 3 weeks of age (Fig. 1B), even though mice can synthesize AA de novo. In contrast, no significant differences were observed in serum AA levels among the different genotype or diet groups at 20 weeks of age, although AA supplementation was continued (Fig. 1C). Thus, AA supplementation in drinking water of breeding pairs and nursing females increased serum AA levels in the progeny, but AA supplementation of weaned mice did not further increase the serum AA levels in both WT and GLUT10G128E mice.

We then compared the changes in body weight and metabolism-related parameters in WT and GLUT10G128E mice fed with CD or HFD and with or without AA supplementation. GLUT10G128E mice gained more weight on a HFD than did WT mice, similar to the results of our previous study (Fig. 1D and E). While AA effectively reduced the HFD-induced body weight gain in both HFD-fed WT mice (Fig. 1D and E), the supplementation more readily prevented HFD-induced body weight gain in GLUT10G128E mice than in WT mice (Fig. 1D and 1E). AA has no effect on body weight in either WT mice or GLUT10G128E mice on CD (Fig. 1D and E).

To analyze the differential effects of AA on reducing HFD-induced body weight gain in WT and GLUT10G128E mice, we assessed the metabolic consequences of AA supplementation. Of note, AA supplementation did not significantly affect food intake, physical activity (walking and resting times), or energy expenditure (VO2, VC02, RER and heat production) in mice of either genotype on a HFD (Fig. S1). We then monitored the changes of fasting blood glucose (FBG) levels in the mice after HFD-feeding. The FBG levels were significantly higher in HFD-fed GLUT10G128E mice than in HFD-fed WT mice after 15 weeks of HFD-feeding (20 weeks of age) (Fig. 2A). We therefore analyzed the metabolic parameters at 20 weeks of age in WT and GLUT10G128E mice on a CD or a HFD and with or without AA supplementation. At this time-point, the HFD-fed WT mice exhibited a trend toward increased FBG when compared with CD-fed WT mice, but the trend did not reach statistical significance (Fig. 2B). We also did not observe significant increases in glycated hemoglobin (HbA1c; an indicator of the average blood sugar levels over the past three months) or insulin levels in HFD-fed WT mice compared with CD-fed controls (Fig. 2C and D). In contrast, FBG levels, HbA1c levels, and insulin levels were significantly increased in HFD-fed GLUT10G128E mice when compared with HFD-fed WT mice (Fig. 2B, C and D). These observations are consistent with our previous findings. Most importantly, AA supplementation attenuated HFD-induced increases in FBG,
HbA1c and insulin levels (Fig. 2B, C and D). Moreover, HFD-fed GLUT10G128E mice exhibited a more profound reduction in glucose tolerance and insulin sensitivity compared with HFD-fed WT mice. Here we showed that AA significantly improved the HFD-induced glucose intolerance and insulin resistance in GLUT10G128E mice, as measured by the glucose tolerance test (GTT) and insulin resistance test (ITT), respectively (Fig. 2E and F). Although AA had no significant effects on FBG, HbA1c or insulin levels in HFD-fed WT mice, the supplementation did significantly improve insulin resistance in HFD-fed WT mice (Fig. 2F). Nevertheless, the improvement in HFD-fed GLUT10G128E mice was more prominent than the improvement seen in HFD-fed WT (Fig. 2F). Taken together, these results again validate that GLUT10G128E mice are highly sensitive to HFD-induced metabolic dysregulation. Most importantly, AA supplementation attenuates the predisposition of HFD-induced metabolic dysregulation in GLUT10G128E mice.

AA supplementation rescues HFD-induced eWAT inflammation and adipokine dysregulation in GLUT10G128E mice

We then sought to elucidate how AA rescues HFD-induced metabolic dysregulation in GLUT10G128E mice. First, we determined the AA effects on overall body fat and lean compositions in WT and GLUT10G128E mice on the HFD. Feeding with a HFD increased the body fat composition in both WT and GLUT10G128E mice (Fig. 3A and B). AA had a trend toward reducing body fat composition in HFD-fed WT mice but did not reach significant significant (Fig. 3A and B). In contrast, AA supplementation significantly reduced the body fat composition in HFD-fed GLUT10G128E mice (Fig. 3A and B). Additionally, HFD reduced the body lean composition in both WT and GLUT10G128E mice, but AA only significantly attenuated the reduction in body lean composition in HFD-fed GLUT10G128E mice (Fig. 3C). We therefore analyzed AA effects on two major fat pads, epididymal WAT (eWAT) and subcutaneous inguinal WAT (sWAT) in CD- or HFD-fed WT and GLUT10G128E mice. AA had no effect on the weight of eWATs in CD-fed WT and GLUT10G128E mice. AA supplementation reduced the weight of eWATs in HFD-fed WT mice, but not in HFD-fed GLUT10G128E mice (Fig. 3D). Notably, the weight of sWAT was highly increased in HFD-fed GLUT10G128E mice compared with HFD-fed WT mice, and AA supplementation has more pronounced effects in reducing the weight of sWAT in HFD-fed GLUT10G128E mice than in WT mice.

