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Differential effects of metformin glycinate and hydrochloride in glucose production, AMPK phosphorylation and insulin sensitivity in hepatocytes from non-diabetic and diabetic mice

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ABSTRACT

The liver is a main target tissue of the biguanide metformin which activates AMP-activated protein kinase (AMPK). We previously reported that administration of metformin glycinate showed a greater decrease of glycated hemoglobin A1c than a placebo in patients with type 2 diabetes mellitus (T2DM). In this study, we compared the effects of metformin hydrochloride, the oral antidiabetic drug of first choice, with those of metformin glycinate in hepatocytes from non-diabetic and diabetic mice and humans. Both formulations were equally potent regard to the reduction of basal and glucagon-induced glucose production and mRNA levels of gluconeogenic enzymes (*Pck1* and *G6pc*) in hepatocytes from C57/Bl6 mice and humans. On the contrary, phosphorylation of AMPK and its substrate acetyl CoA carboxylase (ACC) was faster in hepatocytes treated with metformin glycinate. Likewise, we found stronger reduction in hepatocytes from obese/diabetic *db/db* mice of glucagon-induced glucose output and more sustained AMPK phosphorylation after treatment with metformin glycinate. Importantly, insulin sensitization regarding phosphorylation of AKT (Ser473) was enhanced in hepatocytes from *db/db* mice or humans pretreated with metformin glycinate. In conclusion, our data indicate that metformin glycinate may be an alternative therapy against insulin resistance during obesity and/or T2DM.

1. Introduction

In 2017, ~425 million individuals were estimated to have been diagnosed with diabetes mellitus worldwide, a prevalence of 8.8%, which is predicted to increase up to ~628 million (9.9%) by 2045, mainly due to an increase in type 2 diabetes mellitus (T2DM). The mortality rate of this disease, including indirect mortalities, is estimated to be around 4 million per year for all age groups, which is equivalent to one death every 8 s (International Diabetes Federation, 2017).

Among the peripheral tissues involved in the control of glucose homeostasis, the liver plays a major role since it has the ability to consume and store glucose in the postprandial state and produce glucose during fasting. These processes, modulated in an opposite way by insulin and glucagon, are dysregulated in T2DM leading to hyperglycaemia in both fasted and postprandial states (Petersen et al., 2017). Since chronic hyperglycaemia is the main characteristic feature of the diabetic condition, a tight control of glucose homeostasis is essential to counteract this pathological condition.

The liver is a major tissue of action of the biguanide metformin due to the ability of this drug to inhibit gluconeogenesis by targeting multiple hepatic enzymes and metabolites as recently reviewed by Rena and co-workers (Rena et al., 2017). The effect of metformin in the mitochondria where it inhibits Complex I of the respiratory chain and decreases ATP availability for gluconeogenesis has been reported (Owen et al., 2000). Due to the effect of metformin in increasing AMP:ATP and ADP:ATP ratios, this drug was recognized to activate AMP-

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Abbreviations

ACC	Acetyl coenzyme A carboxylase
AMPK	AMP-activated protein kinase
Bt-cAMP	dibutiryl-cAMP
G6PC	Glucose-6-phosphatase, catalytic subunit
HGP	Hepatocyte glucose production
LDH	Lactate dehydrogenase
Pck1	Gene encoding phosphoenolpyruvate carboxykinase (PEPCK)
T2DM	Type 2 Diabetes Mellitus

activated protein kinase (AMPK) in hepatocytes and livers from rats (Zhou et al., 2001). However, independently of AMPK, cAMP-dependent effects of metformin in the suppression of hepatic glucose production (HGP) have been observed (Miller et al., 2013). More recently, it has been demonstrated that metformin also inhibits mitochondrial glycerophosphate dehydrogenase, thereby preventing glycerol from contributing to gluconeogenic flux (Madiraju et al., 2014).

The formulation of metformin hydrochloride is currently the oral antidiabetic drug of first choice for the treatment of T2DM being prescribed to at least 150 million patients worldwide (Inzucchi et al., 2012). Pharmacological effects of metformin hydrochloride include significant beneficial changes in glucose control, insulin levels and diastolic blood pressure with moderate changes in body weight (Scarpello and Howlett, 2008). Despite all the benefits obtained with metformin hydrochloride and its low risk of hypoglycemic events, the treatment with this drug is associated with gastrointestinal intolerance in 20–30% of patients. In 5% of these cases, patients have to discontinue metformin because of the above mentioned side effects (Kirpichnikov et al., 2002; Spiller and Quadrani, 2004). These adverse effects have been related to high concentrations of the drug in the enterocytes, increases in luminal serotonin and, more recently, through its effect in gut microbiota by increasing *Escherichia* spp. and decreasing *Intestinibacter* spp. (Cubeddu et al., 2000; Dujic et al., 2015, 2016; Forslund et al., 2015).

Our previous studies have reported that a new chemical structure of metformin, metformin glycinate, showed a faster absoption than metformin hydrochloride without differences in the observed side effects and with similar gastrointestinal tolerability between both preparations (Garza-Ocañas et al., 2009, 2011). In addition, the treatment with metformin glycinate led to a greater decrease in glycated hemoglobin A1c concentrations than placebo in a group of drug-naïve adult patients with T2DM (Gonzalez-Ortiz et al., 2012). However, there are no mechanistic studies exploring the molecular actions induced by metformin glycinate in comparison with metformin hydrochloride. In the present study we analyzed the effect of both metformin formulations (glycinate and hydrochloride) in the suppression of glucose production, activation of AMPK and insulin signaling in hepatocytes from control and *db/db* mice, a well characterized mouse model of T2DM. We also provide new insights on the differential effect of both metformin formulations in primary human hepatocytes.

2. Materials and methods

2.1. Reagents

Fetal bovine serum (FBS) and culture media were obtained from Invitrogen (Life Technologies, Gran Island, NY). TRIzol reagent, insulin, glucagon, dibutiryl cAMP and bovine serum albumin (BSA) were from Sigma Aldrich (St. Louis, MO). Bradford reagent, acrylamide and Clarity™ ECL Western Blotting Substrate were purchased from Bio-Rad (Hercules, CA). Both metformin standards, metformin glycinate (also known as DMMET-01) and metformin hydrochloride, were provided by Laboratorios Silanes (Mexico City, Mexico) (Garza-Ocañas et al., 2009, 2011; Gonzalez-Ortiz et al., 2012).

2.2. Animals

C57BL/6J male mice and *db/db* and *db+* male mice (C57BL/KsJ genetic background) were purchased from Charles River Laboratories (Wilmington, MA). Mice were maintained in light/dark (12-h light/12-h dark), temperature (22 °C) and humidity-controlled rooms, and fed *ad libitum* with standard diet with free access to water at the animal facilities of the Instituto de Investigaciones Biomédicas Alberto Sols (CSIC-UAM, Madrid). All animal experimentation was conducted in accord with Spanish and European legislation and approved by the CSIC and Comunidad de Madrid Animal Care and Use Committees.

