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Metformin promotes isolated rat liver mitochondria impairment

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Abstract Metformin, a drug widely used in the treatment of type 2 diabetes, has recently received attention due to the new and contrasting findings regarding its effects on mitochondrial function. In the present study, we evaluated the effect of metformin in isolated rat liver mitochondria status. We observed that metformin concentrations ≥8 mM induce an impairment of the respiratory chain characterized by a decrease in RCR and state 3 respiration. However, only metformin concentrations ≥10 mM affect the oxidative phosphorylation system by decreasing the mitochondrial transmembrane potential and increasing the repolarization lag phase. Moreover, our results show that metformin does not prevent H_2O_2 production, neither protects against lipid peroxidation induced by the pro-oxidant pair ADP/Fe^{2+}. In addition, we observed that metformin exacerbates Ca^{2+}-induced permeability transition pore opening by decreasing the capacity of mitochondria to accumulate Ca^{2+} and increasing the oxidation of thiol groups. Taken together, our results show that metformin can promote liver mitochondria injury predisposing to cell death.

Keywords Liver · Metformin · Mitochondria · Oxidative phosphorylation system · Permeability transition pore · Respiratory chain

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Introduction

Metformin is an oral antihyperglycemic drug widely used in the treatment of type 2 diabetes [1]. The primary action of this biguanide derivative is the suppression of hepatic glucose production and the enhancement of peripheral glucose uptake. Therefore, metformin normalizes plasma glucose levels without any stimulation of insulin production and for this reason it is considered an insulin sensitizer. Despite the success of this antidiabetic agent in the treatment of type 2 diabetes, its mechanism(s) of action remain(s) unclear. Zhou et al. [2] reported that metformin activates AMP-activated protein kinase (AMPK), a major cellular regulator of lipid and glucose metabolism, in hepatocytes. Furthermore, it has been shown that metformin possesses a direct scavenging effect against oxygenated free radicals generated in vitro [3], and decreases intracellular production of reactive oxygen species (ROS) in aortic endothelial cells through the reduction of both NAD(P)H oxidase and/or the mitochondrial respiratory chain pathways [4]. Moreover, it has also been shown that metformin inhibits mitochondrial complex I activity leading to the impairment of mitochondrial function [5–7]. However, it has also been shown that metformin prevents the mitochondrial permeability transition pore (PTP) opening in both permeabilized and intact KB cells [8], and in permeabilized human microvascular endothelial

cells (HMEC-1) [9]. In opposition, Isakovic et al. [10] reported that metformin acts as a PTP inducer in C6 rat glioma cell line.

Mitochondria are intracellular organelles mainly devoted to energy production but they also play a crucial role in the production of reactive species and the onset of cell death [11]. A common mechanism involved in mitochondrial dysfunction is the PTP which is modulated by several physiological factors [12, 13]. Although Ca^{2+} is considered to be the most important inducer, matrix pH, transmembrane electrical potential ($\Delta\Psi_m$), Mg^{2+}, P_i, cyclophilin D, oxidative stress and adenine nucleotides are also effective regulators, while the immunosuppressive agent cyclosporin A (CsA) is considered to be a specific inhibitor of PTP [13–15].

Due to the contradictory results concerning the action of metformin on mitochondrial status, in the present study, we aimed to evaluate the effect of metformin in isolated rat liver mitochondria. Our first task was the evaluation of the impact of increasing concentrations of metformin (0.5, 1, 2, 4, 8 and 10 mM) in the mitochondrial respiratory chain and oxidative phosphorylation system. For that purpose, we analysed the respiratory indexes (RCR and ADP/O), $\Delta\Psi_m$, repolarization level and repolarization lag phase. The second part of this study consisted of the evaluation of the effect of metformin in hydrogen peroxide (H_2O_2) production, lipid peroxidation and Ca^{2+}-induced PTP. The lipid peroxidation was evaluated by analysing TBARS formation and oxygen consumption, while the induction of PTP was characterized by analysing the $\Delta\Psi_m$, Ca^{2+} fluxes and protein thiol groups' oxidation. The choice of rat liver mitochondria as experimental model was based on the fact that liver is the major site for the uptake and metabolism of drugs, liver mitochondria being primary targets for their hepatotoxicity [16].

Materials and methods

Materials

Metformin (1,1-dimethylbiguanide hydrochloride) was obtained from Sigma (Portugal). All the other chemicals were of the highest grade of purity commercially available. In the present study, we used metformin dissolved in distilled H_2O.

