Modulation of mitochondrial bioenergetics in a skeletal muscle cell line model of mitochondrial toxicity

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\section*{A B S T R A C T}

Mitochondrial toxicity is increasingly being implicated as a contributing factor to many xenobiotic-induced organ toxicities, including skeletal muscle toxicity. This has necessitated the need for predictive \textit{in vitro} models that are able to sensitively detect mitochondrial toxicity of chemical entities early in the research and development process. One such cell model involves substituting galactose for glucose in the culture media. Since cells cultured in galactose are unable to generate sufficient ATP from glycolysis they are forced to rely on mitochondrial oxidative phosphorylation for ATP generation and consequently are more sensitive to mitochondrial perturbation than cells grown in glucose. The aim of this study was to characterise cellular growth, bioenergetics and mitochondrial toxicity of the L6 rat skeletal muscle cell line cultured in either high glucose or galactose media. L6 myoblasts proliferated more slowly when cultured in galactose media, although they maintained similar levels of ATP. Galactose cultured L6 cells were significantly more sensitive to classical mitochondrial toxicants than glucose-cultured cells, confirming the cells had adapted to galactose media. Analysis of bioenergetic function with the XF Seahorse extracellular flux analyser demonstrated that oxygen consumption rate (OCR) was significantly increased whereas extracellular acidification rate (ECAR), a measure of glycolysis, was decreased in cells grown in galactose. Mitochondria operated closer to state 3 respiration and had a lower mitochondrial membrane potential and basal mitochondrial O$_2$ level compared to cells in the glucose model. An antimycin A (AA) dose response revealed that there was no difference in the sensitivity of OCR to AA inhibition between glucose and galactose cells. Importantly, cells in glucose were able to up-regulate glycolysis, while galactose cells were not. These results confirm that L6 cells are able to adapt to growth in a galactose media model and are consequently more susceptible to mitochondrial toxicants.

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\section*{Introduction}

Skeletal muscle accounts for around 45% of total body weight and is characterised by a high rate of blood flow. Consequently, it is highly exposed to circulating xenobiotics [24]. In addition, skeletal muscle is one of the most metabolically active tissues, along with the heart and liver, and so requires a large amount of mitochondria for ATP production, making it particularly prone to xenobiotic-induced mitochondrial toxicity [12,23]. Indeed, research has shown that mitochondrial toxicity plays an important role in a number of xenobiotic-induced skeletal muscle myopathies, including antiretroviral (zidovudine) [35] and statin [20,2] myotoxicity. Notably, cerivastatin was withdrawn from the market in 2001 due to over 100 rhabdomyolysis-related deaths [6]. In this instance, skeletal muscle toxicity and mitochondrial perturbation was not identified during preclinical \textit{in vitro} or \textit{in vivo} screening and was only observed after the drug was in the market [20].

It is therefore important that high-throughput assays are implemented early in the research and development process which can effectively detect xenobiotics that impair mitochondrial function. One model that has been developed to improve detection
of mitochondrial toxicants utilises cells grown in two types of media, one supplemented with high glucose (25 mM) and the other with galactose [22]. Cells grown in high glucose media are able to compensate for mitochondrial impairment by utilising glycolysis for ATP generation, and therefore, are more resistant to mitochondrial toxicities. In contrast, cells grown in galactose as the sole sugar are forced to rely on mitochondrial oxidative phosphorylation (OXPHOS) to meet their energy requirements [30,15]. This is due to the slow metabolism of galactose to glucose-1-phosphate, which means that cells grown in galactose likely derive a majority of their ATP from glutamine (if present in the media) metabolism [29,38]. For example, it has been shown that HeLa cells derive 98% of their ATP from glutamine when cultured in galactose [29]. Since cells cultured in galactose (supplemented with glutamine) rely mostly on OXPHOS to produce their ATP, they become more sensitive to mitochondrial toxicants than cells grown in high glucose [22,11]. This model has been successfully used in liver (HepG2) and cardiac (H9c2) cell lines to identify mitochondrial toxicants [22,11,27]. However, to date, it has not been evaluated in a skeletal muscle cell line to assess mitochondrial toxicity.