We have demonstrated that HFD feeding specifically induces inflammation and fibrosis in eWAT (a type of visceral fat) of GLUT10G128E mice, but HFD does not induce a similar response in sWAT of GLUT10G128E mice or the eWAT/sWAT of WT mice, even though the weight of sWAT was significantly increased in HFD-fed GLUT10G128E mice. As central obesity (over-accumulation of visceral fat) is associated with local and systemic inflammation and predisposes individuals to metabolic dysregulation, we then further analyzed the AA effects on eWAT in HFD-fed WT and GLUT10G128E mice. To evaluate the effects of AA supplementation on HFD-induced inflammation in eWAT, we first examined the crown-like structures (CLSs) that surround dead adipocytes and are indicative of inflammation in WAT. No CLSs were observed in eWAT of HFD-fed WT mice, whereas CLSs were frequently found in eWAT of HFD-fed GLUT10G128E mice (Fig. 4A). Moreover, AA supplementation reduced the apparent HFD-induced
increases in CLSs within eWAT of GLUT10\(^{G128E}\) mice (Fig. 4A). In contrast, no CLSs were observed in sWAT of either HFD-fed WT or HFD-fed GLUT10\(^{G128E}\) mice (Fig. S2). Furthermore, AA supplementation had no affect the size or structure adipocytes in sWAT of HFD-fed WT mice or HFD-fed GLUT10\(^{G128E}\) mice (Fig. S2). Thus, we conclude that GLUT10\(^{G128E}\) mice tend to develop HFD-induced eWAT inflammation, and AA supplementation reduces HFD-induced eWAT inflammation.

The eWAT inflammation can change the expression of adipokines and predispose individuals to metabolic dysregulation \(^4,25\). We therefore determined AA effects on systemic adipokine levels in HFD-fed WT and GLUT10\(^{G128E}\) mice by analyzing the serum levels of adipokines that control systemic energy homeostasis, including adipokine, leptin, interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α); these adipokines were previously shown to be highly dysregulated in eWAT of HFD-fed GLUT10\(^{G128E}\) mice \(^9\). Notably, AA supplementation increased the serum levels of adiponectin, a protective adipokine in CD-fed GLUT10\(^{G128E}\) mice (Fig. 4B). The levels of leptin, a cytokine correlated with body fat composition, were increased in HFD-fed WT and GLUT10\(^{G128E}\) mice, and the HFD-fed GLUT10\(^{G128E}\) mice exhibit higher leptin levels than WT counterparts (Fig. 4C). AA supplementation significantly suppressed the elevated serum leptin levels, and this suppression was to a higher extent in HFD-fed GLUT10\(^{G128E}\) mice compared with HFD-fed WT mice (Fig. 4C). Serum levels of two inflammatory cytokines, IL-6 and TNF-α, were highly elevated in HFD-fed GLUT10\(^{G128E}\) mice compared with HFD-fed WT mice (Fig. 4D and E). Most importantly, AA supplementation significantly suppressed HFD-induced serum levels of IL-6 and TNF-α in GLUT10\(^{G128E}\) mice (Fig. 4D and E).

Based on the observations that AA supplementation attenuated HFD-induced CLSs in eWAT and serum levels of IL-6 and TNF-α in GLUT10\(^{G128E}\) mice, we conclude that AA supplementation counteracts the predisposition of GLUT10\(^{G128E}\) mice to HFD-induced eWAT inflammation and adipokine dysregulation.