2.3. Isolation and culture of primary mouse hepatocytes

Primary mouse hepatocytes were isolated from non-fasting C57Bl/6 male mice or *db+* and *db/db* male mice, all of them at 8–12 weeks of age, by perfusion with collagenase as described (Benveniste et al., 1988). Cells were seeded on 6 or 12-well collagen IV pre-coated plates (Corning, New York, NY) and cultured in media containing DMEM and Ham's F-12 medium (1:1) with heat-inactivated 10% FBS, supplemented with 2 mM glutamine, 15 mM glucose, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mM sodium pyruvate (attachment media) and maintained in this medium for 24 h. Then, medium was changed according with the experiments as described in Results and in the Figure Legends.

2.4. Isolation and culture of primary human hepatocytes

Human hepatocytes were isolated by the two-step collagenase procedure from non-tumor areas of liver biopsies from patients submitted to a surgical resection for liver tumors after obtaining patients' written consent at Hospital Virgen del Rocio (Sevilla, Spain) according to Pichard et al. (2006).

2.5. Evaluation of cell viability

Cellular viability was evaluated by lactate dehydrogenase (LDH) (Rojo et al., 2012) or crystal violet (Valdecantos et al., 2015) assays. In the LDH assay, cell cytotoxicity was assessed by measuring the release of cytoplasmic LDH into cell culture supernatants. LDH activity was assayed using the Cytotoxicity Detection Kit (Roche Diagnostics, USA) according to the manufacturer's instructions. Experiments were performed in triplicate. The LDH activity was quantified by measuring the wavelength absorbance at 490 nm. The percentage of cell cytotoxicity was calculated using the following formula: 100 × (experimental LDH release)/(total LDH release). In the Crystal violet assay, after cell incubation with various treatments, the medium was discarded, and the remaining viable adherent cells were fixed with 4% PFA for 10 min. Then, the cells were stained with crystal violet (0.1% wt/vol) for 30 min. After this time, the plates were rinsed with tap water and allowed to dry and, finally, 10% (vol/vol) acetic acid was added to allow solubilization. The absorbance of each plate was red in the spectrophotometer at 590 nm.

2.6. Hepatocyte glucose production (HGP)

Primary hepatocytes from human or mouse origin were isolated and plated as described above. After 24 h, cells were cultured in serum-free, phenol-free DMEM medium with 5.5 mM glucose and 1 mM glutamine for 3 h, after that, cells were washed twice to remove glucose from the culture plates and further stimulated with glucagon (100 nM) in glucose production medium (DMEM without glucose and phenol red, 1 mM glutamine, 2 mM sodium pyruvate, 20 mM sodium lactate) for 6 h or 16 h. In the indicated conditions, metformin glycinate or metformin hydrochloride

was added at the doses shown in the Figures. At the end of the culture time, glucose was measured by the glucose oxidase-peroxidase method (Biosystems, Spain) according with the manufacturer's instruction. Glucose levels were referred to total protein content of each culture plate.

2.7. Quantitative real-time PCR analysis

Total RNA was extracted with TRIzol® reagent (Invitrogen, Madrid, Spain) and reverse transcribed using a SuperScript™ III First-Strand Synthesis System for qPCR following the manufacturer's indications (Invitrogen). qPCR was performed with an ABI 7900 sequence detector. Primer sequences for mouse *G6pc* and *Pck1* are described as follows: *mG6pc,* forward 5′ TCCCCAGAATTCTCCACTTG 3′, reverse 5′ AACAT CGGAGTGACCTTTGG 3'; *mPck1*, forward, 5′ AAGCATTCAACGCCAGG TTC 3′, reverse 5′ GGGCGAGTCTGTCAGTTCAAT 3′, *mActb* (housekeeping gene), forward 5′ AGGAGGAGCAATGATCTTGATCTT 3′, reverse 5′ TCCTTCCTGGGCATGGAG 3'. Primer sequences for human genes were: *hG6pc,* forward 5′ TGTCCAGCTAGCCAACTCCT 3'; reverse, 5′ AAAATTAGCCTGGCATGGTG 3'; *hActb* (housekeeping gene), forward 5′ AGGAGGAGCAATGATCTTGATCTT 3′, reverse 5′ TCCTTCCTGGGC ATGGAG 3'. Data analysis is based on the ΔΔCt method with normalization of the raw data to housekeeping genes as described in the manufacturer's manual (Applied Biosystems). All PCRs were performed at least in triplicate.

2.8. Western blot analysis

After culture and treatments, cells were scraped off in lysis buffer containing 10 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, 30 mM disodium pyrophosphate, 50 mM NaF, 100 μM Na_3VO_4, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml aprotinin (pH 7.6). Cellular lysates were clarified by centrifugation at 12.000 × *g* for 10 min and, after protein content determination by the Bradford method (Bio-rad), they were submitted to Western blot analysis. After SDS-PAGE, gels were transferred to Immobilon membranes (Millipore) and were blocked using 5% non-fat dried milk or 3% bovine serum albumin (BSA) in 0.05% Tween-20, 10 mM Tris-HCl, 150 mM NaCl pH 7.5, and incubated overnight with antibodies as indicated in 0.05% Tween-20, 10 mM Tris-HCl, 150 mM NaCl pH 7.5. Immunoreactive bands were visualized using Clarity™ ECL Western Blotting Substrate (Bio-Rad). Antibodies used were: anti-phospho AKT (Ser473), anti-phospho AMPK (Thr172), anti-AMPK, anti-phospho ACC (Ser79) purchased from Cell Signaling Technology (MA, USA), anti-α-Tubulin purchased from Sigma Aldrich (St. Louis, MO) and anti-p85-

