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Effect of metformin on intact mitochondria from liver and brain: Concept revisited

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ABSTRACT

Metformin is an antihyperglycemic drug which is being examined as a repurposed treatment for cardiovascular disease for individuals without diabetes mellitus. Despite evidence that mitochondrial respiratory complex I is a target of metformin and inhibition of the enzyme is one of the mechanisms of its therapeutic actions, no systematic studies of the metformin effect on intact mitochondria have been reported. In the presented paper, we described the effect of metformin on respiration and ROS release by intact mitochondria from the liver and brain. By comparing the effect of metformin on mitochondria oxidizing different substrates, we found direct inhibition of respiration and stimulation of ROS release when complex I-based respiration is measured (forward electron transfer). Metformin had no effect on respiration rates but inhibited ROS release when mitochondria oxidize succinate or glycerol 3-phosphate in conditions of reverse electron transfer in complex I. In addition, we found that metformin is a weak effector of the active/deactive (A/D) transition of mitochondrial complex I. At high concentrations, metformin increases the rate of spontaneous deactivation of complex I (A→D transition). The results obtained are consistent with the concept of metformin inhibition of complex I and that it can either stimulate or inhibit mitochondrial ROS production depending on the preferential respiratory substrate. This is relevant during the ischemia/reperfusion process, to counteract the ROS overproduction, which is induced by a high level of reverse electron transfer substrates is generated after an ischemic event.

1. Introduction

The glucose-lowering properties of metformin and related compounds were described almost a hundred years ago, however there is still no consensus in the field about the primary mechanism of its action. Since the first clinical use of metformin at the end of the ‘50s (Sterne, 1959, 1960), it has come to be the most prescribed glucose-lowering therapy worldwide (Qaseem et al., 2017). Metformin has also been shown to lower cardiovascular events in diabetic patients, and there is growing evidence suggesting that beyond its hypoglycemic effects, metformin has a protective effect in cerebrovascular disease (Luo et al., 2019; Rena and Lang, 2018; Roussel et al., 2010). It is accepted that at the cellular level, metformin causes inhibition of mitochondrial ATP production, which stimulates AMP-activated protein kinase (AMPK) (Zhou et al., 2001), the key regulator of energy metabolism. This decelerates gluconeogenesis and lowers the blood glucose level, but at the molecular level the exact mechanism and sites of metformin action are not completely understood. While there are several concepts coexisting in the literature (see (Foretz et al., 2014; Hart et al., 2019; Pecinova et al., 2019) for reviews), one of the most accepted mechanisms implicates the inhibition of mitochondrial complex I of the respiratory chain (NADH:ubiquinone oxidoreductase, EC 1.6.5.3) as an opening point of metformin action (Andrzejewski et al., 2014; Bridges et al., 2014; El-Mir et al., 2000; Owen et al., 2000; Palenickova et al., 2011; Pecinova et al., 2017).

Mitochondrial complex I is the enzyme solely responsible for the oxidation of NADH and therefore regeneration of NAD^+ for catabolism. Complex I catalyzes the reduction of membrane ubiquinone (Q) to ubiquinol (QH_2) by NADH provided from the upstream catabolic pathways (TCA cycle, fatty acid oxidation, malate-aspartate shuttle, etc). Redox reactions drive the translocation of protons across the inner mitochondrial membrane from the matrix to the intermembrane space, contributing to the formation of the proton-motive force. Most likely, in the absence of inhibitors, complex I is the major contributor to reactive oxygen species (ROS) generation by the respiratory chain (see (Andreyev et al., 2015; Galkin, 2019; Murphy, 2009) for reviews).

The catalytic properties of complex I are multifarious (see (Dröse

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List of abbreviations:

A/D	transition, active/deactive transition
ADP	adenosine 5-diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
AMPK	AMP-activated protein kinase
BSA	bovine serum albumin
DDM	n-dodecyl-β-D-maltoside;
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N’-tetraacetic acid
FMN	flavin mononucleotide;
hrCN	high resolution Clear Native;
mGPDH	mitochondrial glycerol 3-phosphate dehydrogenase
MSE	mannitol/sucrose/HEPES/EGTA medium
NEM	N-ethylmaleimide;
PMSF	phenylmethylsulfonyl fluoride
RCR	respiratory control ratio
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SET	sucrose/EDTA/Tris medium

et al., 2016; Vinogradov, 1998) for reviews). The energy-generating forward NADH:Q reductase reaction is reversible, and in the presence of the proton-motive force, the enzyme can execute QH_2:NAD^+ oxidoreduction. In this energy-consuming reaction, the flow of protons from the intermembrane space to the matrix drives a “reverse electron transfer” (RET) from ubiquinol towards the nucleotide-binding site where NAD^+ is reduced. Ubiquinol can be provided by oxidation of succinate or glycerol 3-phosphate, and the proton-motive force is generated by the activity of downstream complexes III and IV. Unlike forward electron transfer, RET is associated with the highest rates of ROS generation in intact mitochondria, reaching 2–5% of the total electron flux (Andreyev et al., 2015; Batandier et al., 2006; Kushnareva et al., 2002; Kwong and Sohal, 1998; Liu et al., 2002; Starkov, 2010; Starkov and Fiskum, 2003; Stepanova et al., 2018, 2019; Votyakova and Reynolds, 2001).

An interesting feature of mitochondrial complex I from mammals is the reversible active/deactive (A/D) transition of the enzyme (see for a review (Dröse et al., 2016)). There are two forms of the enzyme present as an equilibrating mixture: active A-form, and deactive dormant D-form. In respiring mitochondria in the presence of substrates (NADH and ubiquinone), complex I is maintained in the A-form. However, when the enzyme is idle, physiological temperatures (>30 °C) increase the rate of equilibration leading to a measurable accumulation of the D-form both in vitro and *in vivo* (Galkin, 2019). There are several modulators of the A/D transition, such as free fatty acids and divalent cations (Dröse et al., 2016). It has been suggested that metformin may bind differently to the A- and the D-form, affecting A/D equilibrium of mitochondrial complex I (Bridges et al., 2014; Matsuzaki and Humphries, 2015).

