

SHORT COMMUNICATION

Inhibitory Effect of Metformin on Oxidation of NADH-Dependent Substrates in Rat Liver Homogenate

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Summary

Metformin is widely used in the treatment of Type 2 diabetes, however, mechanisms of its antihyperglycemic effect were not yet fully elucidated. Complex I of mitochondrial respiration chain is considered as one of the possible targets of metformin action. In this paper, we present data indicating that the inhibitory effect of metformin can be tested also in liver homogenate. Contrary to previous findings on hepatocytes or mitochondria under our experimental conditions, lower metformin concentrations and shorter time of preincubation give significant inhibitory effects. These conditions enable to study the mechanism of the inhibitory effect of metformin in small samples of biological material (50-100 mg wet weight) and compare more experimental groups of animals because isolation of mitochondria is unnecessary.

Key words

Liver homogenate • Glutamate and Palmityl carnitine oxidation • Metformin

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Type 2 diabetes is a progressive metabolic disorder associated with abnormal glucose and lipid metabolism. Metformin (N,N-dimethylimidodicarbonimidic diamide) has been used for the treatment

of diabetes since the late 1950's (Bailey and Turner 1996) and until now is the drug of the first choice. Metformin decreases the hepatic glucose production mainly by inhibiting gluconeogenesis (Hundal *et al.* 2000). We have previously also shown that metformin decreases fatty acids oxidation in liver (Cahova *et al.* 2010).

Recently it was reported that metformin inhibits gluconeogenesis through mechanisms linked to perturbation of mitochondrial function (Foretz *et al.* 2010). Its effect was explained as an inhibition of mitochondrial complex I activity. The inhibitory effect of metformin was demonstrated in hepatocytes incubated with 10 mM metformin for 180 min (El-Mir *et al.* 2000). In isolated mitochondria incubated for 255 min in the presence of 10 mM metformin the oxidation of glutamate+malate+ADP was decreased by 40 % but in disrupted mitochondria the inhibitory effect of metformin was only 10 % (Owen *et al.* 2000). El-Mir *et al.* (2000) could find inhibition by metformin only in intact cells but not in isolated mitochondria. Recently it was demonstrated that metformin attenuates the generation of oxygen reactive species (Ouslimani *et al.* 2005) and inhibits the opening of the mitochondrial membrane permeability transition pore activated by cytosolic Ca²⁺ and ROS, which was associated with prevention of necrotic processes (Carvalho *et al.* 2008, Guigras *et al.* 2004). Despite of these findings, the detailed mechanism of metformin action on mitochondrial respiration and on complex I in particular has not been well defined yet.

