The desert gerbil *Psammomys obesus* as a model for metformin-sensitive nutritional type 2 diabetes to protect hepatocellular metabolic damage: Impact of mitochondrial redox state

Inès Gouaref¹, Dominique Detaille², Nicolas Wiernsperger³, Naim Akhtar Khan⁴, Xavier Leverve⁵, Elhadj-Ahmed Koceir¹*

¹ Bioenergetics and Intermediary Metabolism team, Laboratory of Biology and Organism Physiology, Biological Sciences Institute, University of Sciences and Technology Houari Boumediene, BP 32, ElAlia, Algiers, Algeria, ² Université de Bordeaux, Rhythmo logy and Heart Modeling Institute, Bordeaux, France, ³ CarMeN Laboratory, INSERM U1060, Villeurbanne, France, ⁴ Physiologie de la Nutrition & Toxicologie, INSERM U1236, Université de Bourgogne Franche-Comté (UBFC), Dijon, France, ⁵ University Grenoble Alpes, Laboratoire de Bioénergétique Fondamentale et Appliquée (LBFA), INSERM, U1055, Grenoble, France

* e.koceir@gmail.com

Abstract

Introduction

While metformin (MET) is the most widely prescribed antidiabetic drug worldwide, its beneficial effects in *Psammomys obesus* (*P. obesus*), a rodent model that mimics most of the metabolic features of human diabetes, have not been explored thoroughly. Here, we sought to investigate whether MET might improve insulin sensitivity, glucose homeostasis, lipid profile as well as cellular redox and energy balance in *P. obesus* maintained on a high energy diet (HED).

Materials and methods

*P. obesus* gerbils were randomly assigned to receive either a natural diet (ND) consisting of halophytic plants (control group) or a HED (diabetic group) for a period of 24 weeks. MET (50 mg/kg per os) was administered in both animal groups after 12 weeks of feeding, i.e., the time required for the manifestation of insulin resistance in *P. obesus* fed a HED. Parallel *in vitro* experiments were conducted on isolated hepatocytes that were shortly incubated (30 min) with MET and energetic substrates (lactate + pyruvate or alanine, in the presence of octanoate).

Results

*In vivo*, MET lowered glycermia, glycosylated haemoglobin, circulating insulin and fatty acid levels in diabetic *P. obesus*. It also largely reversed HED-induced hepatic lipid alterations. *In vitro*, MET increased glycolysis but decreased both gluconeogenesis and ketogenesis in the
presence of glucogenic precursors and medium-chain fatty acid. Importantly, these changes were associated with an increase in cytosolic and mitochondrial redox states along with a decline in respiration capacity.

Conclusions
MET prevents the progression of insulin resistance in diabetes-prone P. obesus, possibly through a tight control of gluconeogenesis and fatty acid β-oxidation depending upon mitochondrial function. While the latter is increasingly becoming a therapeutic issue in diabetes, the gut microbiota is another promising target that would need to be considered as well.

Introduction
The epidemic nature of type 2 diabetes mellitus (T2DM) has led to its recognition as an urgent priority by the International Diabetes Institute. Indeed, the prevalence of this disease is predicted to reach 5.4% of the worldwide adult population by the year 2025, i.e., 300 million individuals [1]. T2DM is a multifactorial disease. It results from the interaction of environmental factors and genetic predisposition leading to two major abnormalities: insulin resistance and defective pancreatic β-cell function [2]. During the long-lasting phase that precedes the onset of T2DM, hyperinsulinemia compensates for insulin resistance. Hyperglycaemia then develops with a progressive β-cell failure, but the mechanisms involved remain unknown.

Among the oral antidiabetic agents used for the management of T2DM, Metformin (MET) is mostly considered as first-line drug therapy for patients [3]. Besides its action on glucose homeostasis, which is achieved through a potent reduction of hepatic glucose production due to the inhibition of gluconeogenesis, MET also exerts beneficial effects on the blood lipid profile as well as hepatic steatosis in overweight subjects [4] and ob/ob mice [5]. It is established that phosphorylation of acetyl-CoA carboxylase by AMP-activated protein kinase (AMPK), a master sensor for the fine tuning of cellular energy requirements, is essential for part of these MET-induced improvements [6]. In addition, the internalization of MET was necessary for its biological action, and its effects were specific on insulin signaling pathway [7]. An important breakthrough for understanding the mode of MET action was evidenced by the suppression of glucose output in hepatocytes via AMPK activation [8]. This activation was shown to occur independently of a change in adenylate energy charge [9]. Nevertheless, not all of the MET therapeutic action could be explained by AMPK-dependent mechanisms [10], and there exists now a consensus that MET can activate AMPK due to energetic stress resulting from inhibition of mitochondrial oxidative phosphorylation [11].

Conventional animal models have been employed to elucidate the pathogenesis of T2DM, but they do not recapitulate accurately human disease. In this context, the desert gerbil Psammomys obesus (P. obesus) constitutes a unique model of human android obesity and T2DM [12]. In its native arid environment, the low caloric chenopodiaceae-dominated desert plants serves as its primary diet, but P. obesus with innate insulin resistance usually remains fatty. This feature is mainly explained by a low hydrolysis of liver glucose-6-phosphate [13], leading to normoglycemia and enhanced lipogenesis. When P. obesus are transferred to laboratory conditions and fed a HED ad libitum, the delicate homeostasis of the gerbil becomes impaired and the combination of (i) hyperinsulinemia together with insulin resistance, (ii) increased lipid storage inducing obesity and non-alcoholic steatohepatitis [14], and (iii) hyperglycemia, is responsible for the detrimental effects on β-cells ultimately leading to ketoacidosis [15]. In diabetic P. obesus, peripheral insulin resistant state was evidenced by a reduced
capacity of insulin to activate hormonal receptor tyrosine kinase [16], and the resulting high rate of hepatic gluconeogenesis was characterized by stimulation of insulino-dependent cellular pathways involving phosphoenolpyruvate carboxykinase, glucose 6-phosphatase and pyruvate dehydrogenase [17]. We recently demonstrated that hepatic deterioration, with high oxidative stress and mitochondrial dysfunction, contributed to deleterious outcomes of insulin resistance in diabetic *P. obesus* [18]. Furthermore, food restriction in *P. obesus* reversed the symptoms of frank diabetes without reducing insulin secretion [19]. In the same way, the administration of flavonoid silibinin to obese gerbils provided substantial protection against the progression toward metabolic syndrome by blocking the oxidative process and improving liver steatosis [20].

This current study, using both animal and cellular models, was designed to evaluate the impact of MET on plasma and hepatic profiles as well as metabolic abnormalities and energy homeostasis in *P. obesus* either developing or not diet-induced experimental diabetes.

