

Original Paper

Glibenclamide Mimics Metabolic Effects of Metformin in H9c2 Cells

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Key Words

Glibenclamide • Metformin • ATP • Cardiomyocyte • H9c2 • Metabolism

Abstract

Background: Sulfonylureas, such as glibenclamide, are antidiabetic drugs that stimulate beta-cell insulin secretion by binding to the sulfonylureas receptors (SURs) of adenosine triphosphate-sensitive potassium channels (K_{ATP}). Glibenclamide may be also cardiotoxic, this effect being ascribed to interference with the protective function of cardiac K_{ATP} channels for which glibenclamide has high affinity. Prompted by recent evidence that glibenclamide impairs energy metabolism of renal cells, we investigated whether this drug also affects the metabolism of cardiac cells. **Methods:** The cardiomyoblast cell line H9c2 was treated for 24 h with glibenclamide or metformin, a known inhibitor of the mitochondrial respiratory chain. Cell viability was evaluated by sulforhodamine B assay. ATP and AMP were measured according to the enzyme coupling method and oxygen consumption by using an amperometric electrode, while Fo-F1 ATP synthase activity assay was evaluated by chemiluminescent method. Protein expression was measured by western blot. **Results:** Glibenclamide deregulated energy balance of H9c2 cardiomyoblasts in a way similar to that of metformin. It inhibited mitochondrial complexes I, II and III with ensuing impairment of oxygen consumption and ATP synthase activity, ATP depletion and increased AMPK phosphorylation. Furthermore, glibenclamide disrupted mitochondrial subcellular organization. The perturbation of mitochondrial energy balance was associated with enhanced anaerobic glycolysis, with increased activity of phosphofructo kinase, pyruvate kinase and lactic dehydrogenase. Interestingly, some additive effects of glibenclamide and metformin were observed. **Conclusions:** Glibenclamide deeply alters cell metabolism in cardiac cells by impairing mitochondrial organization and function. This may further explain the risk of cardiovascular events associated with the use of this drug, alone or in combination with metformin.

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Introduction

Concern exists about the cardiovascular safety of the old – yet still widely prescribed – class of antidiabetic drugs, sulfonylureas [1]. Among the sulfonylureas currently used in clinical practice, glibenclamide in particular has been associated with cardiovascular events, including acute myocardial infarction, and mortality [2, 3], consistent with data showing that glibenclamide may have cardiotoxic effects [4]. By contrast, other sulfonylureas, such as gliclazide, seem to be safer in both epidemiological studies [3] and experimental models [4]. Interestingly, further increased cardiovascular risk has been reported for glibenclamide when it is associated with metformin [2], overriding the favourable effects on cardiovascular system [5].

In pancreatic beta cells, sulfonylureas act by binding to and closing ATP-sensitive potassium channels (K_{ATP}), the result being an increase in intracellular K^+ ions, triggering of membrane depolarization, opening of voltage-dependent Ca^{2+} channels, intracellular Ca^{2+} influx and insulin secretion [6].

Cardiac toxicity of sulfonylureas has been related to the closure of specific K_{ATP} channels expressed in the heart [7]. Besides K_{ATP} channels, sulfonylureas also inhibit chloride and calcium channels inducing complex ionic events [8, 9], which regulate cell membrane potential during oxidative stress and ischemic preconditioning. The difference in the profile of cardiovascular activity among glibenclamide and other sulfonylureas may, at least in part, depend on differences in the affinity for the K_{ATP} channels isoforms expressed by cardiomyocytes.

K_{ATP} channels are expressed in both plasma and mitochondrial membranes. Recently, it has been shown that glibenclamide binds to the inner mitochondria membrane of renal tubular cells causing depletion of intracellular ATP [10]. Based on this finding, we hypothesized that glibenclamide could also alter mitochondrial function and ATP production in cardiac cells.

We compared the effects of glibenclamide and metformin (which is known to inhibit mitochondrial respiration and ATP synthesis) on cellular energy balance in the cardiomyoblast cell line H9c2. We found that glibenclamide, similar to metformin, disrupts mitochondrial reticulum organization, impairs mitochondrial oxidative phosphorylation, decreases ATP concentration, and activate phosphofructo-kinase (PFK), pyruvate kinase (PK) and lactic dehydrogenase (LDH), key enzymes of the anaerobic glycolytic pathway.

Materials and Methods

Reagents

Metformin, glibenclamide and gliclazide were provided by Sigma-Aldrich (St. Louis, MO, USA).

Cell Culture

H9c2 cells (RRID: CVCL_286) were obtained from ATCC and cultured in low glucose Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin sulphate at 37°C in 5% CO₂ as previously described [11].

AMP/ATP Ratio Assay

Cells were washed twice with PBS, lysed with 2.5% PCA and sonicated in ice three times for 10 seconds. After centrifugation, supernatants containing PCA were collected and neutralized with K_2CO_3 . ATP and AMP were measured according to the enzyme coupling method, following NADP/NADH reduction/oxidation at 340 nm; 20 µg of total protein were used for both assays. For ATP quantification assay, the medium contained 100 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 50 mM glucose, and 0.2 mM NADP. The assay started after the addition of 4 µg of purified hexokinase plus glucose-6-phosphate dehydrogenase. AMP concentration was assayed using the following medium: 100 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 10 mM phosphoenolpyruvate, 0.15 mM NADH, and 0.2 mM ATP. The assay started after the addition of 4 µg of purified pyruvate kinase plus lactate dehydrogenase and 2 µg of adenylate kinase [12, 13].