**AA supplementation rescues HFD-induced ectopic lipid accumulation in GLUT10\(^{G128E}\) mice**

eWAT inflammation and adipokine dysregulation can contribute to increased serum levels of free fatty acids (FFA) and total cholesterol (TCHO), leading to lipid deposition in other organs, including liver and interscapular brown adipose tissue (iBAT) \(^27-29\). Thus, we set out to determine the effects of AA on HFD-induced ectopic lipid accumulation in GLUT10\(^{G128E}\) mice. We hypothesized that AA supplementation-mediated decreases in eWAT inflammation and adipokine dysregulation in HFD-fed GLUT10\(^{G128E}\) mice may reduce serum levels of FFA and TCHO, and reduce ectopic lipid deposition in liver and iBAT. In our previous work, we demonstrated that HFD-fed GLUT10\(^{G128E}\) mice have increased serum levels of FFA and TCHO and increased lipid accumulation in liver and iBAT, as demonstrated by increased triglyceride content, tissue size and weight, as well as more frequent appearance of fat vacuoles in tissue sections \(^9\). In this study, we observed similar patterns of increased serum levels of FFA and TCHO (Fig. 5A and B). We also observed increased tissue size, weight, and frequency of fat vacuole appearance in tissue sections of liver and iBAT in HFD-fed GLUT10\(^{G128E}\) mice (Fig. 5C-H). We then examined the effects of AA
supplementation on these parameters. Notably, AA supplementation reduced the HFD-induced serum levels of FFA and TCHO in GLUT10G128E mice (Fig. 5A and B). Furthermore, AA supplementation reduced the sizes and tissue weights of liver and iBAT and appearance of fat vacuoles in these tissues from HFD-fed GLUT10G128E mice (Fig. 4C-H). Thus, we conclude that AA supplementation rescues HFD-induced ectopic lipid accumulation in GLUT10G128E mice.

**AA supplementation improves eWAT development in GLUT10G128E mice**

We then determine how AA rescues HFD-induced eWAT inflammation and subsequent metabolic dysregulation in GLUT10G128E mice. As GLUT10G128E mice have compromised eWAT development, which plays a critical role in predisposing the mice to HFD-induced metabolic dysregulation due to the increased inflammation in eWAT and subsequent ectopic lipid accumulation in other tissues, we then tested whether AA supplementation could improve compromised eWAT development in GLUT10G128E mice. The eWAT deposits arise during late-embryonic and neonatal development, we therefore analyzed AA-mediated rescue of early eWAT development by determining the weight and histology of eWAT at 3 weeks of age. AA supplementation in nursing females did not affect body weights of either WT or GLUT10G128E pups at 3 weeks of age (Fig. 6A). However, AA supplementation rescued the reduced eWAT weight (Fig. 6B). AA supplementation also rescued the reduced average adipocyte size in GLUT10G128E eWAT, by reducing the percentage of small adipocytes (< 100 area \( \mu m^2 \)) and increasing the percentage of large adipocytes (> 250 area \( \mu m^2 \)), according to quantification of adipocyte size in GLUT10G128E eWAT sections (Fig. 6C-E). In contrast, AA supplementation did not affect the weight of eWAT nor did it affect the average size or size range of adipocytes in eWAT sections of WT mice (Fig. 6B-E). Thus, AA supplementation rescues weight and adipocyte size in eWAT of GLUT10G128E mice.

We next examined whether AA-mediated rescue of weight of eWAT in GLUT10G128E mice might also involve in increased adipogenesis, in addition to the increased adipocyte size. We have demonstrated that AA supplementation induces adipogenesis in both GLUT10-deficient and control preadipocytes. However, AA supplementation has more pronounced effects in GLUT10-deficient preadipocytes, including mouse embryonic fibroblasts (MEFs) from GLUT10G128E mice as well as GLUT10-knockdown preadipocytes (3T3-L1 cells). We determined the AA effects on adipogenesis in vivo by examining the expression of a preadipocyte marker, preadipocyte factor 1 (Pref-1), and a key adipogenic transcription factor, peroxisome proliferator-activated receptor gamma 1 (PPAR\( \gamma \) 1), in eWATs of WT and GLUT10G128E mice at 3 weeks of age. Pref-1 is highly expressed in preadipocytes and absent after adipocyte differentiation. GLUT10G128E eWATs had higher levels of Pref-1 protein than did WT eWATs, AA supplementation reduced the Pref-1 protein levels in GLUT10G128E eWATs (Fig. 6F). These results suggest that more preadipocytes existed in GLUT10G128E eWATs than WT eWATs, and AA supplementation reduced the preadipocytes in GLUT10G128E eWATs. Furthermore, AA supplementation increased the expression levels of a key adipogenic transcription factor, PPAR\( \gamma \) 1, in eWATs of GLUT10G128E mice and WT mice. These in vivo results were consistent with our previous findings from in vitro cell culture studies. Taken together, these
results suggest that AA supplementation promoted adipogenesis in eWATs of \textit{GLUT10}^{G128E} and WT mice and reduced undifferentiated preadipocytes in \textit{GLUT10}^{G128E} eWATs.

