Pioglitazone Inhibits Mitochondrial Pyruvate Metabolism and Glucose Production in

Hepatocytes

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Abbreviations: T2DM, Type II Diabetes Mellitus; HGP, hepatocellular glucose production; MPC, mitochondrial pyruvate carrier; TZD, thiazolidinedione; PIO, pioglitazone; VEH, vehicle; UK, UK5099; PPAR-γ, peroxisome proliferator-activated receptor gamma; FBP, fructose 1,6-biphosphatase; G6P, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; PC, pyruvate carboxylase.

Key words: Type 2 diabetes, pioglitazone, pyruvate, mitochondria, hepatocyte, glucose metabolism

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Abstract

Pioglitazone is used globally for the treatment of type 2 diabetes mellitus (T2DM) and is one of the most effective therapies for improving glucose homeostasis and insulin resistance in T2DM patients. However, its mechanism of action in the tissues and pathways that regulate glucose metabolism are incompletely defined. Here we investigated the direct effects of pioglitazone on hepatocellular pyruvate metabolism and the dependency of these observations on the purported regulators of mitochondrial pyruvate transport, MPC1 and MPC2. In cultured H4IIE hepatocytes, pioglitazone inhibited $[2\text{-}^{14}C]$-pyruvate oxidation and pyruvate-driven oxygen consumption and, in mitochondria isolated from both hepatocytes and human skeletal muscle, pioglitazone selectively and dose-dependently inhibited pyruvate-driven ATP synthesis. Pioglitazone also suppressed hepatocellular glucose production (HGP), without influencing the mRNA expression of key HGP regulatory genes. Targeted siRNA silencing of MPC1 and 2 caused a modest inhibition of pyruvate oxidation and pyruvate-driven ATP synthesis, but did not alter pyruvate-driven HGP and, importantly, it did not influence the actions of pioglitazone on either pathway. In summary these findings outline a novel mode of action of pioglitazone relevant to the pathogenesis of T2DM and suggest that targeting pyruvate metabolism may lead to the development of effective new T2DM therapies.

Introduction

The thiazolidinedione (TZD) class of drugs is widely used for the treatment of type 2 diabetes mellitus (T2DM) [1] and has been shown to be effective in preventing the conversion of pre-diabetes to diabetes [2]. Pioglitazone (PIO) is an archetypal member of the TZD family that promotes hypoglycemia through enhanced peripheral (muscle and fat) insulin-stimulated glucose uptake and suppressing hepatic glucose output [3, 4]. The metabolic activity of TZDs was initially believed to be secondary to a molecular interaction with peroxisome proliferator-activated receptor gamma (PPAR-γ), a nuclear receptor that regulates gene expression in response to ligand binding [5, 6]. Improvements in insulin sensitivity are thought to occur as a result of PPAR-γ mediated adipose tissue expansion [7] and an increased release of insulin-sensitizing adipokines [8]. However, growing

evidence supports direct PPAR-γ-independent actions of TZDs in insulin-sensitive tissues that are relevant to the pathogenesis and treatment of T2DM [9].

The molecular and biochemical mechanisms surrounding the PPAR-γ sparing activities of TZDs remain largely unknown. Some studies have demonstrated a direct effect of high doses of TZDs on complex I and III function in mitochondria isolated from muscle [10] and liver [11-13], while others have shown that TZDs can bind directly to outer mitochondrial membrane proteins [14, 15]. A potentially unifying hypothesis to explain the direct mitochondrial effects of TZDs and their clinical efficacy is that they modify pyruvate metabolism. Pyruvate is a key molecule in cellular energy metabolism and lies at the intersection of multiple metabolic pathways linking glycolysis to glucose oxidation. In liver, pyruvate metabolism and transport is a critical determinant of gluconeogenic flux and may therefore play an important role in regulating fasting and post-prandial hyperglycemia in patients with T2DM.

Interest in mitochondrial pyruvate transport has increased following the recent discovery of the mitochondrial pyruvate carrier proteins (MPCs), thought to be responsible for import of pyruvate into the mitochondria [16, 17]. Two groups have described a role for the MPCs in the regulation of hepatic glucose metabolism in rodents [18, 19], supporting data that mitochondrial pyruvate transport is an important orchestrator of gluconeogenesis [20]. Divakaruni and colleagues recently provided evidence that TZDs interact with MPCs in skeletal muscle cells and inhibit pyruvate-driven oxygen consumption [21]. However, whether TZDs play a role in liver pyruvate metabolism, or whether the MPCs are required for their activity in hepatocytes, is unknown.

The aim of the present study was to assess: (1) whether PIO negatively regulates pyruvate oxidation and pyruvate-driven mitochondrial ATP production in hepatocytes, (2) the impact of PIO on pyruvate driven hepatic glucose production (HGP) and 3) the role of the MPC proteins in mediating the action of PIO on pyruvate metabolism in hepatocytes. Our data demonstrate that PIO is a specific inhibitor of pyruvate oxidation in hepatocytes and significantly lowers pyruvate-driven glucose production.

However, neither MPC1 nor MPC2 were necessary to mediate the effect of PIO on pyruvate metabolism, suggesting that alternative targets may be involved in the action of TZDs in hepatocytes.

Results

Pioglitazone inhibits pyruvate oxidation in hepatocytes

Although TZDs and related compounds have been reported to inhibit pyruvate-driven uncoupled (maximal) respiration in a number of different cell types [21, 22] the direct effects of PIO have not been specifically tested in liver cells. We chose to focus on PIO because it is the only FDA-approved TZD currently in widespread clinical use and, to our knowledge, no data exists regarding the direct effect of this compound on hepatic pyruvate metabolism or glucose production. We initially investigated basal substrate oxidation, in the absence of chemical uncouplers, in control H4IIE hepatocytes using [2-^{14}C]-pyruvate and [U^{14}C]-palmitate. Four hour PIO treatment inhibited pyruvate oxidation at 10 μM, whilst pharmacological inhibition of pyruvate transport with UK5099 (UK) similarly suppressed pyruvate oxidation, albeit at much lower drug concentrations (50 nM; Fig. 1A). In agreement with one previous publication [21], this finding was reproducible in the murine skeletal muscle L6 cell line (Fig. 1B). Neither PIO nor UK treatment influenced palmitate oxidation in H4IIE cells, which was robustly suppressed by Etomoxir (10 μM; Fig. 1C), confirming that the impairment of pyruvate oxidation in hepatocytes was substrate-specific.

To further validate our radiolabeled substrate oxidation assays we performed complimentary pyruvate-driven respirometry experiments in intact cells using the XF Seahorse. Following 15 minutes incubation with treatments, PIO (10 μM) and UK (5 μM) blunted both the basal and maximal (FCCP-stimulated) oxygen consumption rates (Fig. 1D), whilst the average respiration associated with ATP turnover also tended to be lower in PIO than VEH treated cells (218 ± 18 vs 305 ± 24 pmol·min^{-1}; P=0.09). To exclude the possibility of toxicity-related actions of treatments, cell viability was assessed using a luciferase-reporter assay based on the cellular reduction potential. We found no detrimental effects on cell viability of PIO or UK across the range of concentrations used in this study (Fig.1E).