Sulforodhamine B assay.

5×10^5 H9C2 cells were plated in 96 mm Petri dishes in regular medium. 24 h later, the cell medium was removed, cells were washed twice with PBS and were incubated either in regular medium or in starvation medium. 24 h later cells were treated or not with metformin, glibenclamide or gliclazide. 24 hours later, cell medium was removed and culture plates were fixed with cold 3% trichloroacetic acid at 4°C for 30 minutes, washed with cold water and dried overnight. Finally, the plates were stained with 0.4% sulforodhamine B (SRB) in 1% acetic acid, washed four times with 1% acetic acid to remove unbound dye, dried overnight and solubilized with Tris base 10 mM in 200 ul. Absorbance measurements at 560nm were made using a microplate reader.

Oxygen consumption measurements

Oxygen consumption was measured at 37°C in a closed chamber, using an amperometric electrode (Unisense Microrespiration, Unisense A/S, Denmark). Cells were permeabilized with 0.03mg/ml digitonin for 1 min, centrifuged for 9 min at 1000 rpm and resuspended in the appropriate buffer. The same solution was used in the oxymetric measurements. For each experiment, 200, 000 cells were used. Ten mM pyruvate plus 5 mM malate were added to stimulate the pathway composed by complexes I, III and IV, while 20 mM succinate was added to stimulate the pathway formed by complexes II, III and IV. To observe the ADP-stimulated respiration rates, 0.08 mM ADP was added after pyruvate and malate and succinate addition. The respiratory rates were expressed as nmol O₂/min/106 cells [12, 13].

Fo-F1 ATP synthase activity assay.

To evaluate the Fo-F1 ATP synthase activity, 200, 000 H9c2 cells were incubated for 10 min at 37°C in a medium containing: 10 mM Tris-HCl pH 7.4, 100 mM KCl, 5 mM KH₂PO₄, 1 mM EGTA, 2.5 mM EDTA, 5 mM MgCl₂, 0.6 mM ouabain, and 25 mg/ml ampicillin. Afterward, ATP synthesis was induced by the addition of 10 mM pyruvate plus 5 mM malate or 20 mM succinate, to stimulate the pathways composed by complexes I, III and IV pathway or complexes II, III and IV, respectively. To start the reaction 0.08 mM ADP was added. The reaction was monitored for two minutes, every 30 sec, in a luminometer (GloMax® 20/20n Luminometer, Promega Italia, Milano, Italy), by the luciferin/luciferase chemiluminescent method, with ATP standard solutions between 10⁻⁸ and 10⁻⁵ M (luciferin/luciferase ATP bioluminescence assay kit CLSII, Roche, Basel, Switzerland). Data were expressed as nmol ATP produced/min/10⁶ cells [14].

The oxidative phosphorylation efficiency (P/O ratio) was calculated as the ratio between the concentration of produced ATP and the amount of consumed oxygen in the presence of respiring substrates and ADP. When oxygen consumption is completed devoted to energy production, the P/O ratio is around 2.5 and 1.5 after pyruvate + malate or succinate addition, respectively [15].

Respiring Complexes assays

The activity assay of the four redox complexes was measured on 50 µg of total protein homogenate [16]. Complex I (NADH-ubiquinone oxidoreductase) was assayed following the reduction of ferricyanide at 420 nm. Complex II (Succinic dehydrogenase) activity was measured at 600 nm, in 2 mM EDTA, 0.2 mM ATP, 20 mM succinate, 0.5 mM cyanide, 80 µM dicloroindophenol (DCIP), 50 µM decylubiquinone, 40 µM antimycin A, 10 µM rotenone and 10 mM phosphate buffer, pH 7.2. To measure complex III (Cytochrome c reductase) activity was followed the reduction of oxidized Cytochrome c at 550 nm, in the presence of 0.7 mM NADH, to provide the electrons, through Complex I activity, necessary for cytochrome c reduction. Complex IV (Cytochrome c oxidase) was assayed following the oxidation of ascorbate-reduced Cytochrome c at 550 nm.

Western Blotting Analysis

H9c2 cell extracts were resolved by SDS-PAGE and transferred to a nitrocellulose membrane as previously described [17]. Membranes were incubated overnight at 4 °C and probed with the following antibodies: anti pAMPK and AMPK purchased from Cell Signaling Technologies (Boston, MA), anti ND1 and anti ATP synthase (ATP5B) purchased from Sigma (St. Louis, USA) and anti actin purchased from Abcam (Cambridge, UK). Primary antibodies were detected using a goat anti-mouse or goat anti-rabbit IgG HRP conjugated, and developed with ECL substrate (Thermo Fisher Scientific, IL, USA).

Evaluation of glycolysis.

