8-3-2017

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Original Publication Information

This is the accepted manuscript of the following article:
Grimm, D., Altamirano, L., Paudel, S. et al. "Modulation of Cellular Energetics by Galactose and Pioglitazone." 2017. *Cell and Tissue Research*: 6 pp.
The final publication is available at Springer via http://doi.org/10.1007/s00441-017-2657-1

Modulation of Cellular Energetics by Galactose and Pioglitazone

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Abstract

The Warburg effect is ameliorated by culturing transformed cells in the presence of galactose instead of glucose as the primary carbon source. However, metabolic consequences that are in addition to sensitizing the cells to mitochondrial toxins may occur. As such, the screening of pharmaceutical agents against transformed cells while using galactose must be carefully evaluated. Pioglitazone is used in clinical applications to treat type-2 diabetes, but clearly has other off target effects. Human hepatocellular carcinoma cells (HepG2) were cultured in glucose or galactose-containing medium to investigate the role of pioglitazone on cellular bioenergetics employing calorimetry and respirometry. Compared to cells cultured in 10 mM glucose, HepG2 cells cultured in the presence of 10 mM galactose showed decreased metabolic activity as measured by cellular heat flow. Interestingly, cellular heat flow increased after addition of pioglitazone for cells cultured in glucose, but not for cells cultured in galactose. Our calorimetric data indicate that a reduction in cellular capacity for glycolysis might be the mechanism responsible for the increase in sensitivity to pioglitazone, and likely mitochondrial toxins in general, for cells cultured in galactose. Furthermore, oxygen consumption rates were decreased after addition of pioglitazone to cells grown in glucose, but remained unchanged for cells grown in presence of galactose. Taken together, we demonstrate that pioglitazone induced a reduction in mitochondrial activity that was partially compensated via an increase in glycolysis in the presence of glucose.

Keywords: Metabolic poise, oxidative phosphorylation, respiration, calorimetry, Warburg effect

Introduction

Thiazolidinediones (TZD) are effective insulin sensitizers used to treat type-2 diabetes, and these compounds have been shown to reduce the risk of conversion from impaired glucose tolerance to type-2 diabetes (Day 1999; DeFronzo et al. 2011). Most of the antidiabetic properties of TZDs are believed to be mediated through changes in gene expression patterns after binding to the nuclear peroxisome proliferator-activated receptor (PPAR)-γ (Cariou et al. 2012). However, mounting evidence indicates that at least part of TZD action is independent of PPAR-γ-mediated transactivation and transrepression of target genes (Feinstein et al. 2005). Pleiotropic mechanisms in addition to PPAR-γ binding appear to be crucial for the broad pharmacological profile of TZDs and potentially include several effects on mitochondria, including inhibition of the respiratory complex I (Brunmair et al. 2004), inhibition of the mitochondrial pyruvate carrier (Divakaruni et al. 2013), and binding to CISD1 (Colca et al. 2004; Geldenhuys et al. 2014; Paddock et al. 2007).

First described nearly 60 years ago by Otto Warburg, highly proliferating cancer cells are often characterized by a shift in energy production from oxidative phosphorylation to glycolysis despite the presence of saturating concentrations of oxygen (‘aerobic glycolysis’) (Kim et al. 2006; Vander Heiden et al. 2009; Warburg 1956). In some cases, mitochondrial respiration rates can be rescued if glucose levels are low, and suppression in respiration by high glucose concentration is a phenomenon known as the Crabtree effect (Diaz-Ruiz et al. 2011; Ibsen 1961). For example, substitution of the primary energy substrate glucose by galactose increases mitochondrial activity in several models including adenocarcinoma (HeLa) and hepatocellular carcinoma (HepG2) cells, and sensitizes HepG2 cells to some mitochondrial toxins (Marroquin et al. 2007; Rossignol et al. 2004). The reason for the increase in mitochondrial respiration in presence of galactose is still poorly understood. Glucose and galactose metabolism yields equivalent amounts of ATP and both

sugars are equal as energy sources, but the shift towards respiration might be due to flux limitations through the Leloir pathway (Frey 1996; Petry et al. 1998), since galactose enters glycolysis at a lower rate than glucose (Bustamante et al. 1977). However, in presence of galactose and glutamine, HeLa cells use glutamine to provide about 98% of the ATP used for cell growth (Reitzer et al. 1979).

