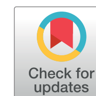

Metformin's Therapeutic Efficacy in the Treatment of Diabetes Does Not Involve Inhibition of Mitochondrial Glycerol Phosphate Dehydrogenase

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Diabetes 2021;70:1575–1580 | https://doi.org/10.2337/db20-1143

Mitochondrial glycerol phosphate dehydrogenase (mGPD) is the rate-limiting enzyme of the glycerol phosphate redox shuttle. It was recently claimed that metformin, a first-line drug used for the treatment of type 2 diabetes, inhibits liver mGPD 30–50%, suppressing gluconeogenesis through a redox mechanism. Various factors cast doubt on this idea. Total-body knockout of mGPD in mice has adverse effects in several tissues where the mGPD level is high but has little or no effect in liver, where the mGPD level is the lowest of 10 tissues. Metformin has beneficial effects in humans in tissues with high levels of mGPD, such as pancreatic β-cells, where the mGPD level is much higher than that in liver. Insulin secretion in mGPD knockout mouse β-cells is normal because, like liver, β-cells possess the malate aspartate redox shuttle whose redox action is redundant to the glycerol phosphate shuttle. For these and other reasons, we used four different enzyme assays to reassess whether metformin inhibited mGPD. Metformin did not inhibit mGPD in homogenates or mitochondria from insulin cells or liver cells. If metformin actually inhibited mGPD, adverse effects in tissues where the level of mGPD is much higher than that in the liver could prevent the use of metformin as a diabetes medicine.

Metformin is a first-line drug for the treatment of type 2 diabetes. After decades of use in millions of patients and many biomolecular studies, the exact mechanism of metformin's antihyperglycemic action still does not seem to be completely understood. Metformin's blood glucose–lowering effect is through its decrease of hepatic glucose production. Many mechanisms for this effect have been proposed. One undisputed mechanism is metformin's activation of AMP-activated protein kinase (AMPK), which suppresses gluconeogenesis in the liver by increasing the phosphorylation of the AMPK catalytic α subunit. Another postulated target of metformin is complex 1 of the mitochondrial electron transport chain and its inhibition, which leads to an increase in the AMP/ATP ratio that is sensed by AMPK subunits (1). Although metformin's activation of AMPK seems to be the best explanation for metformin's mechanism of action, there is still a question of whether this mechanism and other proposed mechanisms for metformin's effects could be indirect. Therefore, the search for the direct target of metformin is still active.

Recently it was reported that metformin lowers blood glucose by a redox mechanism (2) caused by a 30–50% inhibition of mitochondrial glycerol phosphate dehydrogenase (mGPD) (3) that suppresses gluconeogenesis in the liver. mGPD is the rate-limiting enzyme of the glycerol phosphate shuttle, a cytosolic redox NAD^+/NADH shuttle. The mGPD reaction is unidirectional and keeps the glycerol phosphate shuttle operating only in the direction of converting NADH to NAD^+ in the cytosol of cells that possess this redox shuttle (see Figs. 2 and 3 in MacDonald et al. [4]). This shuttle is one of two redox shuttles that use the mitochondrial electron transport chain to oxidize reduced metabolites to oxidized metabolites, which then participate in enzyme reactions that oxidize NADH to NAD^+ in the cytosol of a cell (see Fig. 4 in MacDonald et al. [4]). These shuttles guard against an abnormally high cytosolic $NADH/NAD^+$ ratio inhibiting glycolysis and, thus, cellular glucose metabolism.

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Received 10 November 2020 and accepted 8 April 2021

This article contains supplementary material online at https://doi.org/10.2337/figshare.14394359.

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PHARMACOLOGY AND THERAPEUTICS

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Several factors cast doubt on the idea that metformin's suppression of gluconeogenesis could involve the inhibition of mGPD independent of the fact that 30–50% inhibition of the enzyme is relatively weak. The enzyme activity and protein of the mGPD enzyme in liver are the lowest levels of ten tissues, including the pancreatic islet, where the level is 30- to 60-fold higher than in liver (5–9). In brown adipose tissue, skeletal muscle, brain, and testis, the level of mGPD is reported to be up to 250, 67, 23, and 11–25 times higher, respectively, than in liver (7,8,10–12) (Table 1). Metformin has beneficial metabolic effects in the pancreatic β-cell (reviewed in Yang et al. [13]), enhances thermogenesis markers in brown adipose tissue in mice (14), does not compromise energy status in skeletal muscle during rest or exercise in humans (15), and increases cognition in humans (16) (Table 1). If metformin inhibited mGPD, it could not have beneficial effects in tissues where the level of mGPD is so high.