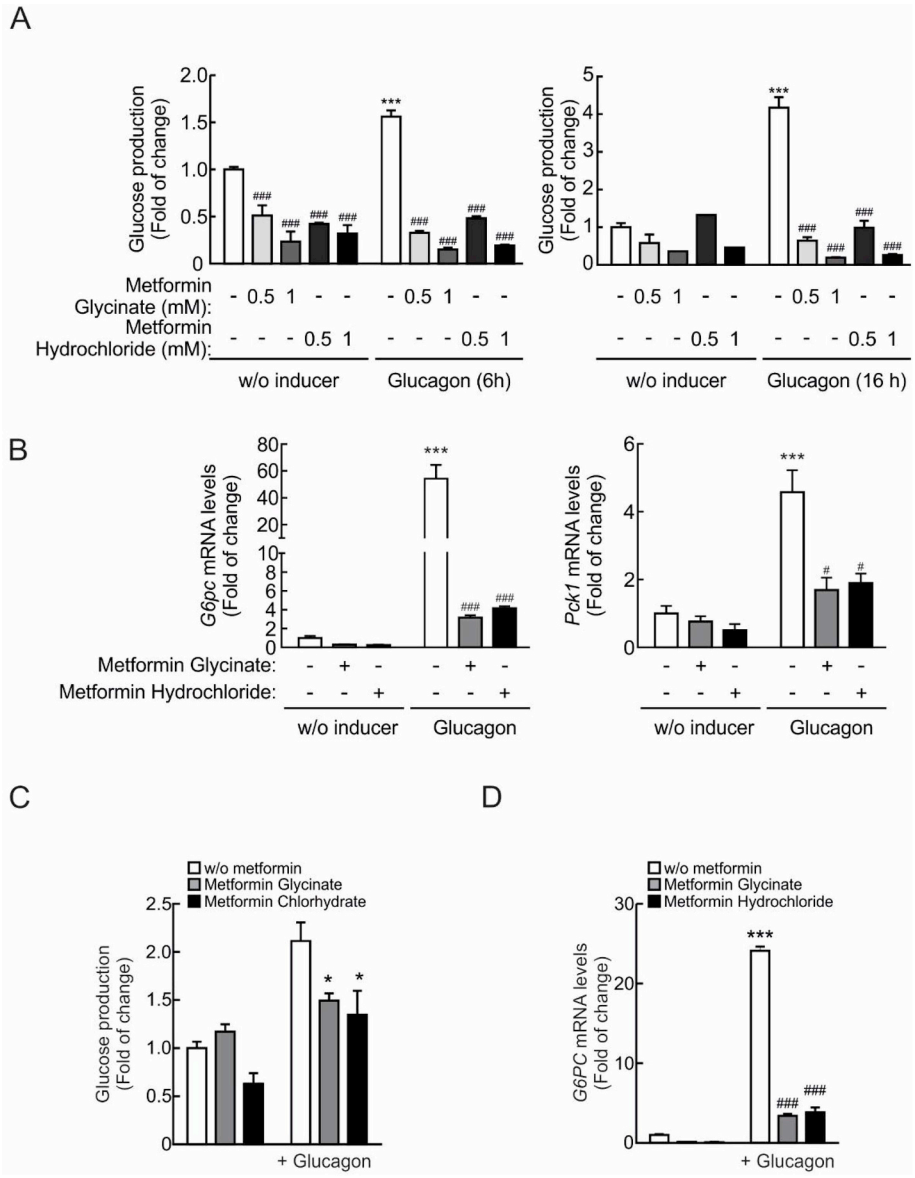

Fig. 1. Metformin glycinate and metformin hydrochloride decreased glucose production and mRNA levels of gluconeogenic enzymes in mouse and human hepatocytes. A, Mouse primary hepatocytes were maintained in low-glucose (5.5 mM) and serum-free conditions for 3 h in the presence or in the absence of the indicated concentrations of metformin glycinate or metformin hydrochloride. Then, hepatocytes were stimulated with glucagon (100 nM) with or without metformin glycinate or metformin hydrochloride in glucose production medium for 6 h (left panel) and 16 h (right panel). Glucose was then analyzed in the culture medium. **B,** *G6pc* and *Pck1* mRNA levels of primary hepatocytes treated for 6 h with 0.5 mM metformin glycinate or metformin hydrochloride in the absence or presence of 100 nM glucagon. **C**, Glucose production assayed in the culture medium after the stimulation of primary human hepatocytes with glucagon (100 nM) with or without metformin glycinate or metformin hydrochloride at 0.5 mM concentration for 6 h. **D,** *G6PC* mRNA levels in primary human hepatocytes treated as in **C**. **A-D,** Values are mean ± SEM. Statistical analysis was performed by two-way ANOVA followed by Bonferroni *post-hoc* test. $^{***}p < 0.001$ versus w/o glucagon; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ versus w/o metformin.

PI3K purchased from Merk-Millipore.

2.9. Data analysis

Data are reported as the mean ± SEM of at least 3 independent experiments performed in duplicate. To determine the effects of genotype or treatments, one-way or two-way ANOVA followed by Bonferroni test, respectively, were carried out. The *p* values presented in figures correspond to post hoc test. All statistical analysis was performed using the GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA). Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Metformin glycinate and metformin hydrochloride similarly suppressed glucose output and gluconeogenic enzymes gene expression in primary hepatocytes from C57/Bl6 mice and humans

The cytotoxicity of metformin glycinate and metformin hydrochloride was analyzed in mouse primary hepatocytes from C57/Bl6 male mice by crystal violet staining and LDH activity. Hepatocytes were plated in DMEM/F12 medium supplemented with 10% FBS and after 24 h in culture, cells were treated with 0.5 or 1 mM of each metformin formulation for 6 or 16 h (Supplementary Fig. 1A and 1B). Under these experimental conditions neither formulation affected the cellular viability of primary hepatocytes.

To evaluate the efficacy of metformin glycinate and metformin hydrochloride to reduce gluconeogenesis, glucose production assays were conducted as well as gluconeogenic enzymes gene expression (*Pck1* and *G6pc* mRNAs) measurement in primary hepatocytes pretreated with either formulation (0.5 and 1 mM) for 3 h prior to the stimulation with glucagon (100 nM) for 6 or 16 h (in the presence of metformins) in accordance with the metformin doses and incubation time-periods previously reported by Cao and co-workers (Cao et al., 2014). As depicted in Fig. 1A, glucagon increased hepatic glucose output to the culture medium in a time-dependent manner and comparable inhibition was observed with the two formulations of metformin. Of note, both metformins equally decreased basal hepatic glucose output in hepatocytes.