The activities of PFK, PK and LDH were measured at room temperature on 20 µg of preterm or term UC-MSC total protein. Enzymatic activity was expressed as mU/mg of total protein (nmol/min/mg of protein). NADH molar extinction coefficient was considered 0.622 mM⁻¹ cm⁻¹, at 340 nm. The reaction mixtures used for the determination of each enzyme activity were the following [18]:

PFK (EC 2.7.1.11): 100 mM Tris-HCl pH 7.4, 2 mM MgCl₂, 5 mM KCl, 2 mM fructose 6-phosphate, 1 mM ATP, 0.5 mM phosphoenolpyruvate, 0.2 mM NADH, and 2 IU/ml of PK and LDH.

PK (EC 2.7.1.40): 100 mM Tris-HCl pH 7.6, 2.5 mM MgCl₂, 10 mM KCl, 0.6 mM phosphoenolpyruvate, 0.2 mM NADH, 5 mM ADP, and 1 IU/ml of LDH.

LDH (EC 1.1.1.27): 100 mM Tris-HCl pH 7.4, 0.2 mM NADH, and 5 mM pyruvate.

Immunofluorescence

H9c2 cells were plated on glass coverslips and treated for 24h with metformin (2, 5 mM MTF), glibenclamide (100 µGLB), or both molecules (2, 5 mM MTF/100 µM GLB). Cells were treated using mitotracker 5 µM, incubated at 37°C for 10 minutes in the dark, washed twice and fixed with 4% PFA. Images were acquired on a Leica TCS SP confocal laser scanning microscope, equipped with 476, 488, 543 and 633 excitation lines with a 60x Plan Apo oil objective. The MitoTracker Red signal was then analyzed, after background subtraction, using ImageJ 1, 46 software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) using a semi-manual method based on the delimitation of the cells. We measured mitochondrial morphological characteristics for each cells, as previously described [19]. Average circularity [19] was used as an indicator for mitochondrial morphological changes.

JC-1 analysis

H9c2 cells were plated in 6-well plates and treated 24h with metformin (2, 5 mM MTF), glibenclamide (100 µM GLB), or both molecules (2, 5 mM MTF/100 µM GLB). At the end of treatment, cells were incubated with (1: 200) JC-1 dye (Thermo Fisher Scientific, Waltham, MA) for 15 min at 37°C in a 5% CO₂ incubator. Cells were washed and subjected to FACS analysis. The ratios of red-to-green fluorescence emissions were quantitated to further estimate the extent of mitochondrial membrane damage.

Statistical Analysis

All experiments were performed at least 3 times. Data are presented as mean ± SEM. Comparisons were drawn by ANOVA (SPSS 16.0; SPSS, Chicago, IL) and P values <0.05 were considered statistically significant.

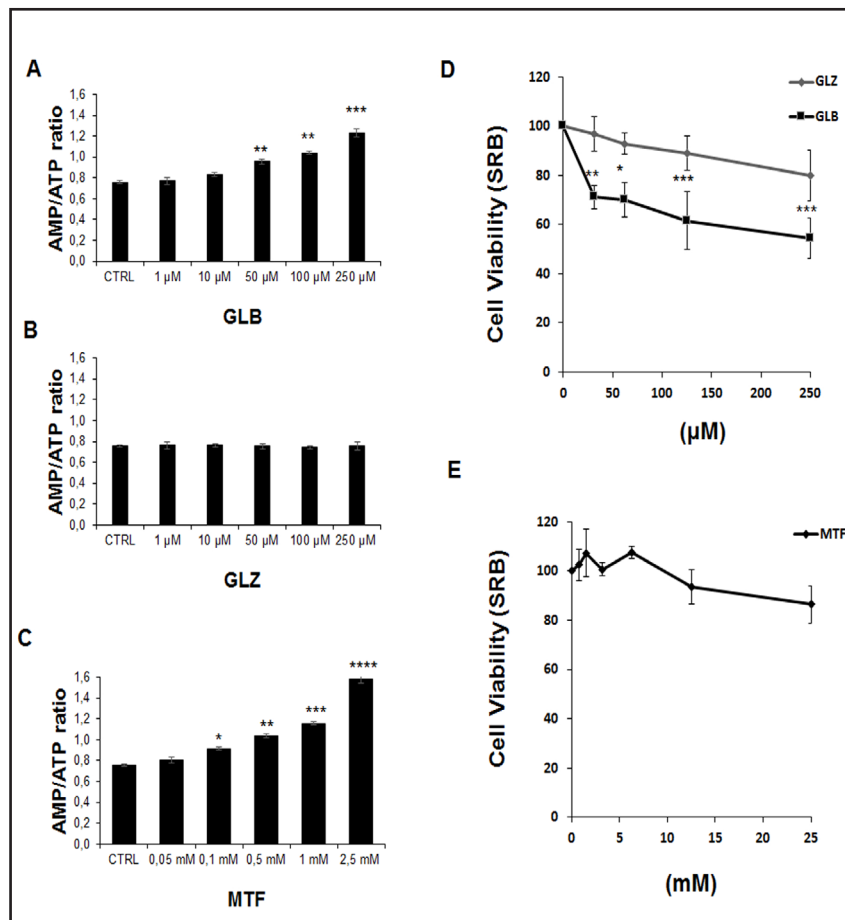
Results

To investigate whether glibenclamide could affect cell metabolism, H9c2 cardiomyoblasts were treated for 24 hr with different doses of glibenclamide, gliclazide, or metformin. Glibenclamide caused a dose-dependent increase of the AMP/ATP ratio, indicating an impairment of the energy balance (Fig. 1 panel A). As expected, gliclazide did not modify the AMP/ATP ratio in H9c2 cells (Fig. 1 panel B). Metformin also increased the AMP/ATP ratio in this cellular model (Fig. 1 panel C). As shown in Fig. 1 panel D, glibenclamide treatment reduced cell viability in a dose-dependent manner, while gliclazide (Fig. 1 panel D) and metformin (Fig. 1 panel E) did not alter the vitality of H9C2.