Another mitochondrial NAD^+/NADH redox shuttle in liver, called the malate aspartate shuttle, is redundant to the action of the glycerol phosphate shuttle. Total-body knockout of mGPD does not inhibit insulin secretion (17) because, like liver, β-cells possess the malate aspartate redox shuttle (4,18) in addition to the glycerol phosphate redox shuttle (see Fig. 4 in MacDonald et al. [4]). In support of the idea of the known redundancy of these two redox shuttles, insulin secretion from pancreatic islets isolated from an mGPD knockout mouse is not inhibited unless the malate aspartate shuttle is inhibited pharmacologically (19) (Table 1). The malate aspartate redox shuttle was discovered in liver (20), where it is much more active than the glycerol phosphate redox shuttle (21,22). Even if metformin did inhibit mGPD, it is unlikely it could inhibit gluconeogenesis via a redox mechanism in liver.

The lactate/pyruvate and glycerol phosphate/dihydroxyacetone phosphate ratios each reflect the NADH/NAD^+ redox ratio in the cytosol of cells. In our mouse with total-body knockout of mGDP, these redox ratios are normal in liver, and there are no or minimal metabolic changes in liver of these adult animals (17). Although metformin is not known to decrease fertility of human males, fertility is markedly decreased in this mGPD knockout mouse (17), consistent with the high level of mGPD in testis (7,8,10) of normal animals (Table 1). In another total-body mGPD knockout mouse, the glycerol phosphate level and the lactate/pyruvate ratio are normal in liver, but they are increased threefold in skeletal muscle compared with normal mice (23) where the malate aspartate shuttle is absent (22). This indicates an abnormally high NADH/NAD^+ ratio will inhibit glucose metabolism in skeletal

Table 1—Reasons to doubt that metformin suppresses gluconeogenesis in liver by inhibiting mGPD or by a redox-dependent mechanism

Tissue	mGPD activity in normal tissue	Effect in tissue of total-body mGPD knockout	Malate aspartate shuttle in tissue	Metformin antidiabetic or other effect on tissue
Liver	Lowest of 10 tissues (5–12)	Normal GP/DHAP level and L/P ratio (17) Normal GP level and L/P ratio (23) No adverse effect (17,23)	Highest in body (20–22)	Excellent, healthy Decreases glucose production
Pancreatic β-cell	30- to 60-fold liver (5–9)	No inhibition of insulin secretion (17) unless Mal Asp shuttle also inhibited (19)	High (4,18,19)	Good, healthy (13)
Skeletal muscle	Up to 67-fold liver (8,10)	Threefold higher GP and L/P ratios (23) indicate glucose metabolism will be inhibited	Very low, absent (22)	Good, healthy (15,31)
Brown adipose tissue	Up to 250-fold liver (8,10,11)	Decreased thermogenesis (23)	Very low (24)	Enhances thermogenesis (14)
Brain	Up to 23-fold liver (8,10,12)	Not studied	High (30)	Increases cognition in humans (16)
Testis (sperm)	Up to 11- to 25-fold liver (7,8,10, 28, 29)	Decreased fertility (17)	Not reported	Fertility okay in humans

Total-body knockout of mGPD has little or no metabolic effect in liver, which has the lowest level of mGPD of 10 tissues and a high activity of the malate aspartate redox shuttle that is redundant to the glycerol phosphate redox shuttle but causes abnormalities in several tissues with high levels of mGPD and low activity of the malate aspartate shuttle. Metformin has healthy effects in several tissues with high levels of mGPD. GP/DHAP and L/P ratios reflect the NADH/NAD^+ redox ratio. References are in parentheses. Asp, aspartate; DHAP, dihydroxyacetone phosphate; GP, glycerol phosphate; L, lactate; Mal, malate; P, pyruvate.

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muscle. Females of this mGPD knockout mouse become hypothermic after fasting (23), indicating a deleterious effect on brown adipose tissue, where the activity of the malate aspartate shuttle is very low or absent in normal mice (24) (Table 1). These detrimental redox changes in tissues of the mGPD knockout mouse are inconsistent with metformin having beneficial effects in these same tissues that in normal animals have a high level of mGPD.

In view of the improbability that a weak inhibition of mGPD and the glycerol phosphate redox shuttle by metformin in liver could inhibit gluconeogenesis in the presence of the much more active malate aspartate redox shuttle in liver, as well as the beneficial effects of metformin in many tissues where the mGPD level is much higher than that in liver, we reassessed whether metformin does inhibit mGPD.

RESEARCH DESIGN AND METHODS

mGPD enzyme activity was measured with four different enzyme assays (Tables 2–4). Methods of the assays were standard and previously described (3,5,7,25). Details of each of the assay procedures are fully described in the Supplementary Material. Preparation of whole-cell homogenates and purification of mitochondria were performed as previously described (7). Statistical significance of potential differences was calculated with Student t test.

