Metformin severely impairs *in vivo* muscle oxidative capacity in a rat model of type 2 diabetes

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Introduction

Metformin is a widely prescribed drug used for the treatment of type 2 diabetes. Its therapeutic action is primarily based on improving insulin sensitivity in the liver, thereby effectively inhibiting gluconeogenesis [1]. In addition, it has been shown that metformin improves insulin sensitivity of skeletal muscle resulting in enhanced peripheral glucose utilization, but the underlying mechanism still remains unclear. Previous studies have demonstrated that metformin specifically inhibits complex I of the mitochondrial respiratory system [2,3]. This seems counterproductive since impaired muscle mitochondrial function has been linked to the pathogenesis of type 2 diabetes [4,5]. However, it is not known whether and to which extend metformin affects muscle mitochondria *in vivo*. In this study we investigated the effects of metformin on *in vivo* and *in vitro* skeletal muscle mitochondrial function in Zucker diabetic fatty (ZDF) rats using ^{31}P magnetic resonance spectroscopy (MRS) and high-resolution respirometry, respectively.

Materials and Methods

12-week old healthy *fa/+* (317.5 ± 15.5 g) and diabetic *fa/fa* (358.8 ± 24.1 g) (i.e. ZDF) rats were treated with either metformin (300 mg/kg body weight/day) dissolved in 1 ml water (n = 6) or 1 ml water as a control (n = 6) for 15 days by oral gavage. At day 14, *in vivo* ^{31}P MRS was performed on the *tibialis anterior* (TA) muscle using a 6.3 T horizontal Bruker MR scanner, an ellipsoid ^{31}P surface coil (10/18 mm) and a circular ^{1}H surface coil (∅ 40 mm; for shimming). ^{31}P MR spectra (Figure 1A) were acquired using an adiabatic BIR4 pulse with a 90° flip angle. A fully relaxed spectrum (TR = 20 s, 32 averages) was measured at rest, followed by a time series of ^{31}P spectra (TR = 5 s, 4 averages) before, during and after muscle contractions. Muscle contractions were induced by electrical stimulation of the TA, via subcutaneously implanted electrodes. The stimulation protocol consisted of a series of stimulation pulses, applied every second, for a duration of 2 min. Recovery was followed for 10 minutes. ^{31}P MR spectra were fit in the time domain using AMARES in the jMRUI software package (jMRUI v2.1). The recovery of PCr was fitted to a mono-exponential function yielding the PCr recovery rate constant, k_{PCr}. One day after *in vivo* MRS, the animals were killed and TA muscles were excised for *in vitro* high-resolution respirometry measurements using a 2-channel high-resolution Oroboros oxygraph-2k (Oroboros, Innsbruck, Austria) at 37 °C. Mitochondria were isolated from one TA muscle and oxygen flux was measured using either pyruvate plus malate (for assessment of complex I-supported respiration) or succinate (complex II-supported respiration) plus rotenone (a complex I inhibitor) as a substrate. An ADP-regenerating system consisting of excess hexokinase (0.1 mg/ml), glucose (12.5 mM) and ATP (1 mM) was used to maintain steady-state maximal ADP-stimulated oxygen consumption rate (OXPHOS = classical State 3). Data are presented as means ± SD. Data were analyzed statistically by applying two-way ANOVA using SPSS. Level of significance was set at $P < 0.05$.

