

Metformin-Induced Inhibition of Mitochondrial Complex I Activity Stimulates Fatty Acid Oxidation and Glycolysis While Blunting Glucose Oxidation in C2C12 Myocytes



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Abstract

Metformin, a commonly prescribed agent for the treatment of type II diabetes, induces multiple beneficial effects including weight loss and lipid reduction in addition to lowering blood glucose levels. Although the precise mechanism is not fully elucidated,^{1,2} metformin has been reported to inhibit mitochondrial complex I and activate AMPK in liver and muscle.^{3,4} In this study, we examined the effect of metformin on cellular energy metabolism in C2C12 skeletal myocytes using the XF24 Analyzer which measures cellular oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) *in vitro*. Fatty acid oxidation, glucose oxidation, and the activity of the mitochondrial respiration complexes I and II were assessed by measuring OCR following exposure of metformin-treated and control cells to the appropriate substrates.

OCR increase in response to acute palmitate addition (fatty acid oxidation) was enhanced by metformin treatment in a dose-dependent manner. Interestingly, acute glucose addition did not increase OCR (glucose oxidation) in metformin-treated cells while it did so in control cells, suggesting that metformin prevented glucose oxidation. Metformin as well as phenformin (another biguanide) significantly decreased cellular respiration while simultaneously increasing ECAR which is indicative of glycolysis. This type of response is similar to that invoked by classical mitochondrial respiration inhibitors such as rotenone. Consistent with previous studies using isolated mitochondria or permeabilized cells, metformin-treated cells did not significantly increase their cellular respiration rate when glutamate/malate was the sole substrate as compared to control. This suggests that metformin inhibited the activity of complex I. In contrast, relative to control cells a small OCR increase was observed in metformin-treated cells when methyl succinate was the only substrate. This suggested that metformin increased the activity of complex II. Finally, phenformin and rotenone were both observed to enhance FAO suggesting a general role of complex I in selective substrate oxidation in these cells. In summary, this type of analysis has shed some light on the basic mechanism of action of metformin. These results demonstrate that metformin may act, at least in part via inhibition of complex I, to enhance fatty acid oxidation while preventing glucose oxidation in C2C12 myocytes.

Materials and Methods

Cell Culture and Chemical Reagents

C2C12 cells were obtained from American Type Culture Collection (Manassas, VA). The cells were maintained in high-glucose DMEM (Invitrogen, Carlsbad, CA) supplemented with 1 mM sodium pyruvate (Invitrogen), 10% FBS (Hyclone, Logan, UT) and 100 µg/ml penicillin-streptomycin (Invitrogen). Sodium palmitate, carnitine, insulin, metformin, phenformin, rotenone, glutamate, malate and methyl succinate were obtained from Sigma (St. Louis, MO). All compounds were prepared according to the manufacturers' instructions. Fatty acid free (FAF) BSA was obtained from Roche Applied Science (Indianapolis, IN). Horse serum was obtained from Invitrogen.

C2C12 Myocytes Differentiation

C2C12 cells were seeded at 10,000 cells per well in 24-well XF plates and incubated for 48 hours in 37°C/10% CO₂ incubator. For differentiation, growth media was replaced with differentiation media (2% horse serum in DMEM). Well differentiated myocytes were observed at day 3 and thereafter. Day 4 through day 6 myocytes were used for all assays.

Fatty Acid Oxidation Assay Using XF24

Palmitate-BSA complex was prepared modifying a protocol described by Harwood et al.⁷ Briefly, sodium palmitate was dissolved in 150 mM sodium chloride and heated to 65 to 70°C to obtain a clear solution. FAF-BSA was dissolved in low buffered KHB at 37°C. The dissolved sodium palmitate was added to the BSA solution to obtain a stock solution of 1 mM palmitate/0.17 mM FAF-BSA. The combined solution was stirred for 1 hour at 37°C and aliquots were frozen and stored at -20°C.

Materials and Methods (continued)

Assay media for FAO is low-buffered KHB buffer consisting of 110 mM NaCl, 4.7 mM KCl, 2 mM MgSO₄, 1.2 mM Na₂HPO₄, 2.5 mM glucose adjusted to pH7.4 supplemented with 0.5mM carnitine and 100 nM insulin unless specified. For induction of FAO, palmitate was injected to a final concentration of 150 µM in myocytes and 200 µM for myoblasts after baseline rate measurement.