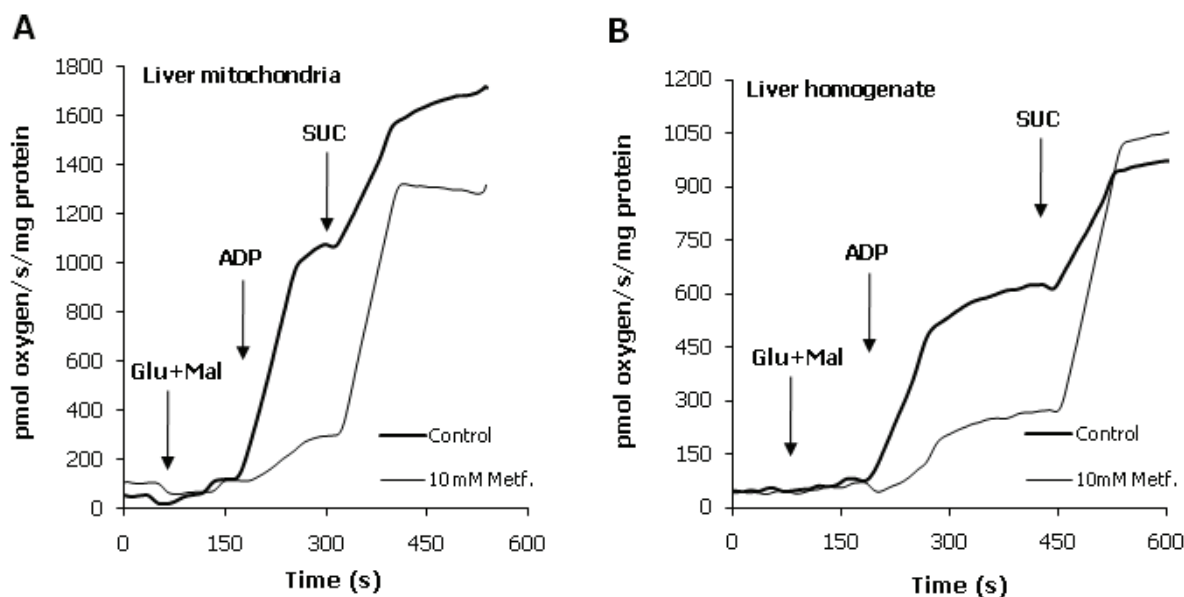


Fig. 1. The effect of 10 mM metformin on the oxidation of glutamate and malate by isolated liver mitochondria (**A**) and liver homogenate (**B**). Respiration of liver mitochondria (0.2 mg protein/ml) or liver homogenate (0.6 mg protein/ml) was measured with or without 10 mM metformin (5 min preincubation) in potassium medium supplemented with 0.5 mg fatty acid free bovine serum albumin per ml and 20 μ M cytochrome c. 10 mM glutamate, 2.5 mM malate, 1.5 mM ADP and 10 M succinate were added as indicated.

More data are thus required to understand the mechanisms through which metformin can inhibit mitochondrial respiratory chain activity.

We tried therefore to compare the effect of metformin on substrate oxidation of mitochondria and liver homogenate and to define conditions for such in vitro studies and we found that the metformin inhibitory effect can be well evaluated also in tissue homogenate which has not been used up to now. In our experimental design we were able to detect significant metformin inhibitory effect after short (3-5 min) preincubation at a lower concentration range (2.5-5 mM) compared with previously published data (Owen *et al.* 2000).

For the oxygen consumption measurements we used High Resolution Oxygraph K2- OROBOROS (Austria). Wistar male rats 200-250 g fed standard laboratory diet were used. Liver homogenate was 10 % (w/v) prepared in 0.25 mM sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4 medium and liver mitochondria were isolated by differential centrifugation according to Schneider and Hogeboom (1950). Oxygen consumption was registered in a medium (potassium medium) containing 100 mM KCl, 10 mM Tris-HCl, 4 mM KH_2PO_4 , 3 mM MgCl_2 , 1 mM EDTA, pH 7.3 at 30 °C, which was prepared with modification according to Gnaiger *et al.* (1995). Palmityl carnitine ((3R)-3-hexadecanoyloxy-4-trimethylazaniumylbutanoate) oxidation was determined as described in our previous papers

(Křiváková *et al.* 2008, Červinková *et al.* 2008, Endlicher *et al.* 2009). The protein concentration was determined according to Bradford (1976).

The data presented in Fig. 1 confirmed that metformin inhibitory effect does not require measurement in intact cell. The inhibitory effect of 10 mM metformin could be demonstrated both in mitochondria and in homogenate after a short 3-5 min preincubation. The sensitivity of both homogenate and mitochondria to metformin was higher compared with previously published data (Owen *et al.* 2000).

In further experiments, we tested the concentration dependence of the inhibitory effect of metformin using glutamate+malate+ADP and palmitylcarnitine+malate+ADP as substrates. We found a significant inhibitory effect of metformin for glutamate+malate+ADP respiration and for respiratory control index already at 5 mM concentration both for homogenate and mitochondria. Maximum inhibitory effect of metformin (about 80 %) was found in a range between 10-20 mM concentration (Fig. 2). Inhibition of “state 3” respiration was parallel with the inhibition of the respiratory control index (Fig. 2B). Decrease of respiratory control may represent another indicator of metformin action on mitochondria connected with decreased ATP production, however, further studies are necessary to elucidate this issue.