Data and Resource Availability

All data generated and analyzed during this study are included in the published article and its Supplementary Material.

RESULTS

No Inhibition of mGPD by Metformin or Phenformin

The effect of metformin concentrations between 10 μmol/L and 1 mmol/L and phenformin concentrations between 250 μmol/L and 10 mmol/L on mGPD enzyme activity were first measured using a timed-and-stopped enzyme assay (5,7). Whole-cell homogenates and isolated mitochondria from both liver and a much richer source of mGPD (the INS-1 832/13 insulinoma cell line [pure β-cells]) were studied. Neither of these drugs inhibited mGPD whatsoever (Table 2).

The effect of 250 μmol/L metformin on mGPD enzyme activity in mitochondria isolated from the INS-1 832/13 cell line and the human liver cell line Huh 7.5 was also tested in three different continuous spectrophotometric enzyme assays (3,5,7,25). Again, no inhibition of mGPD activity was observed (Table 3).

Data in Tables 2 and 3 were from experiments performed using a V_{max} concentration (25 mmol/L) of glycerol phosphate that would permit detection of noncompetitive or uncompetitive inhibition of mGPD if inhibition had occurred (26). To detect competitive inhibition by metformin versus glycerol phosphate, if it occurred, a concentration of glycerol phosphate of 200 μmol/L was used, which is near or slightly lower than its K_m of 400 μmol/L for mGPD (7) and within the range of the concentrations of glycerol phosphate in liver and other tissues (27). When concentrations of metformin or phenformin that are usually considered above therapeutic concentrations (200 μmol/L or 500 μmol/L) in two different mGPD assays (3,5,7) were used, inhibition of mGPD was not seen (Table 4).

DISCUSSION

The laboratory that reported an inhibitory effect of metformin on mGPD enzyme activity described the type of inhibition as noncompetitive (3). Tables 2–4 show results of measurements of enzyme assays of mGPD enzyme activity in homogenates of whole cells or mitochondria from liver or pancreatic β-cells. Assay conditions that would show noncompetitive and uncompetitive inhibition (Tables 2 and 3) or competitive inhibition (Table 4) were used. With low micromolar to millimolar concentrations of metformin, or the stronger biguanide phenformin, neither biguanide caused any inhibition of mGPD enzyme activity (Tables 2–4).

Summary of Conclusions

In analyses with four different assays of mGPD enzyme activity, metformin and/or phenformin showed no inhibition of mGPD (Tables 2–4). The laboratory that reported that metformin inhibited mGPD (3) also reported that metformin suppressed glucose production in liver via a redox-dependent mechanism (2). Even if metformin did inhibit mGPD, its action could not be via a redox mechanism in liver, where, due to the extremely low level of mGPD (5,7,8), the activity of the glycerol phosphate NAD^+/NADH redox shuttle does not compare with the much more active malate aspartate NAD^+/NADH redox shuttle in liver (20–22) that is redundant to the action of the glycerol phosphate shuttle in liver. There are beneficial effects of metformin in tissues where mGPD levels are much higher than in liver (13–16). The laboratory that reported that metformin inhibited mGPD reported that metformin inhibited mGPD enzyme activity 30–50% (3). Even total-body knockout of mGPD has no or minimal effects in liver (17,23), where the normal mGPD level is the lowest of ten tissues (5–12). Total-body knockout of mGPD does cause adverse effects in brown adipose tissue and skeletal muscle (23), where mGPD is normally very high (8,10,11) and malate aspartate is absent or at a very low level (22,24) (Table 1). These factors, supported by the negative results of the four different mGPD enzyme assays, force the conclusion that the mechanism by which metformin suppresses gluconeogenesis in liver cannot involve inhibition of mGPD or a redox-dependent mechanism. It is fortunate that metformin does not inhibit mGPD. If metformin inhibited mGPD, side effects in tissues with high levels of mGPD could prevent its use in the treatment of type 2 diabetes.

