Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase

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Metformin is considered to be one of the most effective therapeutics for treating type 2 diabetes because it specifically reduces hepatic gluconeogenesis without increasing insulin secretion, inducing weight gain or posing a risk of hypoglycaemia. For over half a century, this agent has been prescribed to patients with type 2 diabetes worldwide, yet the underlying mechanism by which metformin inhibits hepatic gluconeogenesis remains unknown. Here we show that metformin non-competitively inhibits the redox shuttle enzyme mitochondrial glycerophosphate dehydrogenase, resulting in an altered hepatocellular redox state, reduced conversion of lactate and glycerol to glucose, and decreased hepatic gluconeogenesis. Acute and chronic low-dose metformin treatment effectively reduced endogenous glucose production, while increasing cytosolic redox and decreasing mitochondrial redox states. Antisense oligonucleotide knockdown of hepatic mitochondrial glycerophosphate dehydrogenase in rats resulted in a phenotype akin to chronic metformin treatment, and abrogated metformin-mediated increases in cytosolic redox state, decreases in plasma glucose concentrations, and inhibition of endogenous glucose production. These findings were replicated in whole-body mitochondrial glycerophosphate dehydrogenase knockout mice. These results have significant implications for understanding the mechanism of metformin’s blood glucose lowering effects and provide a new therapeutic target for type 2 diabetes.

Initial investigations into metformin action found that this compound is a complex I inhibitor at millimolar concentrations. More recent studies suggested that metformin activates AMP-activated protein kinase (AMPK) through decreases in hepatic energy charge (increasing [AMP]:[ATP] concentration ratios) or through the upstream AMPK kinase, LKB1, leading to reduction of gluconeogenic gene transcription. This effect may, however, be due to sensitization of gluconeogenic transcription to insulin through AMPK-mediated decreases in hepatic lipid content.

In contrast to these findings, it has been suggested that metformin suppresses gluconeogenesis independently of AMPK, instead altering hepatic energy charge and inducing allosteric inhibition of glycolytic enzymes or adenylyl cyclase and glucagon-activated gluconeogenic transcription. However, these hypotheses are inconsistent with findings that metformin does not alter hepatic adenine-nucleotide levels.

Given these conflicting results, it is evident that the mechanisms by which guanide/biguanides exert their therapeutic effects remain to be explained. Variability in data on metformin action may arise because of differences in the dose of metformin administered in vivo ranging from 50 to 500 mg kg⁻¹, as well as observation of chronic versus acute responses to the drug.

In tracing the development of this drug class to the rapid-acting parent compound galegine (a mono-guanide), we considered that the acute glucose lowering effects reported might provide a model in which to investigate potential targets of action of the entire class. Within 20 min of intravenous infusion of galegine, plasma glucose and insulin concentrations decreased (Extended Data Fig. 1a, b), and plasma lactate concentrations increased eightfold (Extended Data Fig. 1c), independently of any changes in hepatic gluconeogenic gene expression (Extended Data Fig. 1d). Galegine also increased total AMPK/2 activity (Extended Data Fig. 1e). However, intravenous administration of the AMPK activator A-769662 failed to decrease plasma glucose concentrations or endogenous glucose production (EGP) (Extended Data Fig. 1f, g) despite comparable increases in AMPK activity 20 min after infusion (Extended Data Fig. 1h). Thus although guanide/biguanide treatment may activate AMPK, acute AMPK activation is not sufficient to reduce EGP.

In considering other mechanisms for metformin action, we were struck by the consistent observation of a marked increase in plasma lactate concentrations in guanide/biguanide-treated animals. This increase could not be attributed to reduced activity of enzymes regulating pyruvate metabolism (Extended Data Fig. 2a), as metformin, galegine and phenformin had no effect on pyruvate carboxylase, citrate synthase and alanine aminotransferase activity (Extended Data Fig. 2b–d). We developed the alternative hypothesis that the striking increase in plasma lactate concentrations may be due to metformin-induced increases in the cytosolic redox state, known to manifest as an increased [lactate]:[pyruvate] ratio. To verify this possibility we assessed the acute effects of metformin on fasting plasma glucose concentrations and rates of EGP at doses that are in the range used to treat patients with type 2 diabetes (20 mg kg⁻¹ and 50 mg kg⁻¹) in awake rats. As early as 30 min after administration, there was a marked reduction in fasting plasma glucose concentrations (Fig. 1a) and inhibition of EGP (Fig. 1b and Extended Data Fig. 3a). We measured the [lactate]:[pyruvate] and [β-hydroxybutyrate]:[acetacetate] ratios as surrogates of the cytosolic and mitochondrial redox states, respectively, and found that metformin elicited a significant increase in the cytosolic redox state and decrease in the mitochondrial redox state in both plasma and liver (Fig. 1c–f and Extended Data Fig. 3b, c). Consistent with these findings, metformin treatment increased the liver ([GSH]:[GSSG] ratio, reflecting an increase in the cytosolic redox state (Extended Data Fig. 3d). These data support a mechanism for metformin’s rapid effect on hepatic glucose production through alteration of the redox state. We also observed a trend towards increased plasma glycerol concentrations, suggesting inhibition of glycerol conversion to glucose (Fig. 1g). In contrast, acute metformin treatment did not affect cellular energy charge ([ATP]:[ADP], [ATP]:[AMP], [NADH]:[NAD⁺], [NADPH]:[NADP⁺]) (Extended Data Fig. 3e–h), or liver [cAMP] (Extended Data Fig. 3i). The lack of observed differences in [NADH]:[NAD⁺] and [NADPH]:[NADP⁺] ratios, despite significant metformin-induced alterations in the cytoplasmatic and mitochondrial redox state, can most probably be attributed to the [NADH]:[NAD⁺] ratio moving in opposite directions in these two compartments such that total

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[NADH]:[NAD⁺] may remain the same. Finally, no differences in protein expression of the key gluconeogenic enzymes cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C) and pyruvate carboxylase were observed (Extended Data Fig. 3j–p). There was no significant AMPK activation, as determined by relative levels of phosphorylated AMPK to total AMPK, and no change in the phosphorylation of the downstream target of AMPK, acetyl-coenzyme A carboxylase (ACC), although cyclic AMP response element binding protein (CREB) activation was observed as determined by the ratio of phosphorylated CREB to total CREB (Extended Data Fig. 3j, m–o).