Animals

Male Wistar rats (10 weeks old) were housed in our animal colony (Laboratory Research Center, Faculty of Medicine, Coimbra, Portugal). They were maintained under controlled light (12 h day/night cycle) and humidity with free access (except in the fasting period) to water and powdered rodent chow (URF1; Charles River). Adhering to procedures approved by the Institutional Animal Care and Use Committee, the animals were killed by cervical displacement and decapitation.

Isolation of liver mitochondria

Mitochondria were isolated from liver by conventional methods [17] with slight modifications. Briefly, the liver mitochondria were isolated in a medium containing 250 mM sucrose, 10 mM Hepes, 0.5 mM EGTA and 0.1% fat-free bovine serum albumin (BSA) (pH 7.4). EGTA and BSA were omitted from the final washing medium. The mitochondrial pellet was washed twice and suspended in the washing medium. Mitochondrial protein was determined by the biuret method [18] calibrated with BSA.

Mitochondrial respiration measurements

Oxygen consumption of isolated liver mitochondria was monitored polarographically with a Clark oxygen electrode (YSI Model 5331, Yellow Springs Inst) connected to a suitable recorder in a 1 ml thermostated, water-jacketed closed chamber with magnetic stirring [19]. The reactions were carried out at 30°C in 1 ml of the reaction medium (130 mM sucrose, 50 mM KCl, 2.5 mM $MgCl_2$, 2.5 mM KH_2PO_4, 100 μM EGTA, 5 mM Hepes and pH 7.4). Mitochondria (1 mg/ml) were incubated with metformin 1 min before the addition of 5 mM glutamate/2.5 mM malate.

Membrane potential ($\Delta\Psi_m$) measurements

The mitochondrial transmembrane potential ($\Delta\Psi_m$) was monitored by evaluating transmembrane distribution of the lipophilic cation TPP^+ (tetraphenylphosphonium) with a TPP^+-selective electrode prepared according to Kamo et al. [20] using an Ag/AgCl-saturated electrode (Tacussel, model MI 402) as reference. TPP^+ uptake has been measured from the decreased TPP^+ concentration in the medium sensed by the electrode. The potential difference between the selective electrode and the reference electrode was measured with an electrometer and recorded continuously in a Linear 1200 recorder. The voltage response of the TPP^+ electrode to log[TPP^+] was linear with a slope of 59 ± 1, in a good agreement with the Nernst equation. Reactions were carried out in a chamber with magnetic stirring in 1 ml of the standard medium (130 mM sucrose, 50 mM KCl, 2.5 mM $MgCl_2$, 2.5 mM KH_2PO_4, 100 μM EGTA, 5 mM Hepes

and pH 7.4 supplemented with 2 μM rotenone) containing 3 μM TPP^+. This TPP^+ concentration was chosen in order to achieve high sensitivity in measurements and to avoid possible toxic effects on mitochondria [21]. The $\Delta\Psi_m$ was estimated by the equation:

$$\Delta\Psi m\ (mV) = 59\ \log(v/V)\ -\ 59\ \log(10^{\Delta E/59} - 1)$$

as indicated by Kamo et al. [20] and Muratsugu et al. [22]. v, V, and ΔE stand for mitochondrial volume, volume of the incubation medium and deflection of the electrode potential from the baseline, respectively. This equation was derived assuming that TPP^+ distribution between the mitochondria and the medium follows the Nernst equation, and that the law of mass conservation is applicable. A matrix volume of 1.1 μl/mg protein was assumed. No correction was made for the "passive" binding contribution of TPP^+ to the mitochondrial membranes, because the purpose of the experiments was to show relative changes in potentials rather than absolute values. As a consequence, we can anticipate a slight overestimation on $\Delta\Psi_m$ values. However, the overestimation is only significant at $\Delta\Psi_m$ values below 90 mV, therefore, far from our measurements. Mitochondria (1 mg/ml) were incubated with metformin for 1 min before the addition of 5 mM glutamate/2.5 mM malate. After a steady-state distribution of TPP^+ had been reached (ca. 1 min of recording), $\Delta\Psi_m$ fluctuations recorded. In the PTP experiments, two or three pulses of Ca^{2+} (1st pulse: 50 nmol Ca^{2+}/mg protein; 2nd and 3rd pulses: 40 nmol Ca^{2+}/mg protein) were added, and $\Delta\Psi_m$ was recorded. CsA was added 2 min before Ca^{2+} addition.