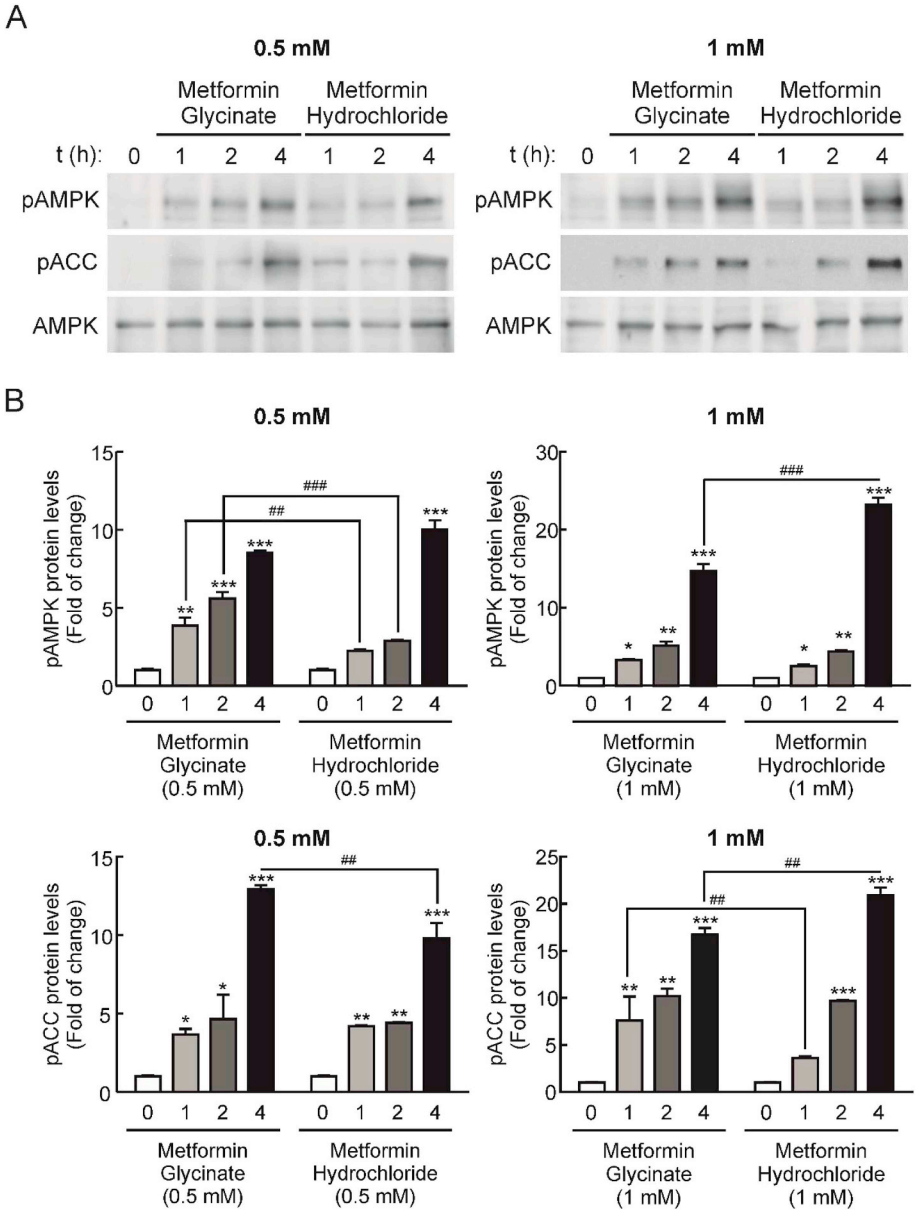

Fig. 2. Stimulation with metformin glycinate or metformin hydrochloride activated AMPK signaling pathway in mouse hepatocytes. A, Primary hepatocytes were maintained in low-glucose (5.5 mM) and serum-free conditions for 3 h and then treated with 0.5 mM (left panel) and 1 mM (right panel) metformin glycinate or metformin hydrochloride for the indicated time periods. Immunoblots showing phospho-AMPK and phospho-ACC levels. Total AMPK levels were used as loading control. **B,** Densitometric quantification of the indicated protein levels. Values are mean ± SEM. Statistical analysis by two-way ANOVA followed by Bonferroni *post-hoc* test. *p < 0.05, **p < 0.01, ***p < 0.001 versus w/o metformin; ##p < 0.01, ###p < 0.001 metformin glycinate versus metformin hydrochloride.

To corroborate these results, we measured mRNA levels of gluconeogenic enzymes under the same experimental conditions. As depicted in Fig. 1B, *G6pc* (encoding glucose 6 phosphatase) and *Pck1* (encoding phosphoenolpyruvate carboxykinase) mRNA levels were induced by glucagon and significantly decreased in hepatocytes treated with metformin glycinate or metformin hydrochloride. No significant differences were found in the effect of both formulations. Likewise, similar inhibition of glucose production was obtained in hepatocytes stimulated with dibutiryl-cAMP (0.5 mM) with the two formulations (Supplementary Figure 2). We also found the same effect of metformin glycinate and metformin hydrochloride in the reduction of glucose output and *G6PC* mRNA levels in primary hepatocytes isolated from human livers (Fig. 1C and D).

3.2. Metformin glycinate promoted an earlier phosphorylation of AMPK than metformin hydrochloride in mouse primary hepatocytes

Several studies in primary hepatocytes have reported that metformin hydrochloride suppresses glucose production through the activation of AMPK signaling pathway (Cao et al., 2014; Meng et al., 2015; Zhou et al., 2001). On that basis, our next goal was to unravel the effect of metformin glycinate in comparison to the hydrochloride in the phosphorylation of AMPK and its substrate acetyl coenzyme A carboxylase (ACC). Since Cao and co-workers (Cao et al., 2014) reported AMPK phosphorylation in primary mouse hepatocytes treated with 0.5 mM metformin hydrochloride, we used 0.5 and 1 mM to compare the effect of both formulations on AMPK-mediated signaling. To achieve this, mouse primary hepatocytes were maintained in low glucose (5.5 mM) and serum-free DMEM medium for 3 h and then stimulated for 1, 2 and 4 h with 0.5 mM (Fig. 2A and B, left panel) or 1 mM (Fig. 2A and B, right panel) of each metformin formulation. Both formulations increased AMPK phosphorylation at Thr172 and ACC phosphorylation at Ser79 in a dose and time-dependent manner. Interestingly, at 0.5 mM, the effect of metformin glycinate was significantly higher than the effect of metformin hydrochloride at 1 and 2 h of treatment. By contrast, when metformins were used at 1 mM concentration, significant differences among the two formulations were found at 4 h, time at which the effect of metformin hydrochloride was significantly higher. The analysis of ACC revealed a significant increase in its phosphorylation in hepatocytes treated with 0.5 mM metformin glycinate at 4 h compared to the effect of metformin hydrochloride. Using 1 mM, the effect of metformin glycinate was accelerated with the highest effect at 1 h whereas the increase in ACC phosphorylation by metformin hydrochloride was detectable at 2 h.

3.3. At low concentrations, metformin glycinate is more effective in reducing glucose output than metformin hydrochloride in primary hepatocytes from diabetic db/db mice

The effect of metformins in hepatocytes from obese and type 2 diabetic *db/db* mice was addressed in this study. For this goal, we first established the optimal conditions for the isolation of viable hepatocytes from *db/db* mice. Hepatocytes isolated from their age-matched *db* + lean littermates were used as control. In all experiments hepatocytes were isolated from mice at 8–12 weeks, age at which *db/db* mice were hyperglycaemic and highly insulin resistant (Arroba et al., 2016). The optimal culture conditions were assessed by cellular viability assays. As shown in Supplementary Figure 3, hepatocytes from *db/db* mice preserved their viability after the treatment with 1–10 mM concentrations of either metformin. Then, mouse primary hepatocytes from *db*+ and *db/db* mice were pre-treated for 3 h with metformin glycinate or metformin hydrochloride at the indicated concentrations ranging from 0.1 to 1 mM followed by the addition of glucagon (100 nM) for 6 h (Fig. 3). Interestingly, in hepatocytes from control *db* + mice the reduction of hepatic glucose output was significantly enhanced by 0.5 mM metformin glycinate compared to the effect of metformin hydrochloride.

These results revealed differences between the C57BLKS/J (Fig. 3) and the C57BL/6J (Fig. 1A) mouse genetic backgrounds. The analysis of glucose released to the culture medium in hepatocytes from *db/db* mice under basal conditions (in the absence of glucagon) showed higher levels compared to those of the hepatocytes from *db* + mice, confirming the diabetic feature of these cells. Interestingly, in hepatocytes from *db/db* mice, both metformin glycinate and metformin hydrochloride reduced glucose output in a dose-dependent manner, being the effect of metformin glycinate significantly enhanced at 0.5 and 1 mM concentrations compared to their respective untreated condition whereas metformin hydrochloride reached statistical significance only at 1 mM concentration. When the effect of the two metformin formulation was compared in hepatocytes from both *db/db* and control *db* + mice, the inhibition of glucose output by metformin glycinate was statistically higher than that of metformin hydrochloride at the dose of 0.5 mM (Fig. 3). By contrast, the effect of both formulations was comparable in *db/db* and *db* + hepatocytes in the 16 h treatment (Supplementary Figure 4).