XF Metabolic Assay

Baseline OCR and ECAR measurements were made using the Seahorse XF24 Extracellular Flux Analyzer as described by Wu et al.⁸

For glucose oxidation assay, 25 mM glucose was injected to cells in DMEM containing 2.5 mM glucose after baseline rate measurement.

For determination of complex I and complex II activity, cells were pre-incubated and baseline rates were determined in KHB buffer free of any substrate. Glutamate/malate or methyl succinate were injected at increasing doses sequentially into each well and test measurements were made at indicated time points. The responses were expressed as percentage increase of OCR or ECAR relative to baseline rate.

Results

Figure 1. Metformin Pretreatment Stimulated Fatty Acid Oxidation in C2C12 Myocytes and Myoblasts

C2C12 myocytes and myoblasts were treated with increasing doses of metformin for 24 hours prior to FAO assay. Myocytes (A) displayed increased OCR over baseline as the dose of metformin increased. In contrast, myoblasts (B) only exhibited increased OCR at the highest dose (2 mM) of metformin. This suggests that FAO induction in myocytes is more sensitive to metformin than in undifferentiated myoblasts.

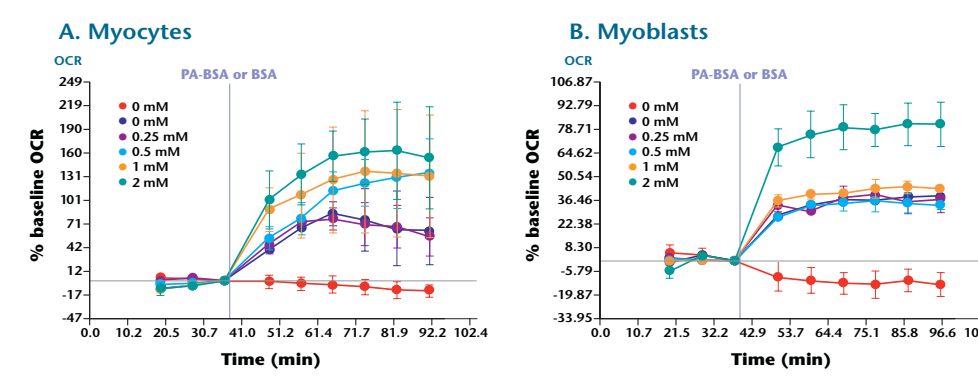


Figure 2. Opposite Effects of Metformin on Fatty Acid Oxidation and Glucose Oxidation

Myocyte cultures, pretreated for 24 hours with 2 mM metformin or vehicle control, were injected with fatty acid and glucose to final concentrations of 150 µM and 25 mM respectively. Palmitate but not glucose addition stimulated OCR in metformin-treated cells; however, both palmitate and glucose addition stimulated OCR in control cells suggesting selective oxidation of palmitate over glucose in metformin-treated cells (A). The FAO and glucose oxidation was quantified by AUC (area under curve) calculation. Note: DMEM containing 2.5 mM glucose was used as FAO and glucose oxidation assay media in this experiment.

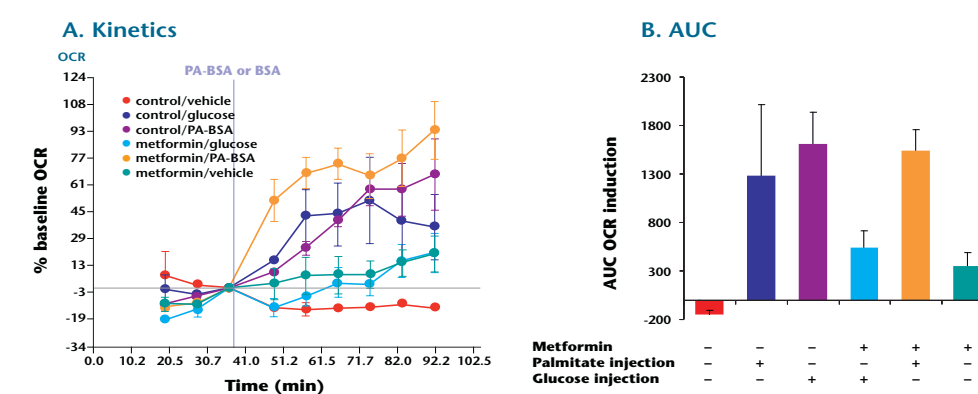


Figure 3. Metformin and Phenformin Inhibited Cellular Respiration Rate and Stimulated Glycolysis Rate of Myocytes

