Original Article

MOLECULAR
METABOLISM

Metformin causes a futile intestinal–hepatic cycle which increases energy expenditure and slows down development of a type 2 diabetes-like state

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ABSTRACT

Objective: Metformin, the first line drug for treatment of type 2 diabetes, suppresses hepatic gluconeogenesis and reduces body weight in patients, the latter by an unknown mechanism.
Methods: Mice on a high fat diet were continuously fed metformin in a therapeutically relevant dose, mimicking a retarded formulation.
Results: Feeding metformin in pharmacologically relevant doses to mice on a high fat diet normalized HbA1c levels and ameliorated glucose tolerance, as expected, but also considerably slowed down weight gain. This was due to increased energy expenditure, since food intake was unchanged and locomotor activity was even decreased. Metformin caused lactate accumulation in the intestinal wall and in portal venous blood but not in peripheral blood or the liver. Increased conversion of glucose-1-^{13}C to glucose-1,6-^{13}C under metformin strongly supports a futile cycle of lactic acid production in the intestinal wall, and usage of the produced lactate for gluconeogenesis in liver.
Conclusions: The reported glucose–lactate–glucose cycle is a highly energy consuming process, explaining the beneficial effects of metformin given continuously on the development of a type 2 diabetic-like state in our mice.

Keywords Futile cycle; Splanchnic bed; Metformin; Mitochondria

1. INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) is one of the most common disorders in industrialized countries, with rapidly increasing patient numbers in the last decades; thus, its successful treatment in the setting of a metabolic syndrome gets more and more important. According to the 2015 guidelines of the ADA (American Diabetes Association) and the EASD (European Diabetes Association), lifestyle modification, i.e. weight control and physical activity, in combination with metformin (1,1-dimethylbiguanide) is the current first-line therapeutic concept for T2DM patients [1,2]. Basically, metformin lowers elevated blood glucose levels, and, with the successful treatment of hyperglycemia, it results in a significantly reduced diabetes-related morbidity [3]. Beyond its glucose lowering effect, metformin-treatment results in significant weight loss (summarized in recent large meta-analyses [4,5]). Surprisingly, the mechanisms responsible for lowering body weight are unknown, even though weight loss alone improves glucose homeostasis in T2DM.

Although being introduced and available for clinical use since the 1950ies, metformin's therapeutic mechanisms are still not understood. One of the earliest possible modes of action, then identified for alkylguanidines that are closely related to the biguanidine metformin, was inhibition of oxygen consumption in liver mitochondria [6]. Later, it was shown in isolated hepatocytes that metformin in high

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Received April 6, 2017 • Revision received May 2, 2017 • Accepted May 2, 2017 • Available online 5 May 2017

http://dx.doi.org/10.1016/j.molmet.2017.05.002

concentrations specifically inhibits complex I of the respiratory chain and reduces gluconeogenesis, probably by inhibiting pyruvate carboxylase inside mitochondria, a rate limiting enzyme of this process which is sensitive to the cellular energy as well as the redox state [7,8]. Stimulated by the finding that the ATP/ADP ratio was lowered by metformin in liver, the AMP-activated protein kinase (AMPK) was subsequently shown to be activated by metformin in isolated hepatocytes [9]. However, later it was shown that in mice lacking both isoforms of the AMPKα subunit as well as the upstream kinase LKB1, the hypoglycemic effect of the drug was still maintained [10,11]. Since then, new alternative mechanisms explaining how metformin may inhibit hepatic gluconeogenesis have been proposed, e.g. suppression of glucagon signaling by interfering with cAMP production [12], altering the hepatic redox state by direct inhibition of mitochondrial glycerophosphate dehydrogenase [13] as well as activation of a neurohumoral gut—brain—liver axis [14].

Most studies still concentrate on the liver as the main target of metformin, arguing that this organ plays the key-role in gluconeogenesis and that intracellular drug concentrations will reach high levels after orally administered metformin is absorbed by the intestine [15]. It was previously shown that an important mode-of-action of the drug is to improve lipid homeostasis by stimulating the AMPK mediated phosphorylation of acetyl-CoA carboxylases, which consequently improves insulin sensitivity [16]. Although elegant, it is important to note that the drug was applied by intraperitoneal injection, thus bypassing the physiological route of orally taken metformin.

However, after oral administration, the highest concentration of metformin is not found in the liver but in the intestinal epithelium [17—19]. Early data using obese fa/fa rats already showed that metformin administration significantly increased glucose consumption in the intestine [20] due to mitochondrial inhibition, which was confirmed later demonstrating increased lactate production in isolated human jejunal preparations [19].

Here, we continuously fed metformin in a therapeutically relevant dose, mimicking a retarded formulation, to mice on a high fat diet in order to investigate how the drug slows down the development of T2DM, but most importantly, how it slows down weight gain, the other well described mode of action in patients.