Similar to acute metformin treatment, chronic metformin treatment lowered fasting plasma glucose concentrations and EGP (Fig. 2a, b). These changes were also associated with an increase in the plasma and cytosolic redox state and a decrease in the mitochondrial redox state (Fig. 2c–f and Extended Data Fig. 4a). Plasma glycerol concentrations were also higher in these animals (Fig. 2g). Similar to what was observed with acute metformin treatment, chronic metformin treatment did not alter hepatic or cytosolic energy charge (Extended Data Fig. 4b–e), and there was only a slight reduction in liver [cAMP] (Extended Data Fig. 4f). Protein expression of PEPCK-C and pyruvate carboxylase were also unchanged; however, AMPK was activated, ACC phosphorylation was increased and CREB activation was decreased (Extended Data Fig. 4g–i).

Given that acute and chronic metformin treatment increased the cytoplasmic redox state and decreased the mitochondrial redox state, we next considered that metformin may block one of the two main redox shuttle systems, the malate–aspartate and glycerophosphate shuttles. Guanidines/biguandies had no effect on total malate–aspartate shuttle activity or on malate dehydrogenase and aspartate aminotransferase activities (Extended Data Fig. 2e–g). We next examined the effect of metformin on the glycerophosphate shuttle (Extended Data Fig. 5a). Guanidines/biguandies did not alter the activity of purified, recombinant cytosolic glycero phosphate dehydrogenase (cGPD) (Extended Data Fig. 5b). In contrast, all guanidines/biguandies inhibited mitochondrial GPD (mGPD) activity by 30–50% in rat mitochondrial lysates (Fig. 3a). These findings are supported by our data showing...
that metformin significantly inhibited mitochondrial respiration from glycerol-3-phosphate (Fig. 3b).

Given the impact of metformin on the cytosolic/mitochondrial redox state, we next examined whether we could influence the effectiveness of metformin by changing the cytosolic redox state by altering the extra-cellular [lactate]:[pyruvate] ratio. Consistent with this hypothesis we found that at lower [lactate]:[pyruvate], metformin and mGPD short interfering RNA (siRNA) treatment to knockdown expression of mGpd no longer significantly reduced glucose production from primary rat hepatocytes (Extended Data Fig. 6a). This finding suggests that an augmented cytosolic redox state is central to metformin-mediated reduction in hepatic gluconeogenesis.

Inhibition of mGPD would prevent glycerol from contributing to gluconeogenic flux, as glycerol must be phosphorylated by glycerol kinase to glycerol-3-phosphate and converted to dihydroxyacetone phosphate by mGPD to contribute to glucose production. Furthermore, inhibition of mGPD would halt the glycerophosphate shuttle and lead to accumulation of cytosolic NADH, which is unfavourable for conversion of lactate to pyruvate by lactate dehydrogenase (LDH). Consistent with this hypothesis, glucose production in hepatocytes from both lactate and glycerol was inhibited by metformin and mGpd knockdown. In contrast, metformin and mGpd knockdown had no effect on gluconeogenesis from dihydroxyacetone, alanine and pyruvate, substrates that enter gluconeogenesis without augmenting the cytosolic [NADH]:[NAD+] ratio (Extended Data Fig. 6b, c). We also observed an increase in glycerol-3-phosphate concentrations in primary hepatocytes after metformin treatment and mGpd knockdown by siRNA, and specifically in liver after acute metformin treatment in vivo (Extended Data Fig. 6d, e), consistent with metformin inhibition of mGPD activity. These data indicate that both acute and chronic metformin treatment inhibit mGPD, limiting lactate and glycolate contributions to hepatic gluconeogenesis.

To examine whether mGPD is the molecular target for metformin in vivo, we treated rats with antisense oligonucleotides (ASOs) to knock down hepatic mGpd and cGpd expression (Extended Data Fig. 7a, b). As seen with metformin treatment, mGPD ASO treatment reduced plasma glucose concentrations, increased cytosolic redox state and decreased mitochondrial redox state. In contrast, GPD ASO treatment had only a mild effect on cytosolic and mitochondrial redox state, did not alter plasma glucose levels and did not affect plasma lactate concentrations (Fig. 4a–c and Extended Data Fig. 7c). These data are consistent with previous studies showing that knockdown of the mGpd gene, but not cGpd, in mice results in a reduction in plasma glucose concentrations.

To investigate further whether mGPD is the primary target of metformin, we examined whether mGPD ASO treatment would abrogate the inhibitory effects of metformin on EGP. mGPD ASO treatment lowered plasma glucose concentrations and inhibited EGP. Moreover, mGPD ASO treatment abrogated the ability of metformin ability to decrease plasma glucose concentrations and inhibit EGP (Fig. 4d, e). mGPD-ASO-treated animals also had increased plasma [lactate]:[pyruvate] and decreased plasma [β-hydroxybutyrate]:[acetooacetate] ratios, whereas metformin had no extra effect on these parameters (Fig. 4f, g). As seen with metformin treatment, mGPD ASO treatment increased the liver [GSH]:[GSSG] ratio, reflecting an increase in the cytosolic redox state (Extended Data Fig. 7d). In contrast the energy charge in the liver was unaltered by mGpd knockdown (Extended Data Fig. 7e–h), although a slight decrease in liver [cAMP] was observed (Extended Data Fig. 7i). There was no appreciable effect of mGpd knockdown on hepatic PEPCK-C and pyruvate carboxylase expression, although AMPK activation, increased ACC phosphorylation and suppression of CREB activation were observed (Extended Data Fig. 7j–o). Chronic metformin treatment of mGPD ASO knockdown rats also had no added effect on plasma glucose concentrations or EGP (Fig. 4h, i). We next examined whether metformin’s effects would be abrogated in a whole-body mGPD knockout mouse and found that these mice, similar to the mGPD-ASO-treated rats, had decreased fasting plasma glucose concentrations and decreased rates of EGP compared with wild-type littermates, and that the effects of acute metformin treatment on plasma glucose concentrations and EGP were also abrogated in mGPD knockout mice (Fig. 4j, k).