Since cells respond to ATP depletion by activating AMPK, we analysed the effect of glibenclamide on AMPK phosphorylation by western blot. Again similar to metformin, and consistent with the increase in the AMP/ATP ratio, glibenclamide increased AMPK phosphorylation (Fig. 2).

The main source of ATP is oxidative phosphorylation, which occurs in mitochondria. Therefore, glibenclamide and metformin effects on mitochondrial oxygen consumption and ATP synthesis were determined. Oxygen consumption and ATP synthase activity decreased in the presence of glibenclamide in a dose dependent manner, after adding both pyruvate plus malate, a mix of substrates activating the complexes I, III and IV pathways, and succinate, which induces oxygen consumption through the complexes II, III and IV pathways (Fig. 3 Panels

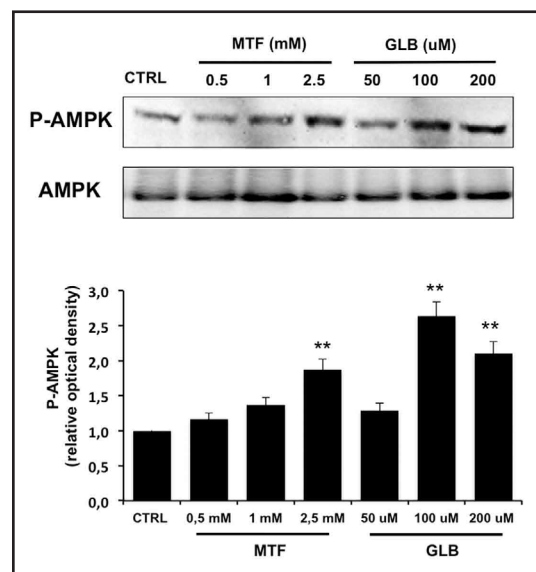
Fig. 1. Glibenclamide increases AMP/ATP ratio and induces toxicity in H9C2 cells. H9C2 cells were treated for 24 hr with different doses of (A) glibenclamide (GLB), (B) gliclazide (GLZ) and (C) metformin (MTF); ATP and AMP concentrations were determined by enzymatic assay, and AMP/ATP ratio was then calculated. H9C2 cells were treated with increasing doses of glibenclamide and gliclazide (D) and metformin (E), 24h later cells were fixed, stained and viability was detected by 96 SRB assay. Bars indicate mean \pm SE. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; GraphPad Software).



A,B). By contrast, metformin treatment impaired oxygen consumption and ATP synthase activity only after the addition of pyruvate plus malate, confirming that complex I is the main metformin target [20] (Fig. 3 Panels C, D). Glibenclamide, as well as metformin, did not display any uncoupling effect. In fact, P/O ratios ranged from 2.5 to 1.5 following the addition of pyruvate plus malate or succinate, respectively (Table 1).

To identify the respiratory chain target of glibenclamide, we assayed the activity of each mitochondrial respiring complex. Glibenclamide inhibited the activity of complexes I, II and III, but not of complex IV (Fig. 4 panels A, B, C, D). It is important to note that the inhibition

Fig. 2. Glibenclamide increases AMPK phosphorylation in H9c2 cells. H9c2 cells were treated for 24 hr with metformin (MTF) and glibenclamide (GLB) at the indicated concentrations and levels of p-AMPK and total AMPK were examined by immunoblotting. Representative western blots and densitometric analysis of the p-AMPK signal normalized against the one of total AMPK are shown. ** = $p < 0.01$ vs. control.