3.4. Effect of metformin glycinate and metformin hydrochloride in AMPK signaling in hepatocytes from db+ and db/db mice

We next evaluated AMPK and ACC phosphorylation in primary hepatocytes from age-matched *db*+ and *db/db* mice treated with metformin glycinate or metformin hydrochloride (0.5 mM) for the indicated time-periods (1–16 h). In hepatocytes from *db* + mice, both

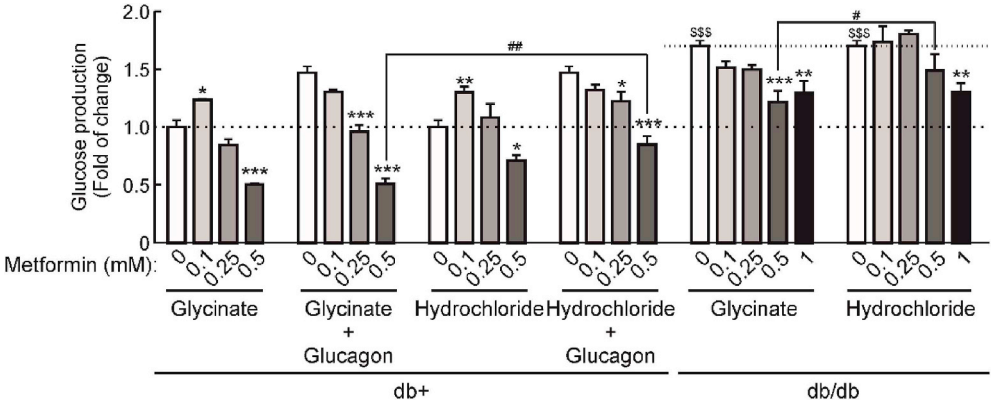

Fig. 3. Effects of metformin glycinate or metformin hydrochloride on glucose production in hepatocytes from *db*+ and *db/db* mice. Mouse primary hepatocytes (*db*+ or *db/db*) were treated with metformin glycinate or metformin hydrochloride at the indicated concentration in low-glucose (5.5 mM) and serum-free medium for 3 h and then with metformins with or without glucagon (100 nM) for 6 additional h. Hepatocyte glucose production (HGP) is represented. Values are mean ± SEM. Statistical analysis was performed by two-way ANOVA followed by Bonferroni *post-hoc* test. *p < 0.05, **p < 0.01, ***p < 0.001 versus w/o metformin (white bars); #p < 0.05, ##p < 0.01, metformin glycinate versus metformin hydrochloride; $$$p < 0.001 basal *db/db* versus basal *db*+.

metformin formulations increased AMPK and ACC phosphorylation in a time-dependent manner, reaching statistically significance at 4, 8 and 16 h in AMPK phosphorylation and at 2, 4, 8 and 16 h in ACC phosphorylation compared to their respective basal conditions (Fig. 4A, B, 4C, 4D). In hepatocytes from *db/db* mice, metformins also increased AMPK and ACC phosphorylation in a time-dependent manner, reaching significant values at 2, 4, 8 and 16 h in hepatocytes stimulated with metformin glycinate and at 8 and 16 h in AMPK phosphorylation and at 4, 8 and 16 h in ACC phosphorylation in hepatocytes treated with metformin hydrochloride compared to their respective basal conditions (Fig. 4E, F, 4G, 4H). When the two formulations were compared, the elevation in AMPK phosphorylation induced by metformin glycinate in hepatocytes from *db/db* mice was significantly higher at the time-period of 16 h (Fig. 4F and H). Regarding ACC, the effect of metformin glycinate was significantly higher at 1 h in *db* + hepatocytes (Fig. 4A and C) and at 2 h in *db/db* hepatocytes (Fig. 4E and G). Of note, only the phosphorylation of ACC at 16 h was significantly higher in *db/db* hepatocytes treated with metformin hydrochloride (Fig. 4F and H). Altogether, these results suggest a more rapid phosphorylation of AMPK and ACC in hepatocytes treated with metformin glycinate.

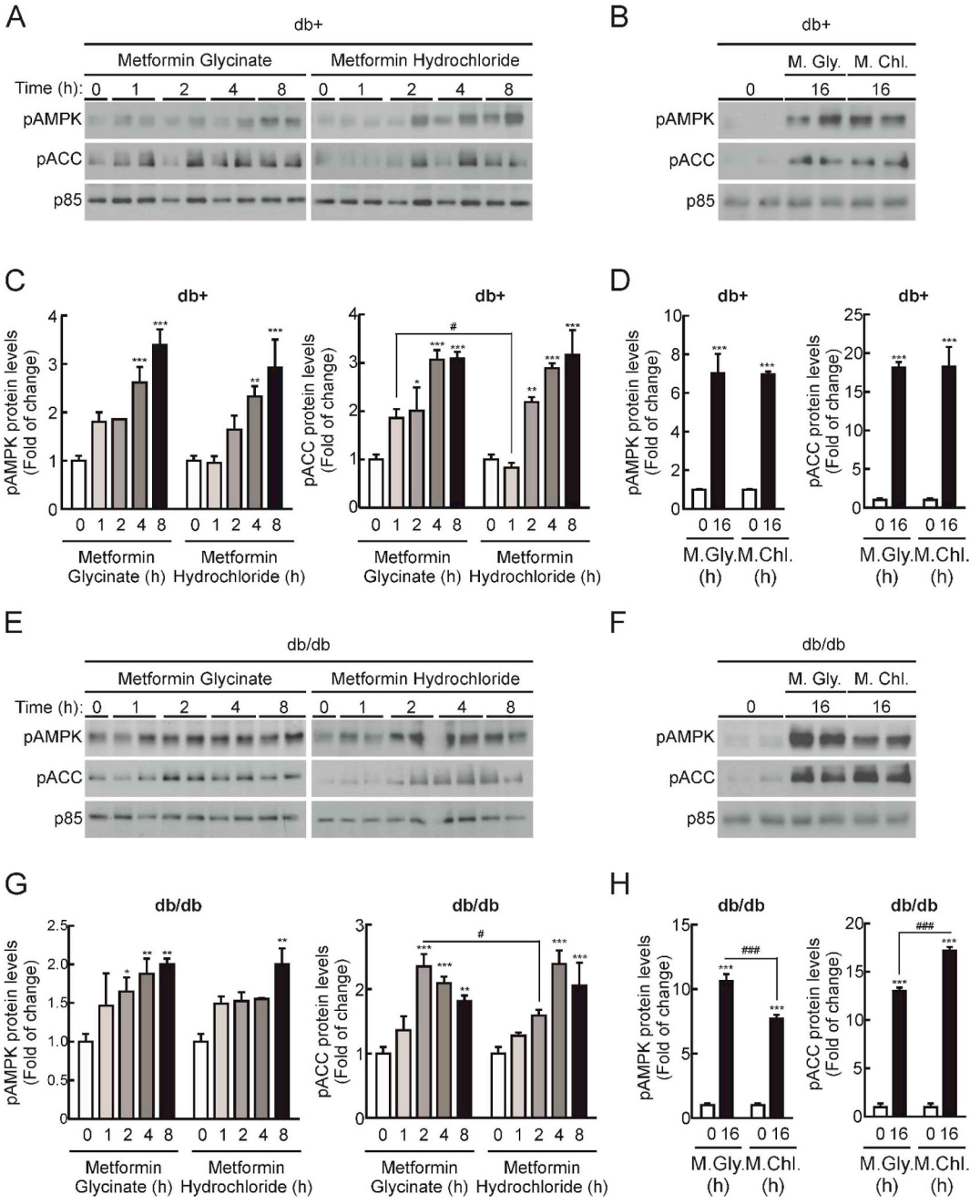

Fig. 4. Metformin glycinate induced a more rapid phosphorylation of AMPK and ACC than metformin hydrochloride in hepatocytes from *db*+ and *db/db* mice. Primary mouse hepatocytes from *db*+ **(A, B)** or *db/db* mice **(E, F)** were maintained in low-glucose (5.5 mM) and serum-free medium for 3 h and then treated with metformin glycinate ormetformin hydrochloride (0.5 mM) for the indicated times. **C-D-G-H,** Densitometric quantification of phosphorylation levels. Values are mean ± SEM. Statistical analysis was performed by two-way ANOVA followed by Bonferroni *post-hoc* test. *p < 0.05, **p < 0.01, ***p < 0.001 versus w/o metformin (white bars); #p < 0.05, ###p < 0.001, metformin glycinate versus metformin hydrochloride.