**Materials and methods**

**Animals, diets, and experimental design**

This study involved one hundred adult male gerbils (*Gerbillidae* family, *Psammomys obesus* species) captured in the Algerian Eastern Sahara. Only animals older than 8 months and weighing between 80 and 120 g were included in the experimental protocol. After being examined by a veterinarian (to ensure that the animals were free of diseases), *P. obesus* were then transferred to the animal house that was maintained under controlled conditions (22–26˚C, 60 ± 10% relative air humidity and 12:12 hours light and dark cycles). They were housed in individual cages with free access to their natural food (ND) that consisted of halophilic plants, primarily belonging to the Chenopodiaceae family (*Traganum nudatum*, *Salsola foetidia*, *Suaeda mollis* and *Artriplex halimus* species). In this study, we used a *Salsola foetida*-based herbal diet directly harvested from the Algerian North Eastern Sahara, in the area of Biskra (34˚25’ North latitude, 5˚55’ West longitude). The ND had a very low calorie content of 0.4 kcal/g (80.8% water, 8.4% carbohydrates, 6.9% ash, 3.5% proteins, 0.4% lipids) and was high in dietary fiber (0.18% total sugar, 1.12% lignin, 2.62% hemicellulose, 2.23% cellulose, 2.62% undetermined substances). A complete analysis of the *Salsola* plants showed high levels of various mineral salts (11.8% sodium, 3.8% potassium, 1% calcium, 2.1% magnesium, 3.8% chlorine, 0.2% phosphorus); this composition was standardized by chemical analysis (Caen University, CNRS Research Centre, U405, France) [21]. Following a 2 week-initial accommodation, the gerbils were randomly placed into two experimental groups (n = 30 per batch): the control group consuming their ND and the *P. obesus* group eating a HED of 3.25 kcal/g (47% carbohydrates, 25% proteins, 7.5% fat), with high energy carbohydrates (33.5% starch and 13.5% total sugar) and fatty acids (0.81% palmitic acid, 0.05% palmitoleic acid, 0.20% stearic acid, 3.76% oleic acid, 2.40% linoleic acid, 0.28% linolenic acid). Although the latter diet is the standard synthetic food for all laboratory rodents, it is considered to be high caloric (compared to ND) for *P. obesus* which subsequently develops diabetes [12]. Food and water consumption were measured daily. Each group was separately subjected to MET treatment that started once HED fed *P. obesus* exhibited blood glucose levels > 90 mg/dL (5 mM). *P. obesus* were deprived of food 12 h prior to the beginning of the experiment, while they were not deprived of water. Blood samples from the retro-orbital venous plexus were performed weekly in EDTA tubes to determine the fasting plasma parameters. MET treatment commenced on week 12 and continued until the end of the experimental period at week 24. Therefore, the duration of MET treatment in both *P. obesus* groups was 12 weeks. Fresh solutions of MET (Merck-Lyon, France) were prepared daily and administrated by gastric intubation at 50 mg/kg of body weight, a
quantity within the clinical dose range [22]. Isotonic saline solution (0.9% NaCl) served as the control or placebo treatment. The food intake was evaluated based on the weight of containers at 2, 4, and 6 h after MET administration, and the body weight was determined twice weekly. Body mass index (BMI) of *P. obesus* was assessed by dividing the weight (g) by the height (cm) squared. The tail of the animal was not taken into consideration for the BMI calculation.

All the experimental procedures, including *P. obesus* capture in the South Eastern Algerian Sahara, were authorized by the Institutional Animal Care Committee of the National Administration of the Algerian Higher Education and Scientific Research (DGRSDT; http://www.dgrsdt.dz). The permits and ethical rules were achieved according to the Executive Decree n° 10–90 completing the Executive Decree n°04–82 of the Algerian Government, establishing the terms and approval modalities of animal welfare in animal facilities. The investigation strictly conforms to the *Guide for the Care and Use of Laboratory Animals* [DHHS Publ. No. (NIH) 85–23, revised 1996, Office of Science and Health Reports, Bethesda, MD 20892].

**Blood metabolic parameters analysis and biochemical assays**

Plasma glucose levels were measured by the glucose oxidase test combination (Boehringer, Meylan, France). Circulating insulin concentrations were determined by a radioimmunoassay using human insulin as the standard, while ketone bodies, triglycerides and total cholesterol were enzymatically assayed. Plasma lactate concentrations were measured by spectrophotometry and non-esterified fatty acids (NEFA) were determined by microfluorimetry. Glycosylated haemoglobin (HbA1c) was measured by turbidimetry using a Cobas Mira Plus automatic analyser (Roche Diagnostic Systems, Basel, Switzerland). The hepatic content in triglycerides, esterified cholesterol and free cholesterol was carried out by a sequential quantitative method [23]. Glycogen was extracted from tissue samples in KOH at 100˚C, assayed after acid hydrolysis according to the method of Chan and Exton [24]. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated at both baseline and end of the study using the following formula: fasting plasma glucose (mM) x fasting insulin (mU/L) / 22.5 [25]. All the enzyme reagents were procured from Roche (Meylan, France). Lactate, octanoate, pyruvate and alanine were purchased from Janssen Cosmetics (Aachen, Germany). Bovine serum albumin (BSA) was purchased from Sigma chemical Co (St Louis, Missouri, USA).

**Metabolic fluxes, intracellular metabolites and adenine nucleotides analysis**

16 h-fasted *P. obesus* were anesthetized by intraperitoneal injection of sodium pentobarbital (5 mg/100 g), and hepatocytes were then isolated according to the method of Berry and Friend [26], as modified by Groen [27]. Liver cells were resuspended in Krebs-Ringer bicarbonate buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 24 mM NaHCO$_3$, 2.4 mM CaCl$_2$, 2% BSA, pH 7.4), saturated with O$_2$/CO$_2$ (95:5%). Using a shaking water bath, isolated hepatocytes (10 mg dry cells per mL) were incubated for 30 min at 37˚C in closed vials containing 2.5 mL of oxygenated buffer with alanine (20 mM) or lactate + pyruvate (10:1 mM) in the presence of medium-chain fatty acid octanoate (2 mM), with or without MET (1 mM and 10 mM). After 30 min, 350 μL of the cell suspension were deproteinized with HClO$_4$ (5% final), then centrifuged at 13,500g for 5 min. The supernatants were neutralized with KOMO buffer (2 M KOH, 0.3 M MOPS) for subsequent assays of glucose, lactate, pyruvate and ketone bodies (3-hydroxybutyrate, acetoacetate) by spectrophotometry [28]. A second sample from the vial was used for the determination of nucleotide content in mitochondrial and cytosolic fractions after separation of cells by a quick spin across a silicone oil layer. ATP and ADP were measured by high performance liquid chromatography as described earlier [29].
Determination of oxygen consumption rates

After incubation of hepatocytes with substrates, in the presence or absence of MET as described above, the cell suspension was quickly saturated with $O_2/CO_2$ and immediately transferred into a stirred vessel equipped with a Clark oxygen electrode. The oxygen consumption rate was measured at 37˚C and expressed as $\mu$mol $O_2$/min/g dry cell.

Data analysis

Statistical analysis was performed using SPSS version 12 (SPSS UK, Surrey UK). Data were expressed as the mean ± SEM, and comparisons between groups were made using student’s $t$ test or one-way analysis of variance (ANOVA) as appropriate. The differences were considered statistically significant at $p < 0.05$.