We further characterized the nature of metformin’s effect on mGPD kinetics, and found that metformin inhibited isolated rat mGPD activity non-competitively with an inhibition constant (K_i) of approximately 38 μM (Extended Data Fig. 5c). Metformin also inhibited purified recombinant human mGPD activity, showing non-competitive kinetics with K_i ~ 55 μM (Fig. 3c and Extended Data Fig. 5d), and non-competitively inhibited activity of pure, Pediococcus sl-glycerophosphate oxidase, a bacterial isoform of mammalian mGPD (Extended Data Fig. 5e).

The glycerophosphate shuttle has an important role in the regulation of hepatic gluconeogenesis, the role of mGPD as a regulator of EGP being strongly supported by the finding that mGPD knockout mice have lowered plasma glucose concentrations and are protected...
from diet-induced hyperglycaemia. We found that metformin inhibits mGPD activity in vitro with an approximate $K_m$ value close to the observed range of plasma metformin levels (10–40 μM) in patients treated with a normally prescribed dose of 1 g of metformin twice a day. Our measurements of metformin concentrations in the plasma and liver of rats treated acutely with 50 mg kg$^{-1}$ metformin gave us a peak average value of 74 μM and 100 μM, respectively, 30 min after administration (Extended Data Fig. 9). These pharmacokinetic data are consistent with the acute metformin-mediated inhibition of hepatic gluconeogenesis within a similar time frame and are consistent with the need to provide dosing of metformin twice a day to patients with type 2 diabetes for optimal plasma glucose lowering. Previous studies assessing metformin action in vivo have typically used metformin doses ranging from 250 mg kg$^{-1}$ to 500 mg kg$^{-1}$, which are supra-pharmacological and result in plasma metformin concentrations greater than 1 mM (Extended Data Fig. 9). In this regard, although metformin inhibited mitochondrial respiration from glycerol-3-phosphate at micromolar concentrations, complex I respiration was affected only at metformin concentrations greater than 1 mM, which are not clinically relevant (Extended Data Fig. 2h).

The kinetics of metformin inhibition of mGPD was found to be non-competitive, which raises the question of how this might be achieved. Computational analyses of the structure of the mGPD isoform, Strep- tococcus sp. $\alpha$-glyceraldehyde phosphate oxidase, mutated in the flavin adenine dinucleotide (FAD) binding pocket to the sequence of the human mGPD, found that metformin shows favourable binding to the FAD-containing pocket (Extended Data Fig. 8a–c). Based on these predictions, FAD, which is non-covalently associated with the enzyme, may dynamically slide in and out of the wider entrance of the tunnel-shaped pocket, and metformin may enter from the smaller end, preventing complete re-entry of FAD and inhibiting electron transfer. Although speculative, this model could explain how metformin could displace the flavin ring and inhibit enzymatic activity without necessarily invoking FAD turnover.

In summary, we have shown that metformin treatment of rats at doses that achieve comparable plasma metformin concentrations observed in patients with metformin-treated type 2 diabetes inhibits mGPD non-competitively and modulates cytosolic and mitochondrial redox state, inducing an effective reduction in EGP. These results identify mGPD as one of the primary molecular targets by which guanidino/biguanides inhibit hepatic gluconeogenesis, and provide a new therapeutic target for type 2 diabetes.

**METHODS SUMMARY**

**Hepatocyte studies.** Primary hepatocytes were isolated from 24-h-fasted Sprague-Dawley rats and treated with or without 100 μM metformin in DMEM supplemented with 10 mM substrate (glycerol at 100 μM). Glucose was measured using Genzyme Glucose-6-P reagent.

**Animal studies.** Studies were performed in 24-h-fasted, awake, unrestrained male Sprague-Dawley rats aged 9–12 weeks. Animals were randomly allocated to treatment groups before collection of any data and the studies were performed unblinded. Acute metformin was administered intravenously and chronic metformin was administered intraperitoneally at 50 mg kg$^{-1}$ daily over 30 days. Continuous 1 mg kg$^{-1}$ min$^{-1}$ arterial infusion of [6,6-2H$_2$]glucose tracer was administered to measure EGP. Plasma...
glucose was measured on a YSI 2700 Biochemistry Analyzer. All other metabolites were measured using enzymatic methods. Whole-body mGPD knockout mouse embryos were a gift from M. J. MacDonald. Overnight-fasted mice were infused with [3-3H]glucose at 0.05 μCi min⁻¹ for 120 min to measure basal glucose turnover. Mice were restrained during the studies, and given acute metformin (50 mg kg⁻¹, intravenously) or saline. All animal studies were conducted with previous approval from Yale University Institutional Animal Care and Use Committee.

**ASO treatment studies.** ASO treatment was administered at 37.5 mg kg⁻¹ twice weekly for 4 weeks by intraperitoneal injection. ASO treatment knocked down mGPD messenger RNA (mRNA) and protein only in the liver (Extended Data Fig. 10).

**Enzyme assays.** mGPD activity in rat liver mitochondrial lysate was measured by cytochrome c as the electron acceptor and measuring gain of absorbance at 550 nm. Activities of rat mGPD isolated from rat mitochondrial lysate using anti-GPD2 antibody K-4, and human mGPD overexpressed in HEK 293 cells from an Ori-gene vector, were measured using 2,6-dichloroindophenol (DCIP) and measuring loss of absorbance at 600 nm. Kinetic measurements were made using a Flexstation 3 Benchtop Multi-Mode Microplate Reader (Molecular Devices).

