Biochimica et Biophysica Acta 1817 (2012) 1768–1775

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Fatty acids revert the inhibition of respiration caused by the antidiabetic drug metformin to facilitate their mitochondrial β-oxidation☆

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ARTICLE INFO

Article history:
Received 12 January 2012
Received in revised form 14 February 2012
Accepted 16 February 2012
Available online 23 February 2012

Keywords:
Metformin
Diabetes
Adipocyte
Fatty acid oxidation
Mitochondria
AMPK

ABSTRACT

While metformin has been widely used to treat type 2 diabetes for the last fifty years, its mode of action remains unclear. Hence, we investigated the short-term alterations in energy metabolism caused by metformin administration in 3T3-L1 adipocytes. We found that metformin inhibited mitochondrial respiration, although ATP levels remained constant as the decrease in mitochondrial production was compensated by an increase in glycolysis. While AMP/ATP ratios were unaffected by metformin, phosphorylation of AMPK and its downstream target acetyl-CoA carboxylase augmented. The inhibition of respiration provoked a rapid and sustained increase in superoxide levels, despite the increase in UCP2 and superoxide dismutase activity. The inhibition of respiration was rapidly reversed by fatty acids and thus respiration was lower in treated cells in the presence of pyruvate and glucose while rates were identical to control cells when palmitate was the substrate. We conclude that metformin reversibly inhibits mitochondrial respiration, it rapidly activates AMPK without altering the energy charge, and it inhibits fatty acid synthesis. Mitochondrial β-oxidation is facilitated by reversing the inhibition of complex I and, presumably, by releasing the inhibition of carnitine palmitoyltransferase. This article is part of a Special Issue entitled: 17th European Bioenergetics Conference (EBEC 2012).

1. Introduction

Metformin is a member of the biguanide family of compounds that has been used for the treatment of diabetes since the 1950s. Biguanides do not alter insulin secretion but rather, they improve insulin sensitivity, reduce plasma triglyceride and LDL concentrations, decrease hepatic gluconeogenesis and inhibit glycogenolysis [1,2]. The mechanism of action by which metformin and its related compounds, such as phenformin, exert their action is still under debate, although the mitochondrial oxidative phosphorylation appears to be one of its main targets. Thus, the pioneering work of Hollunger in 1955 [3] showed that guanidines inhibit mitochondrial respiration. The effect of metformin on the respiratory chain is now well established [4–9]. For many years the prevailing hypothesis stated that mitochondrial impairment attenuated mitochondrial ATP synthesis, leading to a compensatory acceleration of glycolytic flux and increased glucose uptake. In fact, an adverse side effect of biguanides is an increase in the generation of lactate, which can enter the circulation and produce lactic acidosis [2,10]. Notably, phenformin was withdrawn from clinical use in the 1970s following reports of cases of severe lactic acidosis [2,11].

Activation of the AMP-dependent protein kinase (AMPK) is central to the signaling cascade that results from metformin action [12,13]. AMPK is considered a key controller of energy metabolism by "sensing" the cellular ATP levels. When phosphorylated, AMPK is active and stimulates energy generating pathways, while inhibiting anabolic processes or cell growth [14]. Cytosolic adenine nucleotides play a critical role in control of AMPK activity and, thus, AMP not only enhances allosterically the activity of the phosphorylated enzyme but it also protects AMPK from dephosphorylation, an effect antagonized by ATP. It is known that metformin does not interact with this enzyme complex [15] and it has been suggested that a drop in ATP levels, caused by the inhibition of respiration, could be behind its activation [7,16–18]. However, several studies have reported no changes in the AMP/ATP ratio in response to metformin treatment, suggesting that alternative signaling pathways underlie AMPK activation in this context [15,19,20].

The inhibition of respiration by metformin augments the mitochondrial generation of superoxide [21] and, thus, it has been

Abbreviations: ACC, Acetyl-CoA carboxylase; AMPK, Adenosine monophosphate-activated protein kinase; DHE, Dihydroethidium; ECAR, Extracellular acidification rate; FCCP, Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; OCR, Oxygen consumption rate; PI3K, Phosphoinositide-3 kinase; ROS, Reactive oxygen species; SOD, Superoxide dismutase

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doi:10.1016/j.bbabio.2012.02.019

shown to cause oxidative stress at least as an early event [20,22]. However, it should be mentioned that long-term metformin treatment improves the antioxidant status of patients due to, among other parameters, the increase of antioxidant enzymatic activities and serum glutathione levels [23–25]. Reactive oxygen and nitrogen species are known to modulate a variety of signal transduction pathways, either through changes in gene expression or by directly acting on redox sensitive enzymes and receptors [26,27]. In our context, peroxynitrite, a potent oxidant formed by reaction between superoxide and NO, activates c-Src and PI3K leading to AMPK phosphorylation without altering the cellular AMP or ATP content [28,29]. This ROS mediated signaling pathway has been proposed to mediate in the antidiabetic action of metformin [20,28–30]. Intriguingly, a recent study in hepatocytes described AMPK-independent metformin activity and reported its effects to be due to a decrease in energy charge. It was suggested that the increase in the AMP/ATP ratio and the activation of AMPK could represent two parallel, perhaps even redundant, pathways that exert similar overall effects on cellular metabolism [31].