Measurement of hydrogen peroxide (H_2O_2) levels

The H_2O_2 levels were measured fluorimetrically using a modification of the method described by Barja [23]. Briefly, liver mitochondria (0.375 mg) were incubated at 30°C with 5 mM glutamate/2.5 mM malate in 1.5 ml of phosphate buffer, pH 7.4, containing 0.1 mM EGTA, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 145 mM KCl, 30 mM Hepes, 0.1 mM homovalinic acid and 6 U/ml horseradish peroxidase in the absence or presence of metformin. After 15 min, the reaction was stopped with 0.5 ml cold stop solution (0.1 M glycine, 25 mM EDTA-NaOH, pH 12). Fluorescence of supernatants was determined at 312 nm excitation and 420 nm emission wavelengths. The H_2O_2 levels were calculated using a standard curve of H_2O_2.

Measurement of lipid peroxidation

The extent of lipid peroxidation was evaluated by oxygen consumption monitored polarographically at 30°C using a Clark-type oxygen electrode (YSI Model 5331, Yellow Springs Inst) placed in a glass chamber equipped with magnetic stirring [24]. Reactions were carried out in 1 ml of reaction medium containing 175 mM KCl, 10 mM Tris–HCl, pH 7.4 and 1 mg of protein. Reactions were started by the addition of 1 mM ADP/0.1 mM Fe^{2+}, and the changes in oxygen tension were calculated assuming a concentration of 240 nmol O_2/ml. Mitochondria were incubated with metformin 1 min before the addition of ADP/Fe^{2+}. The extent of lipid peroxidation was also determined by measuring thiobarbituric acid reactive substances (TBARS), using the thiobarbituric acid assay, according to a modified procedure as described elsewhere [24]. The amount of TBARS formed was calculated using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ and expressed as nmol TBARS/mg protein.

Measurements of Ca^{2+} fluxes

Mitochondrial Ca^{2+} fluxes were measured by monitoring the changes in Ca^{2+} concentration in the reaction medium using a Ca^{2+}-selective electrode [25]. The reactions were conducted in an open vessel with magnetic stirring in 1 ml of the standard reaction medium supplemented with 5 mM glutamate/2.5 mM malate and 90 nmol/mg protein of $CaCl_2$. The reaction started with the addiction of mitochondria. Metformin was added 1 min after mitochondria addition. CsA was added 2 min before Ca^{2+} addition.

Mitochondrial thiol oxidation

A variation of Ellman's method was used to determine the mitochondrial content in protein thiol groups [26]. At the end of the $\Delta\Psi_m$ experiments, 750 μl of each mitochondrial suspension were frozen and thawed three times. Then, 750 μl of sulphosalicilic acid 4% were added. The samples were submitted to centrifugation at 10,000 rpm for 15 min. The supernatant was removed and the pellet was suspended in 1 ml of phosphate buffer 100 mM, pH 8. The suspension was sonicated and diluted to 2.6 ml in phosphate buffer medium containing 385 mM DTNB [5,5′-dithiobis (2-nitrobenzoic) acid]. After 15 min of reaction, the absorption was measured at 412 nm, and the results were expressed as percentage of control.

Statistical analysis

Results are presented as mean ± SEM of the indicated number of experiments. Statistical significance was determined using the student *t*-test and one-way ANOVA test for multiple comparisons, followed by the posthoc Tukey–Kramer test.

Results

High concentrations of metformin impair mitochondrial respiration

We observed that mitochondria energized with 5 mM glutamate/2.5 mM malate, a substrate for complex I, and pre-incubated with concentrations of metformin ≥8 mM presented a significant reduction on state 3 respiration (ADP-stimulated respiration), while state 4 (substrate alone) was only slightly reduced in the presence of the same concentrations of metformin when compared with the control condition (Fig. 1a and b). Accordingly, RCR was significantly reduced in the presence of metformin concentrations ≥8 mM when compared with control condition (Fig. 1c). However, ADP/O ratio remained statistically unchanged (Fig. 1d).