Results

Effects of MET on BMI, caloric intake and plasma metabolic profile

Adult $P. obesus$ ate over 30.4 ± 2.8 kcal/day (estimated to be around 76 g of food daily or 80% of their body weight) when maintained on an exclusive ND. In such conditions, these rodents do not exhibit hyperglycemia, hyperinsulinemia or plasma lipid impairments. In contrast, the caloric intake was markedly increased in HED fed $P. obesus$ (312 ± 8 kcal/day), and their body weights almost doubled when compared to the control group (Table 1).

Interestingly, both parameters were greatly reduced in diabetes-prone $P. obesus$ after chronic administration of 50 mg/kg of MET. Nonetheless, the weight gain decreased more mildly than the caloric intake, meaning $P. obesus$ remain obese despite MET treatment. BMI was reduced by 37% ($p < 0.05$) in MET-treated diabetic $P. obesus$ compared to untreated diabetic gerbils (Table 1). Moreover, MET did not significantly alter neither the caloric intake nor

| Parameters/Groups                  | Control (ND) | Placebo (ND+MET) | Diabetic (HED) | Diabetic (HED+MET) |
|-----------------------------------|--------------|------------------|----------------|-------------------|
| Caloric intake (cal/100 g wet weight) | 30.4±2.8     | 25.9±1.7        | 312±8         | 124±3***          |
| Body weight (g)                   | 76±5         | 73±1             | 145±3         | 109±5**           |
| BMI (g/cm$^2$)                    | 0.35±0.01    | 0.28±0.01       | 0.67±0.03     | 0.42±0.02*        |
| HbA1c (mmol/mol)                  | 18.8±0.75    | 11.9±0.65*      | 67.5±1.05    | 21.5±0.85***      |
| Glucose (mM)                      | 3.22±0.41    | 3.11±0.18       | 15.2±1.4     | 3.88±0.17***      |
| Insulin (pM)                      | 130±21       | 121±17          | 580±47       | 110±14***         |
| HOMA-IR                           | 2.57±0.14    | 2.21±0.09       | 5.33±0.23    | 2.68±0.11**       |
| Triglycerides (mM)                | 0.80±0.34    | 0.74±0.01       | 1.60±0.61    | 0.83±0.5**        |
| Total cholesterol (mM)            | 1.50±0.30    | 1.38±0.32       | 2.80±0.5     | 1.41±0.2*         |
| HDL-cholesterol (mM)              | 0.65±0.30    | 0.59±0.21       | 1.19±0.04    | 0.95±0.06         |
| LDL-cholesterol (mM)              | 0.50±0.40    | 0.44±0.13       | 0.97±0.30    | 0.29±0.2**        |
| NEFA (μM)                         | 639±65       | 613±15          | 894±132      | 627±89**          |
| Lactate (mM)                      | 0.65±0.09    | 1.05±0.03**     | 0.93±0.14    | 1.13±0.25         |
| Ketone bodies (mM)                | 0.57±0.06    | 0.48±0.04       | 1.33±0.20    | 0.80±0.05**       |

Metformin (MET) was orally administered for 12 weeks at 50 mg/kg/day. Circulating parameters were assayed in 16 h-fasted $P. obesus$, and data are expressed as means ± SEM (n = 30/group).

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$ compared with the corresponding dietary condition without metformin treatment. ND: Natural Diet, HED: High Energy Diet

doi:10.1371/journal.pone.0172053.t001
the body weight of the control animals. As expected, the diabetic *P. obesus* developed metabolic syndrome with multiple anomalies. Both fasting plasma glucose and insulinemia were particularly increased. MET administration mainly lowered plasma glucose levels (-74%, *p* < 0.001) in this group of animals. The decline was progressive throughout the course of the treatment until reaching a stable end-value (3.88 ± 0.17 mM) near the baseline. Concomitantly, MET reduced the amount of HbA1c. The values in plasma glucose and HbA1c were not BMI-dependent (0.50 < or > 0.60 g/cm²), since they correlated strongly with HOMA-IR and insulinemia in HED fed *P. obesus* (*r* = 0.951 and 0.806, respectively). This lost of sensitivity to insulin in obese animals, as demonstrated by the higher value of HOMA-IR and subsequent hyperinsulinemia, was fully reversed by MET (Table 1). All of this confirmed the long-recognized efficacy of MET upon glucose homeostasis in such a diabetogenic setting. We suggest that its glucose-lowering effect was due to an inhibition of hepatic gluconeogenesis (see data below on isolated hepatocytes). It is noteworthy that MET increased plasma lactate in control *P. obesus* to a level similar to that measured in diabetic *P. obesus*, but it did not further aggravate lactate accumulation caused by the HED. Otherwise, MET-treated control animals had a lowered HbA1c compared to baseline (Table 1).

### Effects of MET on lipidemia, ketogenesis and liver metabolic parameters

While no significant impact of MET was found in control *P. obesus*, its benefits on lipid profile and ketone bodies production in *P. obesus* expressing the pathological phenotype represents another key finding in this work. MET treatment abolished net increases of triglycerides, total cholesterol, LDL-cholesterol and NEFA, without changing HDL-cholesterol levels (Table 1). This potent hypolipidemic effect of MET positively correlated with reduced HbA1c (*r* = 0.70, *p* = 0.03) and fasting glucose (*r* = 0.74, *p* = 0.01). In addition, MET treatment markedly decreased plasma ketone bodies (-40%, *p* < 0.01) in diabetic *P. obesus*.

Furthermore, a significant twofold increase of hepatic triglycerides along with liver mass increase indicated a severe tissue dysregulation in diabetic *P. obesus* (Table 2). This harmful fat accumulation was mitigated under MET treatment, which normalized the liver mass/body weight ratio. MET did not alter the hepatic lipid status when given orally to control *P. obesus*, but it increased hepatic glycogen content in the diabetic state vs. control group (Table 2). This finding possibly reveals that MET would be able to stimulate the utilization of glucose as glycogen stores in the liver of injured *P. obesus*.