Pioglitazone inhibits pyruvate-driven ATP synthesis in isolated mitochondria

Prior findings on the inhibitory effects of TZDs on pyruvate metabolism indicated that PIO required intact cells to exert these effects [21]. Therefore, we next investigated whether the inhibitory actions of PIO treatment towards pyruvate metabolism were preserved in isolated mitochondria harvested from H4IIE cells. Consistent with our OCR findings, mitochondrial ATP synthesis from pyruvate + malate was dose-dependently (1- 10 μM) lowered following acute PIO treatment, but was not altered when ATP synthesis was supported by glutamate, succinate or palmitoylcarnitine substrates (Fig. 2A). As a crucial control, UK robustly suppressed pyruvate-driven ATP synthesis without effect on other substrates over the same concentration range. Interestingly, the PIO-induced inhibition of ATP synthesis was overcome when pyruvate concentrations exceeded 5 mM, although higher concentrations of pyruvate appeared inhibitory to ATP synthesis (Fig. 2B). Indeed, the maximal rate of pyruvate-driven ATP synthesis observed across a wide range of substrate concentrations was ~30% and 53% lower with PIO and UK treatment, respectively, compared to vehicle. To highlight the translational potential of these findings, we confirmed these results using mitochondria isolated from human skeletal muscle (Fig. 2F).

Pioglitazone inhibition of pyruvate oxidation does not require the MPC proteins

We next investigated whether expression of the mitochondrial pyruvate carrier proteins MPC1 and MPC2 was paramount to the observed effects of PIO in H4IIE hepatocytes, as has been suggested by others [21]. In agreement with previous observations following stable protein repression with lentiviral shRNA [21] or tissue-specific deletion in mice [18, 19], silencing by siRNA of MPC1 resulted in a concomitant reduction in MPC2 protein levels and vice versa (Fig. 3A-B). Silencing was most effective with siMPC2, which reduced MPC1 and MPC2 protein levels to approximately 20% and 1% of control cells, respectively (Fig. 3A-B), a degree of silencing that is at least comparable to that achieved in several recent studies using similar approaches [21, 23]. In agreement with prior data in hepatocytes [19], basal pyruvate oxidation was approximately 20% lower in siMPC2 cells (Fig. 3C). However, the relative inhibition of pyruvate oxidation by PIO and UK was identical between siSCR and siMPC2 cells (Fig. 3C), strongly arguing against a role for the MPCs in the effects of PIO on pyruvate metabolism.

Because it has been suggested that pyruvate transport in MPC1/2-silenced cells is bypassed by its cytosolic transamination to, and subsequent mitochondrial uptake of, alanine or malate, we investigated whether pyruvate metabolism was more severely compromised in isolated mitochondria from MPC2-silenced cells. Pyruvate-driven ATP synthesis was 36% lower in siMPC2 cells, whilst ATP synthesis from glutamate was unaffected (Fig. 3D). Consistent with our findings from intact cells, the dose-dependent inhibition of pyruvate-driven ATP synthesis by PIO was identical between siSCR and siMPC2 cells (Fig. 3E). These novel data further highlight that 1) MPC proteins are not required for the effect of PIO on pyruvate-driven oxidation and ATP production in hepatocytes, and 2) the lack of effect of MPC silencing on PIO action cannot be explained by alternative routes of pyruvate-derived carbon entry into isolated mitochondria. Interestingly, inhibition of pyruvate-driven ATP synthesis by UK was somewhat less potent in siMPC2 cells compared to siSCR (Fig. 3F), which may be indicative of distinct mechanisms of action of UK compared to PIO.

Pioglitazone does not influence PDH activation status

As MPC silencing did not influence the ability of PIO to inhibit oxidative pyruvate metabolism, we reasoned that PIO could be acting downstream of pyruvate transport. The principle pathway for oxidative pyruvate disposal involves its decarboxylation to acetyl-CoA via the pyruvate dehydrogenase complex (PDH), although whether TZDs directly influence PDH activity has not previously been addressed. To evaluate whether PDH is a target for PIO, we adapted the methodology of Constantin-Teodosiu et al (1991), which provided a sensitive assay of the active fraction of PDH in our cell culture system. Neither the active fraction nor total activity of PDH was altered by two hours of PIO (10 μM) treatment, whilst the pyruvate dehydrogenase kinase inhibitor dichloroacetate (PDH activator) increased the active fraction without altering total activity (Fig. 4). These data are the first direct assessment of the effects of PIO on PDH activity in hepatocytes and demonstrate that the inhibition of pyruvate oxidation with PIO cannot be explained simply by a direct downregulation of PDH activation status.

Pioglitazone inhibits hepatocellular glucose production independent of MPC expression

Although a number of studies have demonstrated that high (≥100 μM) TZD concentrations may inhibit hepatocellular glucose production *in vitro* [24, 25], no data exist regarding the direct effects of pharmacologically-relevant concentrations of pioglitazone on hepatocellular glucose production. In control H4IIE hepatocytes, we observed a dose-dependent suppression of the rate of pyruvate-driven glucose output during overnight incubation with PIO or UK (Fig. 5A-B). In our system, glucose output was detectable within four hours, was linear over at least 24 hours (Fig. 5D) and was robustly inhibited by insulin (Fig. 5C). Low levels of pyruvate / lactate (0.2 / 2 mM), alone or in combination with glutamine / alanine, were insufficient to support detectable rates of glucose production, suggesting that alternative pathways of glucose production were not significant net contributors to glucose production in these cells.

Insulin is thought to regulate glucose production at the transcriptional level by the suppression of rate-limiting gluconeogenic enzymes, but unlike insulin, the inhibition of pyruvate-driven glucose production by PIO occurred in the absence of any alteration of FBP, G6PC, PCK1 or PCX (Fig. 5E). Unlike pyruvate oxidation, and in contrast to one previous report [19], rates of pyruvate-driven glucose production were unaltered in siMPC2 cells (Fig. 6A). Moreover, the inhibition of glucose production by PIO was similar in siSCR and siMPC2 cells, regardless of whether rates were supported solely by pyruvate / lactate, or additionally supplemented with 20 mM glutamine or alanine (Fig. 6C-E). Interestingly, 0.5 μM UK was more potent at suppressing HGP in siMPC2 cells (Fig. 6F-H). Taken together, these data again highlight that the mechanism of action of PIO does not depend on the MPC proteins and further, that it is distinct from the effects of UK5099 and perhaps unrelated to pyruvate transport.

Discussion

The major novel finding of this study is that the TZD pioglitazone inhibits pyruvate metabolism in hepatocytes and in isolated mitochondria, and that this effect is not modulated by the mitochondrial pyruvate carrier proteins MPC1 or MPC2. We show that PIO impairs both oxidative and gluconeogenic pathways of pyruvate metabolism in a dose-dependent manner *in vitro* and *ex-vivo*.

These data are clinically relevant because TZDs are widely used in the treatment of T2DM, but their direct mechanism(s) of action in their target tissues (e.g. skeletal muscle and liver), is largely unknown. These findings may facilitate the development of novel TZD-like compounds that are clinically efficacious but lack the undesirable side effects of traditional TZDs.

In human subjects with T2DM, pioglitazone improves insulin sensitivity in skeletal muscle and liver and reduces hepatic glucose production [4, 26]. Previously these effects were proposed to be related to transcriptional changes in gene networks regulating adipose tissue differentiation and metabolism [27]. However, in isolated tissues and cells a consistent finding has been that TZDs can directly inhibit aerobic fuel consumption via PPAR-γ-independent mechanisms [10, 28] or transcriptional changes [9]. Our data build upon this work by demonstrating that in hepatocytes, pharmacological concentrations of PIO acutely inhibit pyruvate-driven oxygen consumption, coupled mitochondrial ATP production and whole-cell pyruvate oxidation by 30-35%. It has been suggested that PIO may impair substrate oxidation through direct inhibition of the mitochondrial complexes [11]. However, our finding that metabolism of non-pyruvate substrates in either whole hepatocytes or isolated mitochondria was not influenced by PIO argues against any global PIO-mediated inhibition of mitochondrial metabolism, at least in the pharmacological range of PIO concentrations tested here. Therefore, the reduction in $^{14}CO_2$ production in cells offered ^{14}C-pyruvate is more likely attributable to a decreased rate of pyruvate-derived acetyl-group provision to the TCA cycle.