Complex I is a rate-controlling component of the respiratory chain (Bianchi et al., 2004; Genova et al., 1995; Kuznetsov et al., 1997), and even a slight inhibition of complex I (for example by metformin) could result in the slowing down of oxidative phosphorylation. This decreases adenylate energy charge (([ATP]+½[ADP])/([ATP]+[ADP]+[AMP])) and activates AMPK. There is also a significant body of data showing modulation of mitochondrial ROS production by metformin in preparations of cells (Dykens et al., 2008; Guigas et al., 2004; Kajiwara et al., 2018; Kelly et al., 2015; Mackenzie et al., 2013), intact mitochondria (Batandier et al., 2006; Bridges et al., 2014; Matsuzaki and Humphries, 2015; Pecinova et al., 2017), submitochondrial particles (Matsuzaki and Humphries, 2015) or isolated enzyme (Bridges et al., 2014). However, no data simultaneously demonstrating the direct effects of metformin on respiration and ROS release in preparations of intact mitochondria were reported. While there are numerous publications consistent with inhibition of oxidative phosphorylation by biguanidines, no systematic analysis of metformin’s effect on mitochondrial respiration and ROS production was performed. In order to provide new data that contribute to the elucidation and understanding of metformin’s mechanism of action, we characterized the direct effects of metformin on respiration and H_2O_2 release by intact mitochondria from the liver and brain and also tested if metformin affects the A/D equilibrium of complex I.

2. Materials and Methods

2.1. Reagents

The following chemicals were purchased from Sigma: adenosine 5′-phosphate monopotassium salt hydrate (#A5285), alcohol dehydrogenase (#A7011), aminocaproic acid (#A2504), DDM (#D4641), EDTA (#E−5391), FMN (#F2253), imidazole (#792527), L-Malic acid disodium salt monohydrate (#233935), NADH (#N8129), phenylmethylsulfonyl fluoride (PMSF) (#P7626), rac-glycerol-1-phosphate sodium salt (#61668), SDS (#L3771), sodium pyruvate (#P5280), sodium succinate dibasic (#14160), and triphenyl-tetrazolium chloride (#T8877). NativeMark unstained protein standards (#LC0725), Pierce BCA protein assay kit (#23225), and 3–12% acrylamide gradient gel, 8 × 8 cm (#BN1001BOX) were from Thermo Fisher Scientific. Alamethicin (#11425), metformin (#13118), and N-ethylmaleimide (NEM) (#19938) were from Cayman Chemical. Difluorocarboxyfluorescein NHS-ester was from Fluoroprobes (#1223).

2.2. Isolation of intact brain and liver mitochondria

All procedures were approved by the Institutional Animal Care and Use Committee of Weill Cornell Medicine (#2021–0021) and performed in accordance with the ARRIVE guidelines. Intact brain cortex and liver mitochondria were isolated from 10-week C57BL6/J male mice by differential centrifugation (Starkov et al., 2017; Stepanova et al., 2019). For the liver mitochondria, mice fasted overnight before the isolation. Briefly, brain cortex tissue was homogenized with 40 strokes by pestle “B’’ (tight) of a Dounce homogenizer in a tenfold volume of the media comprising 225 mM mannitol, 75 mM sucrose, 20 mM HEPES (pH 7.4), 1 mM EGTA, 1 mg/ml fatty acid free bovine serum albumin (BSA) and centrifuged at 5000 g for 4 min. The supernatant was collected, 0.02% digitonin was added, and the suspension was centrifuged at 10,000 g for 10 min. The pellet was resuspended and washed twice with the same buffer but without BSA. For mouse liver mitochondria isolation, the liver was homogenized in the same buffer and centrifuged at 900 g for 10 min. The supernatant was collected and centrifuged at 8000 g for 10 min, and the pellet was re-suspended in the isolation buffer without BSA and centrifuged at 8000 g for 10 min. The final mitochondrial pellet was re-suspended in a buffer comprising 225 mM mannitol, 75 mM sucrose, 20 mM HEPES-Tris base (pH 7.4) to 10–15 mg/ml mitochondria protein. The final liver pellet was resuspended in the same buffer supplemented with 5 mg/ml BSA. Mitochondrial protein content was determined with BCA Protein Assay Reagent. Mitochondria were stored on ice and used within 3–4 h after isolation.

2.3. Mitochondrial respiration and H_2O_2 release

Mitochondrial respiration and H_2O_2 release were measured using a high-resolution respirometer (Oroboros Oxygraph-2k) equipped with two-channel fluorescence optical setup as described in detail in (Stepanova and Galkin, 2020; Stepanova et al., 2018). Mitochondria (0.1 and 0.05 mg of protein/ml for liver and brain, respectively) were incubated in the medium composed of 125 mM KCl, 0.2 mM EGTA, 20 mM HEPES-Tris (pH 7.4), 4 mM KH_2PO_4, 2 mM $MgCl_2$, 1 mg/ml BSA, 10 μM Amplex UltraRed, 4 U/ml horseradish peroxidase and 10 U/ml SOD

at 37 °C.

The calibration was performed by adding 200–400 pmoles aliquots of fresh standard solution of H_2O_2 ($\varepsilon_{240nm} = 46.3\ M^{-1}\ cm^{-1}$) to the assay chamber. Due to the presence of carboxylesterases that convert Amplex UltraRed to resorufin in the preparation of liver mitochondria, 50 μM PMSF was included in the medium. Liver mitochondria were incubated for 5–10 min in the chamber prior to the addition of substrates to initiate respiration (Miwa et al., 2016).