### Effects of MET on cell energy metabolism and cellular redox states

It is important to recall that diabetic patients on MET treatment display plasma drug concentrations ranging from 10 μM to 40 μM. However, it should also be noted that the liver receives the majority of its blood via the portal vein, which may contain substantially higher amounts of MET [22]. Because of hepatic accumulation, MET concentrations more than 250 μM in the

### Table 2. Comparison of hepatic biochemical parameters between ND-fed and HED-fed *P. obesus* in the absence or presence of metformin.

| Parameters/Groups         | Control (ND)   | Placebo (ND+MET) | Diabetic (HED) | Diabetic (HED+MET) |
|---------------------------|----------------|------------------|----------------|-------------------|
| Hepatic glycogen (g/100 g wet wt) | 2.55±1.0       | 2.75±1.7         | 2.68±0.33      | 3.73±1.72*        |
| Total hepatic lipids (g/100 g wet wt) | 3.19±0.10      | 2.97±0.31        | 4.03±0.16      | 3.01±0.66*        |
| Hepatic glycerides (g/100 g wet wt)  | 0.19±0.05      | 0.179±0.01       | 0.65±0.03      | 0.28±0.03*        |
| Liver mass (% body wt)     | 3.51±0.31      | 2.99±0.15        | 4.03±0.80      | 2.89±0.20**       |

*p* < 0.05

**p* < 0.01 compared with the corresponding dietary condition without metformin treatment.

doi:10.1371/journal.pone.0172053.t002
Liver of diabetic rodents can be reached after a single dose of 50 mg/kg [30]. To study the impact of MET on metabolic pathways in hepatocytes isolated from *Psammomys obesus*, we chose a MET dosing which was more related to the range (upper limit) of in vivo tissue concentrations than the observed blood levels in clinical use. MET used at 1 mM or 10 mM does decrease hepatic glucose output in diabetic *Psammomys obesus*. This dose-dependent effect was observed with different gluconeogenic substrates such as lactate + pyruvate or alanine in the presence of octanoate (Table 3). The basal rate of gluconeogenesis from lactate + pyruvate was higher than that from alanine in HED fed *Psammomys obesus*, though MET exerted its inhibitory effect in both substrate conditions (Table 3).

In addition, MET substantially increased the glycolytic flux from alanine as evidenced by the respective 45% and 110% increases with 1 mM or 10 mM MET. As a result, the L/P ratio, which is in thermodynamic equilibrium with cytosolic NADH/NAD⁺, was augmented by 20% in the presence of 10 mM MET (Fig 1A). Of note, MET failed to significantly inhibit gluconeogenesis in control *Psammomys obesus*, however the glycolytic pathway was activated but to a lesser extent than in diabetic *Psammomys obesus*. After looking at ketogenesis, assessed by the initial oxidation rate of medium-chain octanoate fatty acid, MET did not inhibit the formation of ketone bodies in control animals but fully suppressed their higher production in the diabetic group, regardless of the applied drug concentration (Table 3). The sizeable rise in the β-hydroxybutyrate/acetoacetate ratio, used as a surrogate of the mitochondrial redox potential (Fig 1B and 1C), was consistent with the reductions in either oxygen consumption (Table 4) or cytosolic and mitochondrial ATP/ADP levels in HED fed *Psammomys obesus* (Fig 2).

These responses were in relation to the aforementioned modifications in glucose metabolism, arguing for the well-known action of MET at these high concentrations on the respiratory chain activity. However, if most of these in vitro observations corresponded to in vivo data, we cannot omit the emerging role of intestinal microbiota in this pathophysiological context as discussed below.

**Discussion**

The results presented in this study show that MET prevented the progression of nutritional diabetes in *Psammomys obesus*, a particular strain of herbivorous rodents with a genetic predisposition to
Fig 1. Effect of metformin on both cytosolic and mitochondrial redox states (NADH/NAD$^+$) in *P. obesus* fed ND or HED. Hepatocytes were incubated for 30 min with energy substrates in the absence or presence of metformin at the indicated concentrations before calculating the lactate/pyruvate ratio (A) and β-hydroxybutyrate/acetoacetate (β-HB/Ac Ac) ratio (B and C). Data are means ± SEM of 15 separate experiments. *p < 0.05; **p < 0.01 compared with the group without metformin for each corresponding dietary condition.

doi:10.1371/journal.pone.0172053.g001

Table 4. Effect of metformin on respiratory capacity of isolated hepatocytes from all *P. obesus* groups.

| Energy Substrates | Control (ND) | Placebo (ND) | Diabetic (HED) | Diabetic (HED) |
|-------------------|--------------|--------------|----------------|----------------|
|                   |              | 1 mM MET     | 10 mM MET      | 1 mM MET       | 10 mM MET      |
| Ala+Oct           | 19.60 ± 0.64 | 14.90 ± 0.15** | 11.33 ± 0.51*** | 10.23 ± 0.85   | 8.09 ± 0.13**  | 5.43 ± 0.32*** |
| (L+P)+Oct         | 23.12 ± 0.97 | 17.58 ± 0.24** | 13.36 ± 0.79*** | 11.85 ± 0.43   | 9.37 ± 0.21**  | 6.29 ± 0.66*** |

Hepatocytes were processed as in Table 3 before measuring oxygen consumption rates. Data are means ± SEM (n = 15).

**p < 0.01
***p < 0.001 compared with the corresponding dietary condition without metformin treatment.

doi:10.1371/journal.pone.0172053.t004
develop a metabolic syndrome, and this event was associated with alterations of glycolysis, gluconeogenesis and ketogenesis in vitro. The MET-induced changes in liver metabolic fluxes were combined with an inhibition of oxygen consumption, leading to an increased intracellular redox potential and a lower energy charge.

Previous reports, including ours, have shed light on pronounced physiological disorders in \textit{P}. \textit{obesus} after hypercaloric diet feeding [31, 32, 18]. We further extend these observations herein, showing that 24-week HED fed \textit{P}. \textit{obesus} exhibited hyperglycemia, hyperinsulinemia and altered hormonal response as evidenced by a higher index of insulin resistance. A severe dysregulation in blood lipid profile and hepatic fat content characterized more particularly this diabetes-prone \textit{Psammomys} species. Hence, its metabolic-endocrine system was impaired under restrictive laboratory conditions, emphasizing the contribution of lipotoxicity in such adverse environment [33, 34]. In vivo MET treatment (from week 12 to week 24) prevented the progression of systemic metabolism disturbances and restored insulin sensitivity in diabetic \textit{P}. \textit{obesus}, as evidenced by a controlled glycemia and a lowered HOMA-IR. In T2DM and/or obese patients, the primary mechanism for MET action consists of selective inhibition

---

**Fig 2.** Effect of metformin on both cytosolic and mitochondrial phosphate potential (ATP/ADP) in \textit{P}. \textit{obesus} fed ND or HED. Hepatocytes were incubated for 30 min with alanine + octanoate (A and B) or lactate + pyruvate + octanoate (C and D) in the absence or presence of metformin at the indicated concentrations, before assaying the content in adenine nucleotides within each compartment. Data are means ± SEM of 15 separate experiments. *p < 0.05; **p < 0.01; ***p < 0.001 compared with the group without metformin for each corresponding dietary condition.

doi:10.1371/journal.pone.0172053.g002
of whole body free fatty acid oxidation while leaving the mitochondrial \(\beta\)-oxidation capability unaffected [35]. It was also reported that MET lowered circulating lipids levels by promoting the clearance of VLDL-triglyceride from brown adipose tissue [36]. In MET-treated diabetic \textit{P. obesus}, a remarkable improvement of hepatic triglyceridemia and cholesterolemia, in association with a decline in plasma NEFA, was observed. The reduced availability of plasma fatty acids together with other coordinated effects on the liver, possibly on the adipose tissue, and potential other effects were tightly related to a MET-driven enhancement of fat metabolism in these diabetic \textit{P. obesus}, thereby alleviating their advanced complications. Indeed, the inhibition of lipids storage is essential to restore both insulin sensitivity and glycemia in \textit{P. obesus} fed a regular diet. Importantly, these results were obtained at a dose of MET (50 mg/kg) that was clinically shown to decrease plasma glucose to optimal levels [37].