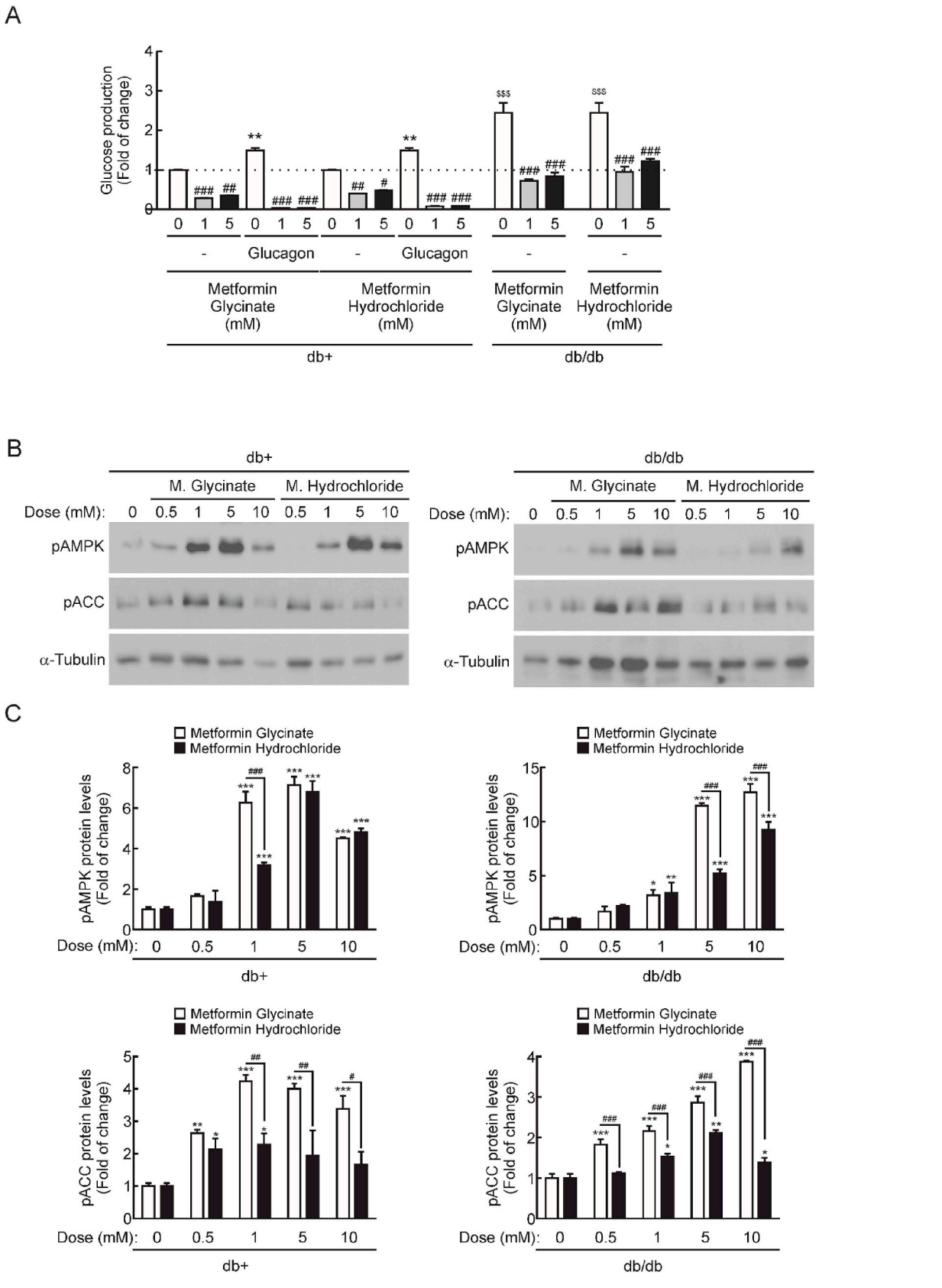

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Fig. 5. Hepatocytes from *db/db* mice required high concentrations of both metformin formulations to activate AMPK signaling compared to hepatocytes from *db* + mice. A, *db*+ and *db/db* mice primary hepatocytes were cultured as detailed in Fig. 1 legend using 1 and 5 mM metformins concentrations. Graph shows glucose production. Values are mean ± SEM. Statistical analysis was performed by two-way ANOVA followed by Bonferroni *post-hoc* test. $^{**}p < 0.01$, $^{***}p < 0.001$ versus w/o glucagon; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus w/o metformin; $^{\$\$\$}p < 0.001$ basal *db/db* versus basal *db*+. **B,** Primary mouse hepatocytes (left panel, *db*+ hepatocytes; right panel, *db/db* hepatocytes) were cultured in serum-free conditions for 3 h and then treated with metformin glycinate or metformin hydrochloride at the indicated concentrations for additional 6 h. Immunoblots showing pAMPK, pACC proteins and α-Tubulin protein as a loading control. **C,** Densitometric quantification of protein levels from experiments corresponding to panel B. Values are mean ± SEM. Statistical analysis was performed by two-way ANOVA followed by Bonferroni *post-hoc* test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus w/o metformin; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ metformin glycinate versus metformin hydrochloride.

3.5. Primary hepatocytes from db/db mice required higher concentrations than cells from control mice of both metformins to reduce glucose output and to activate AMPK signaling

Due to the lower effect of both metformins used at 0.5 mM in decreasing glucose output in hepatocytes from *db/db* mice compared to the non-diabetic *db* + controls (Fig. 3), dose-response analysis was also conducted with higher concentrations of the formulations, as used in other studies in hepatocytes (Foretz et al., 2010; He et al., 2009). As shown in Fig. 5A, both metformins, at 1 and 5 mM, efficiently reduced glucose release in hepatocytes from *db/db* mice reaching values comparable to those of the untreated hepatocytes from *db* + mice (basal condition indicated with a discontinuous line). Phosphorylation of AMPK and ACC was also analyzed under these experimental conditions. In hepatocytes from *db* + mice, AMPK phosphorylation was significantly higher at the dose of 1 mM of metformin glycinate compared to similar dose of metformin hydrochloride (Fig. 5B and C). Interestingly, the dose of 10 mM of both formulations caused a drop in the phosphorylation of AMPK in hepatocytes from *db* + mice, probably due to off-target effects. Regarding ACC phosphorylation in *db* + hepatocytes, the effect of metformin glycinate was significantly higher than that of metformin hydrochloride at 1 and 5 mM concentrations. In hepatocytes from *db/db* mice, maximal effect of AMPK phosphorylation was reached at 5 mM in cells treated with metformin glycinate whereas the maximal effect was detected at 10 mM in cells treated with metformin hydrochloride. Also, the effect of metformin glycinate at 5 and 10 mM concentrations in inducing AMPK phosphorylation was significantly enhanced compared to the effect of the same concentrations of metformin hydrochloride. Importantly, phosphorylation of ACC was also higher in *db/db* hepatocytes treated with metformin glycinate in all concentrations tested, suggesting a lower threshold in AMPK phosphorylation to achieve ACC phosphorylation.