Metformin was added prior to subrates and there was no lag-period in reaching the full effect of inhibtion. The concentration of metformin was determined spectrophotometrically ($\varepsilon_{233nm} = 11.0\ mM^{-1}\ cm^{-1}$) (Majithia et al., 2020).

2.4. Complex I activation, measurement of specific activity and content of complex I

To prepare liver mitochondrial membranes containing complex I in the A-form, an aliquot of frozen membranes was thawed and washed three times with hypotonic MSE medium (112 mM mannitol, 37 mM sucrose, 10 mM HEPES, 0.5 mM EGTA, pH 7.4) by centrifugation at 20,000 g for 10 min and stored on ice. Next, 5 mg/ml of membranes were preincubated with or without metformin at 30 °C in hypotonic SET medium (0.125 M sucrose, 25 mM Tris-HCl, 0.1 mM EDTA, pH 8.5, plus 30 μg/ml alamethicin). Aliquots were taken at the time specified, and residual NADH oxidase activity was assayed in the presence of 1 mM NEM in the standard SET medium (pH 7.5). Only the D-form of complex I is irreversibly inhibited by N-ethyl maleimide (NEM), while the A-form is insensitive to SH-reagents (Babot et al., 2014; Galkin and Moncada, 2007). The accumulation of the D-form can be calculated from the residual NEM-insensitive NADH oxidase activity, which corresponds only to the A-form of complex I (Babot et al., 2014; Maklashina et al., 2003).

NADH oxidase activity of complex I was measured spectrophotometrically as oxidation of 0.1 mM NADH at 340 nm ($\varepsilon_{340nm} = 6.22\ mM^{-1}cm^{-1}$) using the Varian Cary 4000 spectrophotometer in 1.0 ml of SET buffer, pH 7.5, at 25 °C (Ansari et al., 2021). Assay buffer was supplemented with 30 μg/ml alamethicin and 10 μM cytochrome *c*. Activity was fully sensitive to inhibitors of complex I and IV as the rate of NADH oxidation was negligible in the presence of 1 μM rotenone or 1 mM cyanide. Full NADH oxidase activity of permeabilized brain and liver mitochondria was 566 ± 28 and 358 ± 11 nmol NADH/min/mg protein, respectively. Liver mitochondrial NADH oxidase was only 30% rotenone sensitive, and that fraction was subtracted from the total NADH oxidase rate to calculate specific complex I activity.

Separation of respiratory chain complexes from liver and brain mitochondria by high resolution Clear Native page electrophoresis followed by flavin fluorescent scanning and determination of complex I content was performed as detailed in (Ansari et al., 2021).

2.5. Statistical analysis

Data analysis was performed using OriginPro (version 9.3). All data are mean ± SEM (n = 3–4 of biological replicates). Two-tailed *t*-test was used to analyze differences between two groups.

3. Results

For further studies, we used preparations of intact liver and brain mitochondria. First, we compared respiratory activities and H_2O_2 release rates for both preparations. There are three major entry points to the respiratory chain in liver and brain mitochondria: complex I (NADH: ubiquionone oxidoreductase), complex II (succinate dehydrogenase), or mitochondrial glycerol 3-phosphate dehydrogenase (mGPDH), and therefore three experimental conditions were chosen. Oxidation of malate and pyruvate supported complex I-mediated respiration (complexes I-III-IV), succinate provided respiration via complexes II-III-IV and finally, respiration by mGPDH-complexes III-IV was initiated by glycerol 3-phosphate addition. Table 1 shows the quantitative characteristics of mitochondrial respiration and H_2O_2 release during oxidation of different substrates. We found that in comparison with the brain, mitochondria from liver demonstrated lower respiratory activity (except with succinate) and lower H_2O_2 release rate ($n = 3–4$, $p < 0.05$, *t*-test). Note, that in liver mitochondria maximal respiration on glycerol 3-phosphate is only 7% of succinate oxidase, indicating very low activity of mGPDH.

We also compared complex I content in preparations of liver and brain mitochondria based on the flavin determination after Clear Native electrophoresis and in-gel denaturation (Ansari et al., 2021). Densitometry analysis of the FMN fluorescence signal from complex I was performed (Fig. 1) and the calculated complex I content for intact liver and brain mitochondria was determined as 10.8 ± 1.6 and 24.2 ± 1.6 pmol/mg protein, respectively.

Next, we assessed the effect of metformin on mitochondrial respiration. As expected, metformin in the range of 0.5–5 mM showed specific inhibition of only complex I-mediated respiration in mitochondrial preparations from both tissues (Fig. 2A, D). No effect of metformin on succinate or glycerol 3-phosphate-supported respiration was detected, indicating complex I as a specific target of metformin. Note that unlike ADP-stimulated oxygen consumption, non-phosphorylated respiration (state II) was not as sensitive to metformin, probably indicating a significant spare capacity of the respiratory chain.

Next, we measured the effect of metformin on H_2O_2 release by mitochondria using different substrates in non-phosphorylating conditions (State II, no ADP present). These conditions provide measurable rates of mitochondria H_2O_2 release both during forward (malate/pyruvate couple) or reverse (succinate or glycerol 3 phosphate) electron transfer. During the latter, steady-state respiration provides a high ubiquinol/ubiquinone ratio and builds up a proton-motive force to support the reverse electron transfer to complex I and reduction of $NAD(P)^+$ in the matrix. After complete reduction of the matrix nucleotide pool (quinol:NAD^+ oxidoreduction), electrons can “leak” from the reduced enzyme to oxygen and this process is known to support the highest rates of H_2O_2 release in intact mitochondria from different sources (Andreyev et al., 2015; Grivennikova and Vinogradov, 2006; Hinkle et al., 1967; Niatsetskaya et al., 2012; Pryde and Hirst, 2011; Quinlan et al., 2013; Starkov and Fiskum, 2003; Stepanova et al., 2018, 2019; Treberg et al., 2011; Turrens and Boveris, 1980; Votyakova and Reynolds, 2001) (see also (Andreyev et al., 2015; Galkin, 2019) for reviews). Metformin manifested a dual effect on the H_2O_2 release activity of intact mitochondria depending on the substrates used. First, inhibition of complex I during oxidation of malate/pyruvate (shown in Fig. 2 A, D) resulted in an increase of H_2O_2 production (Fig. 3 A, D). The opposite effect was observed in conditions of RET when succinate or glycerol 3-phosphate was used. These two observations indicate that metformin indeed affects complex I so that there is an increase of ROS production in the forward and decrease in the reverse reaction. In the absence of metformin, H_2O_2 release during glycerol 3-phosphate oxidation by liver mitochondria indicates no RET due to the low activity and respiratory control close to 1.