In this respect, one prospective target of PIO could be the pyruvate dehydrogenase complex (PDH), the enzyme responsible for regulating the conversion of pyruvate to acetyl-CoA. Previous efforts to evaluate the effects of TZDs on the PDH have employed the use of methyl-pyruvate to distinguish the activity of MPCs and PDH [21]. This approach is hampered by the apparent difference in the metabolism of methyl-pyruvate to that of pyruvate [29]. For the first time we have determined the direct effect of PIO on the activation status of the PDH, but could find no inhibitory actions of PIO on either the total or active forms of the enzyme complex. It is important to note that whilst this indicates a lack of any direct effect of PIO on the PDH catalytic activation status, it does not strictly dictate that

flux through the enzyme was unaffected. However, should PDH flux indeed be preserved upon PIO treatment, the inhibition of pyruvate oxidation could be attributable to a suppression of non-PDH mediated acetyl-group provision. A recent NMR study of pyruvate metabolism in rat livers suggested that a significant source of pyruvate entry into the TCA cycle was through pyruvate carboxylase, particularly in the fasted state [30]. In this respect it is conceivable that the 35% reduction in pyruvate oxidation we observe is attributable to a suppression of pyruvate carboxylase flux. Indeed, our observation that PIO also suppressed pyruvate-driven glucose production supports the hypothesis that flux through pyruvate carboxylase is reduced by PIO. However, the requirement for anaplerotic pyruvate carboxylase flux in isolated mitochondria was likely obviated in our experiments by the provision of 2 mM exogenous malate, arguing against the direct involvement of pyruvate carboxylase in the PIO-mediated inhibition of pyruvate metabolism.

Thiazolidinedione treatment in humans negatively regulates HGP [4, 26], whilst in non-diabetic rats, troglitazone reduces HGP within 1 h *in vivo* [31]. In isolated hepatocytes from starved rats, troglitazone and pioglitazone were shown to inhibit HGP from lactate and pyruvate [24, 32]. In addition to confirming these data in cell culture, our data also indicate that, unlike insulin, the effect of PIO on HGP is independent of changes in the mRNA expression of rate-limiting HGP enzymes, further supporting the hypothesis that PIO lowers HGP *in vitro* through direct modulation of pyruvate-specific metabolism in the mitochondria. Importantly, we observed a significant effect of PIO at much lower concentrations (2.5 μM) than those used in the studies highlighted above, which are well below the 35 μM C_{max} of PIO observed in rats given a single oral dose of 10 mg/kg [33]. As previously discussed, the reduction in HGP with PIO treatment may be related to changes in pyruvate carboxylase flux. A possibility that could explain the reduction in both oxidative and gluconeogenic routes of pyruvate disposal is that PIO inhibits mitochondrial pyruvate transport. Indeed, PIO was less effective at inhibiting pyruvate-driven ATP production at higher pyruvate concentrations, whereupon the passive component of mitochondrial pyruvate entry becomes more predominant [34]. This finding in itself points towards pyruvate transport, rather than the direct inhibition of pyruvate carboxylase or pyruvate dehydrogenase (K_m towards pyruvate for both enzymes is <500 μM [35, 36]) as the more

likely target for PIO. Recently two new proteins, MPC1 and MPC2, were identified as critical mediators of pyruvate transport in yeast, drosophila and cultured mammalian fibroblasts [16, 18]. It has previously been demonstrated that PIO binds to multiple proteins on the mitochondrial membrane [14], and one study has described a mechanistic link between TZDs, the MPC proteins and pyruvate metabolism [21] which the authors postulated indicated a requirement of the MPCs for the inhibition by TZDs of pyruvate-driven oxygen consumption. Should the effects of PIO on pyruvate metabolism indeed be moderated through an interaction with the MPCs, a reduction in MPC protein levels with knockdown should result in an increased potency of PIO towards pyruvate metabolism [21]. On the contrary, we found that the inhibition of both pyruvate oxidation and pyruvate-driven glucose production by PIO was unaffected by MPC-silencing, suggesting that these proteins are unlikely to be involved in the PIO-induced alteration of pyruvate metabolism.

In the current study we assayed various components of mitochondrial pyruvate metabolism, in the same cellular system and under comparable media conditions. This allows for comment on the extent to which these processes may be regulated by pyruvate availability and / or transport. Assuming a 2:1 stoichiometry for pyruvate conversion to glucose, our data suggest that the calculated rates of both pyruvate oxidation and pyruvate-driven glucose production would require similar rates of mitochondrial pyruvate entry (~275 vs ~300 pmol/mg protein/min). In this respect it is noteworthy that MPC silencing appeared to influence only pyruvate oxidation and pyruvate-supported ATP synthesis, whilst pyruvate-driven glucose production was unaltered. This may be indicative of an effect of MPC knockdown on oxidative pyruvate disposal that is distinct from pyruvate transport. Indeed, it has previously been speculated that the MPC proteins, although likely integral to pyruvate metabolism, may not directly or exclusively regulate mitochondrial pyruvate entry [37]. Further support for this comes from the robust finding, in this study and others [19,21], that both UK5099 and TZDs are able to exert additional inhibitory effects on pyruvate metabolism in MPC-silenced cells, beyond that observed with MPC knockdown alone. Moreover, liver specific knockout mice for both MPC1 and MPC2 have normal glucose tolerance, fasting glucose and rates of hepatic glucose appearance Ra *in vivo* [18, 19]. Overall, the preserved inhibition of pyruvate metabolism by UK5099

in MPC-silenced cells cannot be reconciled with a paradigm in which the MPCs are the only proteins responsible for mitochondrial pyruvate import.

A key novel finding in the current study is that MPC silencing had no effect of hepatocellular glucose production. This is in contrast to McCommis and colleagues [19], who reported a ~70% reduction of glucose production upon MPC2 knockdown in isolated hepatocytes. One potential explanation for the discrepancy in this finding is the influence of glycogenolysis on glucose production. In the aforementioned study [19], glucose production was augmented with ~30 nM glucagon, which has been shown to maximally activate glycogenolysis in murine hepatocytes [38]. Therefore, it is possible that the observed reduction in glucose production in hepatocytes from MPC2-null animals reflects, in part, a reduction in glycogenolysis, rather than compromised pyruvate-driven gluconeogenesis. In support of this, liver glycogen content and rates of glucagon-stimulated glucose production in the absence of pyruvate were significantly lower in MPC2 knockout animals [19]. We chose to evaluate the gluconeogenic component of HGP only, i.e. in the absence of glucagon, as glycogenolysis does not contribute to the inappropriate rise in hepatic glucose output observed in T2DM [39]. The lack of effect of MPC-silencing on HGP in our study is in agreement with the finding that mice expressing a truncated MPC2 protein demonstrated impaired pyruvate oxidation but normal gluconeogenic capacity [40]. Moreover, the pyruvate transport inhibitor UK5099 (K_i=50 nM; [41]) blocked only approximately 50% of pyruvate-driven HGP at a concentration of 500 nM in our study. It has been proposed that this may reflect compensation by alternative gluconeogenic pathways when pyruvate transport is impaired. For example, cycling of pyruvate through cytosolic alanine transaminase may facilitate pyruvate-derived acetyl-CoA production independent of mitochondrial pyruvate transport [18, 19]. However, we found that provision of supplementary alanine did not recover PIO- or UK5099-inhibited rates of glucose production, regardless of the presence/absence of the MPC proteins. The latter is consistent with the observation that pyruvate transamination likely requires mitochondrial pyruvate transport [42]. Alternatively, glutaminolysis may support TCA cycle function and glucose production in response to UK5099 treatment [18, 42] although again, we found no difference in the effects of PIO, UK, or MPC-silencing between HGP with glutamine-deplete and

glutamine-rich media. Finally, it could be argued that our results may be influenced by the potentially transient or incomplete nature of MPC protein silencing. However, although some studies have documented that small quantities of MPCs are sufficient to maintain pyruvate transport activity [43], we routinely achieved 95% silencing of MPC2 protein in this study, which is comparable to numerous studies [21, 23]. Indeed, similarly efficient removal of MPC1 (90%) *in vivo* was sufficient to almost completely abolish the incorporation of $2\text{-}^{14}C$-pyruvate into lipids [43].