Despite being the most commonly prescribed drug for the treatment of type II diabetes for more than five decades, the bioenergetic mechanisms underlying metformin activity remain largely unknown. Furthermore, some of the available data are contradictory. Here, we used differentiated adipocytes to analyze the short-term effects of metformin and extensively characterize the bioenergetic changes caused by the drug. We found that, in the presence of glucose and pyruvate, the inhibitory effects of metformin on respiration can be detected within minutes and, as previously reported, they lead to the activation of AMPK [32,33]. However, these effects were not due to changes in the cellular energy charge, as no changes in the AMP/ATP or ADP/ATP ratios were detected, probably due to the marked increase in glycolysis. AMPK phosphorylation inhibits fatty acid synthesis and stimulates their oxidation [12,14]. Intriguingly, the inhibition by metformin of respiration should hamper their mitochondrial β-oxidation. However, we found that fatty acids caused a striking rapid reversal in the inhibition of respiration which may explain the failure of metformin to inhibit their oxidation. We hypothesize that metformin favors the oxidation of fatty acids, but that it prevents pyruvate oxidation. Moreover, we propose that metformin favors the oxidation of fatty acids to facilitate the removal of plasma fatty acids and improve the lipid profile in the diabetic patient.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated. Cell culture media, antibiotics and bovine serum were from Gibco/Invitrogen (Paisley, UK). The UCP2 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), antibody against mitochondrial porin was from Sigma-Aldrich. Antibodies against AMPK, P-AMPK-Thr172, P-ACC were from Cell Signalling (Invitrogen, Paisley, UK).

2.2. Cell culture and differentiation

3T3-L1 mouse embryo fibroblasts were obtained from the American Type Culture Collection (Mansassas, VA, USA). Cells were cultured in a humidified atmosphere at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated bovine serum supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). When the cells reached confluence, the culture medium was switched to "differentiation medium": DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) plus antibiotics. Two days after they reached confluence (day 2), 1 mM 3-isobutyl-1-methylxanthine (IBMX), 1 μM insulin and 1 μM dexamethasone were added to the medium to induce their differentiation to adipocytes. Three days later (day 5), this medium was replaced by fresh "differentiation medium" containing only 1 μM insulin. From day 7, cells were maintained in "differentiation medium" until day 9, when differentiated adipocytes were subjected to the different treatments in the same medium. For the experiments in the XF24 Seahorse Bioscience instrument, on day 7 of differentiation cells were removed from the plate with PBS/0.1 mM EDTA and were seeded in XF 24-well cell culture plates (9×10^4 cells per well) until day 9 of differentiation.

2.3. Preparation of mitochondria-enriched extracts and whole-cell extract

PBS-washed 3T3-L1 adipocytes were suspended in a buffer containing 250 mM sucrose, 0.1 mM EDTA, 5 mM Hepes pH 7.4 plus 0.1% (v/v) of the protease inhibitor cocktail. Cells were subjected to 3 freeze/thaw cycles and centrifuged at $750\times g$ for 10 min, the supernatant collected and centrifuged at $10{,}000\times g$ for 20 min. The pellet obtained was resuspended in 10–20 μl of the same buffer. Whole-cell extracts were prepared from cells lysed in RIPA buffer (150 mM NaCl, 2 mM EDTA, 50 mM Tris–HCl pH 7.5, 1% (v/v) NP40, 0.1% (v/v) SDS and 1%(v/v) deoxycholate) supplemented with protease and phosphatase inhibitor cocktails. In all cases, the protein concentration was determined by the bicinchoninic acid assay (BCA, Pierce, Rockford, IL, USA) using bovine serum albumin as standard.

2.4. Western blot analysis

30 μg of the mitochondrial extracts or 40 μg of the total cellular extracts were resolved by SDS-PAGE and then transferred to nitrocellulose membranes that were probed with the antibodies at 1:1000 dilution except for the anti-UCP2 antibody that was used at 1:500. Equal loading of the mitochondrial extracts was confirmed by quantifying the content of mitochondrial porin. The immunoblots were developed with the Super Signal West Dura chemiluminescent substrate (Pierce, Thermo Scientific, Rockford, IL, USA) and band intensity recorded using the CCD camera of a Fujifilm LAS-3000 analyzer (Düsseldorf, Germany) and quantified with the Fujifilm MultiGauge programme.

2.5. Measurement of levels of reactive oxygen species and apoptosis assay

ROS levels were determined by incubating the cells with dihydroethidium (DHE). The evaluation of cellular apoptosis was carried out with annexin-V and propidium iodide staining using the Vybrant Apoptosis Assay Kit 2 (Invitrogen, Paisley, UK) and following the manufacturer's instructions. For both assays, adipocytes were trypsinized and resuspended in DMEM without serum at a concentration of one million cells per ml. For ROS detection, cells were incubated in 5 μM DHE for 30 min at 37 °C in the dark, while for apoptosis assays cells were stained for 15 min at room temperature. Samples were washed twice in the same medium and the fluorescence analyzed using an EPICS XL flow cytometer (Beckman Coulter, Brea, CA, USA).