Myocytes were pretreated with metformin (A) for 24 hours before measuring OCR and ECAR as metformin is known to permeate slowly into the mitochondria. The raw values for OCR and ECAR are displayed. Phenformin, a more rapidly permeable biguanide than metformin, induced immediate changes in OCR and ECAR within 20 minutes after compound injection (B). The response is expressed as % of baseline OCR and ECAR. Both compounds decreased OCR and concomitantly increased ECAR which is indicative of a metabolic shift to glycolysis from glucose oxidation. This response pattern is very similar to that observed for mitochondrial respiration inhibitors.

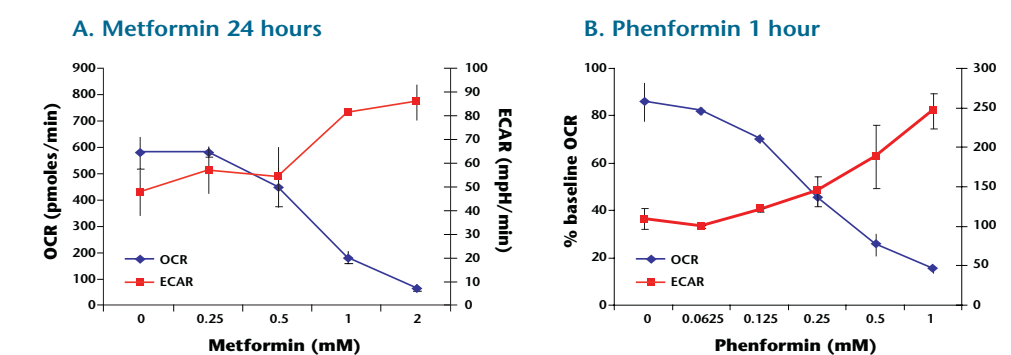
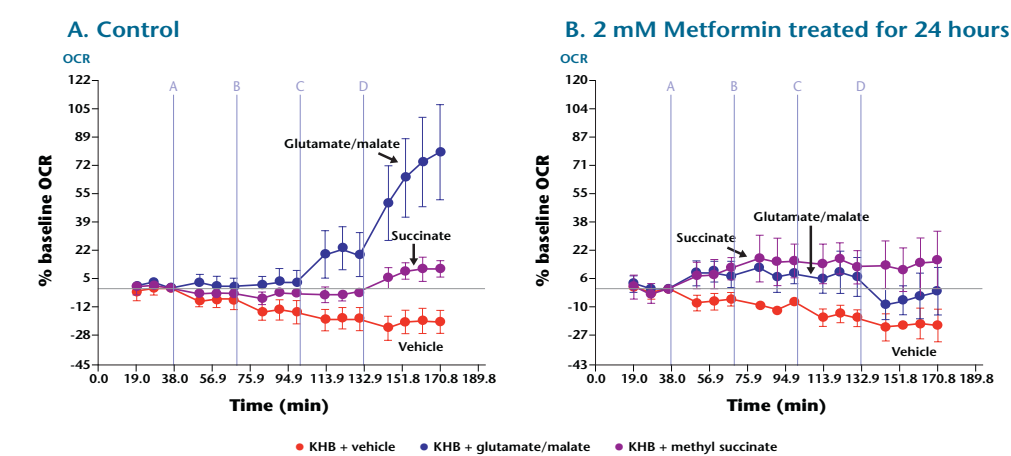


Figure 4. Metformin Inhibited Complex I Activity and Slightly Enhanced Complex II Activity

Myocytes were pretreated with 2 mM metformin or vehicle for 24 hours before XF assay. The assay was performed in substrate-free KHB buffer. Increasing concentrations of glutamate/malate or methyl succinate were injected sequentially to the same population of cells to final concentrations of 1.25, 2.5, 5 and 10 mM. Three rates were measured after each injection. Glutamate/malate did not significantly increase OCR at 10 mM in treated cells (B) but did so in control cells suggesting inhibited complex I activity in the treated cells. In contrast, methyl succinate injection induced a small but consistent increase in OCR starting at the lowest concentration in treated cells (B). This was not observed in control cells.