The acute metabolic effects of PIO we report here are comparable in magnitude to the improvements observed in whole-body insulin sensitivity in T2DM patients after several weeks of PIO treatment [4] and thus, may be clinically relevant. Indeed, our data in isolated human skeletal muscle mitochondria indicate that the effect of PIO on pyruvate-driven ATP production is not restricted to the liver. In man PIO increases whole body (primarily skeletal muscle) insulin sensitivity secondary to increased non-oxidative glucose disposal (i.e. glycogen synthesis) without increasing glucose oxidation [4]. Therefore, a blunting of mitochondrial pyruvate uptake and/or oxidation in skeletal muscle could explain the shift in glucose oxidation towards glycogen storage in T2DM patients treated with PIO. In future experiments, it will be important to study in detail the effects of TZDs in multiple metabolic tissues, including the liver, skeletal muscle and adipose tissue. In summary, we have shown that low concentrations of PIO inhibit pyruvate oxidation and glucose production in hepatocytes, with this effect being independent of the newly identified MPC proteins and unrelated to transcriptional changes in metabolic or mitochondrial genes. Our data suggests the possibility that PIO interacts with a mitochondrial protein that regulates both oxidative and gluconeogenic pyruvate metabolism, but more research will be needed to identify the molecular target of PIO.

Experimental procedures

Cell culture and human muscle biopsies - Rat H4IIE hepatocytes were used in this study. These cells have been shown by many groups to metabolize glucose and lipid in a physiologically relevant manner [44-46] and are sensitive to physiological concentrations of insulin and metformin [45], making them a highly applicable and convenient system for the study of hepatic metabolism in the

context of T2DM. Low passage cells were purchased from ATCC and routinely cultured in low glucose (5 mM) DMEM media (Sigma) supplemented with 10% fetal bovine serum (Invitrogen) in the absence of antibiotics, up to passage 30. To confirm the validity of our experiments in human tissue, vastus lateralis skeletal muscle biopsies were obtained from human subjects after an overnight fast in accordance with the UTHSCSA ethics committee. Human muscle samples were taken with the understanding and written consent of each subject and study methodologies conformed to the standards set by the Declaration of Helsinki.

Drug treatments - For the experiments described below, Pioglitazone stocks (25 mM in 100% DMSO) were prepared by diluting to 1000X final concentrations and further diluted to $1 - 25$ μM working concentrations in assay media. These concentrations are similar to the plasma pioglitazone concentrations achieved in T2DM individuals receiving 45 mg/day [47] and are lower than that observed in rodent plasma after a single oral dose of 10 mg/kg PIO [33]. Vehicle treatment (VEH) consisted of 100% DMSO, identically diluted in assay media. The pyruvate transport inhibitor UK 5099 (UK) was prepared in an identical manner to PIO and diluted in assay media to 0.05 to 50 μM. Cell viability was assessed following 24 hours treatment with various concentrations of each drug using the RealTime-Glo MT assay (Promega).

^{14}C-Pyruvate and ^{14}C-Palmitate Oxidation Assay - We adapted a multi-well assay described by Collins et al. using [2-^{14}C] pyruvate, whereby ^{14}CO$_2$ capture reflects incorporation of the C-2 of pyruvate into the TCA cycle and thus offers a relative index of pyruvate oxidation [48]. Approximately 3 x 10^5 cells were seeded into each well of a 24-well CulturPlate (Perkin Elmer, Waltham, MA) and allowed to adhere overnight. Culture media was then removed and cells were washed twice with PBS and replaced with a glucose- and serum-free media (SFM; DMEM; Sigma) supplemented with 100 μM sodium pyruvate (Sigma) and sodium bicarbonate (3.7 g/L). Treatments were added and the cells were left in a cell culture incubator at 5% CO$_2$, 95% O$_2$ for 2 h. After a 2 h pre-incubation, the media was refreshed with SFM containing 25 mM HEPES (instead of NaHCO$_3$), 30 μCi [2-^{14}C] sodium pyruvate (Perkin Elmer) and treatments and the plate sealed and placed in a

37°C incubator at ambient air. After two hours, 400 µl media was removed and added to 2 ml tubes containing 200 µl perchloric acid (1M). The tube lids, which contained a KOH-saturated filter paper disk for CO_2 capture, were rapidly closed and tubes were gently mixed by vortex for one hour at room temperature. The filter disks were subsequently removed and placed into scintillation vials for counting. An aliquot of unconditioned media was also counted for the determination of specific activity and data was normalized to well protein content (nmol pyruvate / mg protein / min), assessed using the BCA assay (Pierce). Palmitate oxidation was determined as above using 100 µCi U-^{14}C palmitate (Perkin Elmer) and 100 µM palmitate conjugated to BSA (0.3%). Etomoxir (10 µM), an irreversible carnitine palmitoyltransferase inhibitor, was included as a negative control for the palmitate oxidation assay.

Respiration Experiments - Whole-cell oxygen consumption rate (OCR) was assessed using the XF24 Extracellular Flux Analyzer (Seahorse Bioscience). Cells were seeded (7.5 x 10^4 H4IIE) the day before the assay and allowed to grow to 80 – 90% confluence in regular culture media. Media was removed and, after two washes with PBS, replaced with SFM supplemented with 500 µM sodium pyruvate (Sigma), 25 mM HEPES and either VEH, PIO (10 µM) or UK (5 µM). Cells were incubated at room air at 37°C for 2 hours, before being placed into the XF24 Extracellular Flux Analyzer (Seahorse Bioscience) instrument for assessment of oxygen consumption. After assessing the basal pyruvate-stimulated OCR, oilgomycin (4 µM), Carbonyl cyanide-*4-*(trifluoromethoxy)phenylhydrazone (FCCP) (4 µM) and rotenone (1 µM) were added at the indicated time-points for the determination of the OCR associated with ATP turnover, maximal uncoupled respiration and non-mitochondrial respiration, respectively.

Mitochondrial Isolation and ATP synthesis - To investigate the direct effect of PIO on mitochondrial metabolism, the rate of ATP synthesis from multiple substrates was examined in isolated mitochondria. Mitochondria were freshly isolated from cultured hepatocytes (~ 1 x 10^9 cells) or human skeletal muscle (~ 50 – 100 mg) by differential centrifugation using protocols described previously for cells [49] and tissue [50]. Substrate-specific rates of ATP synthesis were determined by

the luminometric method based on firefly luciferase, as described previously by us [50] and others [51]. Isolated mitochondria were incubated with treatments for 15 minutes before dilution in a luciferin-luciferase reaction buffer containing ADP (600 μM) and substrate (500 μM pyruvate + 2 mM malate, PM; 500 μM glutatmate + 2 mM malate, GM; 500 μM succinate, S; 0.7 μM palmitoylcarnitine + 2 mM malate, PCM). The ATP-producing reaction was followed in a 96-well plate for seven minutes after which an internal ATP standard (50 pmol) was added to calibrate results which were subsequently normalized to the protein concentration of the mitochondrial preparations (BCA assay; Pierce). The total amount of mitochondrial protein added to each well was approximately 60 ng.