2.6. Oxidized glutathione determination

Glutathione levels were measured in cell extracts with the GSH/GSSG-412 kit from Oxis Research (Percipio Biosciences, Portland, OR), according to the manufacturer's instructions. To determine GSSG levels, GSH was scavenged with 1-methyl-2-vinylpyridinium. The change in absorbance at 412 nm was recorded for 15 min. GSSG levels were determined from GSSG standards and normalized with protein content.

2.7. Superoxide dismutase activity

Mn- and Cu/Zn-SOD activity was determined with the "SOD Assay Kit II" (Calbiochem, Darmstadt, Germany) according to the manufacturer's instructions. Cells were lysed in a buffer containing 1 mM EGTA, 210 mM mannitol, 70 mM glucose and 20 mM HEPES pH 7.2. SOD activity was determined spectrophotometrically following the absorbance changes at 450 nm.

2.8. Measurement of the adenine nucleotide levels

AMP, ADP and ATP levels were determined by reverse-phase HPLC using a C18 column (Mediterranea SEA 18, Teknokroma, Sant Cugat, Spain) and following essentially the protocol of Vives-Bouza et al. [34]. Cells were washed with cold PBS and quenched with 660 mM $HClO_4$ and 10 mM theophylline. Extracts were homogenized and centrifuged 15 min at 16,000×*g*, supernatant was neutralized using 2.8 M K_3PO_4 and samples stored at −80 °C until HPLC assay. External standards were treated in the same way as the samples.

2.9. Measurements of cellular respiration and estimation of the rate of glycolysis

An XF24 Seahorse Bioscience (North Billerica, MA, USA) instrument was used to measure the oxygen consumption rate (OCR) of differentiated 3T3-L1. 9×10^4 cells were seeded per well on day 7 of differentiation. For the XF24 assay, DMEM growth media was replaced by unbuffered DMEM supplemented with 25 mM glucose, 1 mM pyruvate and 2 mM L-glutamine and cells incubated at 37 °C in a CO_2-free incubator for 1 h. Cells were then placed in the instrument and basal oxygen consumption recorded for 24 min and subsequently 1 μg/ml oligomycin and 400 nM FCCP were added. At the end of the run, 1 μM rotenone and 1 μM antimycin A were added to determine the mitochondria-independent oxygen consumption. When the OCR was determined in the presence of palmitate, unbuffered KHB medium (111 mM NaCl, 4.7 mM KCl, 2 mM $MgSO_4$, 1.2 mM Na_2HPO_4, 2.5 mM glucose and 2.5 mM carnitine) was used. Cells were incubated at 37 °C in a CO_2-free incubator for 1 h and the assay run subsequently. Two palmitate additions were made after recording the basal OCR. The rate of glycolysis was estimated from the extracellular acidification rate (ECAR) [35]. Protein concentration in each well was determined with the BCA method after lysing cells in RIPA buffer.

2.10. Statistical analysis

All values are expressed as mean ± SEM of at least three independent experiments. Differences between groups were determined using either two-tailed unpaired Student's t-tests or the One-Way ANOVA test using the SigmaPlot software. Significant differences between groups are indicated as *P<0.05 and **P<0.01.

3. Results

3.1. Metformin inhibits cellular respiration

Metformin is known to act on mitochondria and inhibit respiration [4–9]. Fig. 1A shows that, after 24 h of treatment, increasing