The ability to alter the energy metabolism using this model has also been employed to identify cells with disease states that have underlying mitochondrial liabilities [30,1]. In addition, it has been used as a method to discover compounds that drive energy metabolism from mitochondrial respiration to glycolysis [15]. For example, Gohil et al. [15] demonstrated that compounds that are able to switch metabolism may have therapeutic potential, since they are able to suppress mitochondrial function and thereby minimise oxidative damage that follows ischaemic injury.

Studies have shown that a number of different cell types (e.g. cancer cells, fibroblasts and myotubes) are able to adapt to growth in galactose media and consequently show a significantly increased oxygen consumption rate and decreased glycolytic rate compared to cells cultured in high glucose [33,22,19]. Since the L6 rat skeletal muscle cell line is widely used as an in vitro model of skeletal muscle [34,18,17], it is potentially an ideal model for identifying mitochondrial toxicities. However, it is not currently known if this cell line is able to adapt to growth in galactose media and subsequently adapt its bioenergetic function as previously described for other cell types. Therefore, in this study we have characterised the effect of replacing glucose with galactose in the media on growth patterns, ATP synthesis capacity and bioenergetic function in the L6 skeletal muscle cell line. We also used classical inhibitors of the mitochondria to further investigate changes in mitochondria function following a switch to galactose media and the mechanism underlying the increased sensitivity of galactose cultured L6 cells to mitochondrial toxicity.

Materials and methods

Materials

The L6.G8.C5 (L6 subclone) cell line was provided by Alan Bevington (University of Leicester). The H9c2 and HepG2 cell lines were purchased from the American Type Cell Collection (ATCC). All chemicals were supplied by Sigma-Aldrich unless otherwise stated. MitoSOX™, MitoTracker™ Red CMXRos and ATP determination kit were purchased from Molecular Probes, Inc., Life Technologies UK Ltd. All materials and reagents for the Extracellular Flux assays were from Seahorse Biosciences (North Billerica, MA).

Cell culture conditions

High glucose media

The high-glucose media consisted of Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen), containing 25 mM glucose and 1 mM sodium pyruvate and supplemented with 5 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 10% FBS, and penicillin–streptomycin (pen–strept; 500 µg/ml).

Galactose media

The galactose media consisted of DMEM without glucose (Invitrogen) supplemented with 10 mM galactose, 2 mM glucose (6 mM final), 5 mM HEPES, 10% FBS, 1 mM sodium pyruvate, and pen–strept as above.

HepG2, H9c2 and L6 cells (ATCC) were grown in either glucose or galactose-containing media (15 ml) and maintained in 5% CO2 at 37 °C. Cells were maintained in 75 cm² flasks and subcultured with trypsin when cells reached 70–80% confluency.

Cell counting

At each subculture, the cells were stained with trypan blue and counted using a haemocytometer. The population doubling (PD) and doubling time (DT) were calculated using the following formula:

\[
PD = (\log_{10} Y_{C0} - \log_{10} Z) / \log_{10} 2
\]

\[
DT = T(h)\log_{10}(2) / (\log_{10} Y_{C0} - \log_{10} Z)
\]

(Y indicates the number of cells harvested; Z indicates the number of cells seeded; T (h) indicates time in hours.) Cumulative population doublings (CPD) were calculated as the sum of the PD with every successive passage.

Measurement of cellular ATP content

L6, H9c2 and HepG2 cells were seeded at a density of 10,000, 7500 and 20,000 cells/well, respectively, in a white 96-well plate with a clear bottom in 200 µl of media. The plates were incubated in a 37 °C humidified incubator with 5% CO2 for 24 h to reach 70–80% confluency. Cellular ATP concentrations were assessed using the ATP Determination kit as instructed in the manufacturer’s protocol. Luminescence was measured using a NOVOSTAR luminometer at 28 °C. For compound treatments, the compound stock solutions were prepared in 1% DMSO and added to the wells to give the indicated final concentrations. Compounds were added 24 h before measurements.