Pyruvate dehydrogenase activation status

PDH activity was determined in cell lysates using an approach based on the methodology of Constantin and colleagues [52]. Briefly, H4IIE cells were treated for two hours with VEH, PIO (10 μM) or DCA (5 mM) in serum- and glucose-free media supplemented with 1 mM sodium pyruvate and 2 mM glutamine in 60 mm dishes. Cells ($\sim 6 \times 10^6$) were washed briefly with PBS, rapidly scraped into PBS and two aliquots were spun in a microcentrifuge for 10s. One resulting pellet was re-suspended in homogenization buffer A (50 mM Tris•HCl, pH 7.8; 200 mM sucrose, 5 mM MgCl$_2$, 5 mM EGTA, 0.1% Triton-X100; pH 7.8) and immediately assayed for active PDH by adding 35 μl lysate to 500 μl reaction buffer (100 mM Tris•HCl, 0.5 mM EDTA, 1 mM MgCl$_2$, 1 mM pyruvate, 1 mM NAD$^+$, 1 mM thiamine pyrophosphate and 0.5 mM Coenzyme A) and removing 150 μl every minute to a fresh vial containing 25 μl 1M perchloric acid. Samples were neutralized (6.25 μl 0.25 M KHCO$_3$) and assayed for acetyl-CoA concentration by radioenzymatic assay [53]. For the determination of total PDH, the second cell pellet was re-suspended in homogenization buffer B, which was similar to buffer A except for the absence of Triton-X100 and the addition of 10 mM glucose, and freeze-thawed twice prior to assay. Complete catalytic conversion of the enzyme to its active form was achieved by pre-incubating samples in a buffer containing 5 mM DCA and 2 U/ml hexokinase, to inhibit the PDH kinases, and 100 μM CaCl$_2$, 10 mM MgCl$_2$ and 500 μM spermine, to activate the PDH phosphatases [54, 55]. Triton-X100 was added immediately prior to assay, which

subsequently followed the protocol described above. PDH activity is expressed as the rate of formation of acetyl-CoA normalized to the protein content of the cell lysate.

Hepatic Glucose Production (HGP) – Hepatocellular glucose production was determined as previously described [45] with modifications [46]. Briefly, H4IIE cells were incubated overnight (15 hours) in glucose- and serum-free DMEM supplemented with pyruvate (2 mM) plus lactate (20 mM) precursors, and containing either VEH, or various concentrations of PIO, UK or insulin. For some experiments, glutamine (2-20 mM) or alanine (20 mM) was added, as indicated in figure legends. Glucose release into the media was subsequently measured using the Amplex Red Glucose Assay Kit (Invitrogen), and calibrated using a standard curve generated by spiking in glucose to assay media incubated overnight in the absence of cells. The rate of glucose production was normalized to the protein content of each well.

MPC1 and MPC2 silencing - Silencing of MPC1 and MPC2 mRNA and protein levels in H4IIE cells was performed using 100 nM Dharmacon SmartPool siRNA [MPC1 (BRP44L) siRNA cat#: L-093029-01-000; MPC2 (BRP44) siRNA cat# L-041059-01-0005), introduced via Neon electroporation. Subsequent experiments were performed 24-36 hours following electroporation. Knock-down of each protein was subsequently confirmed on Western blots using antibodies directed toward MPC1 (Sigma, catalog number: HPA045119) or MPC2 (Cell signaling Technology, catalog number: 46141).

Gene expression analysis - Following the HGP experiments, RNA was extracted and real-time quantitative PCR (qRT-PCR) was performed to assess the expression of rate-limiting HGP genes phosphoenolpyruvate carboxykinase (Pepck), fructose 1,6-bisphosphatase (Fbp1) and glucose 6-phosphatase (G6pc) using Taqman predesigned assays, as described previously [56]. Mitochondria energy metabolism gene expression – including complex I to complex V genes – was assessed separately following 3-days of PIO (10 μM) or VEH treatment using the Mitochondrial Energy

Metabolism RT^2 Profiler PCR Array from Qiagen (Valencia, CA). The online version of the RT2 data analysis software (http://pcrdataanalysis.sabiosciences.com, version 3.5) was used for qRT-PCR array data-analysis.

Statistics - The effect of multiple treatments on pyruvate oxidation, OCR, HGP and ATP production was analyzed using repeat measures one-way anova with Holm-Sidak post-hoc testing accounting for multiple comparisons, unless otherwise stated in the figure legend. A two-way analysis of variance was applied to the pyruvate oxidation and HGP data with siMPC silencing [factor A = siRNA treatment (Scr or siMPC); factor B = Drug treatment (VEH, PIO or UK5099) with post-hoc testing. The number of repeat experiments (n) detailed in each figure legend reflect independent experiments performed using subsequent cell passages, and error bars represent variability between these experiments as standard error of the mean (SEM).

Author contributions: CS designed; performed and analyzed the experiments and wrote the paper. GD and GC performed experiments. RD, MA and LN conceived the study; LN designed the experiments, analyzed the data and wrote the paper. All authors reviewed the results and approved a final version of the manuscript.

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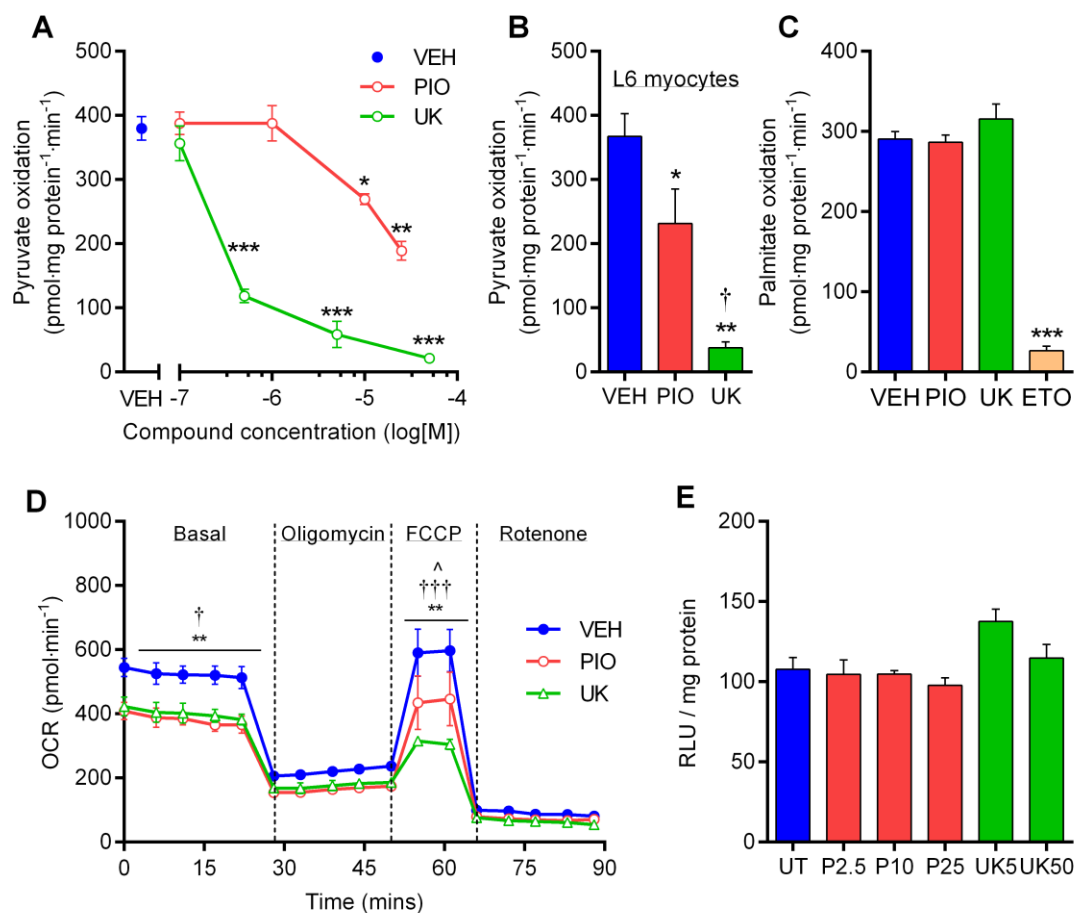
Figure 1. **Pioglitazone inhibits pyruvate oxidation in hepatocytes**

