Chemical Inhibition of Citrate Metabolism Alters Body Fat Content in Mice

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Introduction

Obesity is one of the most relevant health problems in westernized countries, and affects more than 300 million people worldwide [1]. Multiple factors contribute to the obese phenotype, including altered energy intake and fuel dissipation. Recently, impaired mitochondrial metabolism has been suggested as playing a possible role in the obese phenotype; specifically, *in silico* analyses of published literature on obesity and energy metabolism revealed a putative role for mitochondrial aconitase, the enzyme that converts citrate into isocitrate, in aberrant storage of exogenous nutrients [2]. The authors suggest that reduced activity of mitochondrial aconitase would cause impaired oxidative phosphorylation of nutrient intermediates, and might also lead to increased *de novo* synthesis of fatty acids due to excess citrate accumulation subsequent to reduced mitochondrial conversion into isocitrate.

This study aimed to test this hypothesis using fluoroacetate, a competitive inhibitor of citrate metabolism. We chronically exposed C57bl6 mice to this particular substance, which led to significant alterations in citrate metabolism and body composition, specifically a reduction of body fat content.

Material and Methods

Study animals (C57Bl/6, Charles River, Kisslegg, Germany) were housed according to FELASA regulation and kept on a standard diet (Altromin 1324 fortified, Altromin, Lage, Germany) with unlimited access to chow and liquids. Mice (32 to 36 weeks of age) were assigned to two treatment groups (n = 12 per group, 6-male and 6 female). Osmotic pumps (Mini osmotic pump, Alzet, model 2004) were implanted into the peritoneal cavity under ketamine anesthesia. The pumps were filled before operation according the manufacturer's instructions. After adjustment to body mass and pumping rate of 0.25 µl per hour, pumps contained fluoroacetate (2-fluoro-acetate, Sigma) and an isotonic solution (0.9% saline) to attain an amount of 4 mg per kg body mass per day for four weeks. The control group received 0.9% saline only. To check the plasma levels of citrate during treatment, blood was obtained from the retroorbital sinus every seven days during anesthesia using Isoflurane® (1-chloro-2,2,2-trifluoro-ethyl difluoro-methyl ether, Isoflurane®, Baxter, Unterschleissheim, Germany). Blood samples were centrifuged for 10 min at 6,000 rcf at 277 Kelvin, and citrate was measured using a commercial kit following the manufacturer's instructions (r-biopharm, Germany). Determinations were performed in a random-fed state. Body composition was analyzed by NMR (nuclear magnetic resonance spectroscopy; Mini Spect MQ 10 NMR Analyser Bruker, Karlsruhe, Germany).

The protocol for all animal experiments was approved by governmental animal ethic review board. SPSS Version 11.0 was used for statistical analysis. Results were assumed significant at values of $p < 0.05$.

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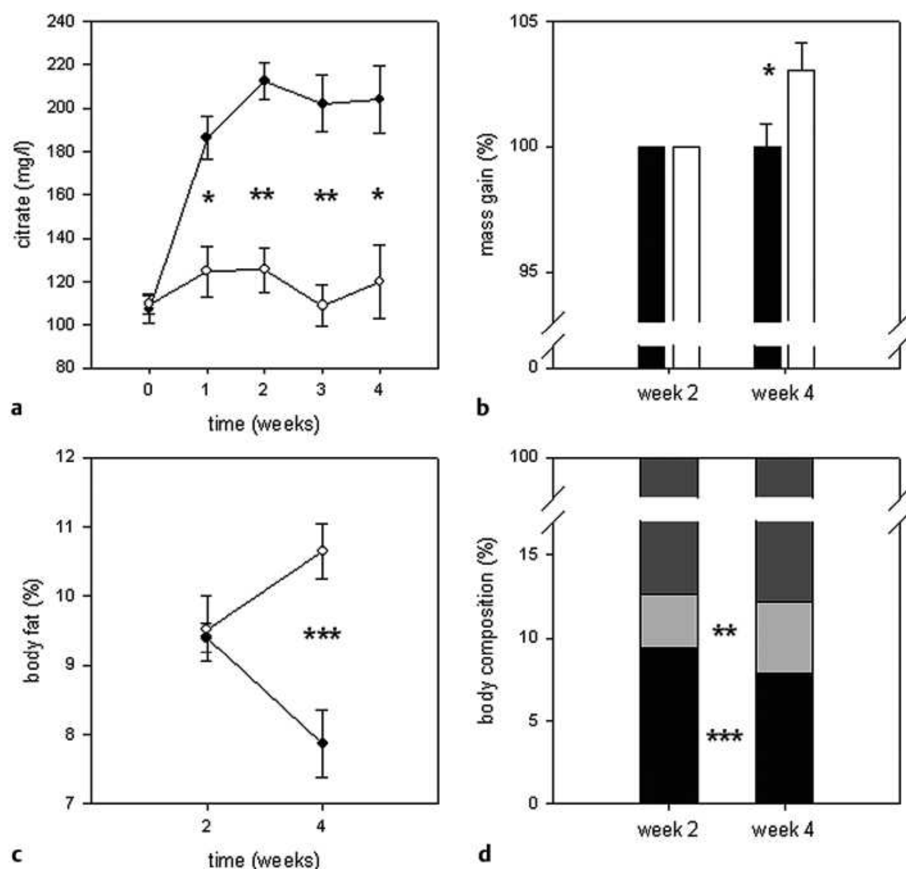