Since metformin acts as a weak inhibitor of complex I, it was important to compare its effects to complex I specific inhibitor rotenone, which is known to stimulate H_2O_2 release during forward electon transfer (*e.g.*, malate/pyruvate couple) and to inhibit RET-dependent H_2O_2 generation. As expected, rotenone inhibited malate/pyruvate-supported respiration by 90% in mitochondria from the liver and brain (Fig. 4A and B). At the same time, H_2O_2 release with these substrates was stimulated by 50–80% (Fig. 4C and D). Rotenone, however, had little effect on succinate or glycerol 3-phosphate-supported respiration (Fig. 4A and B), but inhibited RET-supported H_2O_2 release with these substrates (Fig. 4C and D). The observed slight activation of H_2O_2 release in liver mitochondria in the presence of glycerol 3-phosphate was probably due to the aforementioned absence of RET with this substrate in liver mitochondria.

Table 1
Respiration and H_2O_2 release in mouse liver and brain mitochondria.

Respiration[a], nmol O_2/min/mg protein						
	Complex I		Complex II		mGPDH	
	Liver	Brain	Liver	Brain	Liver	Brain
Non-phosphorylating	9.2 ± 0.5	10 ± 0.6	34.6 ± 2.4	35.0 ± 3.0	11.2 ± 0.7	12.9 ± 2.3
State 3 (+0.4 mM ADP)	31.3 ± 0.3	77.3 ± 15.3	163.9 ± 6.7	105.2 ± 11.4	11.1 ± 1.8	22.5 ± 6.7
RCR	3.4 ± 0.2	7.3 ± 1.2	5.0 ± 0.3	3.0 ± 0.0	1.0 ± 0.1	1.7 ± 0.2
			H_2O_2 release rate, pmol H_2O_2/min/mg protein			
Non-phosphorylating	38 ± 0	145 ± 30	1025 ± 167	677 ± 51	329 ± 11	352 ± 67

[a] Measured, as described in the Materials and Methods section, in 125 mM KCl, 0.2 mM EGTA, 20 mM HEPES-Tris, 4 mM KH_2PO_4, pH 7.4, 2 mM $MgCl_2$, 1 mg/ml BSA, 10 μM Amplex UltraRed, 4 U/ml horseradish peroxidase, 10 U/ml SOD, at 37 °C using 0.05–0.2 mg of protein/ml. For complex I–mediated respiration, 5 mM pyruvate and 2 mM malate were used; for complex II-mediated respiration, 20 mM succinate was used; for mGPDH-mediated respiration, 40 mM glycerol 3-phosphate was used. Respiratory control ratio (RCR) was determined by the rate of state 3 respiration divided by the rate of non-phosphorylating respiration. Values are given as mean ± SEM (n = 3–4).

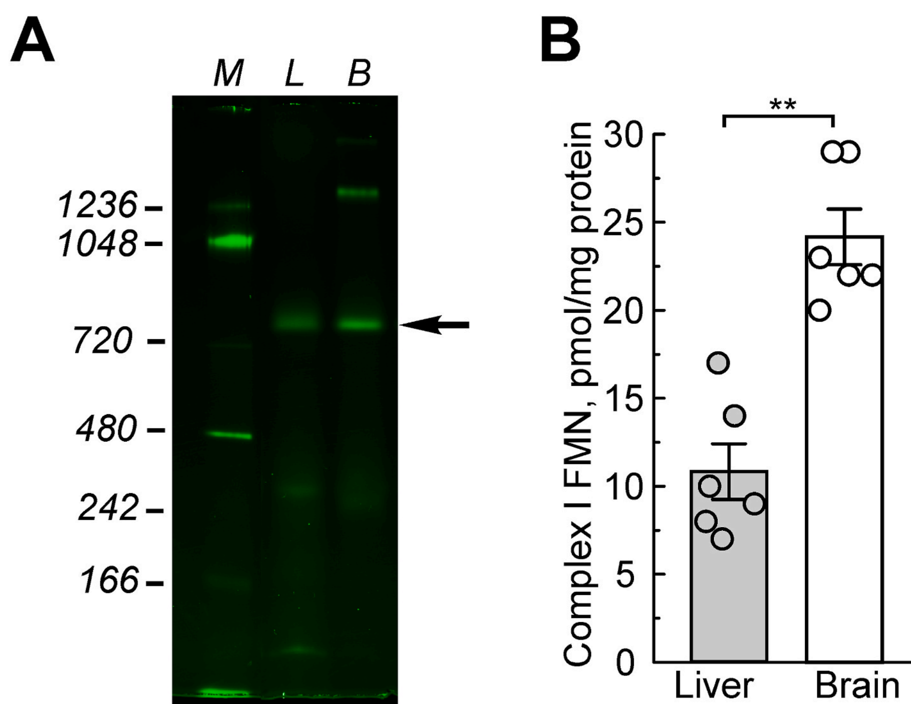

Fig. 1. Determination of complex I content in liver and brain mitochondria. (A) Flavin fluorescence scan of DDM-solubilized intact mitochondria (DDM/protein ratio of 3.6 g/g). Difluorocarboxyfluorescein-labelled markers (M), or identical 25 μg protein aliquots of liver (L) and brain (B) mitochondria samples were applied on the same gel and hrCN electrophoresis was performed. Complex I FMN fluorescence was detected after treatment with 20% SDS (Excitation/Emission = 473/530 nm) as described in the Materials and Methods section. Position of Complex I band is shown by an arrow. (B) Quantitative analysis of the content of complex I FMN in liver and brain mitochondria (grey and white bars, respectively). **p= < 0.01, t-test, values are shown as mean ± SEM. Three independent biological replicates were used for each experiment.