3.6. Comparison between metformin glycinate and metformin hydrochloride in the enhancement of insulin signaling in hepatocytes from db/db mice and humans

Metformin hydrochloride enhances insulin sensitivity by activating AMPK and PI3K/AKT signaling pathways in primary hepatocytes from mice (Fullerton et al., 2013; Sopasakis et al., 2010; Zhou et al., 2001). Therefore, a possible differential effect of both formulations in hepatocytes from *db*+ and *db/db* mice was evaluated. Primary hepatocytes were maintained in low-glucose (5.5 mM) and serum-free conditions for 3 h and then treated with 0.5 mM metformin glycinate or metformin hydrochloride for 6 additional h. Then, cells were stimulated with insulin (1–10 nM) for a further 10 min. At the end of the stimulation, phosphorylation of AMPK, ACC and AKT was analyzed. As depicted in Fig. 6A and B, phosphorylation of AMPK, but not ACC, was significantly enhanced in *db* + hepatocytes pretreated with either metformin formulation before insulin stimulation. Interestingly, under these conditions of pretreatment with either metformin formulation, AKT phosphorylation in response to 10 nM insulin was increased. Then, an analysis for the response of hepatocytes from *db/db* mice was carried out (Fig. 6C and D). Insulin stimulation alone did not change AMPK phosphorylation, but this response was enhanced when *db/db* hepatocytes pretreated with metformin glycinate or metformin hydrochloride. As occurred in hepatocytes from *db* + controls, phosphorylation of ACC was not affected by insulin stimulation either in the absence or in the presence of metformins. Importantly, the phosphorylation of AKT in response to 10 nM insulin was only significantly enhanced in hepatocytes from *db/db* mice pretreated with metformin glycinate.

The relevance of the differential effect of metformin formulations on insulin sensitivity was tested in primary human hepatocytes where a significantly higher effect of metformin glycinate on insulin-induced AKT phosphorylation compared to metformin hydrochloride was detected (Fig. 6E and F).

4. Discussion

It has been recently reported by the authors of this study that administration of metformin glycinate led to a greater decrease in glycated hemoglobin A1c concentrations than placebo in a group of drug-naive adult patients with T2DM (Gonzalez-Ortiz et al., 2012). However, the precise molecular actions driven by metformin glycinate in hepatocytes were unexplored. To address this issue, we analyzed the effect of metformin glycinate in comparison to metformin hydrochloride on the suppression of glucose production in primary hepatocytes isolated from lean normoglycemic (*db*+) or obese and type 2 diabetic (*db/db*) mice. We also studied the effect of both metformin formulations in primary hepatocytes from humans. Notably, to compare the effects of the metformins, hepatocytes were treated with equimolar non-toxic concentrations.

The metformin concentrations and incubation periods recently reported by Cao and co-workers were used in this study (Cao et al., 2014). This issue is relevant since the pretreatment with metformins mimics the usual therapy for type 2 diabetic patients in which metformin is generally administered with food at night prior to the elevation in blood glucagon levels. As expected, metformin hydrochloride suppressed glucagon- or Bt-cAMP-induced glucose production in mouse hepatocytes and importantly, this study has revealed for the first time that metformin glycinate is equally effective in reducing HGP in human and mouse cells. Since HGP is tightly controlled by gluconeogenic enzymes, including G6PC and PEPCK, the gene expression analysis in hepatocytes treated with either metformin formulation also revealed similar reductions in *Pck1* and *G6pc* mRNA levels. The fact that metformin glycinate is as effective as the hydrochloride formulation is relevant since in healthy volunteers metformin glycinate had a more rapid gastrointestinal absorption and similar tolerability than metformin hydrochloride (Garza-Ocañas et al., 2009). As in the *in vitro* system metformin directly impacts the hepatocytes, it could be hypothesized that in humans the enhanced absorption of glycinate formulation and/or its prolonged half-life might accelerate its effect in reaching hepatic cells compared to the hydrochloride formulation (Garza-Ocañas et al., 2011).

Over the last decade, several studies have supported the hypothesis of an AMPK- mediated effect of metformin in the reduction of hepatic gluconeogenesis (Cao et al., 2014; Foretz and Viollet, 2011; Hundal et al., 2000; Viollet et al., 2012; Zhou et al., 2001). By contrast, other studies performed in AMPK-deficient mice have shown AMPK-independent effects of this drug (Foretz et al., 2010; Hou et al., 2018; Miller et al., 2013).