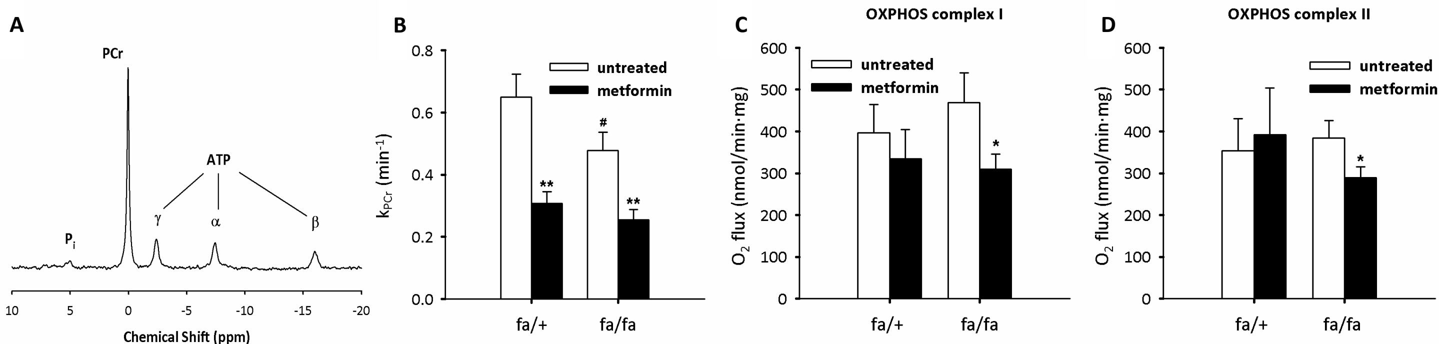

*Figure 1. A) Typical example of a ^{31}P MRS spectrum from TA muscle of an untreated fa/+ rat. B) PCr recovery rate constants as determined from ^{31}P MRS on TA muscle. C) Pyruvate plus malate and D) succinate driven maximal ADP-stimulated O_2 flux as determined by high-resolution respirometry performed on isolated mitochondria from TA muscle. Data are means from n=6 animals; ± SD. # $P<0.001$ relative to fa/+ rats, * $P<0.01$ and ** $P<0.001$ vs. untreated rats of the same genotype.*

Results

Body weight: Metformin treatment had no effect on body weight in *fa/+* rats (340 ± 22 and 344 ± 11 g for untreated and metformin-treated *fa/+* rats, respectively) or in *fa/fa* rats (388 ± 41 and 370 ± 18 g for untreated and metformin-treated *fa/fa* rats, respectively).

^{31}P MRS results: PCr recovery rate constants were 25% lower in untreated diabetic *fa/fa* rats compared with untreated healthy *fa/+* rats (Figure 1B). Metformin treatment decreased PCr recovery rates by two-fold in both healthy *fa/+* and diabetic *fa/fa* rats (Figure 1B).

High-resolution respirometry: Complex I- (Figure 1C) and complex II-supported (Figure 1D) oxygen consumption rates in the OXPHOS state were similar in mitochondria isolated from TA muscle of untreated diabetic *fa/fa* and untreated healthy *fa/+* rats. Irrespective of oxidizable substrate used, there was no effect of metformin treatment on OXPHOS oxygen consumption rates in TA mitochondria from the healthy *fa/+* group. In contrast, complex I- and complex II-supported oxygen consumption rates in the OXPHOS state in mitochondria isolated from TA of metformin-treated diabetic *fa/fa* rats were 33% and 25% lower, respectively, compared with untreated diabetic *fa/fa* rats.

Discussion and Conclusion

Two weeks of metformin treatment led to a severe impairment of *in vivo* skeletal muscle oxidative capacity in TA of both diabetic *fa/fa* and healthy *fa/+* rats. Based on the high-resolution respirometry data, the observation in diabetic *fa/fa* rats, but not in healthy *fa/+* rats, could at least partially be explained by the inhibitory effect of metformin on mitochondrial complex I and II. The discrepancy between the effect of metformin treatment on *in vitro* mitochondrial function in skeletal muscle of diabetic *fa/fa* and healthy *fa/+* rats suggests that disease progression may have caused subtle changes in e.g. the expression level(s) of metformin target(s) in skeletal muscle mitochondria of diabetic *fa/fa* rats, rendering them more sensitive to the inhibitory effect of metformin compared to healthy controls. In conclusion, we showed that metformin treatment severely impaired *in vivo* skeletal muscle oxidative capacity in rats, but this effect could not be fully attributed to inhibition of mitochondrial complex I. Since an impairment of mitochondrial function has been implicated in the pathogenesis of type 2 diabetes, our results suggest that the use of metformin to treat type 2 diabetes might need to be reevaluated.

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