A non-invasive method to directly measure the metabolic response of cells to drugs and changes in substrate availability is the simultaneous measurement of heat flow and oxygen flux (Gnaiger et al. 1990). This approach not only yields baseline estimates of energy flow, but can be used to evaluate the contribution of anaerobic pathways (e.g. glycolysis) to overall cellular energy homeostasis (Guan et al. 1999; Menze et al. 2010). We found a pronounced increase in chemically uncoupled cellular respiration, but a depression in heat dissipation for cells cultured in presence of galactose compared to glucose-cultured cells, indicating a shift in metabolic poise from glycolysis to respiration.

We have investigated the effect of galactose and pioglitazone on bioenergetics and growth of HepG2 cells. Our results demonstrate that (1) galactose treatment shifts metabolic poise from glycolysis to oxidative phosphorylation, (2) pioglitazone treatment inhibits oxidative phosphorylation, (3) in presence of high glucose concentrations cells compensate for pioglitazone induced reductions in respiration via an increase in glycolysis and (4) a significant reduction in cell proliferation after addition of pioglitazone is observed in the presence of glucose, but absent if cells are dependent on galactose as a carbon source. Furthermore, increased metabolic activity induced by pioglitazone is abolished in galactose based medium. This study demonstrates that measuring cellular proliferation as an indicator of drug efficacy in the galactose-utilization model

of cell culture can be misleading if a more detailed technique, such as calorespirometry, is not being performed.

Materials and methods

All chemicals used for respirometry and solution preparations were of the highest grade and purchased from Sigma-Aldrich (St. Louis, MO), or Fisher Scientific (Fair Lawn, NJ). Human hepatocellular carcinoma cells (HepG2) were cultured in glucose free DMEM (Dulbecco's Modified Eagle Medium) supplemented with either 10 mM D-galactose or glucose following the protocol described in Stokich et al. 2014. Microcalorimetry was performed following the procedures detailed in Menze et al. 2010, and respirometry measurements were based on the methods described in Stokich et al. 2014. For detailed descriptions of the employed methods please refer to the supplemental files.

RESULTS

Impact of Pioglitazone on Respiration of HepG2 Cells

Significant reductions in oxygen flux by about 13% were observed after addition of 60 µM pioglitazone to cells respiring in cell culture medium containing 10 mM glucose (n = 6, $p \leq 0.05$; Fig. 1A). A more pronounced inhibitory effect of pioglitazone on cellular respiration was observed if oxygen flux was stimulated via addition of FCCP, and routine respiration rates in control cells increased nearly 3-fold in presence of this potent chemical uncoupler. After the addition of pioglitazone, the uncoupled respiration rate was significantly inhibited by about 30% (n = 6, $p \leq 0.05$; Fig 1A). Moreover, pioglitazone acted as weak chemical uncoupler in intact cells, and proton leak respiration rates in the presence of the F_0F_1-ATPase inhibitor oligomycin ($LEAK_o$) were significantly increased when pioglitazone was present (n = 6, $p \leq 0.05$; Fig 1A). This significant mitochondrial uncoupling effect of pioglitazone was a common finding throughout this study, but we did not observe statistically significant increases in oxygen consumption rates of HepG2 cultured in galactose for two weeks compared to glucose controls (n = 6, $p > 0.05$; Fig 1B). However, maximal uncoupled respiration (FCCP) was significant higher for cells cultured in galactose and the addition of pioglitazone did not reduce uncoupled respiration rates as observed for cells cultured in glucose. Furthermore, no increase in $LEAK_o$ respiration was observed in presence of pioglitazone (n = 6, Fig 1B).

In order to elucidate the action(s) of pioglitazone on the oxidative phosphorylation system (OXPHOS) in more detail, HepG2 cells were permeabilized with digitonin and saturating levels of substrates were added to the sucrose based respiration medium. Two different titration protocols, promoting electron entry into the electron transport chain (ETS) via $FADH_2$, or NADH and $FADH_2$, were employed. In the first titration protocol, oxygen flux fueled by succinate

dehydrogenase (complex II) activity was measured in presence of the NADH:ubiquinone oxidoreductase (complex I) inhibitor rotenone and saturating levels of succinate. When using succinate as complex II substrate the addition of pioglitazone to permeabilized cells significantly stimulated oxygen flux compared to untreated control (n = 6, $p \leq 0.05$; Fig 2A, B). For cells cultured in glucose medium, respiration rates after the addition of ADP did not differ between pioglitazone treated and control cells, demonstrating that pioglitazone does not cause significant reductions in the activities of succinate dehydrogenase or the mitochondrial phosphorylation system (Fig. 2A). As with intact cells a significant uncoupling effect of pioglitazone was observed after the addition of oligomycin (Fig. 2A, B). Interestingly, pioglitazone reduced OXPHOS rates in cells cultured in galactose medium (Fig. 2B).

