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Metformin administration induces hepatotoxic effects in paraoxonase-1-deficient mice

Anabel García-Heredia [a], Marta Riera-Borrull [a], Isabel Fort-Gallifa [a, b], Fedra Luciano-Mateo [a], Noemí Cabré [a], Anna Hernández-Aguilera [a], Jorge Joven [a], Jordi Camps [a, *]

[a] Unitat de Recerca Biomèdica (CRB-URB), Hospital Universitari de Sant Joan, Institut d'Investigació Sanitària Pere Virgili (IISPV), Universitat Rovira i Virgili, Reus, Spain
[b] Laboratori de Referència Sud, Hospital Universitari de Sant Joan, Reus, Spain

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ABSTRACT

Metformin is the first-line pharmacological treatment of diabetes. In these patients, metformin reduces body weight and decreases the risk of diabetes-related complications such as cardiovascular disease. However, whether metformin elicits beneficial effects on liver histology is a controversial issue and, as yet, there is no consensus. Paraoxonase-1 (PON1), an enzyme synthesized mainly by the liver, degrades lipid peroxides and reduces oxidative stress. PON1 activities are decreased in chronic liver diseases. We evaluated the effects of metformin in the liver of PON1-deficient mice which, untreated, present a mild degree of liver steatosis. Metformin administration aggravated inflammation in animals given a standard mouse chow and in those fed a high-fat diet. Also, it was associated with a higher degree of steatosis in animals fed a standard chow diet. This report is a cautionary note regarding the prescription of metformin for the treatment of diabetes in patients with concomitant liver impairment.

1. Introduction

Metformin (dimethylbyguanidine) is the first-line pharmacological treatment of diabetes. In these patients, metformin assists weight loss and reduces the risk of diabetes-related end-points such as microvascular disease, myocardial infarction (large vessel disease) and all-cause mortality. This drug has also been reported to elicit beneficial effects on liver histology, by reducing hepatic steatosis [1]. In normal mice fed with a high-fat diet, metformin has been reported to fully reverse hepatic steatosis and inflammation; effects that appear to be mediated by upregulation of hepatic adenosine monophosphate-activated protein kinase (AMPK) and, as well, to be associated with changes in lipogenic gene expression, such as fatty acid synthase (FASn) [2]. However, clinical studies investigating the effects of metformin on the liver have not reached a consensus [3–6]. Metformin possesses multiple pleiotropic effects [7–10], and one of the most important is to decrease oxidative stress by enhancing the hepatic levels of antioxidant enzymes such as paraoxonase-1 (PON1) [11,12]. PON1 is a lipolactonase synthesized, mainly, by the liver. It degrades oxidized phospholipids and, as such, plays a role in an organism's antioxidant system [13,14]. Preliminary observations from our laboratory suggest that PON1 is an important factor in explaining the beneficial effects of metformin in the liver [15].

Some reports have suggested that metformin may be useful in the treatment of hepatitis or hepatocellular carcinoma [16]. Conversely, however, several cases of metformin-induced aggravation of liver injury have been reported in patients with liver disease [17–20]; liver damage being documented as the elevation of serum liver enzymes, and improvement in liver function being

Abbreviations: ALT, Alanine aminotransferase; AMPK, Adenosine monophosphate-activated protein kinase; AST, Aspartate aminotransferase; BAT, Brown adipose tissue; CD, Chow diet; eWAT, Epididimal white adipose tissue; FASn, Fatty acid synthase; FPLC, Fast protein liquid chromatography; GTT, Glucose tolerance test; HDL, High-density lipoproteins; HFD, High-fat and high-cholesterol diet; iWAT, Inguinal white adipose tissue; CCL2, Chemokine (C–C motif) ligand-2; pAMPK, Phosphorylated AMPK; PON1, Paraoxonase-1; vWAT, Visceral white adipose tissue.

* Corresponding author.
E-mail address: jcamps@grupsagessa.com (J. Camps).

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documented following discontinuation of the drug for 1 week. Unfortunately, the mechanism by which metformin may induce liver injury is unknown. Severe liver impairment is associated with inhibited hepatic and circulating PON1 levels. Indeed, serum PON1 activity is strongly decreased in patients with chronic hepatitis or cirrhosis, and the magnitude of the decrease is related to the extent of liver damage [21,22]. Moreover, a study found a decreased hepatic PON1 activity related to enhanced lipid peroxidation and liver damage in rats with experimental fibrosis [23]. In addition, PON1 over-expression provided strong protection against the development of experimentally-induced liver disease [24].

With all these pointers in mind, the possibility that PON1 deficiency itself is associated with toxic effects of metformin in the liver warrants investigation. The objective of this study was to evaluate whether metformin elicits toxic effects in the livers of PON1-deficient mice fed a standard chow diet or a high-fat diet.