High concentrations of metformin impair the oxidative phosphorylation system

$\Delta\Psi_m$ is at the centre of the cell's interactions, controlling, among other processes, ATP synthesis, mitochondrial Ca^{2+} accumulation and superoxide ($O_2^{\bullet-}$) generation. In the control conditions, the $\Delta\Psi_m$ developed by liver mitochondria after energization with 5 mM glutamate/2.5 mM malate was –216.3 ± 3.4 mV. As shown in Table 1, only the highest concentration of metformin tested, (10 mM) induced a significant decrease in the $\Delta\Psi_m$ and a significant increase in the repolarization lag phase (time necessary for ADP phosphorylation), when compared with control condition. However, the repolarization level (capacity of mitochondria to re-establish the $\Delta\Psi_m$ after the ADP phosphorylation) remained statistically unchanged (Table 1).

Metformin does not protect against oxidative stress

The production of H_2O_2 by mitochondria gives an indication about the propensity of mitochondria to originate and/or exacerbate oxidative stress. At control conditions, energized mitochondria produced 1964 ± 406.3 pmol H_2O_2/mg protein/15 min. We observed that metformin induced a slight, although not significant, increase in H_2O_2 production (Fig. 2). The H_2O_2-scavanging enzyme catalase was used as positive control. As expected, catalase significantly decreased the production of H_2O_2 when compared with the other conditions tested (Fig. 2).

Oxygen consumption and TBARS levels were evaluated to determine the extent of lipid peroxidation induced by ADP/Fe^{2+}. We observed that metformin (independently of the concentrations tested) did not protect against lipid

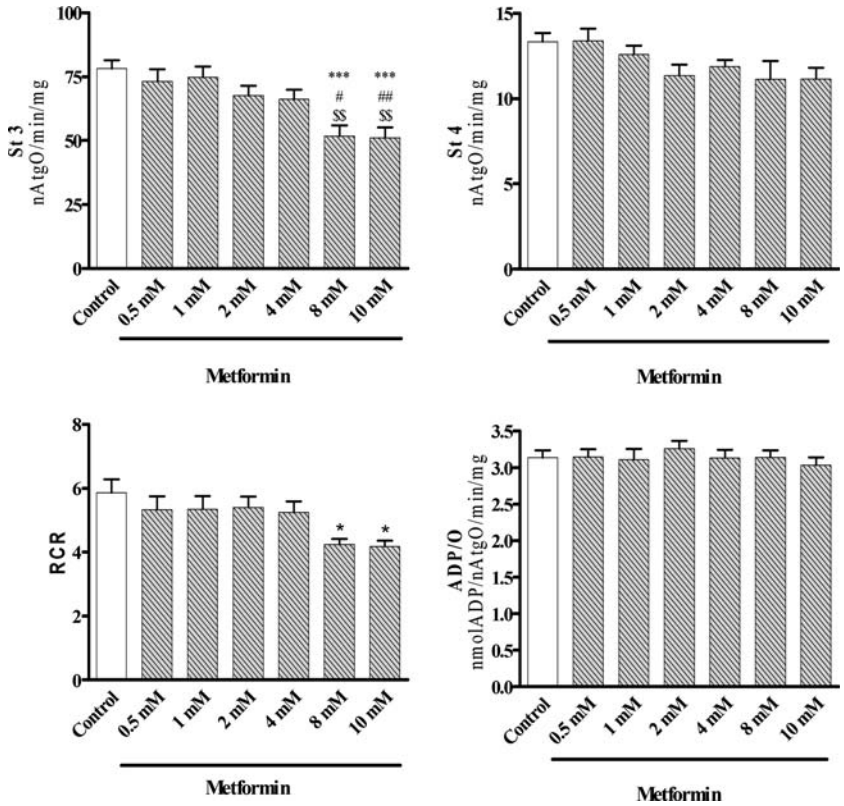

Fig. 1 Effect of metformin on states 3 (**a**) and 4 (**b**) of respiration, RCR (**c**) and ADP/O ratio (**d**) of freshly isolated liver mitochondria. Mitochondria (1 mg) in 1 ml of the reaction medium were energized with 5 mM glutamate/2.5 mM malate. Mitochondria were incubated with metformin for 1 min, at 30°C, before mitochondria energization. Data are the mean ± SEM of six independent experiments. Statistical significance: ***$P < 0.001$; *$P < 0.05$, when compared with control condition (mitochondria without metformin). ##$P < 0.01$; #$P < 0.05$, when compared with mitochondria incubated with 0.5 mM metformin. $$$P < 0.01$, when compared with mitochondria incubated with 1 mM metformin