We next investigated the effect of pioglitazone on complex I activity followed by the concurrent presence of NADH and $FADH_2$ generating substrates (Fig. 3A, B). A combination of malate, glutamate, and pyruvate was added to permeabilized cells to stimulate NADH production by mitochondrial dehydrogenases. In contrast to our results with succinate, no increase in oxygen flux was observed in presence of pioglitazone compared to untreated controls indicating an inhibition of NADH-poised respiration below an activity needed to establish a proton motive force (Δp) large enough to observe the uncoupling effect of pioglitazone (Fig. 3A). The same effect was observed for cells grown in galactose based medium (Fig. 3B). A severe inhibition in complex I activity was confirmed after addition of ADP. In presence of pioglitazone, ADP failed to stimulate oxygen flux, but similar to our results with succinate alone, respiration could be rescued by the addition of succinate. $LEAK_o$ respiration after addition of oligomycin was elevated by 63% in cells treated with pioglitazone if succinate was present and slightly higher in cells grown in galactose based medium (n = 6, $p \leq 0.05$; Fig 3A, B).

Pioglitazone increased heat dissipation in presence of glucose but not galactose.

Although the exact mechanism is unknown, some immortalized cells will shift energy production from glycolysis towards mitochondrial respiration in medium supplemented with galactose instead of glucose. In contrast to respirometry, calorimetry captures the overall metabolic activity including changes in glycolytic activity. HepG2 cells cultured in glucose medium or galactose medium were treated with 60 μM pioglitazone and heat dissipation was monitored every 30 min for 2 h. During this period, heat dissipation dropped for galactose cultured cells, but not for cells measured in glucose medium (Table 1). However, after 30 min of exposure to pioglitazone, heat dissipation of HepG2 cells in glucose-containing medium was increased by about 15%, but no increase was observed for galactose-treated cells. Furthermore, overall heat output was about 17% lower for cells cultured in presence of galactose compared to cells in glucose medium (Table 1). Heat dissipation data demonstrated an overall reduction in metabolic activity and indicated that cells cultured in galactose-based medium might be characterized by an overall decrease in cellular proliferation. This was confirmed by following cellular growth, and the fold-increase in cells per 24 h period decreased by about 40% if cells were grown in galactose based medium compared to glucose. A significant drop in proliferation rate was also observed after the addition of pioglitazone to cells grown in glucose-based medium. However, no additional decrease in proliferation was observed after the addition of pioglitazone to cells grown in galactose-based medium (Table 2).

Discussion

We have investigated the consequences of shifting metabolic poise of HepG2 cells from glycolysis to oxidative phosphorylation on pioglitazone-induced changes in metabolic activity. An increase in mitochondrial capacity was achieved by substituting glucose for galactose in the cell culture medium for two weeks. We found that pioglitazone reduced cellular respiration and increased glycolytic flux in cells cultured in presence of glucose, but not in cells cultured in galactose-based medium. Furthermore, pioglitazone severely inhibited complex I respiration in permeabilized cells, but the overall capacity for oxidative phosphorylation could be largely rescued by $FADH_2$ production in presence of succinate. This capacity for compensation by complex II might be the reason for a less pronounced toxicity as might be expected based on the severely inhibition of complex I activity.