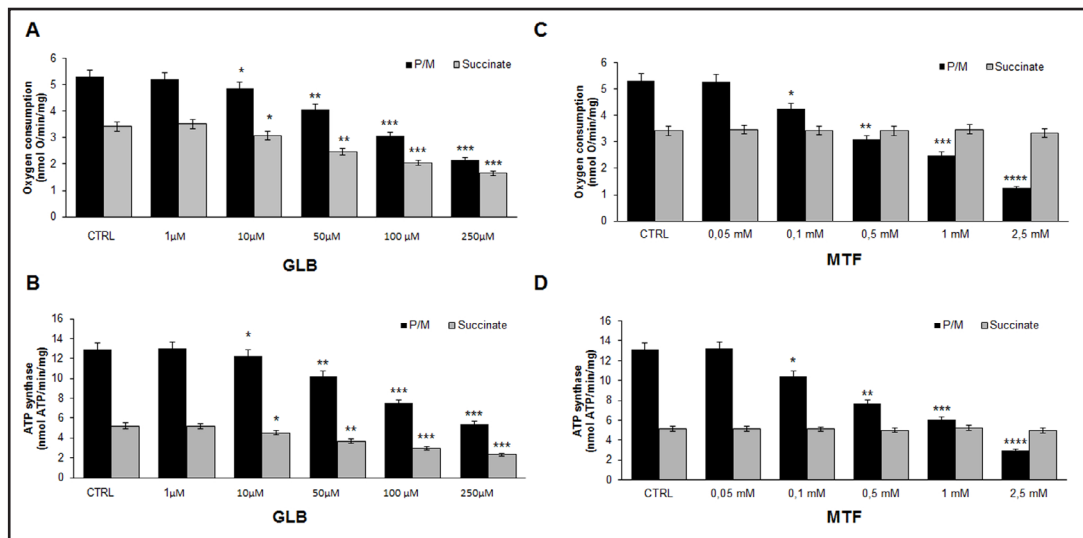


Fig. 3. Oxygen consumption and ATP synthesis in H9c2 cells treated with glibenclamide and metformin. H9c2 cells were treated for 24 hr with metformin (MTF) and glibenclamide (GLB) at the indicated concentrations. Oxygen consumption (A,C) and ATP synthesis via Fo-F1 ATP synthase (B,D) were evaluated at the end of the incubation after adding pyruvate + malate (dark grey columns) or succinate (light grey columns). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$ vs. control.

Fig. 4. Effect of glibenclamide on the mitochondrial respiring complexes. H9c2 cells were treated for 24 hr with 250 μ M glibenclamide (GLB) and complex I (A), complex II (B), complex III (C) and complex IV (D) activity was measured as indicated in Materials and Methods. ** $p < 0.01$ vs. control.

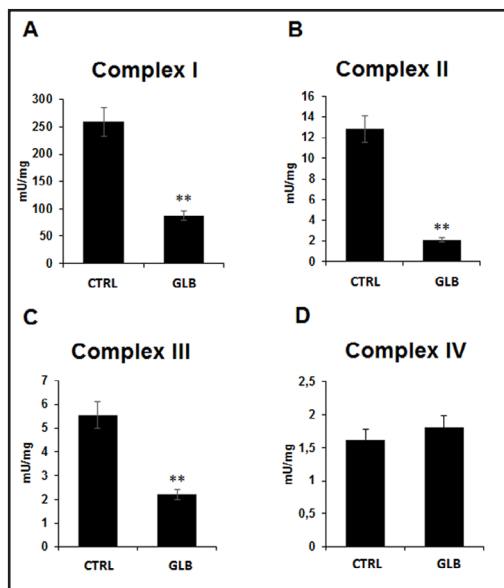


Table 1. Effect of glibenclamide or metformin on P/O ratio. Data are the P/O ratio of H9C2, as the measure of the efficiency of OXPHOS metabolism, in the presence of several concentrations of glibenclamide or metformin. Data are expressed as mean \pm S.D. and represent at least three experiments

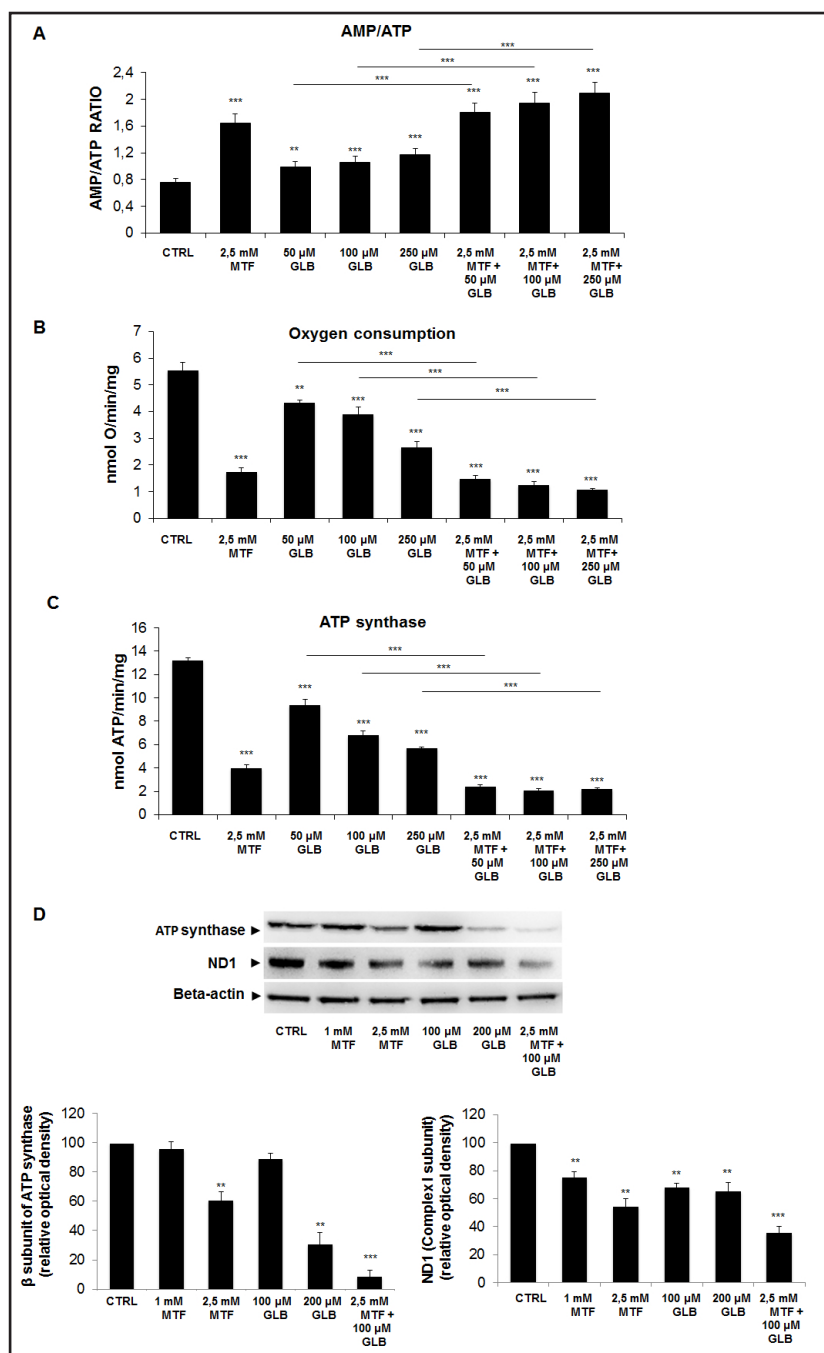
	Glibenclamide (μ M)		Metformin (mM)	
	P/O (Pyr/Mal)	P/O (Succinate)	P/O (Pyr/Mal)	P/O (Succinate)
CTR	2.44 \pm 0.01	1.50 \pm 0.04	CTR 2.45 \pm 0.03	1.49 \pm 0.02
1	2.49 \pm 0.03	1.48 \pm 0.03	0.5	2.50 \pm 0.02
10	2.53 \pm 0.01	1.49 \pm 0.02	0.01	2.49 \pm 0.06
50	2.57 \pm 0.08	1.53 \pm 0.05	0.5	2.57 \pm 0.13
100	2.33 \pm 0.17	1.46 \pm 0.02	1	2.43 \pm 0.00
250	2.52 \pm 0.01	1.38 \pm 0.03	2,5	2.25 \pm 0.18