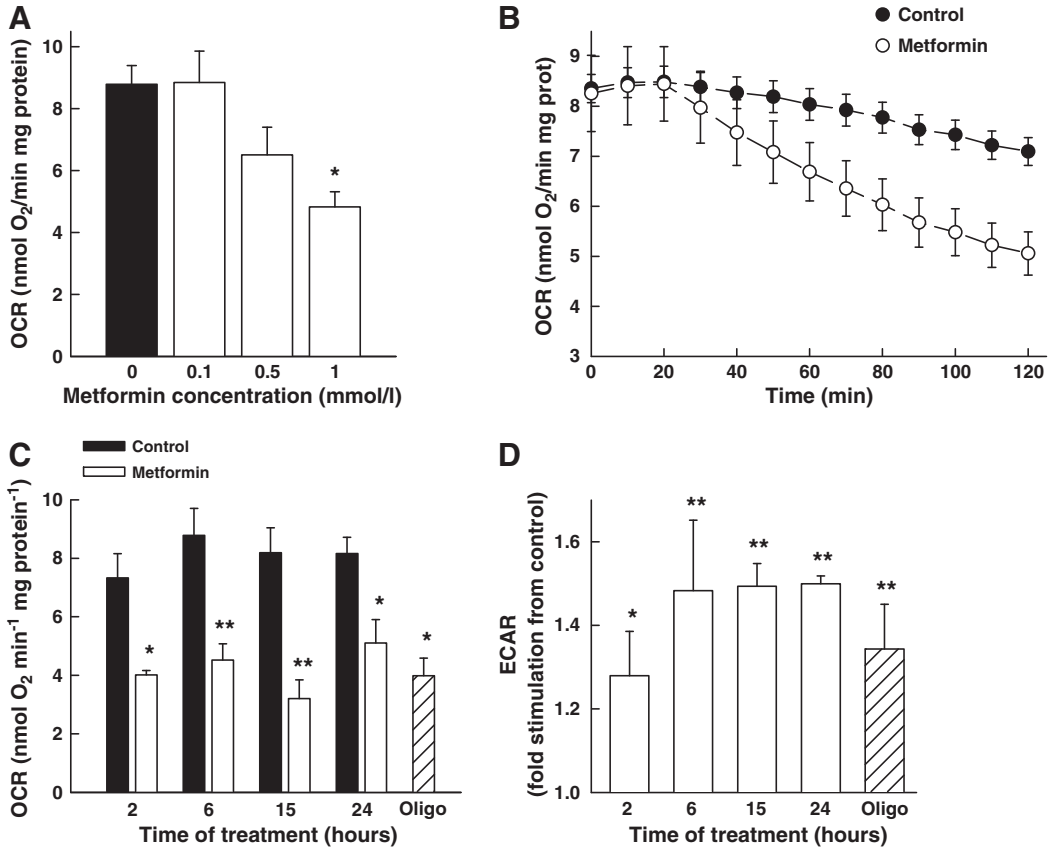

Fig. 1. Effect of metformin on the oxygen consumption rate (OCR) of differentiated 3T3-L1 adipocytes. (A) Titration of the effect of metformin on the rate of respiration after a 24 h incubation of the adipocytes with the drug ($n=3$–7). (B) Kinetics of the inhibition of respiration after the addition of metformin 1 mM ($n=13$–14). (C) Effect of 1 mM metformin on the rate of respiration of 3T3-L1 cells maintained for 2, 6, 15 or 24 h in the presence of the drug. The effect of the ATPase inhibitor oligomycin (1 μg/ml) is included as a reference ($n=5$–6). (D) Effect of the treatment of 3T3-L1 adipocytes on the extracellular acidification rate (ECAR) taken as a measure of the rate of lactate production. ECAR values are expressed as the fold increase in the ECAR compared to the respective control cells ($n=3$–6). Data are means ± the SEM. **P<0.01; *P<0.05 compared to the untreated cells.

concentrations of metformin have a measurable effect on the rate of respiration in 3T3-L1 adipocytes. Thus, 0.5 mM metformin induced a clear decrease (25%) in the basal rate of respiration. The time required for metformin to exert its effects remains a matter of some debate [7]. Hence, we analyzed the time course over which the basal rate of respiration was inhibited by 1 mM metformin. Inhibition of respiration was apparent after 30 min and increased progressively over the 2 h period analyzed (Fig. 1B). In cells maintained in culture in the presence of 1 mM metformin for up to 24 h, the inhibition of respiration persisted for the duration of the test (Fig. 1C). The extent of the inhibition is similar to the one observed in control cells when the ATPase inhibitor oligomycin is present. The effect of metformin on respiration has also been observed in other cell types [7,9,10,20].

The inhibition of respiration by metformin increases the rate of lactic acid production [2]. Under our experimental conditions, inhibition of adipocyte respiration also induced an increase in the extracellular acidification rate (ECAR) (Fig. 1D), an indirect measure of the rate of glycolysis [35]. The ECAR increased by approximately 25% after 2 h, reaching a plateau after 6 h at a level 50% higher than that seen in untreated cells. Notably, oligomycin inhibition of mitochondrial ATP synthesis in control 3T3-L1 cells also increased in glycolysis to a similar extent to metformin.

3.2. Metformin inhibition activates AMPK but has no effect on ATP levels

The effects of metformin are mediated by AMPK activation and increases in the AMP/ATP ratio have been reported in response to metformin inhibition of respiration [7,16–18]. In 3T3-L1 adipocytes, metformin did not alter the cellular energy charge, as reflected by the comparable AMP/ATP and ADP/ATP ratios in control and metformin-treated cells (Fig. 2). It can be envisaged that the increase in glycolytic production of ATP fully compensates for the decrease in the mitochondrial production resulting from the inhibition of respiration. Although the AMP/ATP ratio did not change, metformin did induce AMPK phosphorylation and activation of the corresponding signaling cascade, as witnessed by the phosphorylation of its downstream target acetyl-CoA carboxylase (ACC) (Fig. 2C–E).

3.3. Metformin causes oxidative stress

In a previous analysis of the long-term effects of metformin, we reported increases in superoxide levels in differentiating 3T3-L1 cells [22]. In support of these findings, elevated superoxide levels were already apparent here after 2 h of metformin treatment (Fig. 3A). A similar time course was observed for UCP2, with a significant increase after 2 h (Fig. 3B). Compound C, an inhibitor of AMPK, completely prevented the metformin-induced increase in UCP2 levels (Fig. 3D and E). Interestingly, we observed a significant delay in the increase in SOD levels that coincided with other signs of oxidative stress (Fig. 3F). Thus, under our culture conditions, 15 h of treatment with 1 mM metformin affects the viability since there is an increase in the number of cells that become annexin-V positive (Fig. 3G). Similarly, levels of oxidized glutathione increase steadily during the 24 h treatment with the greatest increase observed at the last time point measured (Fig. 3H).