**Oxygen consumption.** Studies were performed on a Seahorse Bioscience XF24 Analyzer.

**Online Content** Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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1. Hundai, R. S. et al. Mechanism by which metformin reduces glucose production in type 2 diabetes. *Diabetes* **49**, 2063–2069 (2000).
2. Inuzuki, S. et al. Efficacy and metabolic effects of metformin and troglitazone in type II diabetes mellitus. *N. Engl. J. Med.* **338**, 867–872 (1998).
3. El-Mir, M.-Y. et al. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *J. Biol. Chem.* **275**, 223–228 (2000).
4. Owen, M. R., Doran, E. & Halestrap, A. P. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex I of the mitochondrial respiratory chain. *Biochem. J.* **348**, 607–614 (2000).
5. Zhou, G. et al. Role of AMP-activated protein kinase in mechanism of metformin action. *J. Clin. Invest.* **108**, 1167–1174 (2001).
6. Shaw, R. J. et al. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. *Science* **310**, 1642–1646 (2005).
7. He, L. et al. Metformin and insulin suppress hepatic gluconeogenesis through phosphorylation of CREB binding protein. *Cell* **137**, 635–646 (2009).
8. Cool, B. et al. Identification and characterization of a small molecule AMPK activator that treats key components of type 2 diabetes and the metabolic syndrome. *Cell Metab.* **3**, 403–416 (2006).
9. Savage, D. B. et al. Reversal of diet-induced hepatic steatosis and hepatic insulin resistance by antisense oligonucleotide inhibitors of acetyl-CoA carboxylase 1 and 2. *J. Clin. Invest.* **116**, 817–824 (2008).
10. Fullerton, M. D. et al. Single phosphorylation sites in Acc1 and Acc2 regulate lipid homeostasis and the insulin-sensitizing effects of metformin. *Nature Med.* **19**, 1481–1484 (2013).
11. Foretz, M. et al. Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. *J. Clin. Invest.* **120**, 2355–2369 (2010).
12. Miller, R. A. et al. Biguanides suppress hepatic glucagon signalling by decreasing production of cyclic AMP. *Nature* **494**, 256–260 (2013).
13. Hawley, S. A., Gadalla, A. E., Olsen, G. S. & Hardie, D. G. The anti-diabetic drug metformin activates the AMP-activated protein kinase cascade via an adenine nucleotide-independent mechanism. *Diabetes* **51**, 2420–2425 (2002).
14. Yang, L. et al. Metabolomic and mass isotopomer analysis of liver gluconeogenesis and citric acid cycle. I. Interrelation between gluconeogenesis and cataplerosis; formation of methoxamates from aminooxycetate and ketoacids. *J. Biol. Chem.* **283**, 21978–21987 (2008).
15. Krebs, H. A. & Gascoyne, T. The redox state of the nicotinamide-adenine dinucleotides in rat liver homogenates. *Biochem. J.* **108**, 513–520 (1968).
16. Williamson, D. H., Lund, P. & Krebs, H. A. The redox state of free nicotinamide-adenine dinucleotide in the cytoplasm and mitochondria of rat liver. *Biochem. J.* **103**, 514–527 (1967).
17. Bremer, J. & Davis, E. J. Studies on the active transfer of reducing equivalents into mitochondria via the malate-aspartate shuttle. *Biochim. Biophys. Acta* **376**, 387–397 (1975).
18. Cederbaum, A. I., Lieber, C. S., Beattie, D. S. & Rubin, E. Characterization of shuttle mechanisms for the transport of reducing equivalents into mitochondria. *Arch. Biochem. Biophys.* **158**, 763–781 (1973).
19. Garrido, A. & McMurray, W. C. Purification and characterization of glycerol-3-phosphate dehydrogenase (flavin-linked) from rat liver mitochondria. *J. Biol. Chem.* **261**, 8042–8048 (1986).
20. Cole, E. S., Lepp, C. A., Holohan, P. D. & Fonyd, T. P. Isolation and characterization of flavin-linked glycerol-3-phosphate dehydrogenase from rabbit skeletal muscle mitochondria and comparison with the enzyme from rat brain. *J. Biol. Chem.* **253**, 7952–7959 (1978).
21. White, H. B. III & Kaplan, N. O. Purification and properties of two types of diphosphopyridine nucleotide-linked glycerol 3-phosphate dehydrogenases from chicken breast muscle and chicken liver. *J. Biol. Chem.* **244**, 6031–6039 (1969).
22. Sistare, F. D. & Haynes R. C. Jr. The interaction between the cytosolic pyridine nucleotide redox potential and gluconeogenesis from lactate/pyruvate in isolated rat hepatocytes. *J. Biol. Chem.* **260**, 12748–12753 (1985).
23. Sugano, T. et al. Intracellular redox state and stimulation of gluconeogenesis by glucagon and norepinephrine in the perfused rat liver. *J. Biochem.** **87**, 153–166 (1980).
24. MacDonald, M. J. & Marshall, L. K. Mouse lacking NADH-linked glycerol phosphate dehydrogenase has normal pancreatic beta cell function but abnormal metabolism pattern in skeletal muscle. *Arch. Biochem. Biophys.* **384**, 143–153 (2000).
25. Prochazka, M., Kozak, U. C. & Kozak, L. P. A glycerol-3-phosphate dehydrogenase null mutant in BALB/cHeA mice. *J. Biol. Chem.* **264**, 4679–4683 (1989).
26. Harding, J. W. Jr, Pyeritz, E. A., Copeland, E. S. & White, H. B. III. Role of glycerol 3-phosphate dehydrogenase in glyceride metabolism. Effect of diet on enzyme activities in chicken liver. *Biochem. J.* **146**, 223–229 (1975).
27. Harding, J. W. Jr, Pyeritz, E. A., Morris, H. P. & White, H. B. III. Proportionional activities of glycerol kinase and glycerol 3-phosphate dehydrogenase in rat hepatomas. *Biochem. J.* **148**, 545–550 (1975).
28. Brown, L. et al. Normal thyroid thermogenesis but reduced viability and adiposity in mice lacking the mitochondrial glycerol phosphate dehydrogenase. *J. Biol. Chem.* **277**, 32892–32898 (2002).
29. Barberá, A. et al. A high carbohydrate diet does not induce hyperglycaemia in a mitochondrial glycerol-3-phosphate dehydrogenase-deficient mouse. *Diabetologia* **46**, 1394–1401 (2003).
30. Colussi, T. et al. Structure of α-glycerophosphate oxidase from Streptococcus sp.: a template for the mitochondrial α-glycerophosphate dehydrogenase. *Biochemistry* **47**, 965–977 (2008).