\section*{Discussion}

The development of T2DM involves interactions between genetic and environmental factors, and transitions in customary dietary patterns (e.g., switch to HFD) have greatly contributed to the increased prevalence of obesity and accelerated the spread of T2DM epidemic worldwide. At least partly based on its antioxidant properties, AA has been considered as a complementary nutritional treatment for T2DM. However, evidence for the significance and beneficial effect of AA in T2DM has thus far been inconclusive. We suspect that one reason for this inconclusive evidence is that combined genetic and dietary factors may greatly influence AA effects on metabolism. To test the hypothesis, we evaluated the influence of combined genetic and dietary factors on the effectiveness of AA supplementation in mice carrying an orthologous human \textit{GLUT10}^{G128E} variant after feeding with HFD. We chose this combination because genetic polymorphisms in GLUT10 gene are associated with a T2DM intermediate phenotype in non-diabetic population, and \textit{GLUT10}^{G128E} mice have reduced eWAT development and are highly sensitive to HFD-induced metabolic dysregulation \cite{9}. Here, we demonstrate that AA has more pronounced effects in attenuating HFD-induced metabolic dysregulation in \textit{GLUT10}^{G128E} mice compared with WT mice. The mechanism of protection is partly through AA-mediated rescue of eWAT development at early stage, which diminishes later HFD-induced eWAT inflammation and metabolic dysregulation in \textit{GLUT10}^{G128E} mice (Fig. 7). These findings support the idea that the individual gene variants and dietary patterns should be taken into account when considering AA for T2DM prevention and treatment. Furthermore, our study suggests that proper WAT development is essential for metabolic regulation later in life, and higher systemic AA levels might facilitate proper WAT development in individuals carrying certain genetic risk factors. Moreover, many other genetic variants and environmental factors can affect AA requirement \cite{32,33}. Therefore, higher levels of AA intake may be also required to sustain normal physiological function and benefit metabolism in these populations.

We found that AA supplementation significantly increases serum AA levels in pups before weaning and is associated with improved adipogenesis and eWAT development in \textit{GLUT10}^{G128E} mice (Figs. 1 and 5). This finding is consistent with our previous \textit{in vitro} results, which showed AA supplementation rescues impaired adipogenesis of GLUT10-deficient cells by increasing DNA demethylation of key adipogenic transcription factors \cite{9}. Even though the AA supplementation was continued (Fig. 1), we did not observe significantly increased serum AA levels in mice at 20 weeks of age. As mice can synthesize AA de novo \cite{24}, adult mice might have homeostatic mechanisms that will mask the effects of AA supplementation. Nevertheless, the AA supplementation still improved HFD-induced eWAT inflammation, adipokine dysregulation, and metabolic dysregulation in adult \textit{GLUT10}^{G128E} mice (Fig. 3). These results suggest that AA-mediated rescue of WAT development might be largely responsible for the later attenuation of HFD-induced eWAT inflammation and metabolic dysregulation in \textit{GLUT10}^{G128E} mice. Since congenital
lipodystrophy (impaired WAT development) can lead to almost all features of metabolic syndrome \(^{34;35}\), our findings again emphasize the importance of proper WAT development in metabolic regulation. Furthermore, AA supplementation might be able to improve WAT development and metabolic syndrome in lipodystrophy patients.

To analyze the differential AA effects in HFD-fed WT and \(GLUT10^{G128E}\) mice, we first setup the analyzing end-point by monitoring the changes of FBG levels after HFD feeding. The FBG levels were significantly higher in HFD-fed \(GLUT10^{G128E}\) mice than HFD-fed WT mice after 15 weeks of HFD-feeding (20 weeks of age) (Fig. 2A). We therefore analyzed several metabolism-related parameters in 20-week-old WT and \(GLUT10^{G128E}\) mice after feeding with a CD or a HFD and with or without AA supplementation. \(GLUT10^{G128E}\) mice are predisposed to HFD-induced metabolic dysregulation. At that time-point, \(GLUT10^{G128E}\) mice exhibited eWAT inflammation, adipokine dysregulation, increased fat accumulation in liver and other features of metabolic dysregulation. Although the HFD-fed WT mice also had increased body weight and body fat, these effects were not accompanied by obvious signs of metabolic dysregulation, such as eWAT inflammation, insulinemia, glycemia, increased systemic inflammatory cytokines, or the other metabolic features we measured. These results suggest that at the end of our HFD feeding protocol, the WT mice remained metabolically healthier than the \(GLUT10^{G128E}\) mice. In light of our previous work, these results confirm that \(GLUT10^{G128E}\) mice are more susceptible to HFD-induced metabolic dysregulation than WT mice. Most importantly, we demonstrate that AA has more pronounced effects on alleviating metabolic dysregulation in HFD-fed \(GLUT10^{G128E}\) mice than HFD-fed WT mice, suggesting that AA might also have more beneficial effects in more severe metabolic dysregulation populations.