3.4. Metformin does not block the mitochondrial oxidation of fatty acids

Metformin is known to act through the inhibition of substrates linked to complex I [6–8,36]. Thus it has been shown that while it blocks oxidation in the presence of pyruvate/malate, the oxidation of succinate is unaffected. The inhibition of respiration is linked to

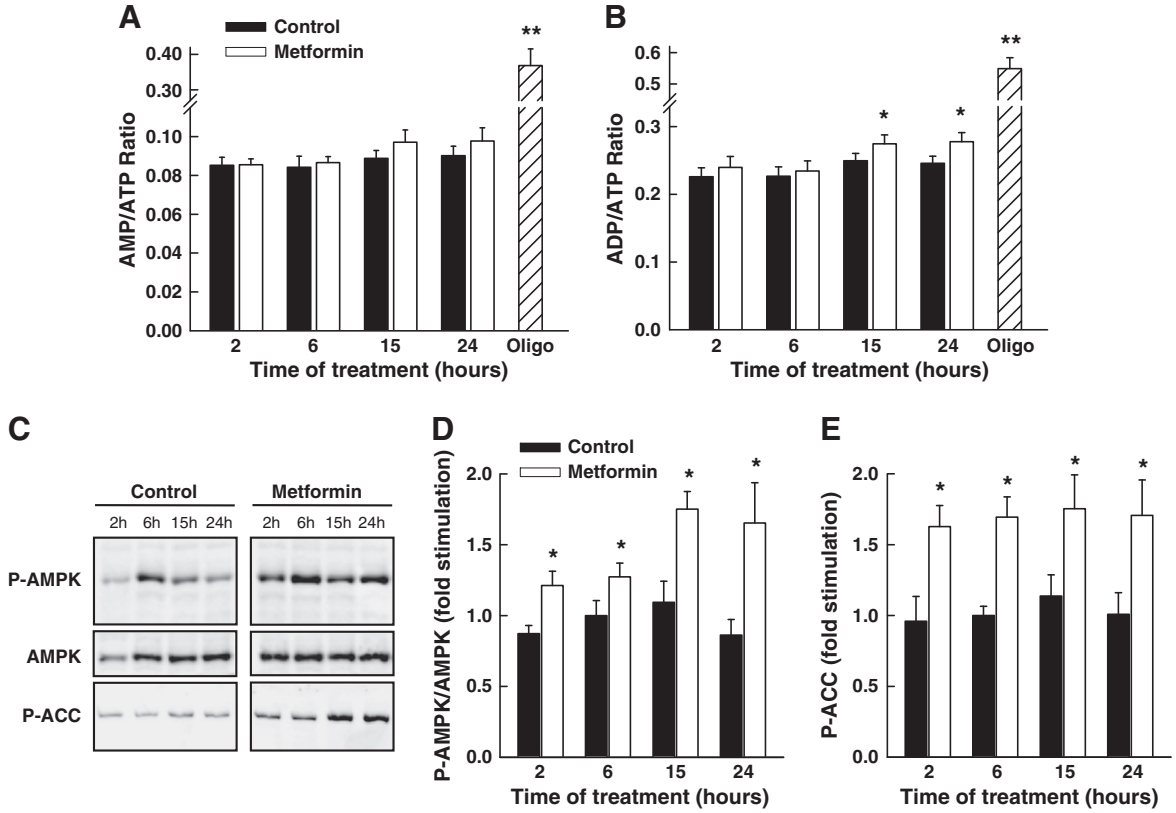

Fig. 2. Effect of metformin on AMP/ATP and ADP/ATP ratios and on the phosphorylation of AMPK and ACC. (A) AMP/ATP ratio in 3T3-L1 adipocytes treated with 1 mM metformin for up to 24 h. (B) Effect of metformin on the ADP/ATP ratio ($n = 10–12$). In panels A and B, ratios obtained with cells treated for 2 h with oligomycin (2 μM) are included as a control. (C) Representative immunoblots of whole-cell extracts showing the changes in the phosphorylation state of AMPK-Thr172 and ACC. (D) Changes in the P-AMPK/AMPK ratio as a result of metformin action ($n = 3–9$). (E) Effect of metformin on the phosphorylation of ACC ($n = 3–5$). In panels D and E, values are expressed as the fold increase in the phosphorylation taking as reference the value of control cells after 6 h of treatment with vehicle. Data are means ± SEM. **P<0.01; *P<0.05 compared to the untreated cells.