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Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to G.I.S. (gerald.shulman@yale.edu).
METHODS

Hepatocyte studies. Primary hepatocytes were freshly isolated by collagenase digestion of livers from hepatic glycogen-deplete, 24-h-fasted Sprague–Dawley rats, and plated on collagen-coated six-well plates. Cells were cultured overnight in recovery media (DMEM supplemented with 4.5 g ml⁻¹ glucose, 1 mM dexamethasone, insulin, 10% FBS, 1% penicillin-streptomycin, 10 mM HEPES and 3.7 mg ml⁻¹ sodium bicarbonate). Cells were then pre-incubated for 2 h in no-glucose DMEM supplemented with 10 mM HEPES and 3.7 mg ml⁻¹ sodium bicarbonate with or without 100 μM metformin, followed by a 2-h incubation in no-glucose DMEM supplemented with 10 mM substrate (except glyceral, which was added at 100 μM). Cells were treated with 20 μM siRNA in recovery media overnight; siRNA transfaction was performed using RNAiFect Transfection reagent (QIAGEN, 301605). siRNA sequences used were control (Sigma Aldrich Mission siRNA Universal Negative Control, SIC001) and mGPD (Sigma Aldrich, SASR_Rnu_00260066, sequence start: 1395; National Center for Biotechnology Information (NCBI) gene NM_012736). siRNA did not induce cytotoxicity, as determined by trypan blue exclusion, CyQuant Assay (Life Technologies, C35011) and cytomegoc virus release into the cytosolic fraction (Extended Data Fig. 6f–h). Glucose in the media was measured using Genzyme Glucose-6-L reagent, and glucose production was normalized to total protein levels as measured by Bradford assay.

Animal studies. Male Sprague–Dawley rats were acquired from Charles River, and were administered regular chow and water ad libitum. Rats were housed individually on a 12:12-h light/dark cycle. Arterial lines were surgically implanted into the carotid artery and venous lines into the jugular vein of rats for the studies. All animal studies included 6–10 rats per group, aged 9–12 weeks and weighed 300–450 g at the time of study. Animals were randomly allocated to treatment groups before collection of any data (for example, weight) or surgical procedures and the studies were performed unblinded. Sample sizes were selected to detect moderate to large (>20%) differences. All studies were performed in awake, unrestrained animals. Acute metformin was administered intravenously in 0.9% saline at 20, 50, 100 and 250 mg kg⁻¹ for 10 min; chronic metformin was administered intraperitoneally at 50 mg kg⁻¹ daily over 30 days. Galegine solutions were prepared in normal saline and infused intra-venously at 25 mg kg⁻¹ h⁻¹ after a 5 min 250 mg kg⁻¹ bolus. Rats were fasted for 24 h before studies were conducted. Rats were anesthetized by intravenous pentobarbital. A continuous arterial infusion of [6,6-H]glucose tracer at a rate of 1 mg kg⁻¹ min⁻¹ was administered throughout the study (after a 5 min, 3 mg kg⁻¹ min⁻¹ prime) to measure EGP. Plasma glucose enrichment was measured by derivatizing deproteinized plasma samples with acetic anhydride and pyridine, and ‘H enrichment was measured by gas chromatography mass spectrometry as previously described. Plasma glucose measured using a YSI 2700 Biochemistry Analyzer from sampled venous blood. All other plasma metabolites were measured from sampled venous blood using enzymatic methods and monitoring changes in NADH absorbance at 340 nm. Liver metabolites were measured from PCA-extracted samples immediately after collection. High-energy intermediates were measured from liver tissue extracted in a methanol, ammonium acetate based buffer by liquid chromatography–tandem mass spectrometry, and values were normalized to an added internal standard, D₅-taurine. Tissues were rapidly acquired and freeze-clamped in situ. Overall, tissues were homogenized and mitochondrial lysate preparation were performed using cytochrome c as the electron acceptor and measuring gain of absorbance at 550 nm (reaction buffer consisted of 10 mM Tris-HCl, 20 mM glycerol, 10 mM NaCl, 5 mM MgCl₂, 1% NP-40) with added cOmplete protease inhibitor tablet (Roche) and measuring loss of absorbance at 600 nm (reaction buffer consisted of 50 mM KH₂PO₄, 95 mM DCIP; reaction conducted at 38 °C). Mitochondria were isolated less than 3 h before conducting activity assays from fresh rat livers. Briefly, rat liver was homogenized using a glass dounce homogenizer in ice-cold isolation buffer (210 mM sucrose, 660 mM mannitol, 30 mM KH₂PO₄, 15 mM MgCl₂, 6H₂O, 3 mM EGTA and 75 mM MOPS). Mitochondria were separated from total homogenate by differential centrifugation. Rat mitochondria was isolated from rat mitochondrial lysate by immunoprecipitation with anti-GPD2 antibody K-14 (Santa Cruz Biotechnology, sc-161680) at a ratio of 1 mg lysate per 10 μl antibody, overnight, at 4 °C. Activity was measured using 2,6-dichloroindophenol (DCIP) as the electron acceptor and measuring loss of absorbance at 600 nm (reaction buffer consisted of 50 mM KH₂PO₄, 95 mM DCIP; reaction conducted at 38 °C; mitochondrial lysate pre-incubated in buffer with or without metformin for 10 min). Human mGPD tagged with Myc-SDK was overexpressed in HEK 293 cells (ATCC CRL-1573) from a pre-designed vector available from Origene. Recombinant human mGPD was isolated by immunoprecipitation from the cell lysates using an anti-DDK antibody (Origene) at 10 μl antibody per 1 mg lysate protein, and activity was measured using DCIP as the electron acceptor. Recombinant, purified Pediococcus sp. γ-glycerophosphate oxidase was obtained from Sigma and activity was determined by indirectly detecting peroxide production by oxidation of γ-phenylendiamine in the presence of phenol and measuring increase in absorbance at 450 nm. Michaelis–Menten, Eadie–Hofstee and Lineweaver–Burk plots were used to determine the kinetics of inhibition. Kinetic measurements used a Flexstation 3 Benchtop Multi-plate Reader (Molecular Devices).

