

F. Isken<sup>1,2</sup>  
T. J. Schulz<sup>3</sup>  
M. Möhlig<sup>1,2</sup>  
A. F. H. Pfeiffer<sup>1,2</sup>  
M. Ristow<sup>1,2,3</sup>

## Chemical Inhibition of Citrate Metabolism Alters Glucose Metabolism in Mice

### Introduction

Diabetes mellitus is one of the most frequent health problems in westernized countries [1]. Type 2 diabetes mellitus is caused by a combination of impaired insulin secretion and decreased insulin sensitivity [2]. Recently, it was suggested that impaired mitochondrial metabolism precedes the development of diabetes mellitus, and may be found in insulin-resistant but otherwise healthy offspring of parents with type 2 diabetes [3]. Furthermore, impaired expression of mitochondrial and mitochondria-related genes has been observed in muscle biopsies from patients with type 2 diabetes [4, 5]. Impaired activity of mitochondrial aconitase (Aco-2) has been hypothetically linked with obesity [6], and hence might as well contribute to a diabetic phenotype.

We aimed to test this hypothesis by employing fluoroacetate, a competitive non-metabolizable inhibitor of aconitase and therefore citrate metabolism. We have chronically exposed C57Bl6 mice to this particular substance, which leads to significant alterations in citrate metabolism and glucose metabolism, indicating specifically an improvement in insulin sensitivity.

### Materials 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. Male Mice (32 to 36 weeks of age) were divided into two treatment groups (n = 6 per group). Osmotic pumps (Mini osmotic pump, Alzet, model 2004) were implanted into the peritoneal cavity under ketamine anaesthesia. The pumps were filled with reagent solutions (see below) before operation according manufacturer's instruction. The pumping rate was adjusted to 0.25 µl per hour so that each mouse received according to its body mass an amount of 4 mg per kg per day for four weeks. The pumps of the experimental group contained fluoroacetate (2-fluoroacetate, Sigma) and an isotonic solution (0.9% saline) while the control group received 0.9% saline only.

After four weeks of the implantation, glucose-tolerance tests and insulin-tolerance tests were performed in overnight-fasted mice as previously described [7]. Mouse plasma insulin levels were determined by ELISA for rat insulin using a mouse insulin standard (both from Crystal Chem Inc., Chicago, Illinois, USA) as described [7]. Plasma citrate during the glucose-tolerance test was measured using a commercial kit following manufacturer's instructions (R-Biopharm, Darmstadt, Germany) as previously described [8].

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

### Affiliation

<sup>1</sup> Department of Clinical Nutrition, German Institute of Human Nutrition Potsdam-Rehbrücke, Nuthetal, Germany

<sup>2</sup> Department of Endocrinology, Diabetes and Nutrition, Charité Berlin, Campus Benjamin Franklin, Berlin, Germany

<sup>3</sup> Department of Human Nutrition, Institute of Nutrition, University of Jena, Jena, Berlin

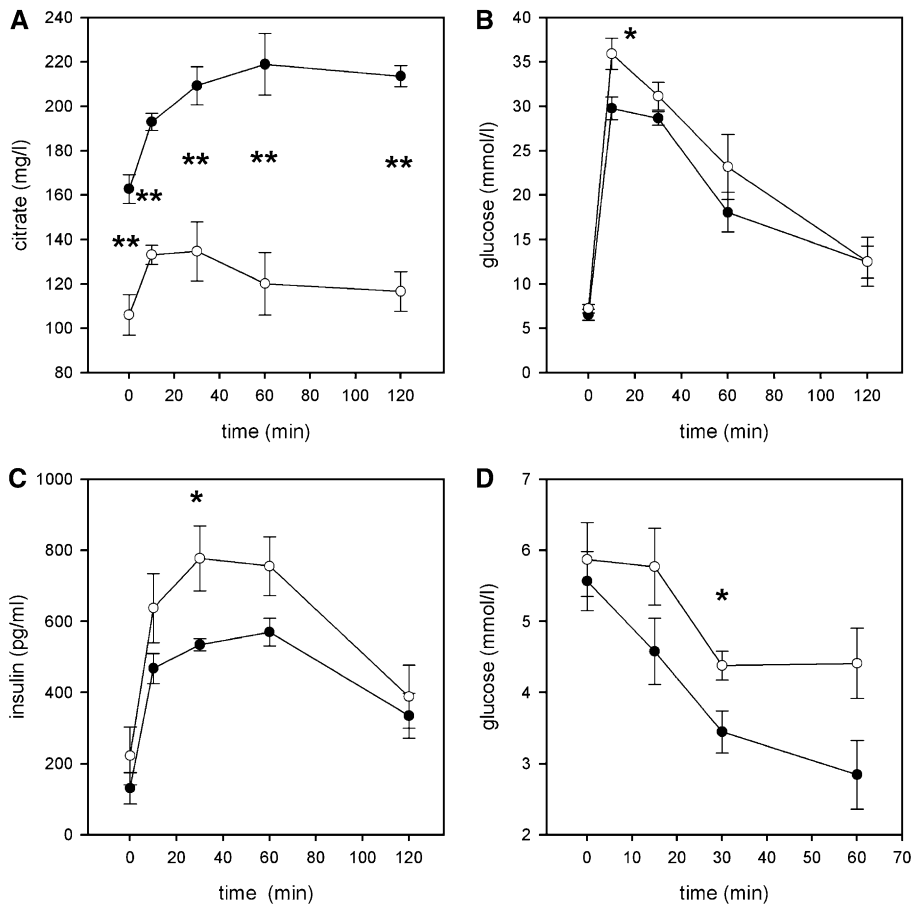
### Correspondence

Michael Ristow · Department of Human Nutrition · Institute of Nutrition · University of Jena · 29 Dornburger St. · 07743 Jena · Germany · Tel.: + 49/3641/94 96 30 · Fax: + 49/3641/94 96 32 · E-mail: michael.ristow@mrstow.org · Website: www.mristow.org