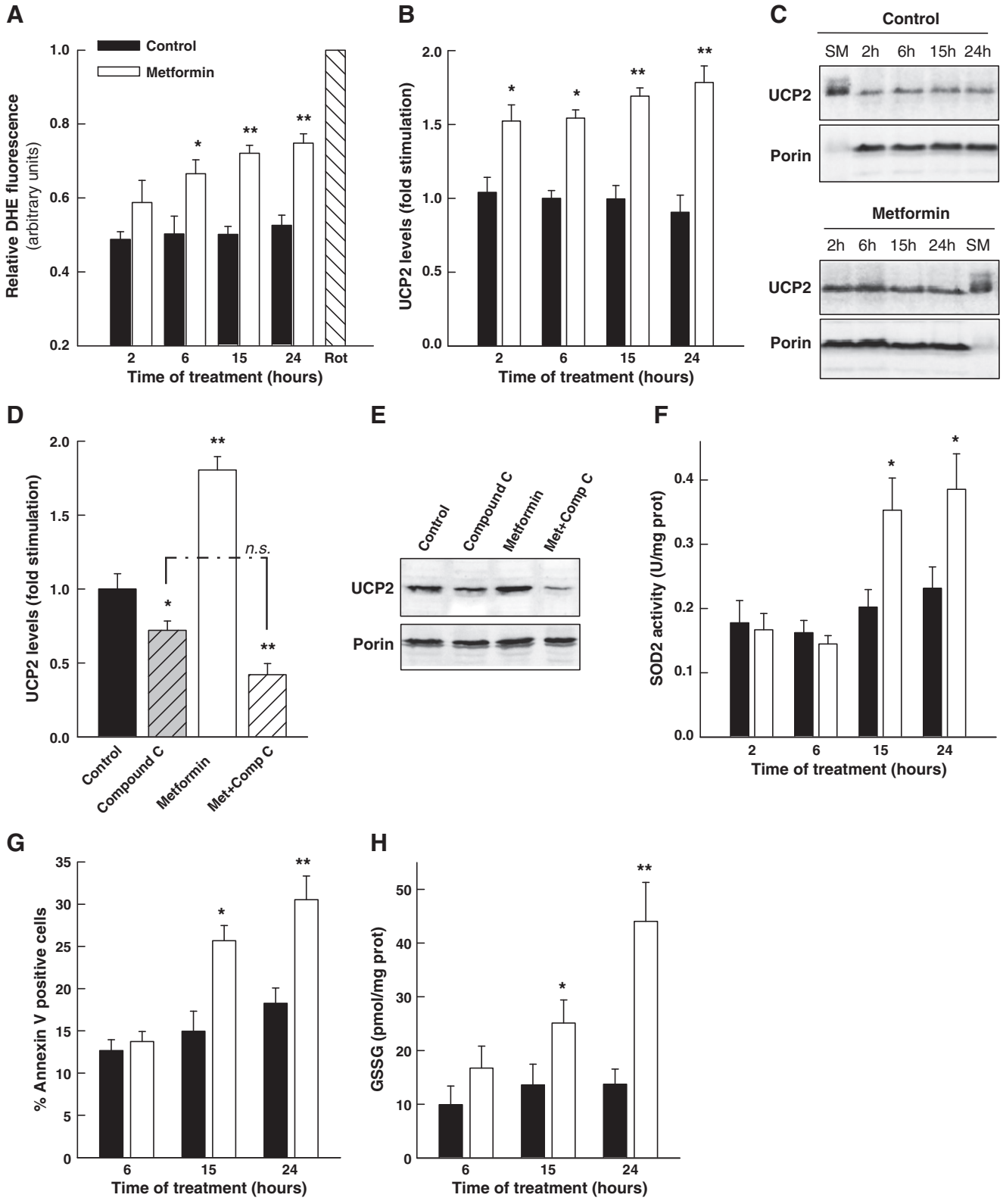

Fig. 3. Effect of metformin on oxidative stress parameters in 3T3-L1 adipocytes. (A) ROS levels as determined by flow cytometry with the probe DHE. Values expressed with respect to the value obtained after incubation of cells with 8 μM rotenone for 60 min ($n=4$). (B, C) Changes in the levels of UCP2 protein using porin as loading control. "SM", control lane loaded with mitochondria isolated from *S. cerevisiae* that express UCP2 recombinantly. Values expressed as the fold increase in the protein levels taking as reference the value of control cells after 6 h of treatment with vehicle ($n=6$–10). (D, E) Effect of the selective AMPK inhibitor, Compound C, on UCP2 protein levels. Cells were treated with 1 mM metformin for 15 h in the presence or absence of 20 μM Compound C. Protein levels are expressed as the fold increase with respect to the untreated control cells. (F) Effect of metformin on the SOD2 activity ($n=4$–6). (G) Effect of metformin on adipocyte induction of apoptosis using annexin-V labeling ($n=3$–8). (H) Effect of metformin on the levels of oxidized glutathione ($n=3$–6). In all cases, data are means ± SEM. $^{**}P<0.01$; $^{*}P<0.05$ compared to the untreated cells.