(A) Rates of ^{14}C-pyruvate oxidation in H4IIE cells incubated for four hours in serum- and glucose-free media supplemented with sodium pyruvate (100 μM) and glutamine (2 mM), treated with various concentrations of PIO and UK. *P<0.05, **P<0.01, **P<0.001 vs VEH.

(B) ^{14}C-pyruvate oxidation in L6 murine skeletal muscle cells treated with VEH, PIO (10 μM) or UK (5 μM). *P<0.05, **P<0.01 vs VEH; †P<0.05 vs PIO.

(C) ^{14}C-palmitate oxidation (100 μM + 2 mM L-carnitine) in H4IIE cells treated with VEH, PIO, UK or Etomoxir (10 μM). ***P<0.001 vs VEH.

(D) Oxygen consumption rates of intact H4IIE cells following four hours treatment with either VEH, PIO or UK during a basal period (500 μM pyruvate) and after the sequential addition of oligomycin (4 μM), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP; 4 μM) and rotenone (1 μM).

(E) Cell viability, expressed as Relative Light Units, following 24 hour treatment with various concentrations of PIO or UK. For all panels, values are mean ± SEM of ≥3 independent experiments.
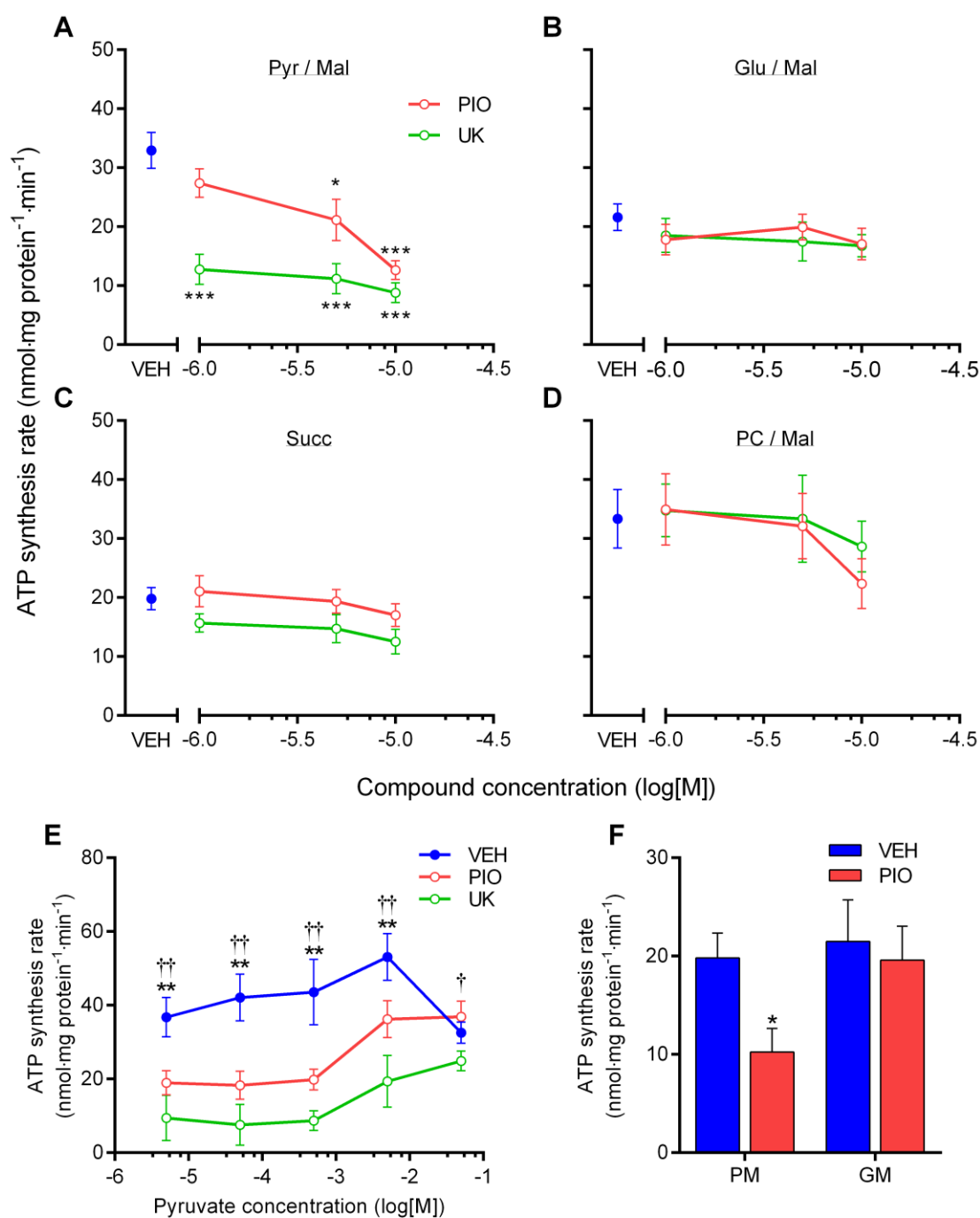
Figure 2. Pioglitazone inhibits pyruvate-driven ATP synthesis in isolated mitochondria

(A-D) Rate of ATP synthesis in mitochondria freshly isolated from H4IIE cells and acutely treated with the indicated concentrations of PIO or UK. Treatments were added 15 minutes prior to the measurement of ATP synthesis, which was supported by ADP (0.6 mM) and 500 μM pyruvate + 2 mM malate (A), 500 μM glutamate + 2 mM malate (B), 500 μM succinate (C), or 0.7 μM palmitoylcarnitine + 2 mM malate (D). *P<0.05, ***P<0.001 vs VEH.

(E) Rate of ATP synthesis in isolated H4IIE mitochondria stimulated by various concentrations of pyruvate and treated with VEH, PIO (10 μM) or UK (5 μM). ** P<0.01 PIO/UK vs VEH, † P<0.05, †† P<0.01 PIO vs UK.