Pioglitazone has been well known to alter mitochondrial bioenergetics. Acute effects of pioglitazone on primary hepatocytes and isolated mitochondria showed reductions in cellular and complex I fueled respiration rates (Brunmair et al. 2004; Sanz et al. 2011). Enzymatic assays on isolated mitochondrial complexes from mouse liver demonstrated decreases in complex I and III activity with increasing pioglitazone concentrations, and the drug was shown to disassemble complex I into 4 distinct subcomplexes (Garcia-Ruiz et al. 2013). We were interested in the effect of pioglitazone on highly proliferating HepG2 cells under *in vivo* conditions, since the relatively low toxicity of pioglitazone seemed difficult to explain based on the pronounced inhibition of complex I. We were especially interested to determine the effects of using the galactose model of cell culture on cellular energy flow in the presence of the known mitochondrial toxin pioglitazone. In absence of anaerobic pathways, the ratio of heat flux to oxygen flux (CR-ratio) will be in the range of the theoretical oxycaloric equivalent (-430 to -480 kJ mol O_2) (Gnaiger et al. 1990). The

CR-ratio for HepG2 cells grown in glucose medium was elevated to -520 kJ/mol O_2 indicating anaerobic contributions to energy production. In response to 60 μM pioglitazone, an increase in the CR-ratio to -720 kJ/mol O_2 was observed, demonstrating an increase in glycolytic activity. Reductions in mitochondrial energy production due to inhibition of respiration by pioglitazone are apparently being counteracted by an increase in glycolytic flux. This increase in glycolytic flux was absence for cells cultured in galactose-based medium. In presence of galactose cells were less glycolytic poised with a CR-ration of -470 kJ/mol O_2 and no increase in the CR-ratio was observed in response to pioglitazone.

While galactose treatment of immortalized cells increases oxidative capacity of mitochondria, (Marroquin et al. 2007) and indeed rescues cells partially from the Warburg effect, sensitization to mitochondrial toxins may be obscured. We did not observe reduction in proliferation rates after pioglitazone addition to cells cultured in galactose. Only by combining proliferation studies with respirometry and calorimetry were we able to gain a further insight into the mechanism of action of pioglitazone. Therefore, caution is warranted in the interpretation of toxicity and efficacy studies of drug molecules in galactose-based culture studies in the absence of thorough metabolic characterization of the treated cells.

Acknowledgments

This work was funded by NSF grant *CHE-160944* to M.A.M, M.K., and N.C. Furthermore, part of this work was supported by a grant from the Mindlin Foundation (*MF16-US04*) to M.A.M. and M.K.

Author contributions

M.A.M., M.K., and N.C. formulated the research goals and aims. M.A.M, M.K., and D.G. designed the experiments. D.G., L.A., and L.W., performed the experiments. D.G. and S.P. performed the statistical analysis of data. D.G., S.P., M.K., and M.A.M wrote the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethics statement

This article does not contain any studies with human participants or animals performed by any of the authors.

Figure legends

Figure 1: Respiration rates of HepG2 cells cultured in glucose (A), or galactose (B) based medium for two weeks prior to respirometry. Oxygen flux is shown for control cells (black bars), and in presence of 60 μM pioglitazone (grey bars). Uncoupled respiration was induced by titrations with FCCP (FCCP), and leak respiration was recorded after inhibition of the F_0F_1-ATPase with oligomycin (Olig). *Indicates statistically significant differences after addition of pioglitazone. #Indicates statistically significant differences between cells cultured in glucose or galactose based medium (n = 6, ± SE).

Figure 2: Respiration rates of HepG2 cells cultured in glucose (A), or galactose (B) based medium for two weeks prior to respirometry. Oxygen flux is shown for control cells (black bars), and in presence of 60 μM pioglitazone (grey bars). Respiration rates of cells permeabilized with digitonin (Dig) were recorded in presence of the Complex I inhibitor rotenone (Rot) and the substrate succinate (Succ). OXPHOS rates were measured after the addition of ADP (ADP), and leak respiration was recorded after inhibition of the F_0F_1-ATPase with oligomycin (Olig). *Indicate statistically significant differences after addition of pioglitazone (n = 6, ± SE).

Figure 3: Respiration rates of HepG2 cells cultured in glucose (A) or galactose (B) based medium for two weeks prior to respirometry. Oxygen flux is shown for control cells (black bars), and in presence of 60 μM pioglitazone (grey bars). Respiration rates of cells permeabilized with digitonin (Dig) were recorded in presence NADH generating substrates (MGP) and OXPHOS rates were measured after the addition of ADP (ADP). Convergent electron entry into the ubiquinone pool was initiated by addition of succinate (Succ) and leak respiration was recorded after inhibition of

the F_0F_1-ATPase with oligomycin (Olig). *Indicate statistically significant differences after addition of pioglitazone. #Indicate statistically significant differences between cells cultured in glucose or galactose containing medium (n = 6, ± SE).