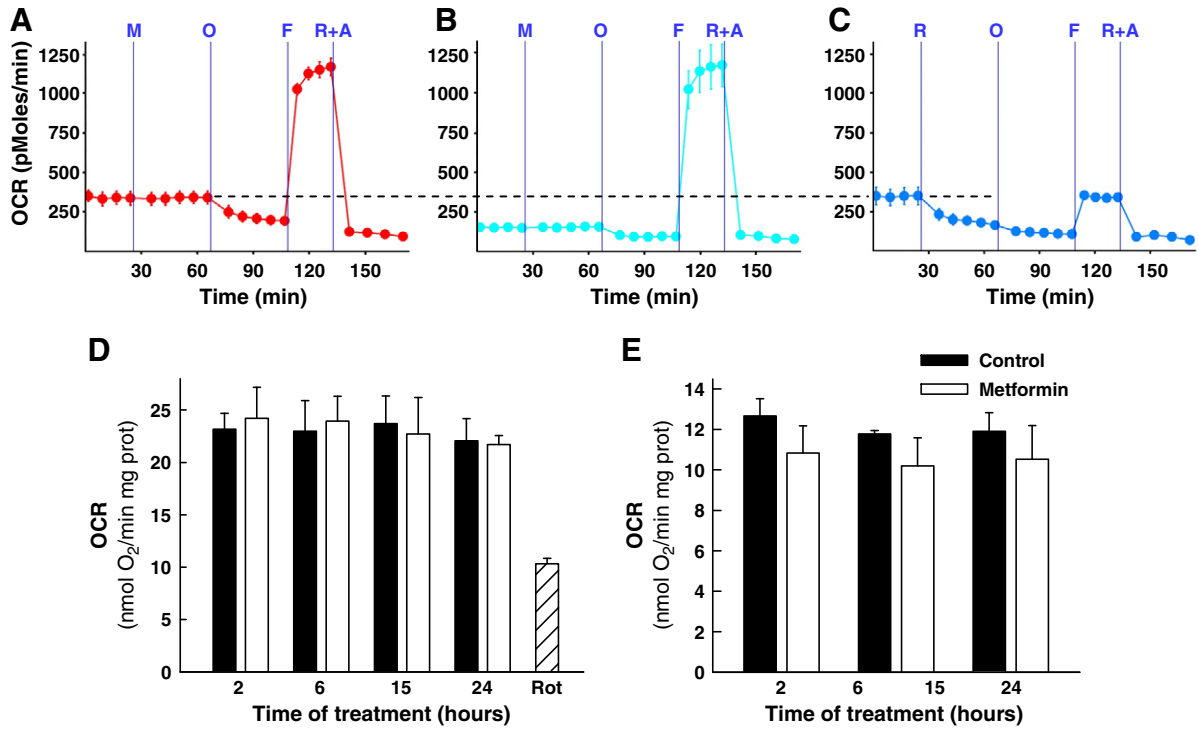

Fig. 4. Reversibility of the inhibition by metformin of respiration in 3T3-L1 adipocytes. (A) Representative experiment with the characterization of the oxygen consumption rate (OCR) of control adipocytes using the XF24 Extracellular Fluid Analyzer. (B) OCR profile of cells treated with metformin for 15 h. (C) Effect on the OCR profile of the addition of rotenone 10 nM (added where indicated with the letter R). In panels A–C, additions to the wells are indicated with the vertical lines. Additions: M, vehicle; O, oligomycin 1 μg/ml; F, 400 nM FCCP; R + A, 1 μM rotenone plus 1 μM antimycin A. (D) Rate of respiration of control and metformin treated cells after the addition of the uncoupler FCCP (400 nM). Hatched bar corresponds to the rate of respiration in the presence of 400 nM FCCP and 10 nM rotenone ($n = 5–6$). (E) Rate of respiration of control and metformin treated adipocytes in low glucose medium without pyruvate and in the presence of 300 μM palmitate (molar ratio 6:1 palmitate to albumin) and 2.5 mM carnitine ($n = 3$). Data are means ± the SEM.

increase glycolysis. However, it is known that metformin promotes catabolism by activation of AMPK [12,13] and particularly fatty acid oxidation. This is a paradoxical situation since β-oxidation of fatty acid requires a competent complex I.

The design of the experiments on the XF24 Extracellular Fluid Analyzer, to investigate the respiration properties of our cell preparations revealed the unexpected finding that despite a marked inhibition of the basal rate of respiration, the rates in the presence of the uncoupler FCCP were identical to those seen in untreated cells (Fig. 4B). This uncoupler-induced reversal of the inhibition was not observed when a low dose of rotenone (10 nM) was used to cause a similar degree of inhibition of respiration (Fig. 4C). Fig. 4D presents the rates of respiration of control and metformin-treated cells in the presence of the uncoupler. To investigate the effect of metformin on fatty acid oxidation in the 3T3-L1 cells, adipocytes were pre-incubated for 1 h in a starvation medium that lacked both pyruvate and glutamine, and in which glucose concentration was lowered to 2.5 mM. Basal respiration in this medium was slightly lower than under the standard conditions although the differences between control and metformin-treated cells were maintained (data not shown). The addition of 300 μM palmitate (molar ratio 6:1 to albumin), in the presence of 2.5 mM carnitine, increased the respiration rates to values slightly higher than those observed in the presence of pyruvate. Strikingly, no differences were observed between the control and metformin-treated cells (Fig. 4E).

4. Discussion

While the inhibition of respiration by metformin is well-documented, it remains unclear whether this represents the primary effect of the drug, accounting for the multiple metabolic alterations seen in target cells. Biguanides target complex I, as witnessed by the inhibition of respiration when the mitochondria oxidize glutamate and malate, an effect not observed when succinate is used as the substrate [6–8,36]. Inhibition of respiration leads to the upregulation of glycolytic enzymes, increasing lactate production. Limiting the mitochondrial capacity to produce ATP may decrease the energy charge (i.e.: AMP/ATP or ADP/ATP ratio), but only if glycolysis is unable to meet the cellular demand for ATP. Here, treatment with metformin (1 mM) inhibited respiration by 50%, although this appeared to be compensated by a 50% increase in ECAR (Fig. 1), since the AMP/ATP or the ADP/ATP ratios remained absolutely constant for several hours (Fig. 2).