2. Methods

2.1. Experimental animals and dietary intervention

Male PON1-deficient mice of the C57BL/6J genetic background were the progeny of those provided to us by the Division of Cardiology of the University of California in Los Angeles [25]. These mice develop a mild degree of spontaneous liver steatosis even on a standard chow diet [26]. At 10 weeks of age, mice were fed a high-fat and high-cholesterol diet [HFD group; n = 16; the diet contained w/w 20% fat and 1.00% cholesterol (Harlan, Barcelona, Spain)], or a chow diet [CD group; $n = 16$; the diet contained w/w 14% protein and 0.03 cholesterol (Harlan, Barcelona, Spain)]. The groups were further divided to receive metformin ($n = 8$) or placebo (regular drinking water; $n = 8$). Metformin (DIANBEN® 850 mg) was added to the water to achieve a dose of 166 mg Kg^{-1} day^{-1}. At 24 weeks of age, animals were sacrificed after an overnight fast. Liver, pancreas, visceral white adipose tissue (vWAT), epididimal white adipose tissue (eWAT), inguinal white adipose tissue (iWAT), and brown adipose tissue (BAT) were removed and weighed. Portions of tissue were stored at −80 °C until needed for histological examination, at which stage the tissues were fixed for 24 h in 10% neutral-buffered formalin, embedded in wax, and microtome sectioned for microscopy. Glucose tolerance tests (GTT) were performed in all mice at one week before sacrifice. Glucose (2 mg g^{-1} of body weight) was administered as an intraperitoneal injection under anesthesia. Measurements of blood glucose concentrations were made at t = 0, 15, 30, 60 and 120 min. Glucose was measured with glucose strips adapted to the Accucheck sensor system (Roche Diagnostics).

Wild-type mice fed with chow diet or HFD and receiving metformin or placebo (n = 8, for each group) were used to investigate the effect of PON1-deficiency in liver histology. All procedures adhered to those described by the Helsinki accord on animal experimentation. The study protocol was accepted by the Ethics Committee on Animal Experimentation of the Faculty of Medicine of the *Universitat Rovira i Virgili* (Reus).

2.2. Biochemical measurements

Following an overnight fast, blood samples were collected from anesthetized animals into blood collection tubes not containing anti-coagulant. Serum glucose, cholesterol and triglyceride concentrations together with alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined by standard clinical laboratory procedures. Analysis of serum lipoprotein profiles was performed using fast protein liquid chromatography (FPLC), to evaluate differences in cholesterol and triglyceride distributions among the lipoprotein fractions in the different experimental groups. Briefly, a pooled serum (200 μL from each experimental group) was fractionated in a Superose 6/300 GL column (GE Healthcare Europe, Glattbrugg, Switzerland) equilibrated with phosphate buffer (NaPi) 50 mM, with NaCl 0.150 M, pH = 7.0 and eluted (500 μl fractions) with the same buffer. Cholesterol and triglycerides were measured in the eluted fractions using photometry, with reagents obtained from Beckman Coulter (Brea, CA, USA) and read with an automated microplate reader (BioTeK Instruments Inc., Winooski, VT, USA).

2.3. Histology analyses

Liver and eWAT sections of 2 μm thickness were stained with hematoxylin and eosin to evaluate histological alterations. Steatosis extent and eWAT adipocyte size were estimated by image analysis software (AnalySIS, Soft Imaging System, Munster, Germany). The degree of steatosis was further evaluated using a semi-quantitative score (percentage) of hepatocytes containing lipid droplets. The scores were arbitrarily dichotomized as 1: <33%; 2: 33–66%; 3: >66%, as previously reported [26]. Chemokine (C–C motif) ligand-2 (CCL2) expression was measured as a marker of inflammation using immunohistochemistry with specific antibodies from Santa Cruz Biotechnology (Heidelberg, Germany). F4/80 antigen was determined as a widely-accepted marker of macrophages, using specific antibodies from Serotec (Oxford, UK). For each sample, we included a negative control that was treated exactly as the test samples throughout, except with the primary antibody omitted from the incubations.

2.4. Western blot analysis

Using a Precellys 24 (Bertin Technologies, France) homogenizer, liver samples were homogenized in a lysis buffer containing an inhibitor of the proteases. FASn, AMPK, and its active form phosphorylated AMPK (pAMPK), were measured using specific antibodies from Cell Signaling Tech. (Danvers, MA, USA). Arginase and caspase-9 were measured using antibodies from Abcam Inc. (Cambridge, UK). Actin expression was used as control (antibodies from Sta. Cruz Biotech, CA, USA).

2.5. Statistical analysis

Results are shown as means ± SD. Between group comparisons were with the Mann-Whitney *U* test. Statistical significance was set at $P \leq 0.05$.

3. Results

3.1. Food intake and weight control

As expected, mice fed with HFD weighed more than animals fed with CD. Metformin administration did not produce any significant change in weight, nor in the cumulative food ingested in any of the animal groups (Fig. 1A). Metformin produced a significant increase of eWAT and vWAT weights, and a small reduction in liver weights in mice fed with CD, but not in animals fed with HFD. Metformin also produced a significant increase in pancreas weight in experimental groups of animals, relative to the group of control animals. We did not observe any significant differences in BAT and iWAT in relation to metformin administration (Fig. 1B).

3.2. Glucose tolerance test

Glucose tolerance was impaired in mice fed HFD compared to animals with CD, as shown by the areas under the curve of the GTT