Fig. **1** Catabolic state following chemical inhibition of citrate metabolism. Panel **a** depicts plasma citrate concentrations over four weeks following implantation of osmotic pumps containing fluoroacetate (filled circles) or isotonic saline as control (open circles). Data are given as means ± SEM; $^{**}p < 0.01$, $^{*}p < 0.05$. Panel **b** shows body mass four weeks after implantation of osmotic pumps filled with fluoroacetate (black bars) and saline (white bars) relative to body mass at two weeks. Data are given as mean ± SEM; $^{*}p < 0.05$. Panel **c** depicts changes in body fat two and four weeks after initiation of treatment with fluoroacetate (filled circles) *vs.* controls (open circles). Data are given as the mean ± SEM; $^{***}p < 0.001$. Panel **d** shows changes in body composition two and four weeks after initiation of treatment with fluoroacetate, fat compartment (black bar), free fluid (light grey bar) and lean body mass (dark grey bar) compartment. Data are given as the mean; $^{***}p < 0.001$ difference between fat compartment; $^{**}p < 0.01$ difference between compartment of free fluid.

Results

Based on the initial hypothesis of Wlodek et al, we aimed to inhibit citrate metabolism by chronically exposing C57bl6 mice to fluoroacetate. This substance has previously been shown to be metabolized to fluorocitrate, which in turn competitively inhibits conversion of citrate into isocitrate by aconitase [3]. The substance was applied into the intraperitoneal cavity using implantable osmotic pumps as indicated in the *Material and Methods* section. To test whether for apparent systemic effects of fluoroacetate, serum citrate concentrations were determined. Indeed, serum citrate levels were found to be significantly increased in mice exposed to fluoroacetate when compared to sham-operated control animals. Specifically, serum citrate concentrations were approximately doubled following continuous exposure to fluoroacetate (Fig. **1 a**). After that, we analyzed body composition and body mass gain in sham-operated control animals as well as in study animals exposed to fluoroacetate. When comparing these two groups starting at two weeks after surgery owing to the average recovery time of ten days in C57bl6 mice, a typical gain in body mass was observed in sham-operated control animals only, while body mass did not increase in fluoroacetate exposed animals (Fig. **1 b**). Non-invasive NMR-based analyses were initiated for further analysis of whether specific compartments may be more strongly affected than others. Using this technique, a significant reduction of body fat content was observed in fluoroacetate treated animals only, while a slight increase in body fat was observed in sham-operated animals within two weeks (Fig. **1 c**). Accordingly, we only observed a compensatory increase in the free fluid compartment in fluoroacetate exposed mice when compared to sham-operated animals (Fig. **1 d**), explaining the overall constant body mass in the group of animals treated (Fig. **1 b**). Finally, to address the question as to whether additional mechanisms may contribute to the phenotype observed, food uptake was quantified in mice exposed to fluoroacetate compared to sham-operated animals; while no differences in food uptake were observed before surgery (3.807 ± 0.090 g (prone to sham) versus 3.832 ± 0.095 g (prone to fluoroacetate), ns), those animals receiving fluoroacetate showed reduced uptake of food (4.083 ± 0.130 g (sham) versus 3.637 ± 0.103 g (fluoroacetate), p = 0.013) after surgery.

Taken together, the findings suggest that chronic exposure to fluoroacetate blocks mass gain and reduces body fat in C57bl6 mice.

Discussion

The present study was initiated to test the hypothesis that impaired activity of mitochondrial aconitase might cause obesity in mammals. Subsequent to chronic exposure to fluoroacetate, an inhibitor of citrate metabolism and hence aconitase [3], we have observed a reduction of body fat content in mice in this study. Therefore, we cannot confirm the above mentioned hypothesis using the specific experimental setup employed.

In agreement with previously published evidence, we find an increase in systemic, i. e. serum, citrate concentrations [4] suggesting a pronounced inhibition of citrate metabolism. Nevertheless,

no induction of *de novo* lipogenesis as suggested by Wlodek et al. was observed. This inconsistency with the initial hypothesis might be due to several reasons: Firstly, inhibition of citrate metabolism, and specifically mitochondrial citrate metabolism, has been shown to severely inhibit ATP generation [3,5]. It is feasible that mild inhibition of citrate metabolism only slightly affects mitochondrial ATP synthesis if at all, while impairment of mitochondrial energy conversion in the current model exceeded the catabolic threshold causing an induction of beta-oxidation owing to a specific loss of body fat content. Further studies have been initiated to analyze these possibilities. Secondly, Wlodek et al. assumed specific inhibition of mitochondrial aconitase, which cannot be replicated using a chemical inhibitor since fluoroacetate and fluorocitrate have additionally been suggested to hamper citrate transport through the mitochondrial membrane by covalent binding to membrane proteins [6]. This may cause biochemical trapping of mitochondrial citrate, preventing a cytosolic increase in *de novo* lipogenesis due to both lack of substrate as well as lack of reduced co-substrates, namely NADPH, following a catabolic state in fluoroacetate exposed mammals [7]. Furthermore, fluoroacetate might additionally exert sub-toxic effects on mice during long-term treatment, as suggested by increases in free fluid as well as a significant reduction in food uptake in comparison to non-treated animals as well as in comparison to the pre-operative state.

Taken together, pronounced and chronic inhibition of citrate metabolism by fluoroacetate reduces body fat content in mammals by increasing availability of systemic citrate anions while decreasing food uptake.

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