The cellular events that trigger the activation of AMPK, and the subsequent metabolic reprogramming, remain the subject of much debate. A decrease in the energy charge has been proposed to be the key event in these processes [7,16–18], although AMPK activation has also been seen in the absence of changes in adenine nucleotide levels [15,19,20]. Our study provides new evidence that increased glycolysis appears to sustain the ATP levels at least during the first hours of exposure to the drug. The discrepancies reported could be due to differences in the capacity of glycolysis to maintain the energy charge in the different cell types. Nonetheless, our results further support the proposal that metformin activation of AMPK does not necessarily involve a change in the energy charge, although it may strengthen the metabolic signal.

There is an alternative hypothesis to explain AMPK activation and that also derives from the metformin effects on the respiratory chain. The inhibition of complex I increases formation of superoxide giving rise to reactive oxygen and nitrogen species. Peroxynitrite, a product of the reaction of superoxide with nitric oxide, has been shown to affect signaling pathways by nitration as well as by oxidation [37]. Thus, it has been proposed that peroxynitrite, formed due to metformin action, may activate AMPK via a c-Src-mediated PI3K-

dependent pathway [20,28–30]. In fact, metformin action does require ROS generation since AMPK activation is prevented if SOD1 or SOD2 is overexpressed [20]. In the same line, restoration of electron flow by introducing a rotenone-insensitive NADH dehydrogenase prevents the metformin-induced AMPK activation [18]. The role of peroxynitrite as intermediate has also been confirmed, since inhibition of nitric oxidase synthase (NOS) with L-NAME also prevents metformin-induced AMPK phosphorylation [20]. In our cellular model, we observed a rapid increase in ROS levels, which is accompanied with the increase in UCP2 levels, that would be acting as part of the antioxidant defense (Fig. 3B). SOD levels also increased, although this did not occur until 12 h later. These findings indicate that UCP2 constitutes the first line of defense and that its levels can be rapidly modulated by its efficient translational regulation [22,38]. While the exact sequence of events in our model remains to be established, it appears that ROS-induced activation of AMPK leads to increased UCP2 levels since AMPK inhibition totally prevented the metformin-induced increase (Fig. 3D). A similar relationship has been described previously in endothelial cells, in which AICAR increased UCP2 levels and reduced superoxide levels [39,40]. We also observed the phosphorylation of AMPK and its downstream target ACC, with a rise evident from the very first time point. We should mention that metformin has previously been shown to activate AMPK in 3T3-L1 adipocytes [32,33].

One of the most striking findings of our work was the capacity of the uncoupler FCCP to rapidly reverse metformin-induced inhibition of respiration in intact cells (Fig. 4B). Thus while metformin alone induced a sustained 50% inhibition of cellular respiration, identical oxygen consumption rates were observed in control and metformin-treated cells in the presence of FCCP (Fig. 4D). This reversible inhibition contrasts with that induced by rotenone at concentrations that produce a similar inhibition of respiration as metformin. However, this puzzling aspect of metformin's behavior is not unprecedented as studies into the effect of guanidine on isolated mitochondria performed in the sixties also demonstrated dinitrophenol-mediated reversal of guanidine inhibition [4,36,41,42]. Derived from those findings came the observation that the inhibition of mitochondrial respiration by phenylethylbiguanide could also be antagonized by free fatty acids [42].

It is surprising that while the mechanism of metformin action remains unclear, the earlier findings described above are nowadays neither taken into consideration nor challenged. Our results clearly demonstrate that the inhibition of respiration by metformin is fully reversible in 3T3-L1 adipocytes, and furthermore, that adipocytes display the same rate of palmitate oxidation (Fig. 4E). This fatty acid-induced reversal of metformin inhibition may be relevant to the drug's mode of action. AMPK phosphorylates and inactivates ACC, inhibiting fatty acid synthesis and stimulating fatty acid oxidation. In addition, reduced synthesis of the ACC product, malonyl-CoA, should relieve the inhibition of fatty acid transfer into the mitochondria and increase fatty acid oxidation [31,43]. AMPK stimulation of fatty acid oxidation facilitates the removal of plasma fatty acids, which is made possible by the fatty acids reversing metformin inhibition of NADH oxidation. Moreover, metformin is known to improve the lipid profile of diabetic patients, reducing plasma triglyceride and LDL concentrations [1,2]. In summary, our results could provide an explanation of how metformin favors the mitochondrial oxidation of fatty acids, while preventing the mitochondrial oxidation of pyruvate that would preferentially be converted to lactate.

Acknowledgements

This work was supported by project grants from the Spanish Ministry of Science and Innovation (BFU2006-08182, SAF2009-07126, SAF2010-20256 and Consolider-Ingenio CSD2007-00020) and the Comunidad de Madrid (S2010/BMD-2402). A.A. was supported by a predoctoral fellowship from the "Master and Back" programme of the autonomous region of Sardinia (Italy). M.M.G.-B. was supported by the "Ramón y Cajal" programme of the Spanish Ministry of Science and Innovation. The authors acknowledge the editorial assistance of Dr. Mark Sefton (BiomedRed SL, Spain). The expert technical assistance of Pilar Zaragoza is gratefully acknowledged.

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