Using this unique \textit{Psammomys} model, Harmel et al. documented the positive effects of MET on hyperlipidemia associated with insulin resistance and T2DM; these changes began in the small intestine and were dependent on AMPK activity [38]. Similarly, MET activated a duodenal AMPK-dependent pathway that lowered hepatic glucose production in rat models of diabetes [39]. Such observations are parallel to the recent recognition of increased intestinal permeability as a consequence of high fat intake or bacterial modifications in gut microbiota [40–42]. Limited findings have described different microbial communities colonizing the digestive tract of \textit{P. obesus} [43, 44]. This intestinal microflora is closely related to the sandy soil where halophyte plants (the dominant food of desert gerbils) grow. The high content in micro-nutrients and cellulose fibres of this herbal diet can therefore interact with the bacteria in order to modulate the host energy metabolism and physiology. As some bacterial strains possess oxidative capacities [45], we suggest that fermentation of dietary fibres by the gut microbiota of \textit{P. obesus} under ND conditions generates short-chain fatty acids (i.e., propionate and butyrate) which should in turn activate intestinal gluconeogenesis, providing a source of glucose during starvation or inducing \textit{de novo} hepatic lipogenesis. Because it was recently established that the bodies of diabetic people have a lower proportion of butyrate-producing bacteria [46], it is likely that both the composition and metabolic functions of the gut microbiota were similarly changed in \textit{P. obesus} ingesting a HED. Assuming that a modulation of the gut microbiota by MET constitutes a novel component of its antidiabetic action [47, 48], one can hypothesize, in the light of our overall findings (e.g., key role of fatty acid metabolism in MET-induced improvement of diabetic phenotypes in \textit{P. obesus}), that MET may alter the gut microbiota diversity of \textit{P. obesus} in parallel with its effects on host (patho) physiology and that the magnitude of this modulatory effect is probably diet-dependent. This hypothesis is highly interesting and asks for further research.

Regarding other issues for MET action, we were struck by the consistent observation of increased plasma lactate amounts in drug-treated animals and patients [49, 50]. It has also been shown that the human intestinal mucosa appears to be an important source of MET-induced lactate production [51], even though delayed glucose absorption still took place more distally along the tract. In our experimental conditions, plasma lactate increased in healthy control \textit{P. obesus} after MET exposure but lactatemia did not further worsen in diabetic \textit{P. obesus} given MET. The plasma level of ketone bodies however remained rather high, presumably due to an accumulation of glycogen in the damaged liver of \textit{P. obesus}. Collectively, our \textit{in vivo} findings suggest the relevance of MET action on the crossroads between lipid and glucose metabolism. This could be partly mediated by intestinal and liver mechanisms without ruling out the implication of circulating redox state since it was found that altered plasma lactate/pyruvate ratios can regulate liver metabolism in mice [52].

Based on \textit{in vitro} results, achieved by contrast with MET concentrations (millimolar) higher than those applied in human therapy, which excluded any role of gut microbiota at the cellular
level, we assured that MET ameliorated hyperglycemia in *P. obesus* through inhibition of gluconeogenesis from various energizing substrates. Lactate + pyruvate supplied reducing equivalents (NADH$_2$, FADH$_2$) from glycolysis to the mitochondrial respiratory chain and, without modifying regulation of the metabolic routes; the cytosolic redox potential was imposed using a concentration ratio for these substrates of 10:1 [53]. Alanine is the principal amino acid taken up by the liver during ingestion of a low protein diet or starvation and, as such, is a key precursor for gluconeogenesis [54]. Octanoate, a medium chain fatty acid, provided reducing equivalents to the mitochondria through β-oxidation, also altering gluconeogenesis in perfused livers [55]. In our experimental conditions, MET inhibited glucose production in a dose-dependent manner, regardless of the glucogenic substrates used (Table 3). Although we did not determine the activity of some key enzymes here, the underlying mechanism could be a lowering in the uptake of substrates by liver cells [56] or an enhanced gluconeogenic flux through pyruvate kinase [57]. Another salient point of this *in vitro* study was the link between ketone body production and fatty acid β-oxidation in MET-treated diabetic *P. obesus*. If the decrease in ketone bodies could be accounted for by a reduction in octanoate oxidation, at least partly (ketogenesis reflecting in fact the flux through fatty acid β-oxidation), the results were rather in favour of an enhanced β-oxidation. Owing to the inhibition of gluconeogenesis by MET, the amount of oxaloacetate available for the reaction with acetyl-CoA increases, and acetyl-CoA is then preferentially channelled from entering the Krebs cycle at the expense of the pathway producing acetoacetate and β-hydroxybutyrate. This kind of process can operate because MET acutely inhibited oxygen consumption rates in hepatocytes from diabetic *P. obesus*, thereby decreasing ATP content and increasing redox potential. To re-oxidize the reducing equivalents in the case of diminished glucose synthesis, MET not only stimulated glycolysis (by increasing lactate) but also enhanced fatty acid β-oxidation concomitantly with an inhibition of ketogenesis. All these statements were consistent with the fact that MET facilitated the removal of plasma NEFA and improved hepatic lipid metabolism in diabetic *P. obesus*.

MET was revealed to inhibit mitochondrial glycerol-3-phosphate dehydrogenase, raising the cytosolic NADH/NAD+ ratio and impairing utilization of redox-dependent substrates for gluconeogenesis, but surprisingly the mitochondrial NADH pool decreased [58]. Here as in other reports [59], the result was quite the opposite. MET reduced this intracellular compartment much more as evidenced by the rise in β-hydroxybutyrate/acetoacetate ratio, a common index of mitochondrial redox state. Despite these discrepancies, possibly reflecting the differences in protocol used for drug administration and/or treatment duration, one may stress that the major target of MET was mitochondria since it selectively inhibited respiratory chain complex I [60, 61]. Taking into account that we highlighted the occurrence of increased oxidative stress along with defective oxidative phosphorylation in diabetic *P. obesus* [18], the current results warrant further investigation for better deciphering the exact mitochondrial role of MET in this rodent strain. When comparing the metabolic action of MET on hepatocytes from normal Wistar rats with that from control lean *P. obesus*, this drug induced mixed effects in the latter. Indeed, gluconeogenesis was barely affected and glycolysis was increased but both pathways were significantly altered in Wistar rats [13]. This may correspond to distinct modes of behaviour, keeping in mind that insulin was relatively high in spite of a very low basal metabolic rate associated with a slow digestion of plants (ND) in the cecum of *P. obesus* [62], thus permitting them to stimulate lipogenesis, when the capacity for glucose oxidation was smaller than in Wistar rats [13, 63].