Table 1 Effect of metformin in the mitochondrial oxidative phosphorylation system ($\Delta\Psi_m$, repolarization level and repolarization lag phase)

	Metformin						
	Control	0.5 mM	1 mM	2 mM	4 mM	8 mM	10 mM
$\Delta\Psi_m$ (–mV)	216.3 ± 3.40	211.3 ± 7.49	213.6 ± 4.85	211.2 ± 5.73	214.3 ± 3.28	211.7 ± 2.32	208.6 ± 3.43**
Repolarization level (–mV)	175.2 ± 3.36	172.8 ± 3.18	174.0 ± 3.76	174.6 ± 3.45	173.5 ± 2.50	175.7 ± 2.58	174.1 ± 4.02
Repolarization lag phase (min)	0.86 ± 0.19	0.96 ± 0.25	0.98 ± 0.23	1.11 ± 0.12	1.10 ± 0.14	1.14 ± 0.18	1.19 ± 0.15**

The oxidative phosphorylation parameters were evaluated using freshly isolated liver mitochondria (1 mg) in 1 ml of the reaction medium supplemented with 3 μM $TPP^+$and energized with 5 mM glutamate/2.5 mM malate. Mitochondria were incubated with metformin for 1 min, at 30°C, before mitochondria energization. Data are the mean ± SEM of six independent experiments. Statistical significance: $^{**}P < 0.01$ when compared to control condition (mitochondria without metformin)

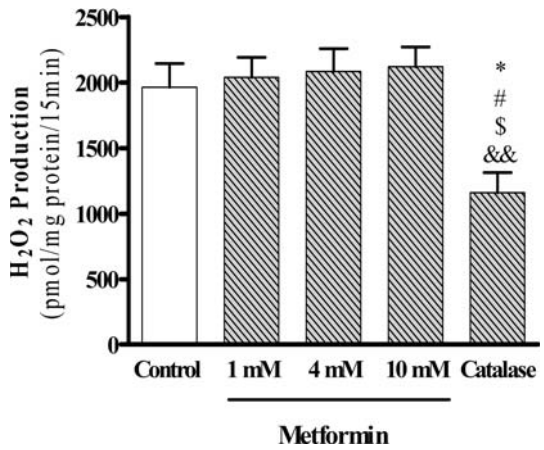

Fig. 2 Effect of metformin on hydrogen peroxide production. Liver mitochondria were incubated under conditions as described in Material and methods section. Data are the mean ± SEM of four independent experiments. $^*P < 0.05$, when compared with control condition; $\$P < 0.05$, when compared with mitochondria incubated with 1 mM metformin; $\#P < 0.05$, when compared with mitochondria incubated with 4 mM metformin; $\&\&P < 0.01$, when compared with mitochondria incubated with 10 mM metformin

peroxidation induced by the pro-oxidant pair ADP/Fe^{2+} (Fig. 3a and b).

Metformin exacerbates PTP induction

The $\Delta\Psi_m$ drop is a typical phenomenon that follows the induction of the PTP. In control conditions (Fig. 4a), we observed that the addition of mitochondrial substrate induced a $\Delta\Psi_m$ of ≈–215 mV. The first pulse of Ca^{2+} (50 nmol/mg protein) leads to a rapid depolarization (decrease of $\Delta\Psi_m$) followed by a smaller repolarization (recover of $\Delta\Psi_m$). The depolarization was due to the entry of Ca^{2+} into the electronegative mitochondrial matrix, followed by the efflux of H^+ in an attempt to restore the $\Delta\Psi_m$. Then, a second pulse of 40 nmol Ca^{2+}/mg protein leads to a rapid depolarization followed by a partial repolarization, and since mitochondria were incapable to sustain the accumulated Ca^{2+}, they depolarized completely, which is an indication of the PTP induction (Fig. 4a). Mitochondria can tolerate a certain amount of Ca^{2+}, but ultimately their capacity to adapt to Ca^{2+} loads is overwhelmed, and mitochondria depolarize completely due to a profound change in the inner membrane permeability. As observed in Fig. 4, metformin exacerbated the opening of PTP in a concentration-dependent manner by decreasing the repolarization capacity of mitochondria after the second addition of Ca^{2+}, i.e., metformin decreased the time needed for pore induction. Since the mitochondrial collapse of the $\Delta\Psi_m$, associated to Ca^{2+} overload, is related to the PTP opening, experiments were performed to further confirm the induction of the PTP opening by studying the Ca^{2+} retention by energized mitochondria. Figure 4b shows that mitochondria incubated with 90 nmol Ca^{2+}/mg protein and energized with 5 mM glutamate/2.5 mM malate, rapidly accumulated the Ca^{2+} added to the reaction medium, but became unable to retain the accumulated Ca^{2+} due to the opening of PTP (Fig. 4b). Interestingly, we observed that metformin exacerbated Ca^{2+}-induced PTP opening in a concentration-dependent manner (Fig. 4b). The presence of 0.85 μM CsA, the specific inhibitor of PTP, added 2 min prior to Ca^{2+}, afforded a clear protection of mitochondria (Fig. 4a and b), since mitochondria was able to accumulate and retain the Ca^{2+} present in the medium. Opening of PTP is also stimulated by the alteration of protein thiol redox status (–SH/–SS–). Figure 5 shows that 50 nmol of Ca^{2+} induced only a slight decrease in the amount of thiol groups. However, 90 nmol of Ca^{2+}/mg protein induced a significant decrease in thiol groups when compared with control condition. Similarly, we observed that mitochondria incubated with metformin and 50 nmol Ca^{2+}/mg protein presented a significant decrease of thiol groups when compared with control condition (Fig. 5). However, metformin per se did not affect thiol groups (data not shown).