It has also been reported that deactivation greatly increases sensitivity of the enzyme to metformin (Bridges et al., 2014; Matsuzaki and Humphries, 2015). Therefore, we tested whether or not metformin can be an effector of the A/D transition. We used permeabilized liver or brain mitochondrial membranes to test the ability of metformin to affect the rate of complex I deactivation (*i.e.*, A to D transition). Incubation of mitochondria containing the A-form of the enzyme without substrates at 30 °C resulted in a slow A to D conversion and increased the fraction of D-form in the preparation (Fig. 5). This can be detected by a kinetic assay measuring the NADH oxidase reaction in the presence of NEM. Only the D-form of the enzyme is sensitive to NEM-treatment, so a gradual decrease of the NADH oxidase rate during incubation at 30 °C corresponds to an increase in the D-form fraction in the preparation. It should be noted that in the absence of NEM, the rate of the reaction after the lag-phase is the same. As shown in Fig. 5, the presence of 5 mM metformin during incubation at 30 °C had very little effect on the time course of D-form accumulation; however, 50 mM metformin accelerated the rate of A to D conversion approximately two-fold.

4. Discussion

Since the original reports of Halestrap and Leverve groups (El-Mir et al., 2000; Owen et al., 2000), it is generally accepted that the biguanidine metformin mechanism of action is mediated by mitochondrial complex I inhibition. Guanidine compounds have long been known to affect catalytic activities of complex I (Hatefi and Galante, 1977; Hatefi et al., 1969; Schafer, 1969). A great number of molecules is known to inhibit mitochondrial complex I and it is a commonly used practice to relate the effects of any inhibitor to those of the enzyme's classic specific inhibitor rotenone. Metformin acts as a weak inhibitor of complex I, which affects the enzyme's activity and ROS generation resembling rotenone (Bridges et al., 2014; El-Mir et al., 2000; Owen et al., 2000; Pecinova et al., 2017). Rotenone, however, is a more potent complex I inhibitor with K_i~1 nM for membrane-bound enzyme (Grivennikova et al., 1997). This is significantly different from the affinity of metformin to isolated enzyme (IC_{50}~20 mM) (Bridges et al., 2014). The therapeutic level of metformin in blood is 5–40 μM (Kajbaf et al., 2016; van Berlo-van de Laar et al., 2011), and therefore it is hard to assign its mechanism of drug action solely to direct inhibition of complex I. Despite our extensive literature search, we were unable to find a

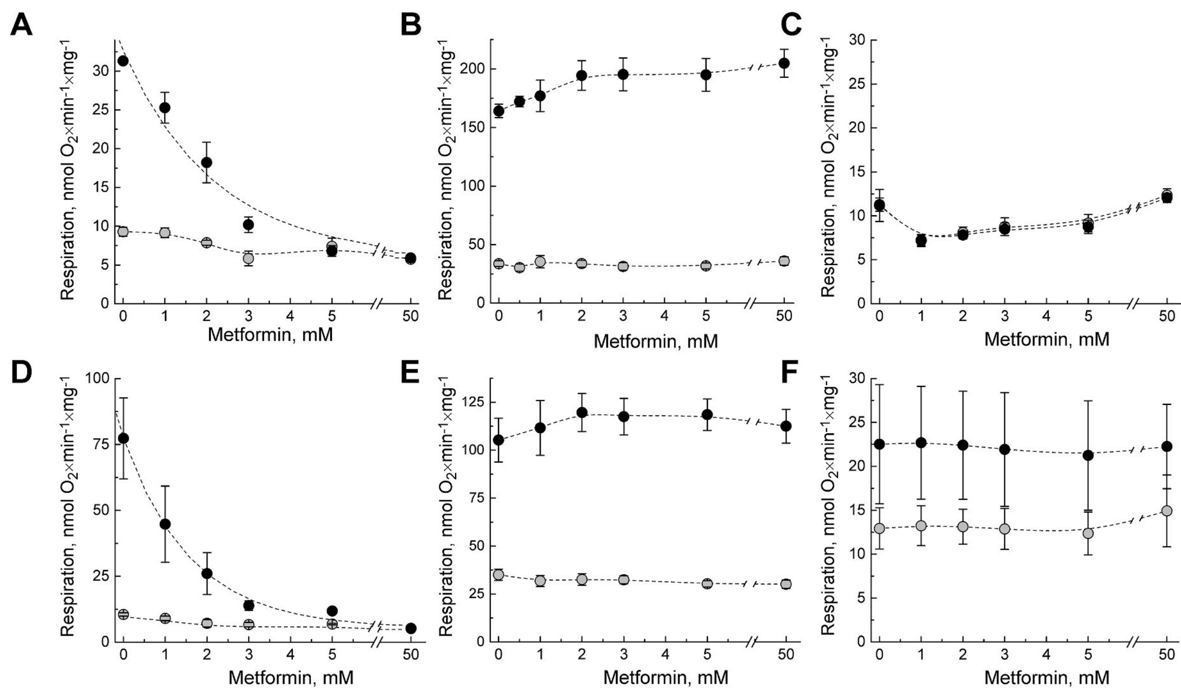

Fig. 2. Effect of metformin on respiration of liver and brain mitochondria (top and bottom panels, respectively). Mitochondria were respiring on different substrates. Activities of liver (A–C) or brain (D–F) mitochondria (0.1 and 0.05 mg of protein/ml, respectively) were measured during non-phosphorylating (no ADP, grey circles) and phosphorylating state 3 (0.4 mM ADP, black circles) in the standard medium as described in the Materials and Methods section. Different concentrations of metformin were added prior to addition of mitochondria. The reaction was initiated by addition of substrates. (A, D) 2 mM malate and 5 mM pyruvate; (B, E) 20 mM succinate (C, F); 40 mM glycerol 3-phosphate. Results are mean ± SEM (n = 3–4).

