# Online Appendix for:

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

Michael J. MacDonald[1], Israr-ul H. Ansari[1], Melissa J. Longacre[1] and Scott W. Stoker[1]

[1]University of Wisconsin School of Medicine and Public Health

3459 Medical Science Center

1300 University Ave.

Madison, WI 53706

Corresponding author: Michael J. MacDonald

Email: *mjmacdon@wisc.edu*

Phone: 608-262-1195

## RESEARCH DESIGN AND MATERIALS DETAILS

**Details of mGPD enzyme assays.** mGPD enzyme activity was measured with four different enzyme assays each with a different electron acceptor. When mGPD enzyme activity was measured according to the method of Gardner (S1, S2) (Table 2) the enzyme reaction mixture contained 25 mM L-glycerol phosphate as the D-L mixture ($V_{max}$ rate), 1 mM KCN, 50 mM Bicine buffer, pH 8.0, and 4 mM iodonitrotetrazolium violet and enzyme source incubated at 37° for 30 min. The enzyme reaction was stopped by adding ethyl acetate. The reaction mixture was centrifuged briefly and the ethyl acetate phase of the mixture containing the reduced iodonitrotetrazolium dye was removed and its absorbance measured at 490 nm.

In addition mGPD enzyme activity was measured in a continuous spectrophotometric assay at 600 nm at 38° in a reaction mixture containing 50 mM potassium phosphate buffer, pH 7.6, 1 mM KCN and 47.5 μM 2,6-dichlorophenolindophenol (DCIP) with or without 25 mM ($V_{max}$ rate) (Table 3) or 0.2 mM L-glycerol phosphate (Table 4) as the DL mixture as previously described (S2-S4) The background rate was recorded for 2 min before starting the enzyme rate by the addition of glycerol phosphate (Tables 3 and 4).

mGPD was also measured in a continuous spectrophotometric assay used by the laboratory that saw inhibition of mGPD by metformin (S5) in a reaction mixture of 10 mM Tris-chloride buffer pH 8.0, 50 mM KCl, 25 μM sodium azide, 1 mM EDTA, 50 μM cytochrome c with or without 25 mM ($V_{max}$ rate) (Table 3) or 0.2 mM L-glycerol phosphate (Table 4) as the DL mixture maintained at 550 nm at 38°. The background rate was recorded for 2 min before starting the enzyme rate by the addition of glycerol phosphate (Tables 3 and 4).

mGPD activity when measured according to Rauchová et al (6) was measured in a continuous spectrophotometric assay in a reaction mixture of 20 mM Tris-chloride buffer, pH 7.4, 50 mM KCl, 2 mM KCN, 1 mM EDTA, 0.1 mM cytochrome c and with or without 25 mM or 0.2 mM L-glycerol phosphate as the DL mixture. After measuring a background rate for 2 min, the reaction was started by the addition of glycerol phosphate. The reaction rate was measured at 550 nm at 30° (Table 3).

In each of the four enzyme assay procedures absorbance values or reaction rates from reaction mixtures containing all ingredients except glycerol phosphate (background rates) were subtracted from reaction mixtures containing all ingredients to give the enzyme rate attributable to mGPD activity. Also, in all four enzyme assay procedures metformin or phenformin, when added, were incubated with the enzyme source in the reaction mixture for 10 min at 30° before measuring either the background rate or starting the enzyme rate with the addition of glycerol phosphate (Because the laboratory that observed inhibition of mGPD by metformin (S5) preincubated the enzyme source with the biguanide for 10 min). In addition, we used metformin from three separate lots in the unlikely event there was a problem with a specific lot.

Preparation of whole cell homogenates and purification of mitochondria were performed as previously described (S4, S7).

**PCR.** Quantitative PCR measurements of mRNA of SLC29A4 were performed as previously described (S4, S7) using a forward primer 5' GCTTCACCTTCGACAGTCAC 3'and a reverse primer 5'TCCGCCGCCTCCTCCAGCTG 3'for human pancreatic islets and a forward primer 5'ACCTGCACACTAGGATCACC 3' and a reverse primer 5'TGGTCGTGAGAGAAGAGCTG 3' for the INS-1 832/13 rat beta cell line.

**Immunoblot.** Immunoblot analyses were performed as previously described (S4, S7) using a rabbit anti-SLC29A4 polyclonal antibody (Catalog no. bs-4176R from Bioss Antibodies) at 1:1,000 dilution.

**Islets.** Human pancreatic islets were 95% pure and were from the Department of Surgery, University of Wisconsin School of Medicine and Public Health via the Integrated Islet Distribution Program.