Discussion

This study shows that only high concentrations of metformin impair liver mitochondrial respiratory chain and

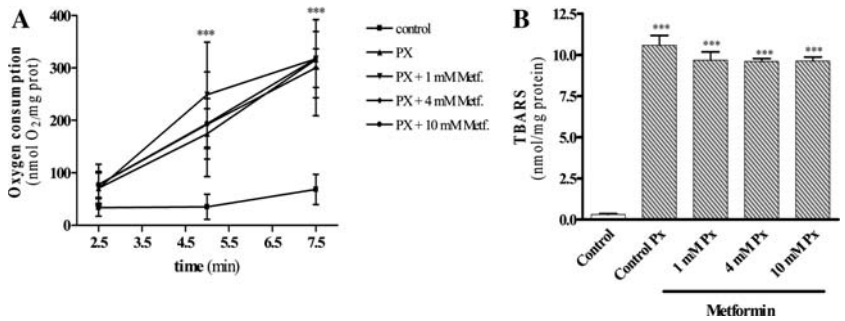

Fig. 3 Effect of metformin on lipid peroxidation (Px): Oxygen consumption (**a**) and TBARS levels (**b**). Mitochondria were incubated at 1 mg/ml under standard conditions as described in the Material and methods section. Mitochondria were incubated with metformin for 1 min, at 30°C, before the addition of ADP/Fe^{2+}. Data shown represent mean ± SEM from six animals for each condition studied. Statistical significance: ***$P < 0.001$, when compared with control condition