We found that AA supplementation significantly decreases body weight, body fat composition and metabolic dysregulation in HFD-fed \(GLUT10^{G128E}\) mice than these in HFD-fed WT mice without affecting food intake (Figs. 1 and 3). In line with our findings, AA supplementation has been shown to reduce body weight and body fat in cafeteria diet-induced obese rats \(^{36}\), obese \(ob/ob\) mice \(^{37}\), and ovariectomized rats \(^{38}\), without affecting food intake. Furthermore, AA has more pronounced effects on reducing body weight gain and metabolic dysregulation in \(ob/ob\) mice and ovariectomized rats compared with their controls. The mechanism by which AA reduces obesity and rescues metabolic dysregulation in different obese models may be complex. For instance, it has been postulated that AA might combat obesity and metabolic syndrome in part through anti-oxidative and anti-inflammatory properties. In obese animals, elevated reactive oxygen species and activated inflammation-related pathways are associated with adipocyte hypertrophy in visceral fat \(^{39;40}\). AA supplementation in cafeteria diet-induced obese rats led to reduced mRNA expression of oxidative and inflammation markers in retroperitoneal fat \(^{41;42}\). Furthermore, evidence from cultured cells suggests that AA is also involved in modulating intracellular fat accumulation \(^{38;43}\). In addition to its roles as an antioxidant and anti-inflammatory agent, AA is an enzyme cofactor that is required for many important biological functions \(^{44}\). In this capacity, AA regulates DNA demethylation, histone demethylation, and synthesis of collagen and carnitine \(^{44;45}\). It will be
especially interesting to determine whether these functions might influence adipose function and metabolism in the context of HFD feeding. Nevertheless, since no obvious signs of inflammation were observed in our HFD-fed WT mice, AA-mediated reductions in lipid accumulation might serve as main reasons for its ability to reduce body weight and body fat in the mice. AA supplementation shows more pronounced effects on attenuating HFD-induced metabolic dysregulation in \textit{GLUT10}^{G128E} mice than in WT mice. We observed that AA significantly increases eWAT development and reduces eWAT inflammation (Figs. 4 and 6). Furthermore, we have demonstrated that AA supplementation has more pronounced effects on promoting adipogenesis in GLUT10-deficient preadipocytes than WT preadipocytes. AA supplementation induces adipogenesis through increasing DNA demethylation in the regulatory regions of two central adipogenesis-regulating transcription factors, \textit{Cebpa} and \textit{Pparg}, and increasing their expression \cite{9}. We suspect that AA-mediated rescuing of eWAT development in \textit{GLUT10}^{G128E} mice at early stage and alleviating eWAT inflammation and lipid accumulation in adipocytes at later HFD-feeding are all contribute to alleviate the metabolic dysregulation in HFD-fed \textit{GLUT10}^{G128E} mice.

In this work, we utilize \textit{GLUT10}^{G128E} mice, which carry an orthologous human \textit{GLUT10}^{G128E} variant with compromised GLUT10 function, as a mouse model of susceptibility to HFD-induced metabolic dysregulation. Genetic polymorphisms in the GLUT10-encoding human gene (\textit{SLC2A10}) locus are associated with a T2DM intermediate phenotype in a non-diabetic population \cite{9}. Furthermore, we previously showed that adipogenesis is not only affected in \textit{GLUT10}^{G128E} MEFs but also in GLUT10-knockdown 3T3-L1 cells \cite{9}. Notably, there are more than 700 identified rare variants in \textit{SLC2A10} gene that are predicted to affect GLUT10 function. Thus, our results suggest that other variants in \textit{SLC2A10} associated with compromised GLUT10 expression or function might also negatively affect eWAT development, and AA could potentially help to attenuate HFD-induced metabolic regulation in carriers of these variants.