(F) Rate of ATP synthesis in mitochondria isolated from human skeletal muscle supported by either pyruvate / malate (PM) or glutamate / malate (GM) and treated acutely with VEH or PIO. *P<0.05 vs VEH. For all panels, values are mean ± SEM of ≥3 independent experiments.
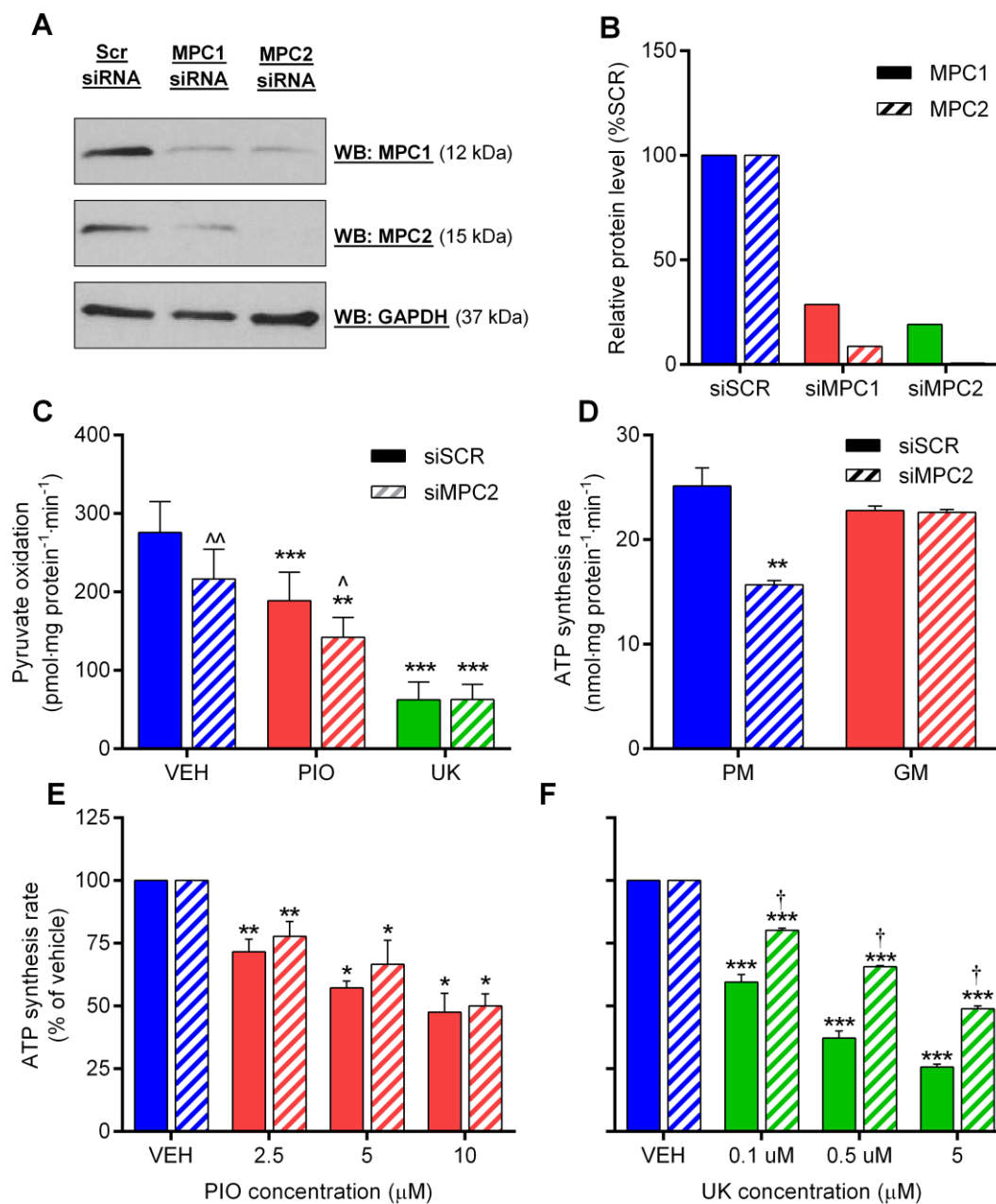
Figure 3. Pioglitazone inhibition of pyruvate oxidation does not require the MPC proteins

(A-B) Representative western blot (A) and densitometry quantification (B) of MPC1 and MPC2 protein expression, normalized to GAPDH, in H4IIE cells following siRNA knockdown of each protein.

(C) Rate of ^{14}C-pyruvate oxidation in siSCR or siMPC2 H4IIE cells treated with either VEH, PIO (10 μM) or UK (5 μM). **P<0.01, ***P<0.001 vs VEH; ^P<0.05, ^^P<0.01 vs siSCR.

(D) Rate of pyruvate or glutamate-driven ATP synthesis in mitochondria isolated from siSCR or siMPC2 H4IIE cells. *$P<0.05$ vs siSCR.

(E-F) Percentage inhibition of pyruvate-driven ATP synthesis by indicated concentrations of PIO or UK in mitochondria isolated from siSCR or siMPC2 H4IIE cells. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs previous PIO concentration; †$P<0.05$ vs siSCR. Values are mean ± SEM of ≥3 independent experiments.
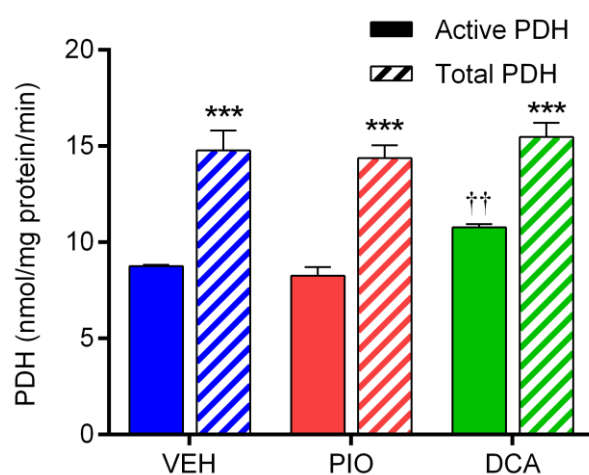