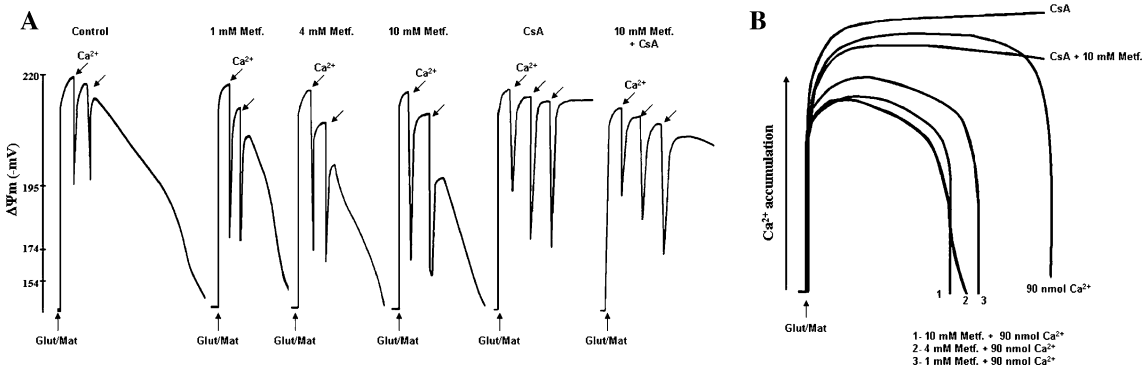

Fig. 4 Effect of metformin on mitochondrial permeability transition pore induction: susceptibility to Ca^{2+} addition [$\Delta\Psi_m$ (**a**) and calcium fluxes (**b**)]. Freshly isolated liver mitochondria (1 mg) in 1 ml of the reaction medium supplemented with 3 μM TPP^+ were energized with 5 mM glutamate/2.5 mM malate. Ca^{2+} (1st pulse: 50 nmol Ca^{2+}/mg protein; 2nd and 3rd pulses: 40 nmol Ca^{2+}/mg protein) was added 1.5 min after mitochondria energization. 0.85 μM CsA was added 2 min before Ca^{2+} addition. The traces are typical of six independent experiments

oxidative phosphorylation system. However, we observed that metformin exacerbates the PTP opening in a concentration-dependent manner. Furthermore, metformin is unable to protect against oxidative stress-related events.

Despite the progress made in recent studies [2, 5, 8, 27], the cellular action of metformin is still not fully understood. Previous data from the literature indicates that metformin inhibits the enzymatic activity of complex I, and impairs cell respiration [5–7]. Owen et al. [6] suggested that the inhibition of mitochondrial complex I contributes to metformin's antidiabetic action by restraining hepatic gluconeogenesis while increasing glucose utilization in peripheral tissues. El-Mir et al. [5] reported that metformin (1–10 mM) mildly inhibits the respiratory chain, but only in intact cells and not in isolated mitochondria or permeabilized cells. However, the lack of effect of metformin on isolated mitochondria has been challenged by results showing that metformin can directly inhibit mitochondrial complex I [6, 28]. Similarly, we observed that metformin affects directly isolated liver mitochondria by decreasing state 3 of respiration (Fig. 1a) and RCR (Fig. 1b). In contrast to Owen et al. [6], who reported that the addition of metformin to rat liver mitochondria oxidizing 5 mM glutamate/1 mM malate gives no inhibition over a period of 5 min at 30°C, even at concentrations as high as 10 mM, we observed an immediate effect of metformin. Furthermore, we observed that the impairment of the respiratory chain occur from concentrations of metformin above 4 mM. In opposition, Brunmair et al. [28] reported that metformin in the range of 3–30 μM already inhibits isolated liver mitochondria. The discrepancies between studies could be due to differences in the methodology used, such as mitochondria isolation procedures and differences in the temperature used in the assays. It has been previously demonstrated that the action of metformin is temperature-dependent [5]. We also observed that high concentrations of metformin impair the oxidative phosphorylation system (Table 1), that is in accordance with a previous study demonstrating that metformin causes mitochondrial depolarization [10].

The plasma concentrations of metformin found in patients are about 10–20 μM [1, 29, 30]. However, the

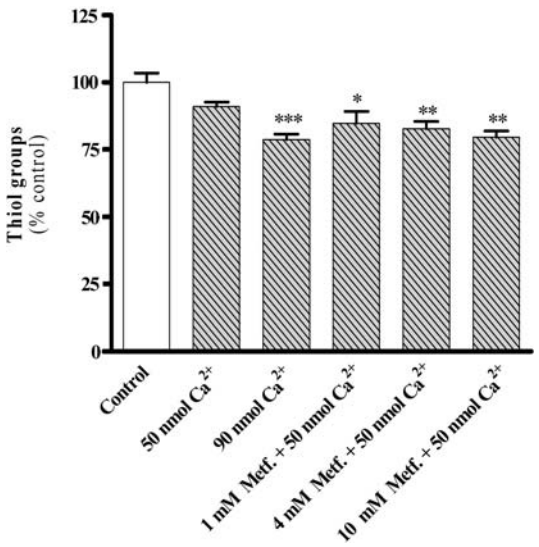

Fig. 5 Effect of metformin on mitochondrial protein thiol groups. Freshly isolated mitochondria (1 mg) in 1 ml of the reaction medium were energized with 5 mM glutamate/2.5 mM malate. Mitochondria were incubated with metformin for 1 min, at 30°C, before mitochondria energization. 90 nmol Ca^{2+}/mg protein was added after mitochondria energization. Data are the means ± SEM of four independent experiments. $***P < 0.001$; $**P < 0.01$; $*P < 0.05$, when compared with control condition

liver receives the majority of the blood via the portal vein, which may contain concentrations of metformin substantially higher than those present in the general circulation [31]. Thus, a significant impact of metformin in liver mitochondria of patients treated with this drug would be predicted. Additionally, the positive charge on metformin could account for its accumulation within the matrix of energized mitochondria driven by the $\Delta\Psi_m$, and such energy-dependent accumulation of metformin within the matrix has been shown to occur experimentally [32]. Indeed, it has been estimated that metformin accumulate 1,000-fold within the mitochondrial matrix [6]. In this line, the range of metformin concentrations used in this study embraces concentrations that approach the therapeutic ones. The highest concentrations of metformin (above the therapeutic range) were used to evaluate the potential concentration-dependent effects of this drug.