\textit{GLUT10}^{G128E} mice need higher systemic AA levels than WT mice in order to maintain proper eWAT development and antioxidant capacity. GLUT10 deficient cells have poor ability to maintain intracellular AA levels \cite{9,19,20}. AA and its oxidized form, dehydroascorbic acid (DHA), can be transported into cells and intracellular compartments by active sodium ascorbic acid transporters (SVCTs) and facilitative glucose transporter members (GLUTs), respectively \cite{47}. GLUT10 is mainly located in intracellular compartments \cite{20}, and GLUT10 mutants do not affect the expression of any other SVCTs or GLUTs \cite{9}. Thus, the reduced intracellular AA levels found in GLUT10-deficient cells are mainly due to a reduced ability recycling DHA to AA in mitochondria \cite{20}. As AA cannot be retained or accumulated in the body, the excess is immediately eliminated through urine \cite{46}. As such, AA supplementation improves eWAT development and improves HFD-induced metabolic changes in \textit{GLUT10}^{G128E} mice. As a result, higher AA levels are needed for \textit{GLUT10}^{G128E} mice to maintain their physiological function and antioxidant capacity.

**Conclusions**
In summary, this study provides a proof of concept that AA has differential effects on metabolism in the presence or absence of genetic and environmental T2DM-risk factors. We expect these findings will stimulate more sophisticated population studies to accurately assess whether AA is an appropriate treatment or preventative agent for T2DM in susceptible populations.

**Methods**

**Mice**

All animal protocols were approved by the Institutional Animal Care and Utilization Committee at Academia Sinica (Protocol #14-12-795). GLUT10<sup>G128E</sup> mice were generated on a C3HeB/FeJ background and were backcrossed to C57BL/6J background as previously described<sup>19</sup>. WT and GLUT10<sup>G128E</sup> mice in this study were on the C57BL/6J background and maintained by heterozygous matings. Mice were housed in a specific pathogen-free controlled environment with a 14-h light/10-h dark cycle at 21–23°C. AA-supplemented groups received AA (3.3 g/L) and 0.01 mM EDTA in the drinking water, which was changed once per week. AA supplementation was provided to breeding pairs, pregnant dams, nursing mothers, and after weaning. For diet treatments, after weaning, mice were fed with a CD for 2 weeks and then placed on a CD or HFD from 5 to 15 weeks of age (Fig. 1A). The standard rodent diet (CD) contained 13% energy from fat (LabDiet 5010 rodent Diet, PMI Nutrition International Inc., Brentwood, MO, USA), and the HFD contained 60% energy from fat (58Y1, Young Li Trading Co., New Taipei City, Taiwan). Male mice were used in this study. No data were excluded in the analyses.

**Glucose And Insulin Tolerance Tests (Gtt And Itt)**

For fasting blood glucose measurements, blood samples were collected from the tail vein after overnight fasting. The GTT and ITT were performed as previously described<sup>9</sup>. Briefly, GTT was analyzed in mice at 16 weeks of age, and mice were fasted for 18 h before receiving an intraperitoneal injection of glucose (2 g/kg). ITT was performed on mice at 18 weeks of age; mice were fasted for 8 h, followed by an intraperitoneal injection of insulin (0.75 U/kg, Humulin R U100, Lilly, Eli and Company, Indianapolis, IN, USA). Blood samples were collected from the tail vein prior to injection, and again at 15, 30, 45, 60, 75, and 90 min post-injection. Blood glucose levels were assessed using a glucometer (Accu-Chek Performa, Roche Medical Diagnostic Equipment Co., Taiwan).

**Measurement Of Food Intake And Metabolic Rate**

Mice were housed individually for measurement of food intake and water intake, using Tecniplast® Metabolic Cage (Tecniplast, Via I Maggio, Italy). The metabolic rate was measured using the CLAMS-home cage (CLAMS-HC) system (Columbus Instruments, Columbus, OH, USA) in the Taiwan Mouse Clinic at Academia Sinica. The first readings were taken after a 48-h acclimation period. Heat production, RER,
oxygen consumption rate (VO2), and carbon dioxide production (VCO2) rates were determined. VO2, VCO2, and heat were measured every 17 min during a 76-h period at the indicated temperature and were normalized to body weight.

**Blood Chemistry And Adipokine Assays**

For blood chemistry and adipokine assays, blood was collected from cardiac puncture at the conclusion of experiments. The TCHO levels were analyzed from serum samples using Fuji biochemical slides and a Fuji Dri-Chem 4000i analyzer (Fujifilm Cooperation, Taipei, Taiwan) in the Taiwan Mouse Clinic at Academia Sinica. Plasma levels of adiponectin, leptin, IL-6, and insulin were measured using mouse ELISA kits (Merck Millipore, Taipei, Taiwan). The plasma free fatty acids were measured using an ELISA kit (ab65341, Abcam, Cambridge, MA, USA), and blood HbA1c was measured using the mouse Hemoglobin A1c (HbA1c) Assay kit (Crystal Chem, Elk Grove Village, IL, USA).