## SUPPLEMENTAL RESULTS

**The plasma monoamine transporter is abundantly expressed in beta cells.** The plasma monoamine transporter (SLC29A4 or PMAT) can carry metformin into cells. Quantitative PCR showed that PMAT mRNA is expressed at 210 ± 52 % (3) and 180 ± 46 % (6) (mean ± SE (N)) of the glutamic acid decarboxylase gene (GLUD) control in human pancreatic islets and the INS-1 832/13 rat beta cell line, respectively. Immunoblot analysis showed that PMAT protein is expressed at levels in human pancreatic islets and the INS-1 832/13 cell line that are similar to human liver (Supplemental Figure 1).

## SUPPLEMENTAL DISCUSSION

**Metformin cannot inhibit gluconeogenesis by a redox mechanism.** In regard to metformin inhibiting gluconeogenesis via a redox mechanism in liver (Even if metformin did inhibit mGPD.), this is not likely because of the redundancy of the malate aspartate redox shuttle to the glycerol phosphate redox shuttle as discussed in the main text of the paper. The group that reported that metformin inhibited mGPD also reported that metformin inhibited gluconeogenesis from reduced substrates lactate and glycerol but not from oxidized substrates pyruvate and alanine in the rat. It was concluded that this was consistent with metformin inhibiting gluconeogenesis by a redox-dependent mechanism in vivo (S8). The malate aspartate redox shuttle is known to be redundant to the glycerol phosphate redox shuttle in liver where the activity of the malate aspartate shuttle is especially high (S9-S11) and the activity of the glycerol phosphate shuttle is low due to the extremely low level of mGPD in liver (S2, S4, S12, S13). Thus, even if metformin inhibited mGPD, metformin inhibiting gluconeogenesis by a redox mechanism is not likely. Another group found that metformin did not inhibit the enzyme activity of mGPD and showed that metformin inhibited gluconeogenesis from both reduced and oxidized substrates in liver cells and concluded that metformin inhibited gluconeogenesis by a redox-independent mechanism (S14).

**Tissues with high levels of mGPD are less able than liver to handle redox changes. The phenotype of a mouse lacking the glycerol phosphate shuttle due to a null mutation in the cytosolic glycerol phosphate dehydrogenase (cGPD) gene is similar to that of the mGPD knockout mouse.** There are three main pathways by which a tissue can maintain a normal cytosolic $NAD^+$/NADH ratio. If this ratio is low in cytosol, glycolysis will be inhibited at the glyceraldehyde phosphate dehydrogenase step that requires $NAD^+$ and glucose metabolism will be inhibited. Several tissues with high levels of mGPD are less equipped than liver for maintaining this ratio. The three pathways are the glycerol phosphate shuttle, the malate aspartate shuttle and the Cori cycle. In the Cori cycle pyruvate is converted to lactate catalyzed by lactate dehydrogenase by anaerobic glycolysis and the reduced product lactate is carried by the blood stream to the liver where it is converted to glucose which then can be returned to the tissue that formed the lactate thus closing the cycle. The pancreatic beta cell is poorly equipped to participate in the Cori cycle

because its level of lactate dehydrogenase is extremely low (S15, S16). In the mGPD knockout mouse insulin secretion is normal because the beta cell has the malate aspartate shuttle (S17, S18). Skeletal muscle that normally has a high level of mGPD is poorly equipped to maintain a normal lactate/pyruvate ratio in the mGPD knockout mouse because normal skeletal muscle has no malate aspartate shuttle (S11). Skeletal muscle and the pancreatic beta cell, unlike liver and kidney, are at an additional redox disadvantage because they do not possess glycerol kinase that can catalyze the formation of glycerol phosphate from glycerol (S19). The cytosolic glycerol phosphate dehydrogenase (cGPD) enzyme reaction is reversible and in the liver of the mGPD knockout mouse cGPD can convert glycerol phosphate to dihydroxyacetone phosphate to maintain a somewhat less abnormal $NAD^+$/NADH ratio via the glycerol phosphate/dihydroxyacetone phosphate ratio.

**A mouse that has a null mutation in the cGPD gene shows little or no redox abnormalities in liver but severe redox abnormalities in skeletal muscle.** For reasons discussed in the paragraph above the phenotype of a mouse with absence of the glycerol phosphate redox shuttle due to the total body absence of cGPD is similar to the total body knockout of mGPD. cGPD and mGPD catalyze the two reactions of the glycerol phosphate shuttle by which NADH formed at the glyceraldehyde phosphate dehydrogenase step in glycolysis is reoxidized to $NAD^+$ via mitochondrial metabolism. The BALB/cHeA mouse due to a naturally occurring null mutation of the gene that encodes cGPD has no cGPD enzyme activity (S19). Insulin secretion in this mouse is normal and the liver lactate/pyruvate ratio reflecting the NADH/$NAD^+$ratio is similar to this ratio in control mice that possess normal levels of cGPD. In contrast, in skeletal muscle of this mouse the lactate/pyruvate ratio is markedly increased and the ATP/ADP ratio is severely decreased at rest, but even more so after exercise and/or a lowfat diet (S19). This is because as discussed in the main text of the paper, the malate aspartate redox shuttle is absent in skeletal muscle.