Table 1: Impact of galactose and pioglitazone treatment on heat dissipation of HepG2 cells.

Condition[1]	30 min	60 min	90 min	120 min
Glu (Vc)	-22.49 ± 1.27	-22.97 ± 0.90	-22.46 ± 0.90	-22.24 ± 1.09
Glu (Pio)	-25.48[*] ± 0.99	-25.28[*] ± 1.10	-24.75[*] ± 0.97	-24.19* ± 0.87
Gal (Vc)	-18.88 ± 1.00	-15.21 ± 0.62	-13.45 ± 0.59	-11.99 ± 0.64
Gal (Pio)	-18.64[$] ± 0.99	-16.27[$] ± 0.72	-14.91[$] ± 0.60	-13.79[$] ± 0.52

[1]Cells were cultured and evaluated in galactose (Gal) or glucose (Glu) based medium, and response to 60 μM of pioglitazone (Pio), or DMSO (vehicle control, Vc) was recorded. The cells were cultured for a minimum of two weeks in presence of the respective sugar prior to calorimetry. [1]all data are shown in μW. *****statistically significant increase in heat dissipation after addition of pioglitazone. [$]statistically significant decrease in heat dissipation compared to glucose plus pioglitazone (n = 5-8, ± SE, $P < 0.05$).

Table 2: Impact of galactose and pioglitazone treatment on proliferation rates of HepG2 cells.

Condition[1]	Fold-increase in cells
Glu (Vc)	2.19 ± 0.15
Glu (Pio)	1.77[*] ± 0.02
Gal (Vc)	1.37[$] ± 0.05
Gal (Pio)	1.17[$] ± 0.15

[1]Cells were cultured for two weeks in presence of galactose (Gal) or glucose (Glu) prior to the proliferation assays. Cells were plated in absence of drugs and 60 μM of pioglitazone (Pio), or DMSO (vehicle control, Vc) was added after 24 h. Cells were enumerated after an additional 24 h of incubation. *****statistically significant decrease in cell proliferation after addition of pioglitazone. [$]statistically significant decrease in cell proliferation compared to cells cultured in glucose (n = 3, ± SE $P < 0.05$).

Figures:

Fig. 1

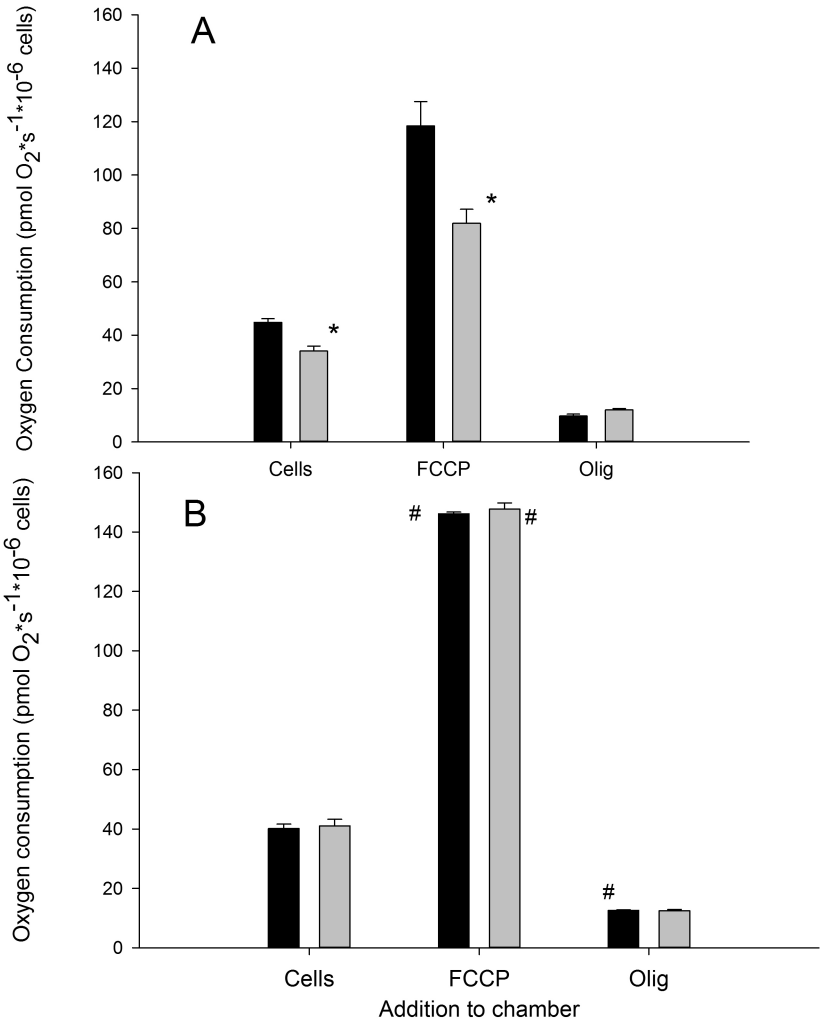


Fig. 2

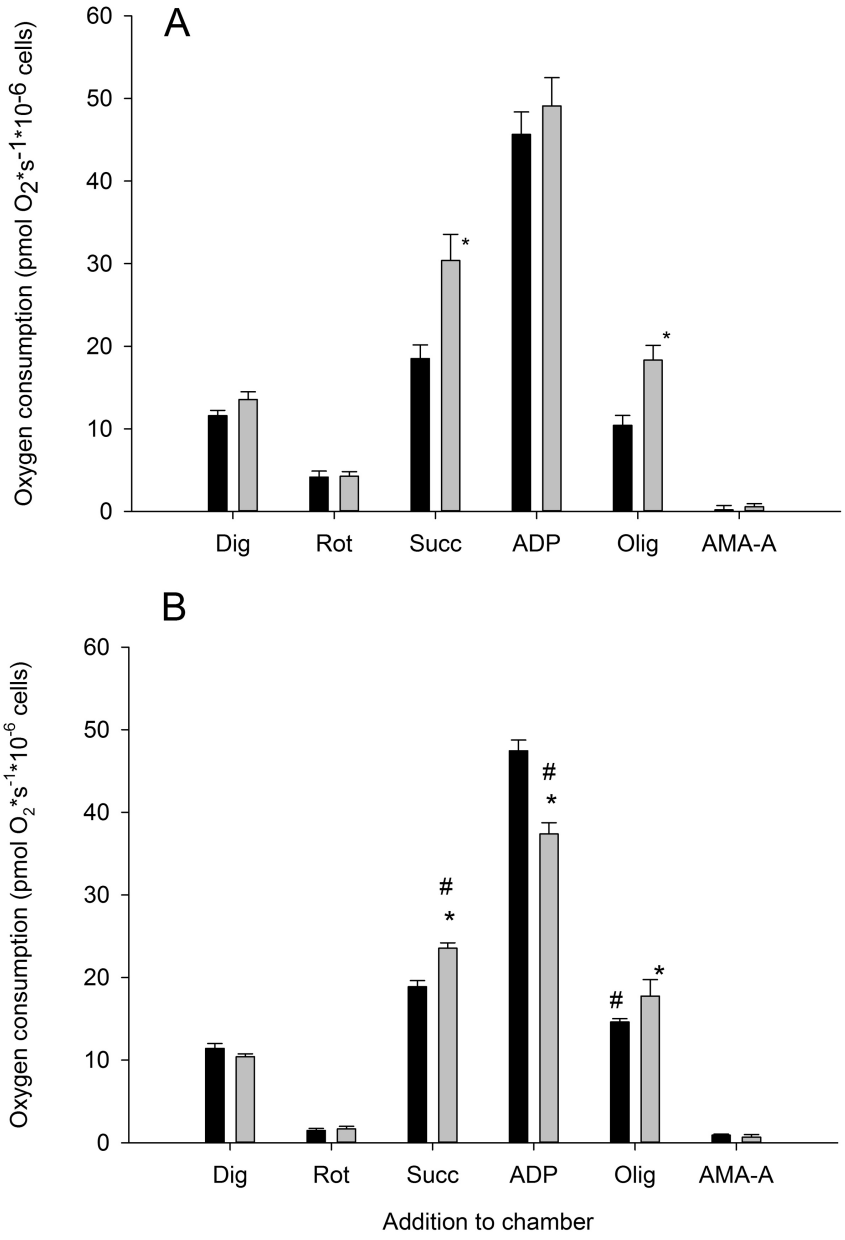
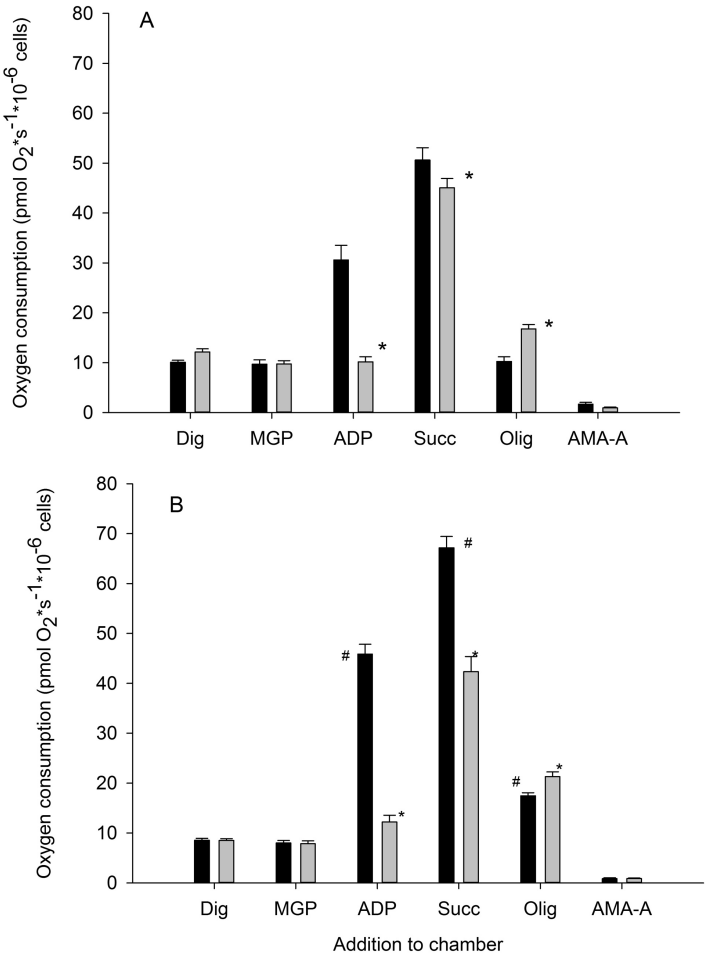