**Serum Aa Measurements**

Serum AA levels were determined using an Ascorbic Acid Assay Kit (Abcam, Cambridge, England, UK).

**Histological Analysis And Immunohistochemistry**

Tissue sections were stained with hematoxylin and eosin (H&E).

**Western Blot**

Total protein lysates from tissues were used for analysis. Proteins were transferred to PVDF membranes (Millipore, Billerica, MA). Membranes were then incubated with the primary antibodies against Pref-1 (DLK1) (Proteintech, Rosemont, IL) or β-actin (GeneTex, Irvine, CA) and the appropriate secondary antibodies. The signal was detected by enhanced chemiluminescence (Millipore Merck, Taipei, Taiwan).

**Body Composition**

Mouse body composition was analyzed with Bruker's minispec LF50 Body Composition Analyzer in the Taiwan Mouse Clinic at Academia Sinica.

**Statistics**

Statistical analyses were performed in GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). Data are presented as mean ± standard error of the mean (S.E.M.). Statistical significance was determined by
one-way analysis of variance (ANOVA) followed by Tukey’s test for multiple comparisons. A compact letter display was used to indicate significant differences in pairwise comparisons. \( P \)-values less than 0.05 were considered statistically significant.

**Abbreviations**

T2DM  
type 2 diabetes mellitus  
AA  
ascorbic acid, vitamin C  
ATS  
arterial tortuosity syndrome  
GLUT10  
glucose transporter 10  
CD  
control diet  
HFD  
high-fat diet  
WT  
wild type  
WAT  
white adipose tissue  
eWAT  
epididymal white adipose tissue  
sWAT  
subcutaneous inguinal WAT  
iBAT  
interscapular brown adipose tissue  
FBG  
fasting blood glucose  
FFA  
free fatty acids  
TCHO  
total cholesterol

**Declarations**

**Competing interests**

The authors declare that they have no competing interests.

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Authors’ contributions

C.L.J. and C.Y.T. performed experiments, analyzed data, and interpreted results. Y.C.L. conceived and supervised the work, designed experiments, analyzed data, interpreted results, secured funding, and wrote the manuscript. All authors have reviewed and approved the final manuscript.

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References


Figures
**Figure 1**

**Ascorbic acid (AA) supplementation reduces HFD-induced weight gain.** (A) Experimental overview. *GLUT10*^{G128E} or WT mating pairs, pregnant dams, nursing mothers, and weaned mice were supplied with drinking water supplemented with or without AA (3.3 g/L). The weaned mice were fed with normal diet (CD) for two weeks and then assigned to a CD or HFD from 5 to 20 weeks of age. (B and C) AA supplementation increases serum AA levels in mice at 3 weeks of age. Serum plasma AA levels were
determined at 3 weeks of age (B) and 20 weeks of age (C) using the Ascorbic Acid Assay Kit. (D and E) AA supplementation reduces body weight gains in both WT and \( GLUT10^{G128E} \) mice. (D) Body weights were determined at indicated ages; \( n = 20 \) mice per group. (E) Fasting body weight at 20 weeks of age. The data are shown as mean ± SEM. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey’s test for multiple comparisons. The compact letter display indicates significant differences in pairwise comparisons; groups with different letters are significantly different.
Figure 2

AA supplementation rescues HFD-induced metabolic dysregulation in *GLUT10*G128E* mice. Mice were treated as described in Figure 1A. (A) HFD feeding significantly increased fasting blood glucose (FBG) levels in *GLUT10*G128E* mice at 20 weeks of age. FBG levels were determined at the indicated time-point in CD- and HFD-fed WT and *GLUT10*G128E* mice. The data are shown as mean ± SEM. Statistical significance was determined by a two-tailed Student’s *t*-test. *P* < 0.05. (B-D) AA supplementation
improves metabolism-related readouts in HFD-fed GLUT10<sup>G128E</sup> mice. Data were collected from mice at the conclusion of feeding (20 weeks of age). (B) fasting glucose, (C) fasting HbA1c levels, and (D) fasting insulin levels. (E and F) AA supplementation had more pronounced effects on improving glucose tolerance and insulin sensitivity in HFD-fed GLUT10<sup>G128E</sup> mice. (E) Glucose tolerance test (GTT) was performed on 16-week-old mice, and (F) insulin tolerance test (ITT) was performed on 18-week-old animals. Right panels in D and E show the areas under the GTT and ITT curves (AUC) respectively. The AUC were calculated using GraphPad Prism 7 software. n = 4 mice per group. The data are shown as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Tukey's test for multiple comparisons. The compact letter display indicates significant differences in pairwise comparisons; groups with different letters are significantly different.