All of the above information reinforces other information that inhibition of mGPD by metformin, even if it could occur, cannot be the mechanism by which metformin benefits type 2 diabetes.

**mGPD is located in spermatozoa in testis.** The level of mGPD is 11- to 25-fold higher in testis than in liver (S4, S12, S13). Since mGPD is concentrated in spermatozoa in testis (S20, S21), the level of mGPD in spermatozoa is much higher than the level in whole testis and even higher than the 11-25-fold ratio compared to liver.

**There are transporters that can carry metformin into cells of various tissues that contain a high amount of mGPD.** A possible explanation for metformin not causing metabolic effects in tissues where the level of mGPD is much higher than in liver, if indeed metformin did inhibit mGPD, might be that metformin is not permeable to pancreatic beta cells and other tissues that contain a high amount of mGPD. Consistent with metformin being able to penetrate these tissues, metformin has beneficial effects in many of these tissues that have much higher levels of mGPD than liver. Metformin is actively transported into liver via the cell membrane organic cation transporters 1 and 3 (OCT1 and OCT3). Both OCT1 and OCT3 are expressed in skeletal muscle that may also be another major site of metformin action (S22). Metformin can also be transported into cells via the plasma membrane monoamine transporter (PMAT or SLC29A4). PMAT and OCT3 have been reported to be present in both human and mice pancreatic islets and in different $\beta$-cell lines (S23, S24). PMAT has an affinity for metformin comparable to that reported for OCT1 (S25, S26). Supplemental Figure 1 shows that the level of PMAT protein is similar in human liver, human pancreatic islets and the rat beta cell line INS-1 832/13. PMAT is abundantly expressed in

brain (S26-S28) and skeletal muscle (S25, S27, S28) where the levels of mGPD are high. That metformin can be taken up by various tissues with much higher mGPD levels than liver and have no adverse or even beneficial effects is also consistent with metformin not inhibiting mGPD.

## SUPPLEMENTAL REFERENCES


S1. Gardner RS. A sensitive colorimetric assay for mitochondrial α-glycerophosphate dehydrogenase. Anal Biochem 1974;59:272-276.

S2. MacDonald MJ. High content of mitochondrial glyceral-3-phosphate dehydrogenase in pancreatic islets and its inhibition by diazoxide. J Biol Chem 1981;256:8287-8290.

S3. Dawson AP, Thorne CJR. Preparation and some properties of l-3-glycerophosphate dehydrogenase from pig brain mitochondria. Biochem J 1969;111:27-34.

S4. MacDonald MJ, Brown LJ. Calcium activation of mitochondrial glycerol phosphate dehydrogenase restudied. Arch Biochem Biophys 1996;326:79-84.

S5. Madiraju A, Erion D, Rahimi Y, Zhang XM, Braddock D, Albright R, Prigaro B, Wood J, Bhanot S, MacDonald MJ, Jurczak M, Camporez JP, Lee H-Y, Cline G, Samuel V, Kibbey RG, Shulman GI. Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. Nature 2014;510:542-546.

S6. Rauchová H, Vokurková M, Drahota Z. Inhibition of mitochondrial glycerol-3-phosphate dehydrogenase by α-tocopheryl succinate. Int J Biochem Cell Biol 2014;53:409-413.

S7. Ansari IH, Longacre MJ, Paulusma CC, Stoker SW, Kendrick MA, MacDonald MJ. Characterization of P4 ATPase phospholipid translocases (flippases) in human and rat pancreatic beta cells: their gene silencing inhibits insulin secretion. J Biol Chem 2015;290:23110-23123.

S8. Madiraju AK, Qiu Y, Perry RJ, Rahimi Y, Zhang XM, Zhang D, Camporez JG, Cline GW, Butrico GM, Kemp BE, Casals G, Steinberg GR, Vatner DF, Petersen KF, Shulman GI. Metformin inhibits gluconeogenesis via a redox-dependent mechanism in vivo. Nat Med 2018;24:1384-1394.

S9. Lardy HA, Paetkau V, Walter P. Paths of carbon in gluconeogenesis and lipogenesis: the role of mitochondria in supplying precursors of phosphoenolpyruvate. Proc Natl Acad Sci USA 1965;53:1410-1415.

S10. Hagopian K, Ramsey JJ, Weindruch R. Enzymes of glycerol and glyceraldehyde metabolism in mouse liver: effects of caloric restriction and age on activities. Biosci Rep 2008;28:107-115.