Detection of mitochondrial O2⁻

MitoSOX™ red was used in live cells. L6 cells were seeded into 6-well plates at 2 x 10⁵ cells/well and grown to 70–80% confluence. MitoSOX was added to a final concentration of 5 µM 30 min prior to the end of the treatment period and cell fluorescence was measured on a CyAn ADP flow cytometer (excitation: 488 nm; emission: 578 nm). Data were also collected in FSC (forward scatter) and SSC (side scatter) and a total of 10,000 events were collected for each sample.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was measured using MitoTracker Red CMXRos (500 nM) in live cells using a CyAn ADP flow cytometer (excitation: 488 nm; emission: 578 nm). Data were also collected in FSC (forward scatter) and SSC (side scatter) and a total of 10,000 events were collected for each sample.
Measurement of L6 mitochondrial bioenergetics using the Seahorse XF24-extracellular flux analyzer

An XF24 Analyzer (Seahorse Biosciences, North Billerica MA) was used to measure bioenergetic function in intact L6 cells in real time. L6 cells were seeded into Seahorse Bioscience XF24 cell culture plates to the indicated density in 250 μl media and allowed to adhere and grow for 24 h in a 37 °C humidified incubator with 5% CO₂. Measurements of extracellular flux were made in unbuffered media. For these experiments, the media was changed 1 h prior to the start of the extracellular flux assay to unbuffered (pH 7.4) DMEM containing 2 mM GlutaMax, 1 mM sodium pyruvate and supplemented with 25 mM glucose or 10 mM galactose and incubated at 37 °C without CO₂. Normalisation to total protein content in the well following the experiment was used to control for variation in cell number between glucose and galactose cultured cells. On completion of the XF assay, the cells were lysed with CellLytic MT lysis reagent (200 μl/well) and protein concentration determined using the Bradford reagent. The protein concentration of a sample was derived by reference to a bovine serum albumin (BSA) standard curve. The OCR data were expressed as pmol/min/mg protein and ECAR as mpH/min/mg protein.

Mitochondrial function of L6 cells cultured in glucose and galactose media was analysed by sequentially adding pharmacological inhibitors of oxidative phosphorylation [10]. The resultant bioenergetic profile (Fig. 1) provides detailed information on the individual components of the respiratory chain. For a detailed and extensive review on the interpretation of the bioenergetic profile, please refer to Hill et al. [16]. Briefly, six parameters of mitochondrial function were calculated from the bioenergetic profile: basal OCR, ATP-linked OCR, proton leak OCR, maximal OCR, reserve capacity and non-mitochondrial OCR (Fig. 1). These parameters of mitochondrial function were used to derive the respiratory flux control ratios: coupling efficiency, respiratory control ratio (RCR), Stateapparent and phosphorylating respiration. State apparent is used to determine the apparent respiratory state of the cells. As suggested by other studies, it is unlikely that the classical state 3 (ADP and substrate are not limited and respiration is maximum) or state 4 (ADP reaches equilibrium and respiration slows), observed in isolated mitochondria [4], are replicated in intact cells. Instead an intermediate turnover state, known as State 3.5 (the Stateapparent) has been proposed for intact cells [10]. In these experiments, the assumption is made that state 3 respiration is equivalent to the rate measured after addition of FCCP (‘state 3FCCP’ or ‘state 3u’) and state 4 is the rate measured after addition of oligomycin (‘state 4oligomycin’ or ‘state 4o’). These assumptions allow for calculation of the apparent respiratory state of the cells using the following equation:

Stateapparent = 4 – (Basal – Oligo) (FCCP–Oligo)

where ‘Basal’ represents the basal OCR, ‘Oligo’ represents the oligomycin-insensitive OCR (proton leak) and ‘FCCP’ represents the FCCP-stimulated OCR (maximal OCR). Using the same assumptions regarding the relative state 3 and state 4 respiration, the respiratory control ratio (RCR) can be calculated as the state 3u rate divided by state 4o rate (maximal OCR/oligomycin-insensitive OCR). RCR indicates the tightness of the coupling between respiration and oxidative phosphorylation and is sensitive to changes in substrate oxidation and proton leak, but not ATP turnover [4]. Coupling efficiency is the proportion of the oxygen consumed to drive ATP synthesis compared with that driving proton leak and is calculated as the fraction of basal mitochondrial OCR used for ATP synthesis (ATP-linked OCR/basal OCR) [4]. Finally, the fraction of respiration that is used under routine conditions to produce ATP (phosphorylating respiration) was estimated as the ratio between ATP-linked OCR and maximal OCR (FCCP) (ATP-linked OCR/Maximal OCR), as described in Domenis et al. [9].