of complex III may be related with the inhibition of complex I, as this latter represents a functional component of the complex III assay (see Materials and Methods section). Thus, despite a similar final effect on oxidative phosphorylation, metformin and glibenclamide acted differently on the respiratory

chain complexes in H9c2 cells.

Then, we tested the effect of combined treatment with glibenclamide and metformin on H9c2 cells. Compared to glibenclamide alone, the addition of 2.5 mM metformin to increasing concentrations of glibenclamide (50-250 μ M) significantly increased the AMP/ATP

Fig. 5. Combined effect of glibenclamide and metformin on energetic status and oxidative phosphorylation of H9c2 cardiomyoblasts. H9c2 cells were treated for 24 hr with 2.5 mM metformin (MTF), increasing doses of glibenclamide (GLB), and glibenclamide/metformin association (GLB/MTF); following treatments (A) the AMP/ATP ratio, (B) oxygen consumption, and (C) ATP synthesis were evaluated. (D) H9c2 cells were treated for 24 hr with metformin (MTF), glibenclamide (GLB) and metformin/glibenclamide association using the indicated concentrations. β subunit of ATP synthase and ND1 (a subunit of complex I) expression were analysed by immunoblotting. Representative western blots and densitometric analysis of the signals normalized against the one of total beta-actin are shown. ** $p < 0.01$ *** $p < 0.005$ vs. control.



ratio, decreased oxygen consumption and ATP synthase activity (Fig. 5, Panels A, B, C). To determine the expression of respiratory chain proteins we performed western blot analysis of ND1, a subunit of Complex I and of ATP synthase β subunit. Glibenclamide and metformin decreased the expression of both proteins, solely and in association (Fig. 5, Panel D) showing an additive effect.

Respiratory chain function may be also influenced by alterations of mitochondrial distribution in the cell. Therefore, we evaluated the organization of the mitochondrial reticulum by confocal analysis in the presence of glibenclamide, metformin or their combination after 24h of treatment. After glibenclamide or metformin treatment, the mitochondrial reticulum appeared less defined than in control cells. Furthermore, mitochondria showed a more

Fig. 6. Mitochondrial reticulum morphology after treatment with glibenclamide and metformin. (A) Representative confocal images showing the morphology and distribution of the mitochondrial reticulum in H9c2 cardiomyoblasts after 24h treatment with metformin (2,5 mM MTF), glibenclamide (100 μ M GLB), or both molecules (2,5 mM MTF/100 μ M GLB). Scale bar 20 μ m. (B) The bar diagram showed mitochondrial morphological changes analyzed by the means of circularity. (C) The bar diagram shows the ratio of JC-1 red to green fluorescence under each treatment condition. * $p < 0,05$, ** $p < 0.01$ vs control. Bars indicate mean \pm SE.