Although some studies found that MET reduced body weight gain in patients suffering from diabetes [64], we did not observe any effect on fat deposition in diabetic *P. obesus*. Meanwhile, the caloric intake was substantially lessened by MET. This drug could thus indirectly influence the central nervous system through a modulation of hypothalamic feeding circuits [65]. Indeed, by accumulating into the gut, MET increased incretins level, including glucagon-
like peptide-1 (GLP-1), in obese patients [66] and T2DM animal models [67]. In that respect, authors disclosed an upregulation of GLP-1 release in metabolically challenged obesus rats [68]. These last observations reinforce the idea that MET might exert its glucose-lowering action via the gut as newly proposed in human beings, at least when administrated orally [69]. An explanation for the reduced food intake seen in diabetic obesus under MET treatment could be linked to this phenomenon but direct evidence for this was not available.

**Conclusions**

If healthy obesus did not fulfill the criteria for metabolic syndrome, by means of adaptation to a hypocaloric food (ND), transferring them to a HED for 24 weeks led to profound metabolic
disorders which were acutely corrected by MET. Our in vivo and in vitro findings confirmed its effectiveness to ameliorate ketoacidosis and lipid metabolism in diabetic *Psammomys obesus*; these events likely relied on hepatocellular redox status and mitochondrial activity (see Fig 3 for summary).

This study likewise agreed with the successful use of MET for patients intolerant to glucose or with moderate hyperglycemia. Such data are of utmost importance for establishing innovative therapeutic strategies, and any comparison between the natural history of diabetes in *Psammomys obesus* and human pathology should help explain, at least partially, the higher risk of developing morbid obesity for people with unhealthy dietary habits. In addition, part of the MET taken orally by *Psammomys obesus* might directly interact with the microbiota of the gastrointestinal tract before entering the circulation and peripheral tissues to protect host cells against severe damages. This open question linked to the dependency and interaction of MET effects with microbiome in metabolic diseases needs to be further elucidated.

**Acknowledgments**

This article is dedicated to the late Professor Leverve who initiated this study. The authors would like to acknowledge the financial support of the Algerian Agency for the Research & Development in Health (PNR No. 208/ANDRS and PNR No.41/ANDRS/2011) and the Algerian Ministry of Higher Education Program (No. F 0022014 0100). Authors are indebted to Dr C. Bouziane for fruitful discussion and to Pr T. Merghoub (Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA) for her very valuable help in correcting this manuscript.

**Author Contributions**

Conceptualization: EAK DD XL.

Data curation: IG EAK.

Formal analysis: IG EAK.

Investigation: IG EAK XL.

Methodology: IG EAK XL.

Software: EAK DD NAK.

Validation: DD EAK.

Visualization: EAK DD NAK.

Writing – original draft: NW NAK DD EAK.

Writing – review & editing: XL.

**References**

1. King H, Aubert RE, Herman WH. Global Burden of Diabetes, 1995–2025: prevalence, numerical estimates, and projections. Diabetes Care 1998; 21(9): 1414–1431. PMID: 9727886
2. Ferrannini E, Mari A. B–Cell function in type 2 diabetes. Metabolism 2014; 63(10): 1217–1227. doi: 10.1016/j.metabol.2014.05.012 PMID: 25070616
3. Inzucchi SE, Bergenstal RM, Buse JB, Diamant M, Ferrannini E, Nauck M. Management of hyperglycaemia in type 2 diabetes: a patient-centered approach. Position statement of the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD). Diabetologia 2012; 55(6): 1577–1596. doi: 10.1007/s00125-012-2534-0 PMID: 22526604
4. Salpeter SR, Buckley NS, Kahn JA, Salpeter EE. Meta-analysis: metformin treatment in persons at risk for diabetes mellitus. Am J Med 2008; 121(2): 149–157. doi: 10.1016/j.amjmed.2007.09.016 PMID: 18261504

5. Song YM, Lee YH, Kim JW, Ham DS, Kang ES, Cha BS, et al. Metformin alleviates hepatosteatosis by restoring SIRT1-mediated autophagy induction via an AMP-activated protein kinase-independent pathway. Autophagy 2015; 11(1): 46–59. doi: 10.4161/15548627.2014.94271 PMID: 25484077

6. Fullerton MD, Galic S, Marcinko K, Sikkema S, Pulinikunnel T, Chen ZP, et al. Single phosphorylation sites in Acc1 and Acc2 regulate lipid homeostasis and the insulin sensitizing effects of metformin. Nature Med 2013; 19(12): 1649–1654. doi: 10.1038/nm.3372 PMID: 24185692

7. Khan NA, Wiensperger N, Quemener V, Moulinoux JP. Internalization of metformin is necessary for its action on potentiating the insulin-induced Xenopus laevis oocyte maturation. J Endocrinol 1994; 142(2): 245–250. PMID: 7930997

8. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP activated protein kinase in mechanism of metformin action. J Clin Invest 2001; 108(8): 1167–1174. doi: 10.1172/JCI13505 PMID: 11602624

9. Cao J, Meng S, Chang E, Beckwith-Fickas K, Xiong L, Cole RN, et al. Low concentrations of metformin suppress glucose production in hepatocytes through AMP-activated protein kinase (AMPK). J Biol Chem 2014; 289(30): 20435–20446. doi: 10.1074/jbc.M114.615721 PMID: 24928508

10. Miller RA, Birnbaum MJ. An energetic tale of AMPK-independent effects of metformin. J Clin Invest 2010; 120(7): 2367–2370.

11. Foretz M, Guigas B, Bertrand L, Pollak M, Viollet B. Metformin: from mechanisms of action to therapies. J Clin Invest 2001; 108(8): 1167–1174. doi: 10.1172/JCI13505 PMID: 11602624

12. Kaiser N, Cerasi E, Leibowitz G. Diet-induced diabetes in the sand rat (Psammomys obesus). Diabetes Res 2012; 2012: 430176. doi: 10.1155/2012/430176 PMID: 22675340

13. Shafrir E, Ziv E. Cellular mechanism of nutritionally induced insulin resistance: the desert rodent Psammomys obesus and other animals in which insulin resistance leads to detrimental outcome. J Basic Clin Physiol Pharmacol 1998; 9(2–4): 347–385. PMID: 10212843

14. Spalding B, Connor T, Wittmer C, Abreu LL, Kaspi A, Ziemann M, et al. Rapid development of non-alcoholic steatohepatitis in Psammomys obesus (Israeli sand rat). PloS One 2014; 9(3): e92656. doi: 10.1371/journal.pone.0092656 PMID: 24651520

15. Marquie G, Duhault J, Jacotot B. Diabetes mellitus in sand rats (Psammomys obesus). Methods Mol Biol 2012; 933: 89–102. doi: 10.1007/978-1-62703-068-7_7 PMID: 22893403

16. Shafrir E. Molecular background of nutritionally induced insulin resistance leading to type 2 diabetes: from animal models to humans. Int J Exp Diabetes Res 2001; 2: 299–319. doi: 10.1155/EDR.2001.299 PMID: 11795838

17. Shafrir E. Molecular background of nutritionally induced insulin resistance leading to type 2 diabetes: from animal models to humans. Int J Exp Diabetes Res 2001; 2: 299–319. doi: 10.1155/EDR.2001.299 PMID: 11795838