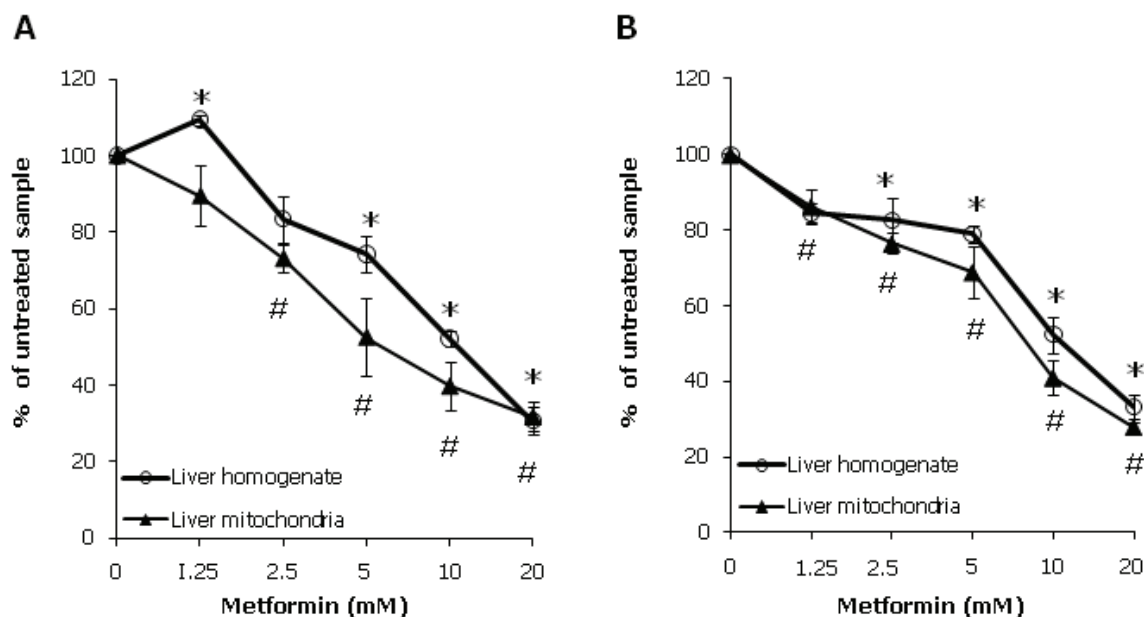


Fig. 2. The effect of metformin concentration on glutamate+malate oxidation (A) and respiratory control index (B) in the rat liver homogenate and mitochondria. Glutamate and malate-dependent respiration of rat liver homogenate and mitochondria was measured as described in Fig. 1 after 5 min preincubation with 0, 1.25, 2.5, 5, 10, 20 mM metformin. Respiratory control index was calculated from values obtained in Fig. 2A as the ratio of respiration rate in the presence and absence of ADP. * $P < 0.05$ vs. untreated sample (homogenate). # $P < 0.05$ vs. untreated sample (mitochondria). The presented data represent average from 4 independent experiments \pm S.E.M. and they are expressed as percent of untreated sample values. The oxygen consumption of liver homogenate and mitochondria were 609 pmol/s/mg protein and 1100 pmol/s/mg protein, resp. Control values of the respiratory control index of liver homogenate and mitochondria were 7.7 and 7.6, resp.