Lipid peroxidation is a process whereby free radicals "steal" electrons from the lipids, resulting in cell damage and increased production of free radicals [33]. Recently, Majithiya and Balaraman [34] reported that metformin protects liver, kidney and aorta tissues against lipid peroxidation promoted by streptozotocin-induced diabetes. It has also been shown that metformin decreases the level of lipid peroxidation markers in low-density and high-density lipoprotein particles obtained from adult type 2 diabetic patients [35]. However, our in vitro study shows that metformin does not protect against mitochondrial lipid peroxidation induced by the pro-oxidant pair ADP/Fe^{2+} (Fig. 3a and b), suggesting that the decrease in lipid peroxidation observed in the previous studies are not the result of a direct action of metformin but, probably, the result of its antihyperglycemic effects or the activation of AMPK [36]. Previously, Bonnefont–Rousselot et al. [3] reported that metformin in vitro is able to scavenge hydroxyl ($HO^{\bullet}$) but not $O_2^{-\bullet}$, and that H_2O_2 did not react with metformin. Our results show that metformin does not prevent H_2O_2 production (Fig. 2) and, instead, it elicits a slight increase in the levels of this reactive specie, probably resulting from the inhibition of mitochondrial complex I (Fig. 1a and b). A recent study showed that metformin in confluent C6 cultures causes massive induction of caspase-dependent apoptosis, associated with c-Jun N-terminal kinase (JNK) activation, mitochondrial depolarization and oxidative stress [10]. Previously, Kowaltowski et al. [37] suggested that the rise in the production of endogenous mitochondrial ROS, including H_2O_2, facilitates the process of PTP opening. Indeed, we observed that metformin exacerbates Ca^{2+}-induced PTP opening (Figs. 4 and 5) probably due to the slight increase in H_2O_2 production (Fig. 2), mitochondrial depolarization (Table 1) and inhibition of the respiratory chain (Fig. 1a and c). It is well known that the collapse of $\Delta\Psi_m$ and the uncoupling of the electron transport chain promotes the PTP [11, 38]. The PTP causes a non-selective permeabilization of the inner mitochondrial membrane typically promoted by the accumulation of excessive quantities of Ca^{2+} ions and stimulated by a variety of compounds or conditions [39]. In this study, the classic inductor Ca^{2+} was used to induce the PTP opening (Figs. 4 and 5). Ca^{2+} uptake is concentration-dependent, with the higher concentrations of metformin leading to a lower amount of Ca^{2+} being accumulated by mitochondria, which indicates a continuous cycling of Ca^{2+} due to the PTP induction. These results are in accordance with recent data demonstrating that metformin-triggered apoptosis is completely prevented by the specific inhibitor of the PTP, CsA [10]. We also observed that the effect of metformin in Ca^{2+}-induced PTP opening is prevented by CsA (Figs. 4 and 5). The PTP opening allows the equilibration of ions and respiratory substrate between cytosol and mitochondrial matrix leading to the reduction of $\Delta\Psi_m$ [40] that is linked to depletion of GSH [41]. Indeed, the mitochondrial pore induction is intimately associated with GSH and protein thiol redox state [42]. When protein thiol groups from the inner mitochondrial membrane are oxidized, the pore complex suffers conformational changes resulting in a large nonselective permeabilization of the mitochondrial membrane [43]. Accordingly, we observed that metformin potentiates the effect of Ca^{2+} on thiol groups' oxidation (Fig. 5).

Altogether, our results show that metformin can promote liver mitochondria injury and predispose to cell death,

which is in accordance with previous case reports [44, 45] describing the existence of metformin-induced hepatotoxicity. Since liver mitochondria of diabetic patients under metformin treatment are potentially exposed to high levels of this antidiabetic agent, the potential side effects of metformin should be carefully considered.

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