18. Boudouba S, Sanz MN, Sanchez-Martin C, Detaille D, Villanueva GR, Koceir EA. Hepatic mitochondrial alterations and increased oxidative stress in nutritional diabetes prone Psammomys obesus model. Exp Diabetes Res 2012; 2012: 430176. doi: 10.1155/2012/430176 PMID: 22675340

19. Collier GR, Walder K, Lewandowski P, Sanigorski A, Zimmet P. Leptin and the development of obesity and diabetes in Psammomys obesus. Obes Res 1997; 5(5): 455–458. PMID: 9385621

20. Bouderba S, Sanchez-Martin C, Detaille D, Villanueva GR, Koceir EA. Beneficial effects of silybin against the progression of metabolic syndrome, increased oxidative stress, and liver steatosis in Psammomys obesus, a relevant animal model of human obesity and diabetes. J Diabetes 2014; 6(2): 184–192. doi: 110.10111/1753-0407.12083 PMID: 23953934

21. Daly M. Early use of solid food by a leaf-eating gerbil (Psammomys obesus). J Mammal 1975; 56(2): 509–511. PMID: 1141778

22. He L, Wondisford FE. Metformin action: concentrations matter. Cell Metab 2015; 21(2): 159–162. doi: 10.1016/j.cmet.2015.01.003 PMID: 25651170

23. Shibko S, Koivistoimen P, Tratnyek CA, Newhall AR, Friedman L. A method for sequential quantitative separation and determination of protein, RNA, DNA, lipid, and glycogen from a single rat liver homogenate or from a subcellular fraction. Anal Biochem 1967; 19(3): 514–528. PMID: 4292701

24. Chan TM, Exton JH. A rapid method for the determination of glycogen content and radioactivity in small quantities of tissue or isolated hepatocytes. Anal Biochem 1976; 71(1): 96–105. PMID: 1275237

25. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985; 28(7): 412–419. PMID: 3899825
26. Berry MN, Friend DS. High yield preparation of isolated rat liver parenchymal cells: a biochemical and fine study. J Cell Biol 1969; 43(3): 506–519. PMID: 4900611

27. Groen AK, Sips HJ, Vervoorn RC, Tager JM. Intracellular compartmentation and control of alanine metabolism in rat liver parenchymal cells. Eur J Biochem 1982; 122(1): 87–93. PMID: 7060572

28. Bergmeyer HU. Methods in Enzymatic Analysis, 3rd ed. Academic Press, New York, 1974.

29. Ichai C, Guignot L, El-Mir MY, Nogueira V, Guigas B, Chauvin C, et al. Glucose 6 phosphate hydrolysis is activated by glucagon in a low temperature-sensitive manner. J Biol Chem 2001; 276(30): 28126–28133. doi: 10.1074/jbc.M101682000 PMID: 11371550

30. Wilcock C and Bailey CJ. Accumulation of metformin by tissues of the normal and diabetic mouse. Xenobiota 1994; 24(1): 49–57. doi: 10.3109/00498259409043220 PMID: 8165821

31. Khalhali A, Haddar A, Semiane N, Maliek A, Abdelmalek A, Castex F, et al. Obesity, insulin resistance and diabetes in the sand rat exposed to a hypercaloric diet: possible protective effect for IL-1β. C R Biol 2010; 335(4): 271–278.

32. Sahraoui S, Dewachter C, de Medina G, Naeije R, Aouichat Bouguerra S, Dewachter L. Myocardial structural and biological anomalies induced by high fat diet in Psammomys obesus gerbils. PLoS One 2016; 11(2): e0148117. doi: 10.1371/journal.pone.0148117 PMID: 26840416

33. Atek-Mebarki F, Hichami A, Abdoul-Azize S, Bitam A, Koceir EA, Khan NA. Eicosapentaenoic acid modulates fatty acid metabolism and inflammation in Psammomys obesus. Biochimie 2015; 109: 60–66. doi: 10.1016/j.bioch.2014.12.004 PMID: 25528298

34. Brandes R, Tsur R, Arad R, Adler JH. Liver cytosolic fatty acids binding proteins in rats and Psammomys obesus: modulation in diabetes. Comp Biochem Physiol B1986; 83(4): 837–839.

35. Del Prato S, Marchetto S, Fipitone A, Zanon M, Vigili de Kreutzenberg S, Tiengo A. Metformin and free fatty acid metabolism. Diabetes Metab Rev 1995; 11: S33–S41. PMID: 8529483

36. Geerling JJ, Boon MR, van der Zon GC, van den Berg SA, van den Hoek AM, Lombardino TJ, et al. Desert gerbil gut microbiota in high-fat diet-fed rats are dynamic and region dependent. Am J Physiol Gastrointest Liver Physiol 2015; 21(5): 506–511. doi: 10.1093/ajpgi/dfs399 PMID: 25849133

37. Graham GG, Punj J, Arora M, Day RO, Doogue MP, Duong JK et al. Clinical pharmacokinetics of metformin. Clin Pharmacokinet 2011; 50(2): 81–98. doi: 10.2165/11534750-000000000-00000 PMID: 21241070

38. Harmel E, Grenier E, Bentjoudi Ouadda A, El Chebly M, Ziv E, Beaulieu JF, et al. AMPK in the small intestine in normal and pathophysiological conditions. Endocrinology 2014; 155(3): 873–888. doi: 10.1210/en.2013-1750 PMID: 24424053

39. Duca FA, Côté CD, Rasmussen BA, Zadeh-Tahmasebi M, Rutter GA, Filippi BM, Lam TK. Metformin activates a duodenal Ampk-dependent pathway to lower hepatic glucose production in rats. Nat Med. 2015; 21(5): 506–511. doi: 10.1038/nm.3787 PMID: 25849133

40. Ley RE, Turnbaugh P, Lozupone CA, Knight RD, Gordon JL. Obesity alters gut microbial ecology. Proc Natl Acad Sci USA 2005; 102(11): 11070–11075. doi: 10.1073/pnas.0504978102 PMID: 16033867

41. Qin J, Li Y, Cai Z, Li S, Zhu J, Zhang F, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature 2012; 490(7418): 55–60. doi: 10.1038/nature11450 PMID: 23023125

42. Hamilton MK, Boudry G, Lemay DG, Raybould HE. Changes in intestinal barrier function and gut microbiota in high-fat diet-fed rats are dynamic and region dependent. Am J Physiol Gastrointest Liver Physiol 2015; 308(10): G940–G951. doi: 10.1152/ajpgi.00029.2015 PMID: 25747351

43. Kuznetsova TA, Kam M, Khokhlova IS, Kostina NV, Dobrovolskaya TG, Umarov MM, et al. Desert gerbils affect bacterial composition of soil. Microb Ecol 2013; 66(4): 940–949. doi: 10.1007/s00248-013-0263-7 PMID: 23857378