Western blotting. Liver lysates were made by homogenizing pulverized, freeze-clamped liver tissue in lysis buffer (50 mM Tris-HCl pH 7.4, 1 mM EGTA, 150 mM NaCl, 5 mM MgCl₂, 1% NP-40) with added Complete protease inhibitor tablet (Roche) per 50 ml buffer. SDS samples were prepared by adding 2× SDS samples buffer (20% glycerol, 120 mM Tris-HCl pH 6.8, 4% SDS, 0.02% bromphenol blue, 4% beta-mercaptoethanol) to one volume of lysate and boiling at 95 °C for 5 min. Samples were run on Novex 4–12% Tris-Glycine gels (Invitrogen) and gels were transferred to PVDF membranes by semi-dry transfer. Antibodies used to determine protein expression were as follows: PEPC-K C-3000 (Santa Cruz Biotechnology, sc-161680), Phospho-AMPk Thr172 (Santa Cruz Biotechnology, sc-161680), Phospho-AMPKα Thr172 (Santa Cruz Biotechnology, sc-161680), Phospho-AMPK Thr172 (Cell Signaling, 9198), CREB 48H2 (Cell Signaling, 9197), Phospho–AMPk Thr172/R173 (Cell Signaling, 2355), AMPKα (Cell Signaling, 3532), GAPDH 1D6H11 (Cell Signaling, 5174) and anti-GPD2 K-14 (Santa Cruz Biotechnology, sc-161680).

Real-time quantitative PCR with reverse transcription. RNA was isolated using a Qiagen RNeasy kit (Qiagen). mRNA was reverse transcribed to cDNA using MuLV reverse transcriptase (New England Bio Labs). The abundance of transcripts was assayed by real-time PCR on a 7500 Fast Real-Time PCR System (Applied Biosystems). Each run was evaluated in duplicate for both the gene of interest and actin as a control. The expression data for the gene of interest and actin were normalized for the efficiency of amplification determined by the standard curve included for each data acquisition. Primer sequences used were as follows: mGPD forward, 5'-AGGACGGCAAAGTTGGACGC-3'; mGPD reverse, 5'-TCTGACAACAGCGACTTC-3'; cGPD forward, 5'-AACAGATCGGGGAGAAAG-3'; cGPD reverse, 5'-GGCCGTGTGCTCACTCCTCTTA-3'; actin forward, 5'-CCAGCATCTGTTGTGACCTTC-3'; actin reverse, 5'-CATGAGGTTAGTCTGTCAGGTC-3'.

Enzyme assays. Enzyme assays measuring mGPD activity using rat liver mitochondrial lysate preparation were performed using cytochrome c as the electron acceptor and measuring gain of absorbance at 550 nm (reaction buffer consisted of 10 mM Tris-HCl, 50 μM cytochrome c, 25 μM sodium azide, 1 mM EDTA, 50 mM KCl; buffers were bubbled with N₂ gas before assay; lysates were pre-incubated in buffer with or without treatment for 10 min; the reaction was conducted at 38 °C). Mitochondria were isolated less than 3 h before conducting activity assays from fresh rat livers. Briefly, rat liver was homogenized using a glass dounce homogenizer in ice-cold isolation buffer (210 mM sucrose, 660 mM mannitol, 30 mM KH₂PO₄, 15 mM MgCl₂, 6H₂O, 3 mM EGTA and 75 mM MOPS). Mitochondria were separated from total homogenate by differential centrifugation. Rat mGPD was isolated from rat mitochondrial lysate by immunoprecipitation with anti-GPD2 antibody K-14 (Santa Cruz Biotechnology, sc-161680) at a ratio of 1 mg lysate per 10 μl antibody, overnight, at 4 °C. Activity was measured using 2,6-dichloroindophenol (DCIP) as the electron acceptor and measuring loss of absorbance at 600 nm (reaction buffer consisted of 50 mM KH₂PO₄, 95 mM DCIP; reaction conducted at 38 °C; mitochondrial lysate pre-incubated in buffer with or without metformin for 10 min). Human mGPD tagged with Myc-SDK was overexpressed in HEK 293 cells (ATCC CRL-1573) from an over-expressed vector under control of the CMV promoter. Recombinant human mGPD was isolated by immunoprecipitation from the cell lysates using an anti-DDK antibody (Origene) at 10 μl antibody per 1 mg lysate protein, and activity was measured using DCIP as the electron acceptor. Recombinant, purified Pediococcus sp. γ-glycerophosphate oxidase was obtained from Sigma and activity was determined by indirectly detecting peroxide production by oxidation of γ-phenylendiamine in the presence of phenol and measuring increase in absorbance at 450 nm. Michaelis–Menten, Eadie–Hofstee and Lineweaver–Burk plots were used to determine the kinetics of inhibition. Kinetic measurements used a Flexstation 3 Benchtop Multi-plate Reader (Molecular Devices).
Molecular docking and binding simulation. Molecular docking simulations used the program Glide (Schrodinger), with a modified version of the crystal structure of α-glycerophosphate oxidase from Streptococcus sp. (Protein Data Bank 2RGH) as the target enzyme. Sequence alignments with human mitochondrial glycerol-3-phosphate dehydrogenase (hG3PD, NCBI reference sequence NM_000408.4) were used to mutate the FAD binding site coordinates of 2RGH to the corresponding hG3PD sequence. As a positive control, a docking simulation of FAD to this target protein yielded a result essentially identical to the actual FAD position observed in the 2RGH structure. Subsequent docking simulations were conducted using metformin, buformin, phenformin and galegine.

Oxygen consumption. Mitochondria were prepared immediately before each study on a Seahorse Bioscience XF24 Analyzer. Liver was rapidly excised from an overnight-fasted rat anaesthetized under isoflurane. Liver was then homogenized in mitochondrial assay solution (70 mM sucrose, 220 mM mannitol, 5 mM KH₂PO₄, 5 mM MgCl₂, 2 mM HEPES, 1 mM EGTA, 0.2% BSA). Mitochondria were purified by differential centrifugation and the mitochondrial pellet was used for respiration studies in mitochondrial assay solution without BSA. All reagents were diluted in mitochondrial assay solution without BSA and loaded into the ports of the flux plate (port A: 10 mM pyruvate, 2.5 mM glutamate, 2.5 mM malate; port B: 2 μM rotenone; port C: 5 mM succinate or 10 mM glycerol-3-phosphate; port D: 1 μg ml⁻¹ oligomycin).