2. MATERIALS AND METHODS

2.1. Animals and experimental protocols

Animals (male C57BL6/J mice) were housed in a 12 h light-dark circle (06:00 on, 18:00 off, including a period of dawn) at constant temperature of 22 °C and a humidity of 60 rH.

Controls received standard chow (Altromin Spezialfutter, Lage, Germany, #TPF-1314: 5% fat, 4.8% disaccharide, 23% protein). HFD treated mice were fed a high fat, high sucrose diet (HFD; Altromin Spezialfutter, Lage, Germany, #105712: 35% fat, 19% disaccharide, 19% protein). HFD + Met and HFD + lateMet received the same HFD supplemented with 0,5% of metformin (1,1-Dimethylbiguanide Hydrochloride, 97%, Sigma Aldrich, Darmstadt, Germany, #D150959). Metformin was added to the diet during the manufacturing process to assure equal distribution over the HFD. Mice had access to chow and water ad libitum unless otherwise specified.

A detailed description about the different cohorts of mice used in this study can be found in the supplementary experimental procedures. All animal procedures were performed in accordance with the German Laws for Animal Protection and were approved by the local animal care committee (Landesamt für Natur-, Umwelt und Verbraucherschutz, LANUV, Recklinghausen, Germany; Az 37.09.298).

2.2. Metabolic characterization and glucose tolerance test

Mice were weighed every two weeks, starting at 6 weeks of age. Intraperitoneal glucose tolerance tests (GTT) were performed every 2 weeks, starting at 12 weeks of age, after 16 h of fasting by an intraperitoneal injection of 2 g/kg glucose (Supplementary Figure 1). Glucose was measured in tail venous blood at 15, 30, 60 and 120 min after injection (Glucomen LX, Berlin-Chemie, Berlin, Germany).

2.3. Indirect calorimetry and physical activity measurement

At the age of 6 weeks, this cohort of animals was divided into two groups and fed HFD + Met or HFD (n = 16 each) for 12 weeks. At the age of 18 weeks, mice were kept for 48 h (starting at 10 am) in an open circuit measurement system (PhenoMaster, TSE Systems GmbH, Bad Homburg, Germany) after having been acquainted to the new environment. Light phase was from 7:30 PM until 6:30 AM. The following parameters were obtained: CO_2 production, O_2 consumption, home cage activity, food intake, water intake and feeding events, and values for energy expenditure (EE) and respiratory exchange ratio (RER; CO_2 production/O_2 consumption) were derived from these measurements.

2.4. Inhibition of mitochondrial complex I and integrity of the intestinal mucosa in the presence of metformin

Mice were sacrificed by cervical dislocation; the small intestine (duodenum) was removed and immediately cooled on a metal plate kept on ice to slow down self-digestion as much as possible. Samples from the duodenum were immersed in Tissue Tek (O.C.T.™ Compound — Sakura Finetek, Staufen, Germany), immediately frozen in liquid nitrogen, and sectioned using a Leica CM1950 cryostat (Leica Microsystems, Wetzlar, Germany) at −20 °C. In order to quantitate NADH dehydrogenase (Complex I) activity, the following method was used (Diaphorase activity, modified from [21]: directly after preparation, 2—3 µm sections were incubated in 0.1 M Tris, pH 7.4, 4 mM NADH, 0.1 M Tris—HCl, pH 7.4, 0.2 mM nitroblue tetrazolium chloride for 10 min at room temperature. Sections were then briefly rinsed with distilled water, dried, and mounted in aqueous medium. Sections incubated without NADH were used as controls. Before, it was established that after 10 min, staining had not reached maximal intensity, thus allowing determination of Complex I activity *in situ* in different samples processed in a highly parallel way. Images were taken at 20× or 40× magnification using an Olympus BX-40 microscope (Olympus, Hamburg, Germany) and staining intensity was analyzed using a thresholding tool-based method (Using ImageJ [22], and the image processing package Fiji for Image J [23,24].). The thresholding tool settings were established in samples from HFD mice and used for quantification of all samples. The ratio of staining-positive to total area of 2—4 samples of the duodenum from HFD and HFD + Met mice (n = 2) were determined.

Staining procedures for hematoxylin-eosin (HE) and PCNA (Proliferating cell nuclear antigen) are described in the supplementary experimental procedures.

2.5. Quantitation of glucose, lactate, and metformin by LC-MS/MS

Mice were fed with HFD or HFD + Met lacking disaccharides, that was supplemented with 1 g of glucose-1-^{13}C (# 297046; Sigma Aldrich, Darmstadt, Germany) per 10 g of food. After 3 h, cheek punch blood samples of variable size were collected directly in 100 µl acetonitrile and diluted 1:10 with acetonitrile; serum samples generated from ventricular cavity blood were diluted 1:10 or 1:100 with acetonitrile. Of these, 10 or 20 µl were analyzed on a triple quadrupole mass spectrometer (4000 QTRAP, AB Sciex, Darmstadt, Germany). The following