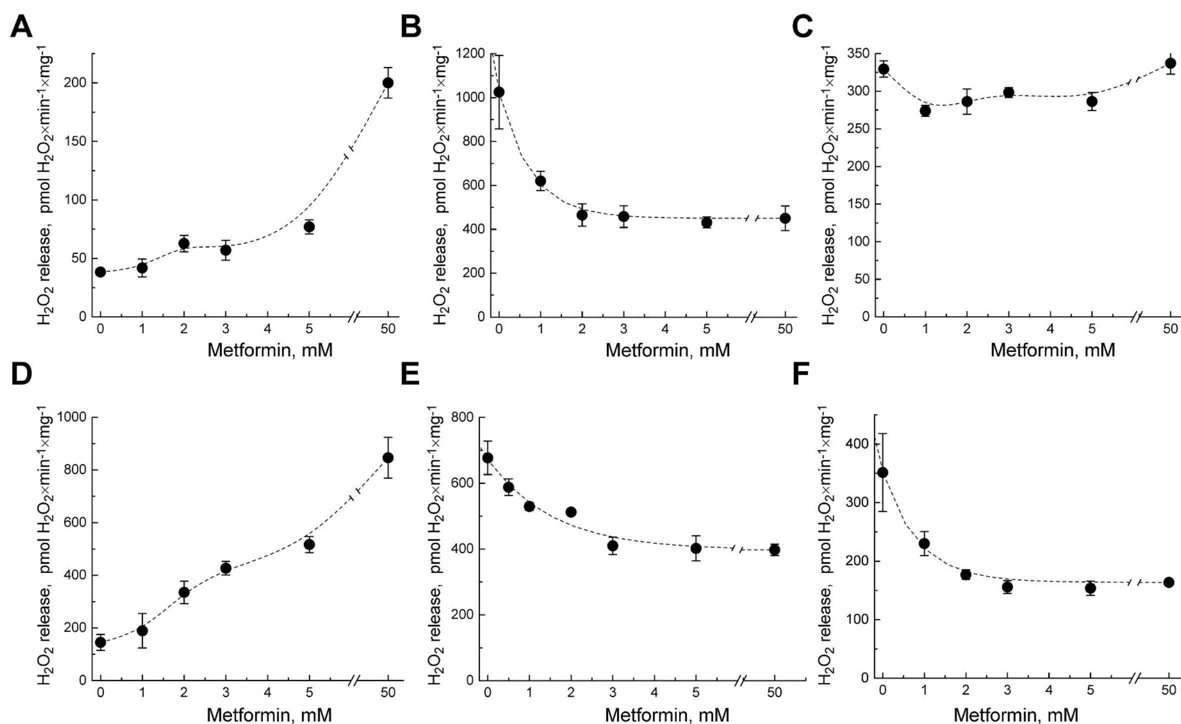

Fig. 3. Effect of metformin on H_2O_2 release by liver and brain mitochondria respiring on different substrates in non-phosphorylating conditions. H_2O_2 formation was followed by the catalase-sensitive horseradish peroxidase-dependent formation of resorufin from Amplex UltraRed in the same conditions as in Fig. 2. Activities of liver (A–C) or brain (D–F) mitochondria were measured during non-phosphorylating respiration (no ADP present). The reaction was initiated by addition of substrates. (A, D) 2 mM malate and 5 mM pyruvate; (B, E) 20 mM succinate; (C, F) 40 mM glycerol 3-phosphate. Results are mean ± SEM (n = 3–4).

systematic examination of metformin inhibition in a simple system of intact mitochondria. Therefore, a clear analysis of the effect of metformin on oxygen consumption and H_2O_2 release by intact mitochondria seemed desirable. Metformin accumulates in the liver (Graham et al., 2011; Wilcock et al., 1991) and recent studies have shown that it can penetrate the blood–brain barrier, influencing neuronal energy