Statistical analysis. All data are expressed as mean ± s.e.m. P values less than 0.05 were considered statistically significant differences as determined by unpaired two-tailed Student’s t-test or ANOVA. Data shown are representative of repeated experiments performed as indicated; where not indicated, a single experiment was performed. All replicates are biological replicates unless otherwise indicated.

31. Faupel, R. P., Seitz, H. J., Tarnowski, W., Thiemann, V. & Weiss, C. The problem of tissue sampling from experimental animals with respect to freezing technique, anoxia, stress and narcosis. A new method for sampling rat liver tissue and the physiological values of glycolytic intermediates and related compounds. Arch. Biochem. Biophys. 148, 509–522 (1972).
32. Ayala, J. E. et al. NIH Mouse Metabolic Phenotyping Center Consortium. Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice. Dis. Model. Mech. 3, 525–534 (2010).
33. Carmignani, M. et al. Novel hypotensive agents from Verbesina caracasana. 8. Synthesis and pharmacology of (3,4-dimethoxycinnamoyl)-N²-agmatine and synthetic analogues. J. Med. Chem. 44, 2950–2958 (2001).
Extended Data Figure 1 | Effect of acute galegine treatment and acute AMPK activator treatment in vivo. A 20-min infusion of galegine in rats (a) decreased fasting plasma glucose concentrations, (b) decreased fasting plasma insulin concentrations, and (c) increased plasma lactate concentrations, (d) independently of any changes in gluconeogenic gene expression. e, AMPK was activated by acute galegine treatment. f, Twenty-minute infusions of A-769662 in rats had no effect on fasting plasma glucose concentrations or (g) endogenous glucose production, in spite of (h) comparable activation of AMPK. Data are mean ± s.e.m. (saline, n = 6; galegine, n = 8; A-769662, n = 5 biological replicates). *P < 0.05, **P < 0.01, ***P < 0.001 by unpaired t-test.
Extended Data Figure 2 | Effect of guanide/biguanide treatment on enzymes involved in pyruvate metabolism, redox regulation and the malate–aspartate shuttle, and on complex-I-mediated respiration. 

**a**, Lactate enters metabolism through LDH by a redox-dependent reaction into the pyruvate pool. Pyruvate lies at the intersection of alanine influx, glycolysis, citric acid cycle and gluconeogenic flux. **b**, Guanides/biguanides did not affect pyruvate carboxylase (PC) activity, compared with the known inhibitor coenzyme A, and (*c*) did not affect citrate synthase activity. **d**, Alanine aminotransferase activity was also unaffected. **e**, Guanides/biguanides did not affect pyruvate carboxylase (PC) activity, compared with the known inhibitor coenzyme A, and (*c*) did not affect citrate synthase activity. **d**, Alanine aminotransferase activity was also unaffected. *P < 0.05, **P < 0.01, ***P < 0.001 by unpaired t-test."
Extended Data Figure 3 | Effect of acute metformin (20 mg kg$^{-1}$ and 50 mg kg$^{-1}$, intravenously) treatment on EGP, liver redox, energy charge and liver gluconeogenic protein expression in Sprague-Dawley rats.

a, Acute metformin (20 mg kg$^{-1}$) treatment significantly lowered EGP, (b) increased the liver [lactate]:[pyruvate] ratio and (c) decreased liver [β-hydroxybutyrate]:[acetoacetate]. d, Acute metformin (50 mg kg$^{-1}$) treatment increased liver [GSSG]:[GSH], (e) but had no effect on the liver [ATP]:[ADP] or (f) [ATP]:[AMP] ratios. g, The [NADH]:[NAD$^+$] and (h) [NADPH]:[NADP$^+$] ratios also remained unchanged. i, Acute metformin treatment had no effect on liver [cAMP] levels. j, Acute metformin (50 mg kg$^{-1}$) treatment had no effect on the expression of key gluconeogenic enzymes or AMPK in the liver. k, PEPCK-C protein expression and (l) pyruvate carboxylase protein expression were unchanged, although (m) activated CREB as determined by the ratio of phosphorylated CREB to total CREB levels was slightly increased. n, There was no activation of liver AMPK, as reflected by the ratio of phosphorylated AMPK to total AMPK levels, and (o) no change in the phosphorylation of AMPK downstream target ACC. Data are mean ± s.e.m. (n = 6 biological replicates). *P < 0.05, **P < 0.01, ***P < 0.001 by unpaired t-test.
Extended Data Figure 4 | Effect of chronic metformin (50 mg kg⁻¹ per day, intraperitoneally for 30 days) treatment on liver redox, energy charge and expression of gluconeogenic regulators. a, Chronic metformin treatment increased the liver [GSSG]:[GSH] ratio, but (b) had no effect on the liver [ATP]:[ADP] or (c) [ATP]:[AMP] ratios. d, The [NADH]:[NAD⁺] and (e) [NADPH]:[NADP⁺] ratios also remained unchanged. f, Chronic metformin treatment slightly reduced liver [cAMP] levels. g, Chronic metformin treatment had no effect on the protein levels of principal gluconeogenic enzymes in the liver; (h) PEPCK-C protein expression and (i) liver pyruvate carboxylase protein levels both remaining unaltered. j, Activated CREB as determined by the ratio of phosphorylated CREB to total CREB levels was decreased. k, Chronic metformin treatment activated liver AMPK as indicated by the increased ratio of phosphorylated AMPK to total AMPK levels and (l) increased phosphorylation of ACC. Data are mean ± s.e.m. (n = 6 biological replicates). *P < 0.05, **P < 0.01, ***P < 0.001 by unpaired t-test.
Extended Data Figure 5 | Effect of metformin on the glycerophosphate shuttle. NADH made in the cytosol by glycolysis cannot cross the mitochondrial membrane and contribute electrons to the electron transport chain (ETC) for ATP synthesis. Two mechanisms, the reversible malate-aspartate shuttle and (a) the unidirectional glycerophosphate shuttle, oxidize NADH in the cytosol and transport electrons into the mitochondria through metabolic intermediates. The glycerophosphate shuttle is composed of cytosolic and mitochondrial glycerophosphate dehydrogenases, two structurally distinct enzymes. b, Metformin had no effect on cGPD, which consists of two subunits and catalyses the conversion of dihydroyacetone phosphate (DHAP) to glycerol-3-phosphate (G-3-P), oxidizing one NADH.