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Table 2—No inhibition of mGPD by metformin or phenformin when enzyme activity is measured with a V_{max} concentration of glycerol phosphate

	Enzyme source			
	INS-1 832/13 mitochondria	INS-1 832/13 whole-cell homogenate	Liver mitochondria	Liver whole-cell homogenate
Metformin experiments				
Control specific mGPD activity (nmol dye reduced/min/mg protein)	164 ± 4.6 (8)	39 ± 0.71 (6)	8.3 ± 0.6 (16)	1.2 ± 0.07 (8)
	mGPD activity with metformin (% of control)			
Metformin concentration				
None (control)	100 ± 2.5 (8)	100 ± 0.7 (6)	100 ± 1.5 (16)	100 ± 5.5 (8)
10 μmol/L	104 ± 0.9 (4)	99 ± 2.5 (6)	98 ± 4.4 (12)	95 ± 6.3 (8)
50 μmol/L	102 ± 3.7 (4)	102 ± 1.6 (6)	99 ± 2.8 (8)	106 ± 5.3 (8)
100 μmol/L	104 ± 3.5 (4)	101 ± 1.8 (6)	99 ± 2.4 (4)	116 ± 4.6 (8)
250 μmol/L	104 ± 2.7 (4)	107 ± 0.6 (4)	98 ± 4.4 (4)	110 ± 4.0 (4)
0.5 mmol/L	101 ± 2.5 (4)	101 ± 1.0 (6)	104 ± 1.6 (8)	115 ± 6.0 (4)
1 mmol/L	97 ± 1.2 (4)	102 ± 0.6 (6)	109 ± 5.5 (4)	121 ± 5.7 (4)
Phenformin experiments				
Control specific mGPD activity (nmol dye reduced/min/mg protein)	194 ± 4.7 (4)	34 ± 0.97 (4)	6.6 ± 0.15 (4)	1.6 ± 0.64 (10)
	mGPD activity with phenformin (% of control)			
Phenformin concentration				
None (control)	100 ± 2.4 (4)	100 ± 1.0 (8)	100 ± 2.3 (4)	100 ± 3.6 (10)
250 μmol/L	93 ± 2.3 (4)	100 ± 1.5 (4)	101 ± 0.5 (4)	111 ± 6.8 (4)
0.5 mmol/L	94 ± 2.1 (4)	98 ± 1.6 (4)	100 ± 2.7 (4)	110 ± 2.1 (5)
1 mmol/L	92 ± 2.4 (4)	100 ± 2.4 (4)	103 ± 1.8 (4)	99 ± 4.1 (6)
10 mmol/L	89 ± 2.2 (4)	95 ± 1.5 (6)	109 ± 1.4 (3)	105 ± 3.0 (4)

The electron acceptor was iodonitrotetrazolium blue (5,7). Data are mean ± SE (*N*).

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Table 3—No inhibition of mGPD enzyme activity by metformin in three different enzyme assays

	mGPD activity (% of control) at metformin concentration of:	
Enzyme source	None (control)	250 μmol/L
INS-1 832/13 mitochondria	100 ± 5.9 (4)*	102 ± 1.3 (4)*
INS-1 832/13 mitochondria	100 ± 2.2 (4)†	110 ± 3.3 (4)†
INS-1 832/13 mitochondria	100 ± 0.6 (4)‡	112 ± 1.5 (4)‡
Huh 7.5 liver cell mitochondria	100 ± 3.1 (4)*	103 ± 2.2 (4)*
Huh 7.5 liver cell mitochondria	100 ± 6.4 (4)†	106 ± 1.9 (4)†
Huh 7.5 liver cell mitochondria	100 ± 3.6 (8)‡	105 ± 3.6 (8)‡

The assays used cytochrome *c* in two different methods or dichlorophenolindophenol as electron acceptor. Enzyme activity was measured with a V_{max} concentration of glycerol phosphate. Data are mean ± SE (*N*). *Electron acceptor: cytochrome *c* (3). †Electron acceptor: cytochrome *c* (25). ‡Electron acceptor: dichlorophenolindophenol (5).

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Table 4—No inhibition of mGPD enzyme activity by metformin or phenformin when enzyme activity is measured with a concentration of glycerol phosphate of 200 μmol/L

Inhibitor	mGPD activity (% of control)
None (control)	100 ± 3.7 (8)*
Metformin (250 μmol/L)	102 ± 4.8 (8)*
Metformin (500 μmol/L	94.2 ± 2.7 (4)*
None (control)	100 ± 4.1 (16)†
Metformin (250 μmol/L)	98 ± 2.7 (7)†
Phenformin (250 μmol/L)	103 ± 4.5 (8)†

A concentration of glycerol phosphate of 200 μmol/L is lower than the K_m for mGPD (7) and within the range of its concentration in tissues that would show competitive inhibition if inhibition occurred. Experiments were performed with a preparation of mitochondria isolated from the INS-1 832/13 insulinoma cell line. Data are mean ± SE (*N*). *Electron acceptor: dichlorophenolindophenol (5). †Electron acceptor: cytochrome *c* (3).

Funding. This work was supported by the Nowlin Family Trust of the In-Faith Community Foundation and the estate of the late Ramona Garde.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. M.J.M. designed the research and analyzed the data. I.H.A. performed experiments and researched the data. M.J.L. performed the experiments and researched the data. S.W.S. performed experiments. M.J.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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