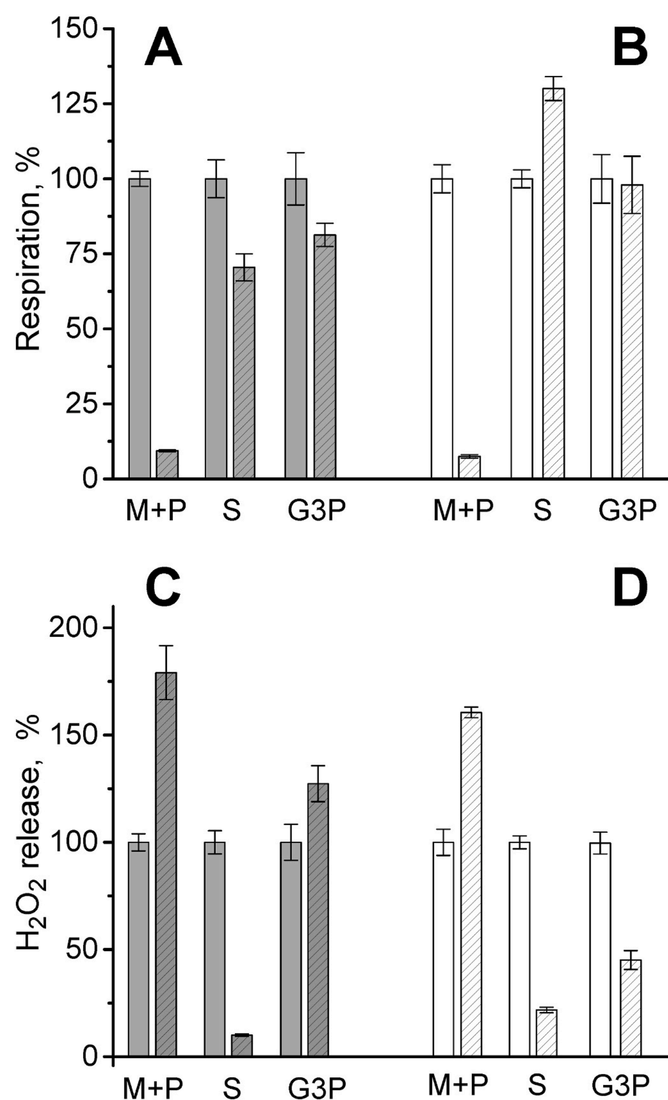

Fig. 4. Effect of rotenone on respiration and H_2O_2 release by liver and brain mitochondria using different substrates. Respiration of liver (A, grey bars) or brain (B, white bars) mitochondria was measured as in Fig. 2. 100% corresponds to respiratory rate in the presence of 0.4 mM ADP (State 3). H_2O_2 formation by the liver (C, grey bars) or brain (D, white bars) mitochondria was followed in the non-phosphorylating conditions (no ADP) as in Fig. 3. 100% corresponds to H_2O_2 release rate in the absence ADP (State 2). In all panels, hatched bars indicate the presence of 2 μM rotenone. Reaction was initiated by addition of substrates: 2 mM malate and 5 mM pyruvate (M + P), 20 mM succinate (S), 40 mM glycerol 3-phosphate (G3P). Results are mean ± SD (n = 3–4).

metabolism (Moreira, 2014; Ying et al., 2014); therefore, liver and brain mitochondria preparations were analyzed.

In accordance with previous studies (Andrzejewski et al., 2014; Bridges et al., 2014; El-Mir et al., 2000; Owen et al., 2000; Palenickova et al., 2011), the addition of metformin inhibited respiration on malate/pyruvate, substrates of NAD^+-dependent dehydrogenases that generate NADH in the matrix, providing forward electron flow via complex I. Both liver and brain mitochondria preparations were affected, and the estimated IC_{50} of inhibition was around 2 mM. This is lower than the value for isolated bovine enzyme (20 mM) but significantly higher than IC_{50} of 0.2–0.3 mM shown for intact cells (Bridges et al., 2014) probably due to membrane-related events (Detaille et al., 2002) (see also (Fontaine, 2014)). No metformin effect on succinate or glycerol 3-phosphate-mediated respiration was found. These observations indicate a direct inhibition of complex I by metformin. At the same time, inhibition of complex I resulted in the blockage of electron transfer to the quinone pool and an increase of H_2O_2 production most likely via complex I FMN or via the α-ketoglutarate dehydrogenase complex of Krebs cycle, connected to complex I via the NADH-pool (Bunik and Sievers, 2002; Starkov et al., 2004; Tretter and Adam-Vizi, 2004).

Metformin had the opposed effect on mitochondrial H_2O_2 release when succinate or glycerol 3-phosphate was used as respiratory substrates. While no effect on oxygen consumption was observed, metformin prevents reduction of complex I FMN by RET from the downstream, leading to decreased ROS production. Such a dual effect is very similar to the action of rotenone, which is known to increase ROS production in the forward and decrease ROS production in the reverse reaction as shown here and observed in other studies (Cadenas et al., 1977; Grivennikova and Vinogradov, 2006; Kushnareva et al., 2002; Lambert and Brand, 2004; Quinlan et al., 2013; Stepanova et al., 2018; Votyakova and Reynolds, 2001). Our results on metformin's effect on RET-mediated ROS production are in good agreement with previous observations of Batandier et al. where lower RET-linked H_2O_2 release was found in intact mitochondria isolated from metformin-perfused rat liver (Batandier et al., 2006). The opposite, stimulating effect of 24 h incubation with low-doses of metformin on mitochondrial respiration observed in cardiomyocytes (Emelyanova et al., 2021) is probably associated with an increase in mtDNA content and stimulation of expression of genes involved in oxidative phosphorylation. In this study, direct acute inhibition of complex I-mediated respiration by metformin in isolated cardiac mitochondria was also observed. However, almost no effect of metformin on ROS production during succinate-supported respiration of cardiac mitochondria was found, which was most likely due to the presence of rotenone in the measuring assay (Emelyanova et al., 2021).

It is worth mentioning that non-competitive inhibition of mGPDH activity by metformin with K_i ~55 μM has been reported previously (Madiraju et al., 2014). We were unable to demonstrate any sensitivity of the glycerol 3-phosphate-mediated respiration to metformin in concentrations up to 50 mM, which is in accord to more recent studies where the specific activity of mGPDH was assessed (MacDonald et al., 2021; Pecinova et al., 2017). Therefore, it can be concluded that mGPDH is not a target of the antihyperglycemic action of metformin.