A
B
Oxygen consumption (pmol O2*s-1*10-6 cells)
Oxygen consumption (pmol O2*s-1*10-6 cells)
60
50
40
30
20
10
0
Dig
Rot
Succ
ADP
Olig
AMA-A
*
#
Addition to chamber

Fig. 3


A
B
Oxygen consumption (pmol O_2*s^{-1}*10^{-6} cells)
80
70
60
50
40
30
20
10
0
Dig
MGP
ADP
Succ
Olig
AMA-A
*
#
Addition to chamber

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Supplemental Files - Material and Methods

Chemicals

All chemicals used for respirometry and solution preparations were of the highest grade and purchased from Sigma-Aldrich (St. Louis, MO), or Fisher Scientific (Fair Lawn, NJ). Water for solution preparation was purified with a Milli-Q Reagent Water System (Billerica, MA) to an electrical resistance of 18 mΩ.

Cell culture

Human hepatocellular carcinoma cells (HepG2) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and grown in 75 cm^2 cell culture flasks (Corning Incorporated, Corning, NY). Standard cell culture medium to maintain HepG2 cells was composed of Opti-MEM I reduced serum medium (ThermoFisher, Grand Island, NY) supplemented with 5.5% fetal bovine serum (FBS) (Atlanta Biologicals Inc., Flowery Branch, GA), 100 units/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B (MP Biomedicals, Santa Anna, CA). Galactose treatment groups were cultured for a minimum of four weeks in glucose free DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10 mM D-galactose, 2 mM glutamine, 1 mM pyruvate (all from ThermoFisher, Grand Island, NY), plus 10 % dialyzed or complete FBS as indicated in the figure legends (Gal-DMEM). Gal-DMEM medium prepared with complete FBS contained >0.2 mM glucose (data not shown). For experiments directly comparing the impact of high glucose and galactose, cells were cultured in the above medium supplemented with 10 mM glucose instead of galactose (Glu-DMEM). The cells were maintained in a humidified atmosphere of 6.5% CO_2 and 93.5% air at 37 °C and the culture medium was renewed every 3 - 4 days. The cells were subcultured every seven days or before reaching 90% confluency. To

subculture, cells were dissociated using 0.25% trypsin and 1 mM EDTA in balanced salt solution (ThermoFisher, Grand Island, NY) and reseeded at 1.5 • 10^6 cells per 75 cm^2 cell culture flasks.