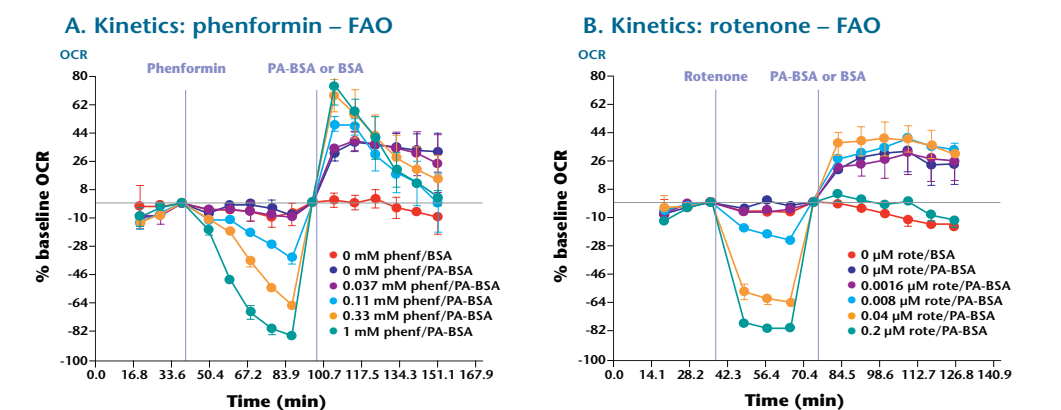


Summary

1. Metformin stimulated FAO and prevented glucose oxidation in C2C12 myocytes.
2. Substrate studies confirmed that metformin inhibits complex I activity in this *in vitro* model system.
3. Inhibition of complex I activity in general may lead to selective substrate oxidation as observed in these experiments.

Figure 5. Comparison of Enhanced FAO by Phenformin as well as Complex I Inhibitor Rotenone

Phenformin (A) or rotenone (B) was injected into myocyte cultures and OCR was measured repeatedly for 4–6 rates. Both compounds decreased OCR in a dose-dependent manner. Subsequent to the compound additions, palmitate-BSA was added in the same experiment to the same treated cells. This counteracted the OCR decrease resulting in increased FAO relative to the rate prior to palmitate injection. These data suggest that inhibition of complex I by either phenformin or the classic inhibitor rotenone can increase FAO. Note: The FAO response kinetics are distinctive for each inhibitor.



References

1. Collier CA, et al. Am J Physiol Endocrinol Metab. 2006. 291(1):E182-9. Metformin counters the insulin-induced suppression of fatty acid oxidation and stimulation of triacylglycerol storage in rodent skeletal muscle.
2. Hinke SA, et al. Br J Pharmacol. 2007. 150(8):1031-43. Methyl succinate antagonises biguanide-induced AMPK-activation and death of pancreatic beta-cells through restoration of mitochondrial electron transfer.
3. Owen MR, Doran E, Halestrap AP. Biochem J. 2000. 348 Pt 3:607-14. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain.
4. El-Mir MY, et al. J Biol Chem. 2000. 275(1):223-8. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I.
5. Musi N, et al. Diabetes. 2002. 51(7):2074-81. Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes.
6. Zhou G, et al. J Clin Invest. 2001. 108(8):1167-74. Role of AMP-activated protein kinase in mechanism of metformin action.
7. Harwood HJ Jr, et al. J Biol Chem. 2003. 278(39):37099-111. Isozyme-nonselective N-substituted bipiperidylcarboxamide acetyl-CoA carboxylase inhibitors reduce tissue malonyl-CoA concentrations, inhibit fatty acid synthesis, and increase fatty acid oxidation in cultured cells and in experimental animals.
8. Wu M, et al. Am J Physiol Cell Physiol. 2007. 292(1):C125-36. Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells.