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**Fig. 1** Increased insulin sensitivity following chemical inhibition of citrate metabolism. **Panel A** depicts plasma citrate concentrations after a glucose injection four weeks following implantation of osmotic pumps containing fluoroacetate (filled circles) or isotonic saline as control (open circles). Data are given as the mean  $\pm$  SEM; \*\* =  $p < 0.01$ . **Panel B** shows plasma glucose concentration after i.p. glucose load four weeks succeeding implantation of osmotic pumps filled with fluoroacetate (filled circles) and saline (open circles). Data are given as mean  $\pm$  SEM; \* =  $p < 0.05$ . **Panel C** depicts changes in plasma insulin in the same experiment. Data are given as the mean  $\pm$  SEM; \* =  $p < 0.05$ . **Panel D** shows changes in plasma glucose four weeks after initiation of treatment with fluoroacetate (filled circles) and controls (open circles) following an insulin injection. Data are given as mean-SEM; \* =  $p < 0.05$ .

## Results

Based on our initial hypothesis, we aimed to inhibit citrate metabolism by chronically exposing C57bl6 mice to fluoroacetate. This substance has been previously 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, and have been shown to effectively inhibit citrate metabolism in previous studies [8]. To test whether systemic effects of fluoroacetate might be observed in this specific experimental setup, 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 significantly elevated following continuous exposure to fluoroacetate (Fig. 1A, 0 min). When exposing mice to i.p. glucose injections, serum levels of citrate were found to be persistently (up to 120 min) elevated in fluoroacetate-treated animals only, while sham-operated animals showed a transient increase in serum citrate only (Fig. 1A). Subsequently, blood glucose excursions were determined in such animals following i.p. injections of glucose. While peak glucose levels were slightly higher in sham-operated control animals directly after injection of glucose only (Fig. 1B), no significant differences regarding the area-under-the-curve (AUC) of glucose serum levels were observed (FL-Ac:  $2384.0 \pm 147.9$  mmol/l  $\times$  min vs. controls:  $2772.5 \pm 231.3$  mmol/l  $\times$  min,  $P = 0.195$ ). Subsequently we determined insulin serum levels following i.p. glucose injection. Serum insulin excursions

were found to be significantly reduced in fluoroacetate-treated animals in comparison to sham-operated animals (Fig. 1C), suggesting an increase in insulin sensitivity. Furthermore, the AUC in regard to insulin secretion was found to be reduced in fluoroacetate-treated animals (FL-Ac:  $56779.7 \pm 1939.3$  pg/ml  $\times$  min versus controls:  $75791.0 \pm 7922.7$  pg/ml  $\times$  min,  $P = 0.038$ ). Finally to test whether exogenously applied insulin might also induce differences in serum glucose, insulin tolerance tests were performed. These assays revealed an increased sensitivity towards exogenous insulin in fluoroacetate-treated animals (Fig. 1D), which was confirmed by comparison of the corresponding AUCs (FL-Ac:  $230.6 \pm 16.0$  mmol/l  $\times$  min versus controls:  $295.4 \pm 15.4$  mmol/l  $\times$  min,  $P = 0.019$ ).

Taken together, these findings suggest that chronic exposure to fluoroacetate increases insulin sensitivity and glucose metabolism in C57bl6 mice.

## Discussion

The present study was initiated to test the hypothesis that impaired activity of mitochondrial aconitase might impair glucose metabolism in mammals. Subsequent to chronic exposure to fluoroacetate, an inhibitor of citrate metabolism and hence aconitase [9], we observed here an increased insulin sensitivity and glucose tolerance in mice. Therefore, we cannot confirm the above mentioned hypothesis using the specific experimental setup employed.

Consistent with previously published evidence [10] we find an increase of systemic, i.e. serum, citrate concentrations over a time period of four weeks [8] and crimping after a glucose load suggesting a pronounced inhibition of citrate metabolism. We have previously shown that inhibition of citrate metabolism following chronic exposure to fluoroacetate reduces body fat content in mice [8]. This was previously found to be in contradiction with a recent hypothesis on citrate metabolism and its proposed role in the induction of obesity [6]. Since body mass, and especially body fat content, has been positively correlated with insulin sensitivity in mice and humans [11,12], the findings of the current study might also be influenced by differences in body fat content in the animals evaluated [8]. Hence, besides the putative increase of insulin sensitivity by decreased citrate metabolism itself, as suggested by the current findings, a significant proportion of the data might be attributed to differences of the adipose tissue compartment subsequent to treatment by fluoroacetate [8]. Nevertheless, the present findings somewhat contradict the earlier reports in humans on decreased expression of mitochondrial genes in patients with type 2 diabetes [3]. Even if our mice have reduced fat mass, a strong inhibition of citrate metabolism should counteract the advantages of reduced adiposity, and should altogether lead to unaltered glucose metabolism, rather than significantly increasing the insulin sensitivity. Hence further studies are needed and warranted regarding the role of citrate metabolism, and specifically mitochondrial acinase, in glucose metabolism of mice, and possibly humans.

In conclusion, in addition to the previously described reduction in body fat mass, pronounced and chronic inhibition of citrate metabolism by fluoroacetate increases insulin sensitivity and enhances glucose metabolism in mice.

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