**Figure 3**

**AA supplementation reduces body fat composition and WAT weights in HFD-fed mice.** Mice were treated as described in Figure 1A. Data were collected from mice at the conclusion of feeding (20 weeks of age). (A) Representative photographs of mice and eWAT from experimental mice. Each square on the green mat is 1 cm × 1 cm. (B) Body fat and (C) body lean compositions. (D) eWAT weights. The data are shown as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Tukey's test for multiple comparisons. The compact letter display indicates significant differences in pairwise comparisons; groups with different letters are significantly different.

**Figure 4**

**AA supplementation rescues HFD-induced eWAT inflammation and adipokine dysregulation in GLUT10<sup>G128E</sup> mice.** Mice were treated as described in Figure 1A. Data were collected from mice at the conclusion of feeding (20 weeks of age). (A) AA supplementation reduces crown-like structures (CLSs) in eWAT of HFD-fed GLUT10<sup>G128E</sup> mice. CLSs are a hallmark of eWAT inflammation. The eWAT sections were examined by H&E staining. A black arrow points to a presumptive CLS surrounding an adipocyte. (B-E) AA supplementation rescues HFD-induced adipokine dysregulation in GLUT10<sup>G128E</sup> mice. The serum levels of (F) adiponectin, (G) Leptin, (H) IL-6, and (I) TNFa were determined. The data are shown as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Tukey's test for multiple comparisons. The compact letter display indicates significant differences in pairwise comparisons; groups with different letters are significantly different.
Figure 5

**AA supplementation rescues HFD-induced ectopic lipid accumulation in GLUT10\textsuperscript{G128E} mice.** Mice were treated as described in Figure 1A. Data were collected from mice at the conclusion of feeding (20 weeks of age). (A-B) AA supplementation reduces HFD-induced free fatty acid (FFA) levels and total cholesterol (TCHO) levels in GLUT10\textsuperscript{G128E} mice. (A) FFA levels and (B) TCHO levels in serum were determined. (C-H) AA supplementation rescues HFD-induced ectopic lipid accumulation in liver and iBAT. Representative photographs of (C) livers and (D) iBAT from experimental mice. Each square on the green mat is 1 cm × 1 cm. (E) Liver weights. (F) iBAT weights. H&E staining of (G) liver sections and (H) iBAT sections. The data are shown as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Tukey's test for multiple comparisons. The compact letter display indicates significant differences in pairwise comparisons; groups with different letters are significantly different.

Figure 6

**AA supplementation improves eWAT development in GLUT10\textsuperscript{G128E} mice.** Mice were treated as described in Figure 1A. Data were collected from pups at 3 weeks of age. (A and B) AA supplementation increases percentage of eWAT weight of GLUT10\textsuperscript{G128E} mice. (A) body weight and (B) eWAT percent of total body weight were measured. (C-E) AA supplementation increases adipocytes size in eWAT of GLUT10\textsuperscript{G128E} mice. (C) Representative photographs of hematoxylin and eosin (H&E) staining of eWAT sections. (D) The cross-sectional area of adipose cells in eWAT is presented as the percentage of cells in the given size range and (E) average size of cells in eWAT. In D and E, n = 6 mice per group; more than 1000 adipocytes were analyzed in each mouse. (F) AA supplementation reduces Pref-1 protein levels in eWAT of GLUT10\textsuperscript{G128E} mice. The protein levels of Pref-1, C/EBP\textalpha, and PPAR\gamma 1 in eWAT were analyzed by western blotting. Protein samples from 6 mice per group were pooled. The protein levels were quantified, normalized to β-actin levels, and compared to WT no-AA-treated controls. The data are shown as mean ± SEM from triple repeats of western blotting experiments of the pooled samples. Statistical significance was determined by one-way ANOVA followed by Tukey's test for multiple comparisons. The compact letter display indicates significant differences in pairwise comparisons; groups with different letters are significantly different.
Figure 7

Model of AA-mediated attenuation of HFD-induced metabolic dysregulation in \textit{GLUT10}^{G128E} mice. AA supplementation in pregnant dams, nursing mothers and weaned mice rescues eWAT development in \textit{GLUT10}^{G128E} pups and attenuates later of HFD-induced eWAT inflammation and metabolic dysregulation in \textit{GLUT10}^{G128E} mice.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AArescueSI.pdf