44. Quadri I, Hassani II, l’Haridon S, Chalopin M, Haëcne H, Jebbar M. Characterization and antimicrobial potential of extremely halophilic archaea isolated from hypersaline environments of the Algerian Sahara. Microbiol Res 2016; 186–187: 119–131. doi: 10.1016/j.micres.2016.04.003 PMID: 27242149

45. Chakraborti CK. New-found link between microbiota and obesity. World J Gastrointest Pathophysiol 2015; 6(4): 110–119. doi: 10.4291/wjgp.v6.i4.110 PMID: 26600968

46. Arora T, Bakchved F. The gut microbiota and metabolic disease: current understanding and future perspectives. J Intern Med 2016; 280(4): 339–349. doi: 10.1111/joim.12508 PMID: 27071815

47. Shin NR, Lee JC, Lee HY, Kim MS, Whon TW, Lee MS, et al. An increase in the Akkermansia spp. population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice. Gut 2014; 63(5): 727–735. doi: 10.1136/gutjnl-2013-303839 PMID: 23804561

48. Lee H, Ko G. Effect of metformin on metabolic improvement and gut microbiota. Appl Environ Microbiol 2014; 80(19): 5935–5943. doi: 10.1128/AEM.01357-14 PMID: 25038099
49. Rapin JR, Wiernsperger N. Metformin as a cardiovascular drug: evidences, mechanisms and insight into safety. In: Mithieux G, Wiernsperger N, editors. Metformin: Mechanistic insights towards new applications, Kerala: Transworld Research Network; 2008, p. 131–62.

50. Boucaud-Maître D, Ropers J, Porokhov B, Altman JJ, Bouhanick B, Doucet J, et al. Lactic acidosis: relationship between metformin levels, lactate concentration and mortality. Diabet Med 2016; 33(11): 1536–1543. doi: 10.1111/dme.13098 PMID: 26882092

51. Bailey CJ, Wilcock C, Scarpello JH. Metformin and the intestine. Diabetologia 2008; 51: 1552–1553. doi: 10.1007/s00125-008-1053-5 PMID: 18528677

52. Nocito L, Kleckner AS, Yoo EJ, Jones AR IV, Liesa M, Corkey BE. The extracellular redox state modulates mitochondrial function, gluconeogenesis, and glycogen synthesis in murine hepatocytes. PLoS One 2015; 10(3): e0122818. doi: 10.1371/journal.pone.0122818 PMID: 25816337

53. Sistare FD, Haynes RC Jr. The interaction between the cytosolic pyridine nucleotide redox potential and gluconeogenesis from lactate/pyruvate in isolated rat hepatocytes. J Biol Chem 1985; 260(23): 12748–12753. PMID: 4044607

54. Felig P. Amino acid metabolism in man. Annu Rev Biochem 1975; 44: 933–955. doi: 10.1146/annurev.bi.44.070175.004441 PMID: 1094924

55. Cano N, Catelloni F, Fontaine E, Novaretti R, Di-Costanzo Dufetel J, Reynier JP, et al. Isolated rat hepatocyte metabolism is affected by chronic renal failure. Kidney Int 1995; 47(6): 1522–1527. PMID: 7643520

56. Radziuk J, Zhang Z, Wiernsperger N, Pye S. Effects of metformin on lactate uptake and gluconeogenesis in the perfused rat liver. Diabetes 1997; 46(9): 1406–1413. PMID: 9287039

57. Argaud D, Roth H, Wiernsperger N, Leverve X. Metformin decreases gluconeogenesis by enhancing the pyruvate kinase flux in isolated rat hepatocytes. Eur J Biochem 1993; 213(3): 1341–1348. PMID: 8504825

58. Madiraju AK, Erion DM, Rahimi Y, Zhang XM, Braddock DT, Albright RA, et al. Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. Nature 2014; 510(7506): 542–546. doi: 10.1038/nature13270 PMID: 24847880

59. Owen MR, Doran E, Halestrap AP. Evidence that metformin exerts its antidiabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. Biochem J 2000; 348(P13): 607–614.

60. Fullgencio JP, Kohl C, Girard J, Pégrior JP. Effect of metformin on fatty acid and glucose metabolism in freshly isolated hepatocytes and on specific gene expression in cultured hepatocytes. Biochem Pharmacol 2001; 62(4): 439–446. PMID: 11448453

61. El-Mir MY, Nogueira V, Fontaine E, Averet N, Rigoulet M, Leverve X. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. J Biol Chem 2000; 275(1): 223–228. PMID: 10617608

62. Frenkel G, Kraicer PF. Metabolic pattern of sand rats (Psammomys obesus) and rats during fasting. Life Sci 1972; 11(5): 209–222.

63. Schäfer H. Glucose disappearance rates into peripheral tissues of adult rabbits (Sylvilagus floridanus), Wistar rats (Rattus rattus) and sand rats (Psammomys obesus) in relation to body weight and blood glucose concentration. Comp Biochem Physiol Part A 1990; 95(2): 209–213.

64. Glueck CJ, Fontaine RN, Wang P, Subbiah MT, Weber K, Illig E, et al. Metformin reduces weight, centripetal obesity, insulin, leptin, and low-density lipoprotein cholesterol in non-diabetic, morbidly obese subjects with body mass index greater than 30. Metabolism 2001; 50(7): 856–861. doi: 10.1053/meta.2001.24192 PMID: 11436194

65. Lv WS, Wen JP, Li L, Sun RX, Wang J, Xian YX, et al. The effect of metformin on food intake and its potential role in hypothalamic regulation in obese diabetic rats. Brain Res 2012; 1444: 11–19. doi: 10.1016/j.brainres.2012.01.028 PMID: 22325091

66. Mannucci E, Ognibene A, Cremasco F, Bardini G, Mencucci A, Pierazzuoli E, et al. Effect of metformin on glucagon-like peptide 1 (GLP-1) and leptin levels in obese non-diabetic subjects. Diabetes Care 2001; 24(3): 489–494. PMID: 11289473

67. Yasuda N, Inoue T, Nagakura T, Yamazaki K, Kira K, Saeki T, et al. Metformin causes reduction of food intake and body weight gain and improvement of glucose intolerance in combination with dipeptidyl peptidase IV inhibitor in Zucker fa/fa rats. J Pharmacol Exp Ther 2004; 310(2): 614–619. doi: 10.1124/jpet.103.064964 PMID: 15039452

68. Hansen AM, Bödvarsdottr TB, Nordestgaard DN, Heller RS, Gotfredsen CF, Maedler K, et al. Upregulation of alpha cell glucagon-like peptide 1 (GLP-1) in Psammomys obesus—an adaptive response to hyperglycaemia? Diabetologia 2011; 54(6): 1379–1387. doi: 10.1007/s00125-011-2080-1 PMID: 21347622
69. Buse JB, DeFronzo RA, Rosenstock J, Kim T, Burns C, Skare S, Baron A, Fineman M. The primary glucose-lowering effect of metformin resides in the gut, not the circulation: results from short-term pharmacokinetic and 12-week dose-ranging studies. Diabetes Care 2016; 39(2): 198–205. doi: 10.2337/dc15-0488 PMID: 26285584