There are several studies from different research groups showing the effect of metformin not only on the specific activity of complex I, but also on temperature-dependent reversible A/D transition of mitochondrial complex I (Bridges et al., 2014; Matsuzaki and Humphries, 2015). Therefore, a direct assessment of metformin's effect on the deactivation rate was performed. The A/D conformational changes emerged as an important component of the mitochondrial response to alteration of tissue oxygen level (see (Galkin, 2019) for review). It has been proposed that metformin can trap complex I in a D-like conformation, therefore shifting the A/D steady-state ratio. In the conventionally used NADH oxidase assay, this can look like apparent suppression of the activity. We analyzed the effect of metformin on the temperature-dependent rate of A/D equilibration, when the idle enzyme was preincubated at 30 °C. In this condition, a gradual increase of the fraction of the D-form with a half-time of around 15 min is observed. If metformin specifically affects the A/D steady-state ratio, then an acceleration of deactivation should have been detected when the time course of deactivation is assessed. It should be stressed that we used a very high concentration of metformin during deactivation at 30 °C when the enzyme is idle. These metformin concentrations were enough to inhibit mitochondrial respiration, but were diluted when aliquots of the membranes were added to the activity assay. We found that 50 mM metformin accelerates the deactivation of complex I by around two-fold. This concentration is at least three-orders of magnitude higher than the maximal levels detected in tissues after metformin administration. However, the hydrophilic metformin molecule is positively charged at neutral pH. Therefore, acceleration of the

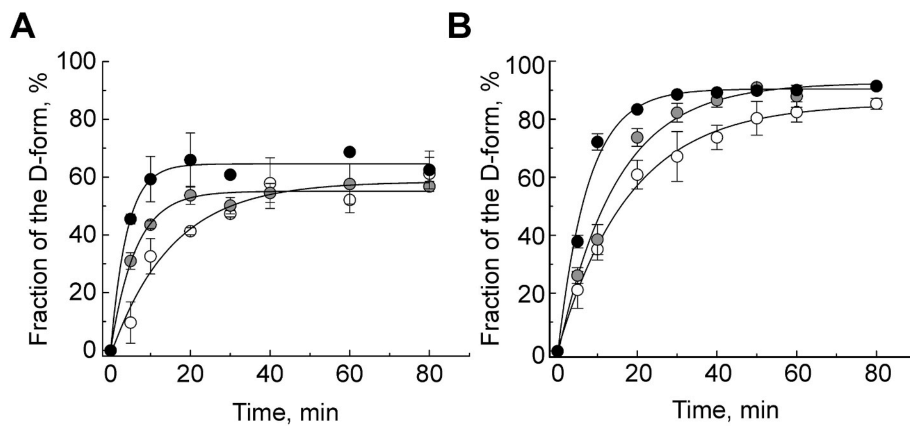

Fig. 5. Effect of metformin on A/D transition of complex I in mitochondria. Permeabilized liver (A) or brain (B) mitochondrial membranes (5 mg/ml) were resuspended in hypotonic SET assay buffer at pH 8.5 and incubated at 30 °C in the absence (open circles) and in the presence of 5 mM or 50 mM metformin (grey and black circles, respectively). Aliquots were taken at the time specified, and the NADH oxidase kinetic assay was performed to determine the fraction of the D-form as described in the Materials and Methods section. Results are mean ± SEM (n = 3–4).

A→D transition by metformin may be of similar nature to the effect of divalent cations (Ca^{++} or Mg^{++}) which decelerate the turnover-dependent activation of mitochondrial complex I, *i.e.* D→A conversion (Kotlyar et al., 1992; Stepanova et al., 2015). Our findings do not exclude interaction of metformin with complex I reaction turnover intermediates but does not support the concept of complex I D-form trapping by metformin. Temperature sensitivity of the metformin inhibition of cellular respiration of intact hepatocytes and oocytes found in initial studies (El-Mir et al., 2000) can be explained by the disruption of inhibitor transport via the plasma membrane (Detaille et al., 2002) rather than direct effects on the temperature-dependent A/D transition.

A short note concerning the physiological relevance of the data reported here is worth discussing. Metformin manifested a protective effect in the *in vivo* models of brain (Arbelaez-Quintero and Palacios, 2017) and cardiac (Higgins et al., 2019) ischemia/reperfusion tissue injury. Metformin's protective role involves several cellular pathways such as activation of AMPK, decrease of oxidative stress, endothelial nitric oxide synthase activation, upregulation of superoxide dismutase, inhibition of mitochondrial permeability transition pore opening, activation of angiogenesis and neurogenesis, autophagia, and apoptosis (Arbelaez-Quintero and Palacios, 2017; Higgins et al., 2019). Limited inhibition of mitochondrial complex I in various *in vivo* models of ischemia/reperfusion of the brain and heart was associated with a decrease in tissue injury (Ambrosio et al., 1993; Chen et al., 2006; Chouchani et al., 2013; Kim et al., 2018; Lesnefsky et al., 2004; Niatsetskaya et al., 2012; Riepe and Ludolph, 1997; Stewart et al., 2009). We propose that mild inhibition of complex I, associated with the attenuation of mitochondrial ROS production in conditions of reverse electron transfer shown here can be considered as the main mechanism of metformin protection against acute tissue injury observed in several *in vivo* ischemia/reperfusion models (Benjanuwattra et al., 2020; Cahova et al., 2015; Leech et al., 2020; Mohsin et al., 2019). At the same time, a relatively mild increase of ROS production with the substrates of forward electron transfer may stimulate mitohormesis and be cytoprotective, leading to an increment in the health of metformin users (De Haes et al., 2014).

Both the mechanism of metformin therapeutic action and the exact mechanism of its inhibition of complex I remain to be elucidated. The results presented here add a new set of reference data in our attempt to dissect the molecular mechanism by which metformin may modulate mitochondrial metabolism leading to the hypoglycemic effect.

CRediT authorship contribution statement

Belem Yoval-Sánchez: designed the experiments; performed the experiments and analyzed the data, Formal analysis, Data curation, Writing – review & editing. **Fariha Ansari:** Formal analysis, Data curation, Writing – review & editing, performed the experiments . **Dmitry Lange:** did, Conceptualization, Writing – review & editing. **Alexander Galkin:** , the data, Writing – review & editing, Writing – review & editing, Writing – original draft, designed the experiments, Writing – review & editing, wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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