In the present study we found that the glycinate formulation was

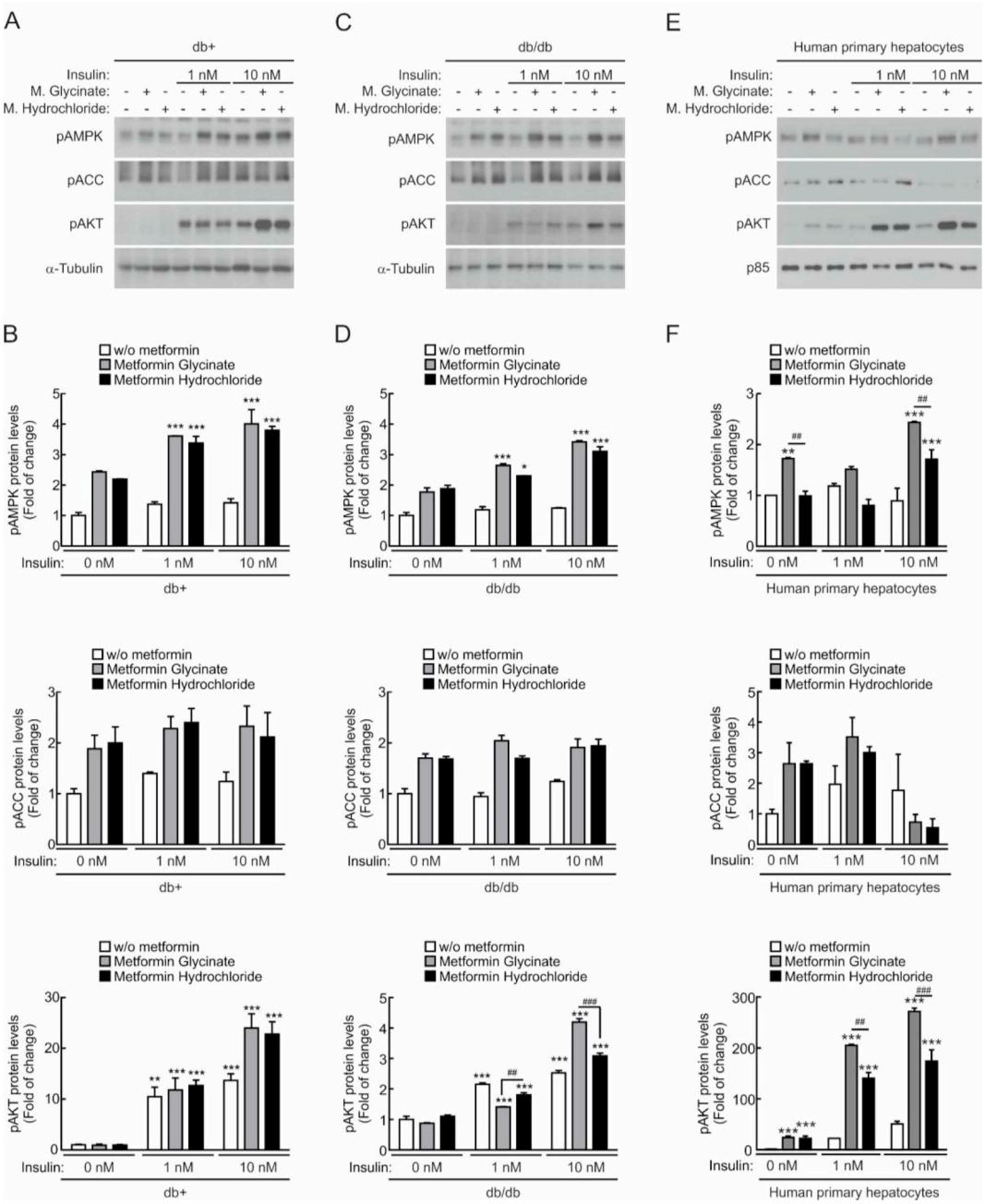

Figure 6. Hepatocytes derived from *db/db* mice are more sensitive to insulin stimulation upon pretreatment with metformin glycinate. A-C-E, Primary hepatocytes derived from *db+* (A) and *db/db* (C) mice or from human liver (E) were maintained in low-glucose (5.5 mM) and serum-free conditions for 3 h and then treated with metformin glycinate or metformin hydrochloride (0.5 mM) for 6 additional h. Insulin (1 nM or 10 nM) was added to the indicated wells for the last 10 min pAMPK, pACC and pAKT protein levels were analyzed. **B-D-F,** Densitometric quantification of the protein levels corresponding to the experiments in panels A, C or E, as indicated. Values are mean ± SEM. Statistical analysis was performed by two-way ANOVA followed by Bonferroni *post-hoc* test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus each w/o insulin condition; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, metformin glycinate versus metformin hydrochloride.

more rapid in inducing AMPK phosphorylation in comparison with the hydrochloride, and this effect might be responsible, at least in part, of its potential benefits in patients. Of note, the dose of 0.5 mM was previously reported by Cao and co-workers (Cao et al., 2014) to promote the maximal AMPK activation in hepatocytes. AMPK also plays a significant role in the regulation of lipid metabolism via its downstream effector ACC. Thus, inhibition of ACC by AMPK-mediated phosphorylation depletes malonyl-CoA content causing a reduction of fatty acid synthesis in favour of higher mitochondrial fatty acid oxidation (Coughlan et al., 2014; Hardie, 2003). In the current study, phosphorylation of ACC by 0.5 mM metformin glycinate was also accelerated, suggesting that this new formulation might have additional benefits in hepatic lipid metabolism. Further research will be necessary to address this important issue in depth.

To analyze the differential effects of metformin glycinate and hydrochloride in a diabetic context primary hepatocytes were isolated from *db/db* mice, a congenital obese animal model that recapitulates the pathogenesis of T2DM very similarly to that found in humans (Hummel et al., 1966). Importantly, the results shown in this study clearly revealed that hepatocytes from *db/db* mice preserved their diabetic feature since higher basal levels of glucose output were detected in comparison with their corresponding controls derived from lean mice littermates (*db+*). Unlike metformin hydrochloride, the glycinate formulation used at 0.5 mM concentration decreased glucose

production in *db* + hepatocytes. Likewise, *db/db* hepatocytes treated with 0.5 mM metformin glycinate displayed an early response in inducing AMPK phosphorylation in comparison with metformin hydrochloride, suggesting an accelerated effect of glycinate that may be responsible of the enhanced AMPK phosphorylation found after 16 h of treatment. Whether these temporal differences in hepatocytes could be extrapolated to implement a new and superior therapeutic intervention with metformin glycinate in type 2 diabetic patients deserves further studies.

In an attempt to achieve a drop in glucose production within the range found in the control (*db* +) hepatocytes, hepatocytes from *db/db* mice were treated with high concentrations of either metformin formulation (1–5 mM) previously tested for hydrochloride (Foretz et al., 2010; He et al., 2009). Under these conditions, a higher reduction in glucose output was found although without differences between metformin formulations. Nevertheless, at the dose of 5 mM, the effect of metformin glycinate in inducing AMPK and ACC phosphorylation was higher than the effect of the hydrochloride. These results suggest that in this case both treatments might activate AMPK above a threshold necessary to reduce glucose production or, alternatively, the existence of other AMPK-independent effects such as decrease in cAMP production (Miller et al., 2013) or inhibition of mitochondrial Complex I (Hou et al., 2018) that might be equally modulated by both metformins. Of note, when either formulation was used at 10 mM concentration the phosphorylation of AMPK dropped exclusively in *db* + hepatocytes, reflecting possible off-target effects under this non-pathologic condition.

In addition to the suppression of HGP, it has been pointed out that metformin improves hyperglycemia in T2DM by targeting hepatic insulin (Gomez-Samano et al., 2012; Gunton et al., 2003; Musso et al., 2012; Natali and Ferrannini, 2006). In this regard, Fullerton and colleagues (Fullerton et al., 2013) have shown that metformin hydrochloride improved insulin-stimulated phosphorylation of AKT and FoxO1, as well as the suppression of gluconeogenic enzymes gene expression and glucose production in insulin resistant hepatocytes loaded with palmitate. In this study it has been clearly demonstrated that insulin was able to induce AMPK phosphorylation only when *db* + or *db/db* hepatocytes were pretreated with metformin glycinate or hydrochloryde, and this response was coincident with an enhancement of AKT phosphorylation in *db* + hepatocytes stimulated with 10 nM insulin. More importantly, in *db/db* hepatocytes the enhancement of insulin-mediated AKT phosphorylation was found only in those pretreated with metformin glycinate, strongly suggesting a better insulin sensitizing effect of this new formulation under insulin resistant conditions. Of relevance, the enhanced insulin-sensitizing effect was also detected in human primary hepatocytes pretreated with metformin glycinate providing new evidences on the benefits of this formulation in human liver cells and supporting a possible clinical relevance of these data. In this regard, future experiments in *db/db* mice will address the *in vivo* relevance of our findings.

5. Conclusions

In conclusion, taking into account the tolerability and the efficacy of metformin glycinate in lowering glycated hemoglobin A1c in healthy volunteers (Gonzalez-Ortiz et al., 2012), the data reported here present preclinical mechanistic evidences suggesting that metformin glycinate might represent an alternative therapy, with additional benefits over metformin hydrochloride, in the setting of insulin resistance caused by obesity and/or T2DM.

Conflict of interest

J.G.-C. and F. F. are part-time employees of Laboratorios Silanes. No competing financial interests exist for all other authors.

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Transparency document

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2018.11.019.

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