S11. Lehninger AL. In *Biochemistry, The Molecular Basis of Cell Structure and Function.* Worth Publishers, Inc., New York, NY, USA, 1970, pp. 408, 412, 485-493.

S12. Lee Y-P, Lardy HA. Influence of thyroid hormones on L-α-glycerophosphate dehydrogenases and other dehydrogenases in various organs of the rat. J. Biol. Chem. 1965;240:1427-1436.

S13. Koza RA, Kozak UC, Brown LJ, Leiter EH, MacDonald MJ, Kozak LP. (1996) Sequence and tissue- dependent RNA expression of mouse FAD-linked glycerol-3-phosphate dehydrogenase. Arch Biochem Biophys 336:97-104.

S14. Alshawi A, Agius L. Low metformin causes a more oxidized mitochondrial NADH/NAD redox state in hepatocytes and inhibits gluconeogenesis by a redox-independent mechanism. J Biol Chem 2019;294:2839-2853.

S15. Sekine N, Cirulli V, Regazzi R, Brown LJ, Gine E, Tamarit-Rodriguez J, Girotti M, Marie S, MacDonald MJ, Wollheim CB, Rutter GA. Low lactate dehydrogenase and high mitochondrial glycerol phosphate dehydrogenase in pancreatic beta-cells. Potential role in nutrient sensing. J Biol Chem 1994;269:4895-4902.

S16. MacDonald MJ, Kelley PC, Laclau M. Histochemical evidence for pathways insulin cells use to oxidize glycolysis-derived NADH. Metab. 2002;51:318-321.

S17. MacDonald MJ. Evidence for the malate aspartate shuttle in pancreatic islets. Arch Biochem Biophys 1982;213:643-649.
S18. MacDonald MJ, Fahien LA, Brown LJ, Hasan NM, Buss JD, Kendrick MA. Perspective: emerging evidence for signaling roles of mitochondrial anaplerotic products in insulin secretion. Am J Physiol Endocrinol Metab 2005;288:E1-15.
S19. MacDonald MJ, Marshall LK. Mouse lacking NAD+-linked glycerol phosphate dehydrogenase has normal pancreatic beta cell function but abnormal metabolite pattern in skeletal muscle. Arch Biochem Biophys. 2000;384:143-153.
S20. Mohri H, Mohri T, Ernster L. Isolation and enzymic properties of the midpiece of bull spermatozoa. Exp Cell Res 1965;38:217-246.
S21. Rajkovic M, Middendorff R, Wetzel MG, Frkovic D, Damerow S, Seitz HJ, Weitzel JM. Germ cell nuclear factor relieves cAMP-response element modulator tau-mediated activation of the testis-specific promoter of human mitochondrial glycerol-3-phosphate dehydrogenase. J Biol Chem 2004;279:52493-52499.
S22. Graham GG, Punt J, Arora M, Day RO, Doogue MP, Duong JK, Furlong TJ, Greenfield JR, Greenup LC, Kirkpatrick CM, Ray JE, Timmins P, Williams KM. Clinical pharmacokinetics of metformin. Clin Pharmacokin 2011;50:81-98.
S23. Cataldo LR, Mizgier ML, Busso D, Olmos P, Galgani JE, Valenzuela R, Mezzano D, Aranda E, Cortés VA, Santos JL. Serotonin- and dopamine-related gene expression in db/db mice islets and in MIN6 β-cells treated with palmitate and oleate. J Diabetes Res. 2016; 2016: 3793781.
S24. Kobayashi M, Yamato E, Tanabe K, Tashiro F, Miyazaki S, Miyazaki J. Functional analysis of novel candidate regulators of insulin secretion in the MIN6 mouse pancreatic β cell line. PLoS One. 2016;11:e0151927.
S25. Zhou M, Xia L, Wang J. Metformin transport by a newly cloned proton-stimulated organic cation transporter (plasma membrane monoamine transporter) expressed in human intestine. Drug Metab 2007;35:1956-1962.
S26. Wang J. The plasma membrane monoamine transporter (PMAT): structure, function, and role in organic cation disposition. Clin Pharmacol Ther 2016;100:489-499.
S27. Dahlin A, Xia L, Kong W, Hevner R, Wang J. Expression and immunolocalization of the plasma membrane monoamine transporter in the brain. Neuroscience. 2007;146:1193-1211.
S28. Duan H, Wang J. Selective transport of monoamine neurotransmitters by human plasma membrane monoamine transporter and organic cation transporter 3. J Pharmacol Exp Ther 2010;335:743-753.

**Supplementary Figure 1. The plasma monoamine transporter protein (SLC29A4 or PMAT) is expressed in human pancreatic islets and the rat INS-1 832/13 beta cell line at levels equal to human liver.** Immunoblot with 20 µg whole cell protein per lane.