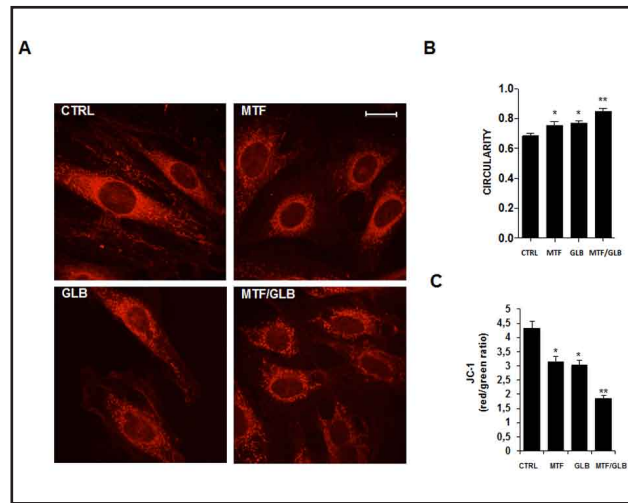
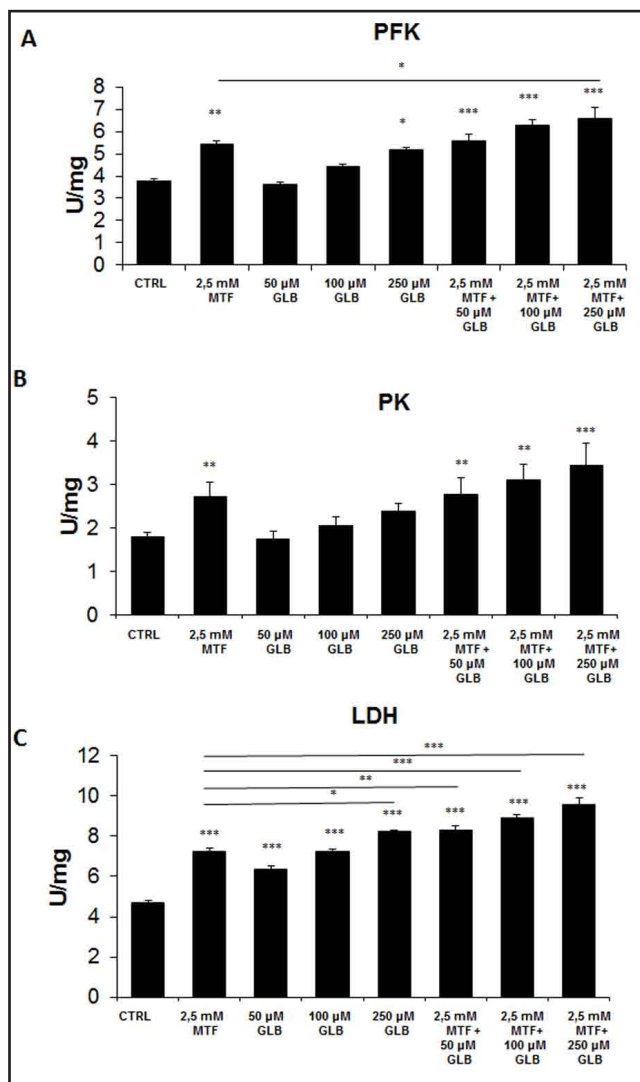


Fig. 7. Enhancement of the glycolytic flux in H9c2 treated with glibenclamide and metformin. H9c2 cells were treated for 24 hr with 2.5 mM metformin (MTF), increasing doses of glibenclamide (GLB) (0 μ M-250 μ M) and their combination. (A) The activity of phosphofruktokinase (PFK), (B) pyruvate kinase (PK) and (C) lactate dehydrogenase (LDH) was then assessed. * $p < 0,05$; ** $p < 0.01$; *** $p < 0.005$ and **** $p < 0.001$; vs corresponding control.

punctuated distribution following combined treatment compared to the single treatments, indicating a synergistic effect of glibenclamide and metformin (Fig. 6 panel A). Moreover, to deeply characterize mitochondrial asset following treatment, mitochondrial network integrity was determined analysing circularity parameter (Fig. 6 panel B). H9C2 treated cells were labelled with mito-traker dye and subjected to confocal microscopy analysis. As shown in Fig. 6 panel C, glibenclamide, metformin and their association significantly reduced mitochondrial interconnectivity and elongation compared to the control.

We measured also mitochondrial membrane potential, which was expressed as the absolute red/green ratio. As expected, metformin induced mitochondrial depolarization. Interestingly also glibenclamide caused a significant reduction in the red/green fluorescence



ratio indicating a drop in mitochondrial membrane potential (Fig. 6 panel C). The association of the molecules elicited a decrease in membrane potential stronger than the one triggered by either drug alone.

Finally, we examined glycolysis in H9c2 cells by measuring the activity of the PKF, PK and LDH, three key enzymes of the anaerobic glycolysis flux. As presented in Fig. 7, the activity of PKF, PK and LDH increased in the presence of metformin as well as of glibenclamide. Interestingly, we observed a further increment with the combination of the two molecules.

Discussion

This study shows that glibenclamide affects energy metabolism of H9c2 cardiomyoblasts, an established cell model for the evaluation of drug cardiotoxicity [11, 21]. Treatment of H9c2 cells with glibenclamide leads to a profound depletion of ATP, secondary to altered mitochondrial organization and oxidative phosphorylation. Overall, this metabolic perturbation is similar to the one induced by metformin, a well-known inhibitor of complex I of the respiratory chain [22].