Statistical analysis

All data are presented as mean and standard error of the mean (SEM) or standard deviation (SD) as appropriate. Statistical analyses were performed using Graphpad Prism® 6 software (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance of the data was assessed by paired Student’s t-test, one-way Analysis of Variance (ANOVA) (with Dunnett’s multiple comparison test) or two-way ANOVA (with Sidak’s or Tukey’s multiple comparison test) as appropriate. A P-value of < 0.05 was considered statistically significant. IC₅₀ values were calculated in Graphpad Prism using a nonlinear regression curve fit with 4 parameters. The maximum (DMSO control) and minimum values (total cell kill) were fixed.

Results and discussion

Maintenance of cell growth in galactose media

A number of cell types, including H9c2 and HepG2, have previously been shown to successfully adapt to galactose media [22,27] with 7 days sufficient for cells to adjust their cellular bioenergetics (i.e. an increase in OXPHOS) [15,1,37,9]. Cryopreserved L6, H9c2 and HepG2 cells previously grown in high glucose media were thawed and grown for a minimum of 7 days in either high glucose (25 mM) or galactose (10 mM) media. No significant

![Fig. 1. Representative OCR profile of the mitochondrial function assay using L6 cells cultured in glucose media. The example is from control L6 cells cultured in glucose. Baseline OCR was measured 3 times for 3 min each separated by a 2 min wait and 2 min mix. Following the measurement of basal respiration, oligomycin (800 nM) was injected into each well, followed by 3 cycles of; 2 min mix, 2 min wait and 3 min measurement to determine O₂ consumption resulting from proton leak. The difference between the basal OCR and the oligomycin-insensitive OCR yields the amount of O₂ consumption that is ATP-linked. The balance of the basal OCR is comprised of O₂ consumption due to proton leak and non-mitochondrial sources. Then, FCCP (800 nM) was injected into each well, followed by 3 cycles of 2 min mix, 2 min wait and 3 min measurement to determine the maximal O₂ consumption that is possible at complex IV. The difference between FCCP rate and basal OCR yields an estimate of the reserve capacity of the cells. Finally, antimycin A (200 nM) was injected into each well, followed by 3 cycles of; 2 min mix, 2 min wait and 3 min measurement to determine the rate of O₂ consumption from non-mitochondrial sites.](image-url)
change in cell morphology or cell death rate (trypan blue extraction) was observed in L6, H9c2 or HepG2 cells grown in galactose compared with glucose (data not shown). In all three cell lines there was a trend for higher growth rates in glucose than in galactose media (Fig. 2A–C), reflected in increased doubling times for all three cell types (Fig. 2D). Consequently, there were a significantly greater number of L6 cells after 24 and 48 h growth in glucose (Fig. 2E). A slower growth rate in galactose media has been demonstrated with HepG2 cells [7,9], skin fibroblasts [15] and HeLa cells [33]. Slower growth is partly due to the difference in uptake of galactose compared with glucose since GLUT transporters have a higher affinity for glucose than galactose [38,19]. Galactose is also metabolised at about an 8-fold slower specific rate than glucose, because galactokinase, the rate-limiting step in galactose metabolism, is considerably less active than hexokinase, the first enzyme and rate-limiting step in the oxidation of glucose [38]. Consequently, the levels of glucose-6-phosphate, an important substrate essential for cell proliferation via the pentose phosphate pathway (PPP) production, are lower in galactose compared to glucose media [7,39]. Therefore, in galactose media, the key PPP intermediates, NADPH and ribose, are formed at a lower rate than in glucose media and thus the growth rate is slower [38].