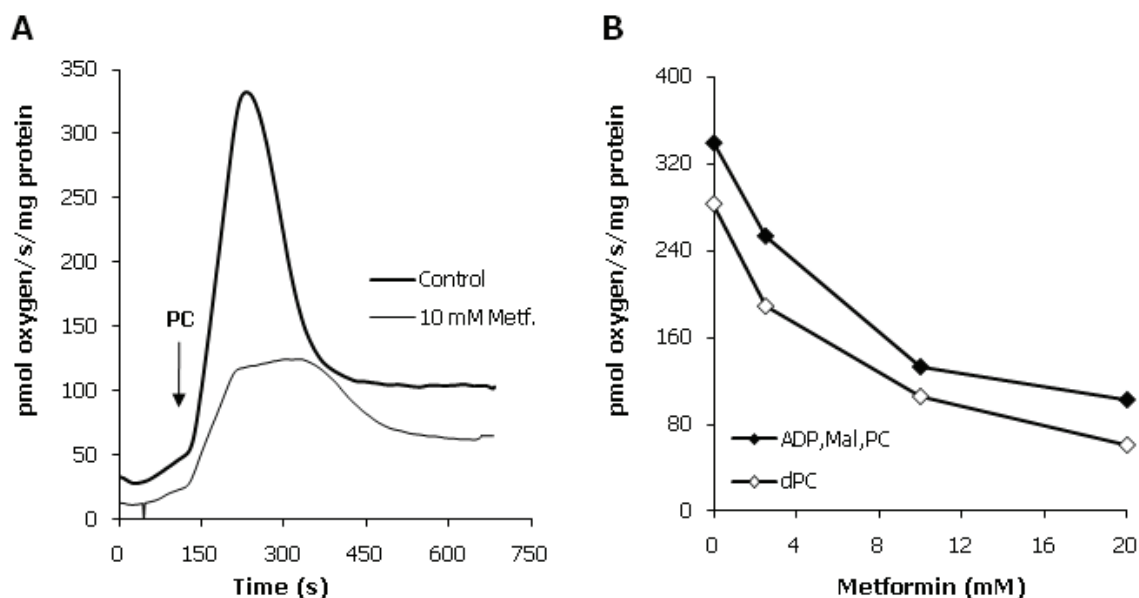


Fig. 3. The palmityl carnitine oxidation in rat liver homogenate A: the effect of single dose of metformin; B: concentration-dependent effect of metformin on palmityl carnitine oxidation. Palmityl carnitine oxidation was determined under condition described at Fig. 1, but BSA was not added. 12.5 μ M palmityl carnitine was added to the incubation medium with homogenate malate and ADP after 5 min equilibration with metformin. dPC were calculated as the difference of oxygen consumption in the presence of malate+ADP with and without palmityl carnitine. Data are representative values from two independent experiments.

Palmityl carnitine oxidation which involves both NADH- and flavoprotein-dependent dehydrogenases was inhibited at the same concentration range as oxidation of

glutamate+malate (Fig. 3). Similarly, 60 % inhibition of palmityl carnitine oxidation by 10 mM metformin after 5 min preincubation, as with glutamate+malate was found

(Fig. 3A). We evaluated both the maximum rate of palmityl carnitine oxidation in the presence of malate and ADP and the net increase of respiration induced by palmityl carnitine addition (Fig. 3A). Data in Fig. 3B show that both these parameters are inhibited by metformin similarly as glutamate+malate oxidation.

In agreement with previous findings, we found that succinate oxidation is not affected by metformin (see Fig. 1AB). Our data show that combination of two substrates, NADH-dependent glutamate and flavoprotein-dependent succinate, can fully saturate respiratory chain activity by electrons. Glutamate+malate (NADH-dependent substrate) alone can not saturate respiratory chain capacity and succinate (flavoprotein-dependent substrate) was used to reach maximum values of the respiratory rate. However, when the complex I activity is decreased by metformin complex II activity can fully compensate this decrease of electron flow (see Fig. 1AB), both in liver homogenate and isolated mitochondria.