H9c2 cardiomyoblasts express the SUR2A/Kir6.1 and SUR2A/Kir6.2 K_{ATP} channels isoforms [5]. To understand whether metabolic impairment by glibenclamide was K_{ATP} channels dependent, we also exposed the cells to equimolar concentrations of gliclazide, a second-generation sulfonylurea like glibenclamide, but with no affinity for the K_{ATP} channels subtypes of H9c2 cells [7]. ATP levels were not modified by gliclazide, providing indirect evidence that metabolic toxicity of glibenclamide occurs via binding to K_{ATP} channels. Metformin and glibenclamide are very different molecules with different mechanism of action. Similarly to metformin [23, 24] glibenclamide increases AMPK phosphorylation and mitochondrial dysfunction.

It has already been demonstrated that mitochondria express SUR and that glibenclamide can inhibit potassium fluxes in mitochondria of renal cells [10]. The functional characterization of a sulfonylurea receptor SUR2 splice variant, in fact, has been reported also in cardiac mitochondria [25]. H9c2 cells express SUR2A/Kir6.1 and SUR2A/Kir6.2 [5], which are bound by glibenclamide but not gliclazide [7]. This result suggests that binding to SUR2A/Kir6.1 and SUR2A/Kir6.2 is necessary for glibenclamide-mediated ATP depletion. It remains to be determined whether inhibition of the respiratory chain by glibenclamide is directly mediated by mitochondrial K_{ATP} channels or whether it is the consequence of intracellular events triggered by glibenclamide after binding to cytoplasmic and/or mitochondrial K_{ATP} channels [26]. Mitochondria reticulum integrity is necessary to maintain cell energy balance. We found that glibenclamide disrupts mitochondria reticulum integrity similarly to inhibitors of the respiratory chain such as piericidin and myxothiazol that cause fission of mitochondria [27]. Our data show that metformin also causes mitochondria to switch from an elongated, tubular shape to a punctate one. This perturbation in mitochondrial organization could be related to the energy balance dysfunction observed with both molecules.

Metformin directly inhibits complex I [28, 29] while glibenclamide inhibits complexes I-II-III. These findings suggest that glibenclamide and metformin combined treatment could potentiate each other respiratory chain destabilization. Here metformin but also glibenclamide reduced the expression of ND1 and ATP synthase β subunit. This result could be related to the impairment of oxidative phosphorylation. Since ND1 and ATP synthase β subunit are codified by two different subcellular compartments, respectively mitochondria and nucleus, a transcriptional mechanism is unlikely. An alternative explanation could be that glibenclamide binds to the mitochondria inner membrane.

Metformin action on mitochondria is well established. Metformin decreases the functionality of complex I [20]. The inhibition of complex I could be a generic effect resulting from molecular changes occurring in the inner mitochondrial membrane following the binding of metformin to phospholipids [22, 30]. Interestingly it has been shown that glibenclamide can bind to the inner membrane of mitochondria [10]. This mechanism could

interfere with the conformations of important mitochondrial proteins, including respiratory chain complexes.

Clinical trials and registries have consistently shown that metformin does not increase cardiovascular morbidity and mortality of diabetic patients; in fact, a favourable effect of metformin on diabetes-related cardiovascular risk has been reported [5]. Consistently, most experimental studies have demonstrated that metformin prevents ischemic cardiac damage, this protection being ascribed to various pathways, not necessarily downstream of the reduction in complex I activity, such as activation of AMPK and phosphoinositide 3-kinase/AKT, enhanced intramyocardial availability of adenosine, blunting of oxidative stress, and inhibition of mitochondrial permeability transition pore opening [24, 31]. By contrast, therapy with glibenclamide has been associated with negative cardiovascular outcomes in epidemiological analysis [2, 3] and glibenclamide has been shown to worsen ischemic injury of cardiomyocytes in cell and animal models [31]. This toxicity has been primarily related to the closure of K_{ATP} channels [7]. Here we describe that glibenclamide also elicits a drop in ATP concentrations in cardiac cells like metformin. Nonetheless, glibenclamide, but not metformin decreased H9c2 cell viability in our experiments. Further work is needed to better understand mechanisms of this difference and to dissect the actions of glibenclamide and metformin on H9c2 cells. The combined effect of glibenclamide and metformin will deserve particular attention, as clinical investigations pointed to an increased cardiovascular risk when diabetic patients are given both drugs [2].

In our model, gliclazide did not modify the AMP/ATP ratio. Even though this finding cannot be extrapolated to cell types other than the H9c2 line, it is in good agreement with the literature suggesting that gliclazide is safer than glibenclamide as far as cardiovascular outcomes are concerned and, thereby, represents a valid therapeutic option for type 2 diabetes [32].

Conclusion

The experiments presented here add new data on the effects of glibenclamide on cardiac cells, showing that this drug interferes with mitochondrial organization and inhibits oxidative phosphorylation. This may further explain the risk of cardiovascular events associated with glibenclamide use. Furthermore, changes in energy metabolism should be taken into account by future investigations addressing the effects of glibenclamide on cardiac cells or the whole heart.

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Disclosure Statement

The authors have nothing to disclose.

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