Previous studies have demonstrated that HepG2 and H9c2 cells maintain equivalent levels of ATP when cultured in glucose or galactose media [22,27]. Similarly, no significant difference was found in ATP content between L6 cells grown in glucose or galactose media (Fig. 2F). This is also in agreement with studies using human primary skeletal muscle cells, in which no difference in the ATP content between myotubes cultured in high glucose and galactose was found [1].
Sensitivity of cells grown in galactose to mitochondrial inhibition

Since cells grown on galactose rely mostly on mitochondrial OXPHOS for their ATP synthesis they are reportedly more sensitive to mitochondrial toxins than when grown in glucose [22,27]. This hypothesis was tested in glucose and galactose-grown L6, H9c2 and HepG2 cells treated with rotenone (500 nM) or antimycin A (AA; 150 nM), classical inhibitors of the mitochondrial complex I and III, respectively. Effects on ATP content were used to measure the susceptibility of the cells to OXPHOS inhibition.

In all three cell types grown on glucose media, ATP concentrations were reduced to approximately 40–55% of control following culture with AA for 24 h (Fig. 3A). In contrast, all cell types grown in galactose were more highly susceptible and ATP levels were depleted to <6% of control values. As further confirmation that the L6 cells had adapted to galactose, rotenone reduced ATP levels of L6 and HepG2 cells to 1% of control values compared to glucose-grown cells which maintained ATP levels at 35–48% of the control (Fig. 3B).

It should be noted that from these experiments it is not possible to determine whether ATP is depleted due to a reduction in the generation of ATP or whether an increase in the rate of cell death also played a role. To determine the temporal relationship between ATP level and cell death, a time course measuring ATP levels and cell death markers (such as Annexin V/PI) would have to be carried out. However, these results still indicated that in the presence of galactose these three cell lines were more sensitive to ETC inhibitors than in glucose. We therefore carried out AA dose responses for each cell line in either glucose or galactose containing media (Fig. 4). The AA IC50 values differed considerably between each cell line in both glucose and galactose media. All three cell types showed increased sensitivity to AA when grown on galactose media (Fig. 4A-C). The differential effect based on the IC50 ratio of glucose to galactose, ranged from 5.9 (L6) to 17.7 (HepG2) and on the basis of ATP levels, clearly demonstrates that on galactose, OXPHOS is the predominant source of ATP. It therefore serves as an ideal early screening assay to identify compounds that may potentially induce mitochondrial toxicity. The variation in glucose/galactose IC50 also shows that some cell types are more sensitive to inhibitors of oxidative phosphorylation. Thus, the cell type employed may be an important factor in the sensitivity of the assay when using an IC50 ratio to investigate mitochondrial perturbation. Therefore, more research using a number of cell types and mitochondrial toxicants is required to ascertain the importance of cell type.

It should be noted that not all the effects of mitochondrial toxicants will be detected using a 24 h toxicity assay, as was used here. While this assay may be able to accurately detect compounds that inhibit the ETC (thiazolidinediones), uncouple OXPHOS (bupivacaine) and redox cycle (quinones) within the mitochondria, it is unlikely to detect compounds that target mtDNA replication (NRTIs) and gene expression, which may need longer exposure times [5,11,26].

Bioenergetics of L6 cells grown in galactose

Having demonstrated that L6 cells were more sensitive to AA in galactose media we next characterised the bioenergetic status of these cells using a seahorse extracellular flux (XF) analyser. In this system mitochondrial respiration (OCR) is used to measure OXPHOS and extracellular acidification (ECAR) as a measure of glycolysis [14].

Consistent with previous studies on other cell types [29,15], we found that L6 cells grown in glucose derived ATP from both aerobic glycolysis and mitochondrial glutamine and pyruvate oxidation (Fig. 5A and B). However, when L6 cells were cultured in galactose, OXPHOS levels increased by at least 50% and basal ECAR activity was ~25% lower than in glucose cultured cells (Fig. 5B and D). This is consistent with the fact that galactose metabolism is unable to derive sufficient ATP from glycolysis, forcing the cells to rely almost exclusively on mitochondrial OXPHOS (via glutamine and pyruvate oxidation) to produce ATP [29,30,33]. The response of ECAR (glycolytic rate) in each media type to mitochondrial toxicants provided detail on the mechanism of increased galactose cell sensitivity after mitochondrial perturbation (Fig. 5C and D). Following oligomycin treatment, mitochondrial ATP synthesis is blocked and glucose cultured cells were able to up regulate glycolysis to meet their demand for ATP. However, galactose cultured cells were unable to up regulate glycolysis and so ECAR remained unchanged. The treatment with FCCP and AA demonstrated that the majority of ECAR measured in galactose cultured cells is derived from a small ‘proton leak’ from the respiring mitochondria [31]. The uncoupling of the electron transport chain by FCCP results in an increase in proton leak across the inner mitochondrial membrane, which accounts for the sharp increase of ECAR in the galactose cultured cells (Fig. 5C). The inhibition of the ETC by AA severely depleted galactose cell ECAR, but had no effect on glucose cell ECAR. This further confirms that glucose cultured cells are able to stimulate glycolytic flux following mitochondrial stress to provide the requisite ATP for survival, while galactose cultured cells are unable to and consequently die following mitochondrial perturbation [22].
Effect of galactose on mitochondrial function in L6 cells