Microcalorimetry

Heat dissipation of HepG2 cells was measured as described before (Menzeet al. 2010). Briefly, the LKB 2277 thermal activity monitor (Bromma, Sweden) was charged with a 4 mL sealed ampoule filled with 2.5 mL of HepG2 cells in suspension to measure heat dissipation. Cells were diluted in culture medium equilibrated with 10% CO_2 and 90% air to yield 500,000 cells per 2.5 mL of medium. The medium formulations (Gal-DMEM and Glu-DMEM) used to measure cellular heat dissipation were identical to the ones used for cell culture. The reference vessel contained 2.5 mL of water and all calorimetric measurements were performed at 37 °C. A 15 min period for thermal equilibration was allowed after the ampoule was lowered into the calorimeter, and the heat flow (μW) was recorded for 2 h. Pioglitazone stock solutions (6 mM) were prepared in dimethyl sulfoxide (DMSO). Heat dissipation of cells without pioglitazone was measured in presence of DMSO alone (vehicle control) and treated cells were exposed to a final concentration of 60 μM pioglitazone.

Respirometry on HepG2 cells

Respiration was measured at 37 °C using 1 × 10^6 cells per mL in each chamber of the Oxygraph-2K (OROBOROS Instruments, Innsbruck, Austria). Routine respiration of intact cells was measured in either Opti-MEM I reduced serum medium, DMEM supplemented with glucose, or DMEM supplemented with galactose. The media formulations in these experiments were identical to the media used to culture cells. In some experiments, cellular respiration was uncoupled by successive titrations of carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; 0.5 μM steps), and leak respiration was measured in the presence of oligomycin (2 μg/mL).

Oxygen consumption of permeabilized cells was measured in 2 mL of MiR05 (110 mM sucrose, 60 mM potassium lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 3 mM $MgCl_2$, 0.5 mM EGTA, 0.1% BSA, 20 mM HEPES-KOH, pH 7.1). In order to supply mitochondrial substrates, cells were permeabilized by the addition of digitonin dissolved in dimethyl sulfoxide (DMSO) at 10 mg/mL (final concentration 10 μg × 10^{-6} cells). This digitonin concentration was found to be sufficient to permeabilize the plasma membrane of HepG2 cells with minimal impact on the integrity of the outer mitochondrial membrane as tested by addition of cytochrome *c*. Electron flow through complex I was stimulated by adding 2 mM malate, 10 mM glutamate, and 5 mM pyruvate. To engage the phosphorylation system, 1 mM ADP was added followed by the addition of 10 mM succinate to supply electrons to the ubiquinone pool via succinate dehydrogenase. Leak respiration in presence of ADP and ATP was measured after addition of oligomycin (2 μg/mL), and contribution of complex I to leak respiration was recorded after addition of rotenone (0.5 μM). Non-mitochondrial oxygen consumption was recorded after addition of 2.5 μM of antimycin A. DATLAB software (OROBOROS Instruments, Innsbruck, Austria) was used for data analysis and acquisition.

Impact of pioglitazone on cell proliferation

Approximately 500,000 cells were plated per well on 12-well plates, placed in a humidified atmosphere of 6.5% CO_2 and 93.5% air at 37 °C, and grown in glucose or galactose containing medium. After 24 h cell counts were performed on untreated control cells. Samples for proliferation assays were exposed to pioglitazone concentrations of 0 or 60 μM and after an additional 24 h of culture time, treated and untreated cells were enumerated using a hemocytometer. Membrane integrity was assessed by diluting samples in a 1:1 ratio with trypan blue prior to cell counts and only trypan blue negative cells were used to calculate cellular

proliferation rates. Fold increase in cell numbers were expressed as numbers of cells recovered after 24 h of plating divided by cell numbers recovered after 48 h.

Statistical analyses

Data were analyzed with a one-way analysis of variance (ANOVA) on ranks followed by comparison of experimental groups with the appropriate control group (Holm-Sidak method), or 2-way ANOVA followed by comparison of experimental groups with the appropriate control groups (Holm-Sidak method). SigmaPlot 12.5 (Systat Software Inc., San Jose, CA) was used for the analyses.