We may thus conclude that liver homogenate

represents a very useful system for evaluation of metformin inhibitory effect on mitochondrial function. The results are comparable with those obtained on isolated mitochondria and the whole procedure is less labour- and time-consuming. Our measurements on homogenate also confirm previous findings indicating that intact cells are not required for the manifestation of metformin inhibitory effect. We further show that the respiratory control index, an important indicator of mitochondrial function, could also be well detected on samples of tissue homogenates.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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References

- BAILEY CJ, TURNER RC: Metformin. *New Engl J Med* **334**: 574-579, 1996.
- BRADFORD MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-254, 1976.
- CAHOVÁ M, DRAHOTA Z, OLIVARNYK O, KUČERA O, DANKOVÁ H, KAZDOVÁ L: The effect of metformin on liver mitochondria and lipid metabolism in NAFLD. *Diabetologia* **53** (Suppl 1): S304, 2010.
- CARVALHO C, CORREIA S, SANTOS MS, SELCA R, OLIVEIRA CR, MOREIRA PI: Metformin promotes isolated rat liver mitochondrial impairment. *Mol Cell Bioch* **308**: 75-83, 2008.
- ČERVINKOVÁ Z, RAUCHOVÁ H, KŘIVÁKOVÁ P, DRAHOTA Z: Inhibition of palmityl carnitine oxidation in rat liver mitochondria by tert-butyl hydroperoxide. *Physiol Res* **57**: 133-136, 2008.
- EL-MIR M-Z, NOGUEIRA V, FONTAINE E, AVERET N, RIGOLET M: Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain Complex I. *J Biol Chem* **275**: 223-228, 2000.
- ENDLICHER R, KRIVAKOVA P, RAUCHOVA H, NUSKOVA H, CERVINKOVA Z, DRAHOTA Z: Peroxidative damage of mitochondrial respiration is substrate-dependent. *Physiol Res* **58**: 685-692, 2009.
- FORETZ M, HEBRARD S, LECLERC J, ZARRINPASHNEH E, SOTY M, MITHIEUX G, SAKAMOTO K, ANDREELLI F, VIOLLET B: 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.
- GNAIGER E, STEINLECHNER-MARAN R, MÉNDEZ G, EBERL T, MARGREITER R: Control of mitochondrial and cellular respiration by oxygen. *J Bioenerg Biomembr* **27**: 583-596, 1995.
- GUIGAS B, DETAILLE D, CHAUVIN C, BATANDIER C, DE OLIVEIRA F, FONTAINE E, LEVERVE X: Metformin inhibits mitochondrial permeability transition and cell death: a pharmacological in vitro study. *Biochem J* **382**: 877-884, 2004.
- HUNDAL RS, KRSSAK M, DUFOUR S, LAURENT D, LEBON V, CHANDRAMOULI V, INZUCCHI SE, SCHUMANN WC, PETERSEN KF, LANDAU BR, SHULMAN GI: Mechanism by which metformin reduces glucose production in type 2 diabetes. *Diabetes* **49**: 2063-2069, 2000.
- KŘIVÁKOVÁ P, LÁBAJOVÁ A, ČERVINKOVÁ Z, DRAHOTA Z: Inhibitory effect of t-butyl hydroperoxide on mitochondrial oxidative phosphorylation in isolated rat hepatocytes. *Physiol Res* **56**: 137-140, 2007.

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- OUSLIMANI N, PEYNET J, BONNEFONT-ROUSSELOT D, THEROND P, LEGRAND A, BEAUDEUX JL: Metformin decreases intracellular production of reactive oxygen species in aortic endothelial cells. *Metabolism* **54**: 829-834, 2005.
- OWEN MR, DORAN E, HALESTRAP AP: Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochem J* **348**: 607-614, 2000.
- SCHNEIDER WC, HOGEBOM GH: Intracellular distribution of enzymes. 5. Further studies on the distribution of cytochrome-c in rat liver homogenates. *J Biol Chem* **183**: 123-128, 1950.;
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