Similar to previous reports [10,28] mitochondrial function of L6 cells cultured in glucose and galactose media was defined by sequentially adding mitochondrial inhibitors to delineate the components of the respiratory chain. As shown in Fig. 6, basal OCR, proton leak, ATP-linked OCR and maximal OCR were significantly higher in galactose than in glucose cultured cells. In contrast, the reserve capacity of cells grown in glucose was significantly greater than for galactose. Cells in galactose media were unable to maintain maximal OCR levels following the addition of FCCP, with the OCR decreasing to basal levels prior to AA treatment (50–70 min; Fig. 5A). This implies that at basal levels, the galactose cultured cells were operating closer to maximal OCR capacity and any increase was unsustainable, resulting in a lower reserve capacity. There was also a trend for a higher non-mitochondrial OCR in galactose cultured cells compared to glucose cultured cells, but this did not reach statistical significance.

The parameters of mitochondrial function were used to derive the respiratory flux control ratios, coupling efficiency, respiratory control ratio (RCR), State3/State4 and phosphorylating respiration (Table 1). The advantage of the flux control ratios is that they are internally normalised allowing for accurate comparison between glucose and galactose cultured cells [4]. Table 1 shows there was no significant difference in coupling efficiency between glucose and galactose cultured cells, indicating that sugar substrate change had no effect on the efficiency of mitochondrial respiration (Table 1). In both glucose and galactose media, ~66% of cellular OCR was related to ATP synthesis. The coupling efficiency of rat skeletal muscle mitochondria has been shown to be ~65% [32], which is very similar to what was obtained with L6 cells cultured in glucose and galactose media. RCR was significantly greater in glucose than galactose cultured cells (Table 1), which means the mitochondria of glucose cultured cells have a greater potential for substrate oxidation and ATP turnover than galactose cultured cells. In addition, a higher RCR in glucose cultured cells correlates with the lower proton leak seen in this study [4]. As with reserve capacity, the lower RCR of cells cultured in galactose is attributable to the fact that the basal OCR level of galactose cultured cells is close to the maximal OCR capacity of the cells.

Taken together, these results show that the mitochondria of galactose cultured cells were operating at a higher level of respiration at basal levels than glucose cells and so were closer to ‘state 3’ configuration. This was not due to an increased efficiency of the mitochondrial ETC however, implying a change in OXPHOS complex synthesis or activity regulation. The reason for an increased mitochondrial OCR in L6 cells grown in galactose media compared to glucose media was not investigated in this study. It has been shown that substrate oxidation correlates with an increased respiratory flux and is thereby responsible for changes in state3u [4]. Possible explanations for an increased substrate oxidation include a general increase in mitochondrial content (through mitochondrial biogenesis), an increased synthesis of electron-transport chain complexes, an increase in processing enzymes, an increased flux of glutamine oxidation through the TCA cycle and ETC or a combination of each [33]. For example, complex IV activity was found to be increased in primary human muscle cells in galactose cultured cells, although there was no increase in mitochondrial content [1]. In contrast, it has been shown that HepG2 cells increase their mitochondrial content through mitochondrial biogenesis in response to galactose media [9]. Thus, each cell line/primary cell adapts mitochondrial and
Galactose

Effect of galactose on mitochondrial membrane potential and superoxide generation