Figure 4. Pioglitazone does not influence PDH activation status

Pyruvate dehydrogenase activation status was assayed in whole cell lysates following two hours of incubation of H4IIE cells in serum- and glucose-free media (1 mM sodium pyruvate) with VEH, PIO (10 µM) or dichloroacetate (5 mM). Complete PDH activation was achieved through pre-incubation of cell lysate with $CaCl_2$ (100 µM), $MgCl_2$ (10 mM), DCA (5 mM), spermine (500 µM), glucose (10 mM) and hexokinase (2 U/ml). ***$P<0.001$ total vs active; ††$P<0.05$ vs VEH and PIO. Values are mean ± SEM of ≥3 independent experiments.
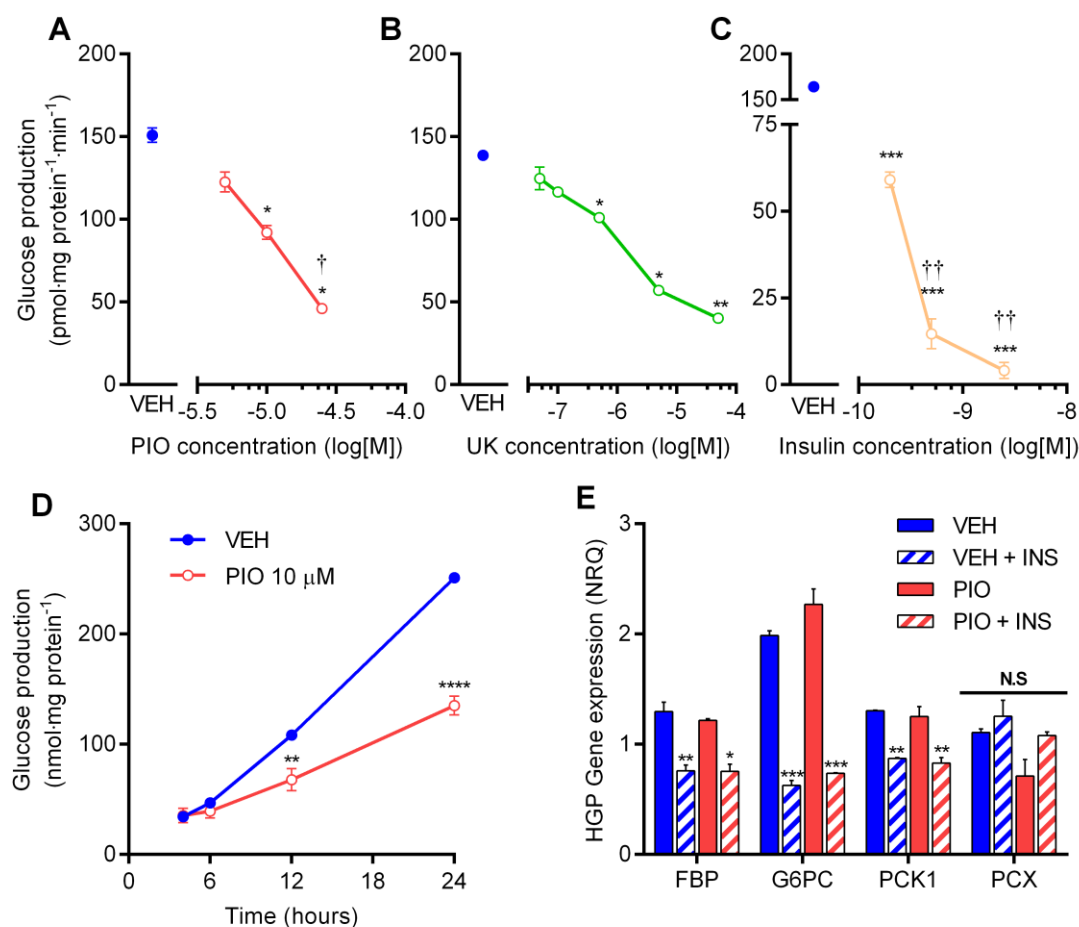
Figure 5. Pioglitazone inhibits hepatocellular glucose production

(A-C) Rate of glucose production in H4IIE cells incubated overnight in glucose- and serum-free media supplemented with pyruvate (2 mM), lactate (20 mM) and glutamine (2 mM) and treated with various concentrations of PIO (A), UK (B) or insulin (C). *P<0.05, **P<0.01, ***P<0.001 vs VEH; †P<0.05, ††P<0.01 vs prior concentration.

(D) Effect of PIO and insulin on the mRNA expression of key gluconeogenic genes in H4IIE cells following overnight treatment in serum and glucose-free media. FBP, fructose 1,6-biphosphatase; G6P, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; PCX, pyruvate carboxylase. *P<0.05, **P<0.01, ***P<0.001 vs VEH. N.S: not significant. Values are mean ± SEM of ≥2 independent experiments.
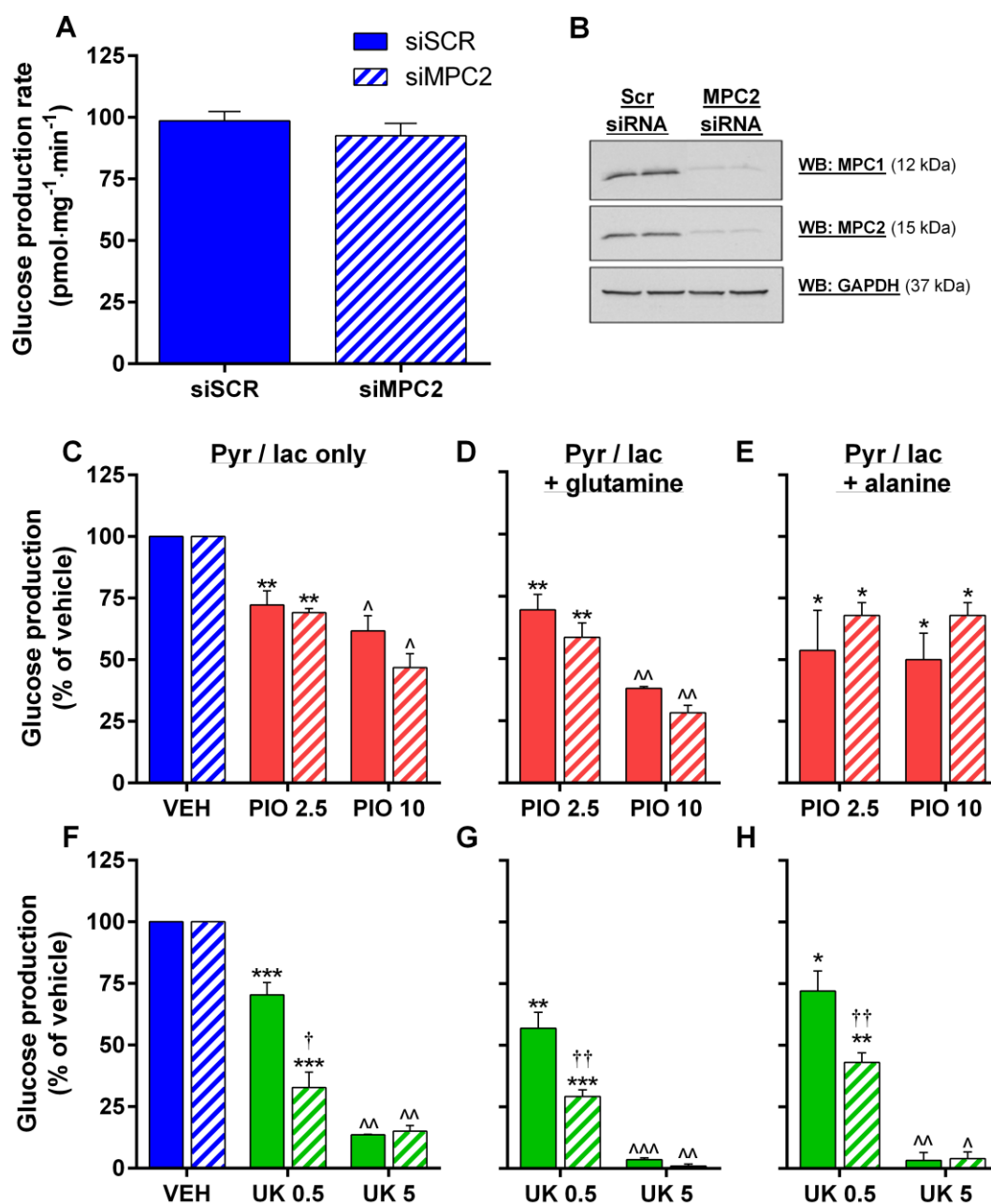
Figure 6. MPC silencing does not influence hepatocellular glucose production

(A) Rate of glucose production in siSCR and siMPC2 H4IIE cells supported by pyruvate (2 mM) and

lactate (20 mM).

(B) Representative western blot of MPC1 and MPC2 protein expression H4IIE cells following siRNA

knockdown of MPC2.

(C-H) Rates of glucose production in siSCR and siMPC2 H4IIE cells supported by pyruvate (2 mM) and lactate (20 mM) alone (A and F) or supplemented with 20 mM glutamine (D and G) or alanine (E and H) and treated with the indicated concentrations of PIO (C-E) or UK (F-H). *P<0.05, **P<0.01, ***P<0.001 vs VEH, ^P<0.05, ^^P<0.01, ^^^P<0.001 vs both other treatments. †P<0.05, ††P<0.01 vs siSCR. Values are mean ± SEM of ≥2 independent experiments.