c, Metformin inhibited the activity of rat mGPD, a FAD-linked enzyme that transmits electron pairs to the electron transport chain through the quinone pool, purified from liver by immunoprecipitation. Inhibition of rat mGPD was non-competitive. Data shown are the average of five separate experiments.
d, Metformin inhibited pure, recombinant human mGPD non-competitively, and decreased $V_{\text{max}}$ without affecting the Michaelis constant ($K_m$). Data shown are representative of two experiments.
e, Metformin also inhibited the activity of the bacterial mGPD isoform, Pedicoccus sp. $\alpha$-glycerophosphate oxidase, showing non-competitive kinetics. Data are mean ± s.e.m. ($n = 4$ or 5 technical replicates). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by unpaired t-test.
Extended Data Figure 6 | Effect of metformin and knockdown of mGPD by siRNA on glucose production from various substrates in primary hepatocytes, and metformin-mediated increase in glycerol-3-phosphate concentrations. a, Metformin treatment (100 μM) and siRNA knockdown of mGpd in rat primary hepatocytes inhibited glucose production at higher [lactate]:[pyruvate] ratios, but lower redox state induced by decreased [lactate]:[pyruvate] abrogated the ability of metformin and mGpd knockdown by siRNA to decrease glucose production. Decreasing the redox state itself inhibited glucose production. b, Metformin inhibited glucose production only from lactate and glycerol, not from substrates that did not increase the cytosolic redox state. c, mGpd knockdown by siRNA showed a similar substrate-selective inhibition of glucose production. d, Both metformin and mGPD siRNA treatment increased [glycerol-3-phosphate] levels in hepatocytes, and (e) acute metformin (50 mg kg⁻¹, intravenously) treatment in vivo increased liver [glycerol-3-phosphate] levels without significantly altering [glycerol-3-phosphate] levels in other tissues, suggesting an impasse at the mGPD catalytic step. f, siRNA treatment did not induce cytotoxicity, as determined by trypan blue exclusion, (g) CyQuant proliferation assay and (h) the absence of cytochrome c release into the cytosolic fraction from mitochondria of treated cells. Data are mean ± s.e.m. (n = 5 technical replicates, n = 3 for cytotoxicity tests (f–h)). *P < 0.05, **P < 0.01, ***P < 0.001 by unpaired t-test.
Extended Data Figure 7 | Effect of mGPD and cGPD ASO treatment on liver redox, high-energy intermediates and expression of gluconeogenic regulators. a, mGPD ASO effectively reduced expression of liver mGPD protein and (b) cGPD ASO effectively reduced liver cGPD protein levels. c, mGPD ASO treatment increased plasma lactate concentrations significantly, but cGPD ASO knockdown had no effect on plasma lactate concentrations. d, mGPD ASO knockdown increased the liver [GSSG] [GSH] ratio, (e) had no effect on the liver [ATP] [ADP], (f) [ATP] [AMP], (g) [NADH] [NAD+], or (h) [NADPH] [NADP+] ratios. (i) although liver [cAMP] levels were slightly decreased. j, ASO-mediated knockdown of mGPD did not affect expression of gluconeogenic enzymes, (k) PEPCK-C and (l) pyruvate carboxylase protein levels remaining unchanged in the liver. m, Activated CREB was decreased, and (n) mGPD ASO knockdown led to activation of liver AMPK as indicated by increased phosphorylated AMPK and (o) increased ACC phosphorylation. Data are mean ± s.e.m. (n = 6 biological replicates). *P < 0.05, **P < 0.01, ***P < 0.001 by unpaired t-test.
Extended Data Figure 8 | Computational binding model of guanides/biguanides to mGPD from *Streptococcus sp*. Modelling of guanide/biguanide binding to mGPD after modification of key residues to fit the human sequence, show (a) FAD binding and predicted movement in the pocket, (b) metformin binding and (c) phenformin binding to the FAD-containing pocket.
Extended Data Figure 9 | Plasma and tissue metformin concentrations in rats treated with metformin. a, Acute metformin (50 mg kg$^{-1}$, intravenously) administration led to peak plasma metformin concentrations of approximately 74 μM; 100 and 250 mg kg$^{-1}$ doses increased plasma metformin concentration to 345 and 1300 μM, respectively. b, Acute metformin (50 mg kg$^{-1}$, intravenously) led to liver metformin concentrations of approximately 100 μM, and metformin levels in other tissues were comparatively low. Data shown are representative of two experiments. Data are mean ± s.e.m. (n = 3 biological replicates for plasma concentrations, n = 5 biological replicates for tissue levels). *P < 0.05, **P < 0.01, ***P < 0.001 by ANOVA.
Extended Data Figure 10 | Effect of mGPD and cGPD ASO treatment on liver toxicity and tissue-specific knockdown of mGPD expression by mGPD ASO. 

a, mGPD ASO treatment had no effect on body weight after treatment at 37.5 mg kg\(^{-1}\) ASO twice a week for 4 weeks. 
b, All ASOs screened in this study, mGPD ASO 1, mGPD ASO 2, cGPD ASO 1 and cGPD ASO 2, elicited no significant liver toxicity as determined by plasma AST/ALT levels after 4 weeks. 
c, Treatment with mGPD ASO 2 for 4 weeks during the mGPD ASO with acute metformin study also had no effect on plasma AST/ALT. 
d–k, mGPD ASO treatment led to cleavage of mGPD mRNA transcript exclusively in the liver, only slightly decreasing transcript levels in white adipose tissue and having no effect on mGPD mRNA in other tissues. e–k, mGPD ASO treatment specifically reduced protein expression of mGPD in the liver, with no significant effect on mGPD protein levels in the pancreas, kidney, muscle, white adipose tissue or brown adipose tissue. Data are mean ± s.e.m. (n = 6 biological replicates). \( ^* P < 0.05, \quad ^{**} P < 0.01, \quad ^{***} P < 0.001 \) by unpaired t-test.