State 3u is characterised by a decrease in the protonmotive force (Δp), an increase in proton leak and an acceleration of electron transport [4]. The Δp describes the movement of protons against their electrochemical gradient into the mitochondrial inner membrane space, and has two components, ΔpH (the pH gradient across the inner membrane) and ΔΨm (mitochondrial membrane potential) [8]. As the dominant component, ΔΨm contributes 80–85% to the Δp, and hence it is an important measure of how the mitochondria are functioning [13,36]. The ΔΨm of L6 cells cultured in glucose and galactose media was

bioenergetic capabilities differently to galactose media and so further research would be required to understand the mechanism of adaptation of L6 cells to galactose media.

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Table 1

| Normalised respiratory flux control ratios | L6 cells | Glucose | Galactose |
|------------------------------------------|---------|---------|----------|
| Stateapparent                            | 3.37 ± 0.03 | 3.19 ± 0.03* |
| Respiratory control (RCR)                | 4.26 ± 0.36  | 3.43 ± 0.19** |
| Phosphorylating respiration              | 0.47 ± 0.01  | 0.57 ± 0.02** |
| Coupling efficiency                      | 0.66 ± 0.02  | 0.66 ± 0.02 |

Stateapparent was calculated to determine the apparent respiratory rate in glucose and galactose cells to provide an estimate of the relative mitochondrial work being used by the cells under basal conditions. The galactose cells Stateapparent (3.19) was significantly closer to state 3 than glucose cells (3.37) were, indicating that galactose cells had an increased mitochondrial workload compared to glucose cells. In addition, the phosphorylating respiration of galactose cells (0.57) was significantly higher than glucose cells (0.47), demonstrating that galactose cells use a greater proportion of the maximal respiratory capacity (maximal OCR) for ATP production than glucose cells under basal conditions.

* P < 0.05.
** P < 0.01 glucose vs. galactose.

Fig. 5. Cellular bioenergetics of L6 cells cultured in glucose and galactose. L6 cells were cultured for a minimum of 7 days in either glucose (25 mm) or galactose media (10 mm). Real time measurements (mean ± SEM, n=5) of OCR (A) and ECAR (B) were measured using the XF24 analyzer and oligomycin (800 nm), FCCP (800 nm) and AA (200 nm) injected sequentially as shown (A and C). (B) Basal OCR and ECAR values of glucose and galactose cells were plotted to illustrate the difference in metabolic profile (mean ± SEM, n=5).

Fig. 6. Measurement of mitochondrial function in L6 cells cultured in glucose and galactose. The AUC for each mitochondrial parameter was calculated for L6 cells cultured in glucose and galactose cells for a minimum of 7 days and derived from the data in Fig. 5 A as detailed in the methods section (Fig. 1). Bars represent mean ± SEM; n=5 (*P < 0.05, **P < 0.01 glucose vs. galactose).
assessed using MitoTracker Red CMXRos. As shown in Fig. 7A, the \( \Delta \psi_m \) of L6 cells cultured in galactose media was \( \sim 10\% \) lower than cells cultured in glucose media. Since galactose cultured cells had a lower \( \Delta \psi_m \) and a higher proton leak than glucose cultured cells, this supports the suggestion that galactose cultured cells were operating closer to state 3 than glucose cultured cells. To achieve net forward flux through each electron transport component a thermodynamic disequilibrium is required, i.e. the free energy available from electron transfer must be greater than that required to pump protons across the membrane against the \( \Delta \psi \) [4]. Thus, the electron transport chain responds to a drop in \( \Delta \psi \), resulting from an increased proton re-entry through the ATP synthase or proton leak, with an increased flux. Therefore, in galactose grown cells, the decrease in \( \Delta \psi \) (as shown by the decrease in \( \Delta \psi_m \)), increase in proton leak and increase in ATP-linked OCR compared to glucose grown cells, is indicative of an accelerated electron transport flux, and subsequent increase in ATP production via mitochondrial OXPHOS.

The \( \Delta \psi_m \) functions as a regulator of mitochondrial superoxide production [36] and so the effect of culturing L6 cells in glucose and galactose media on mitochondrial \( O_2^{\cdot-} \) production was assessed using MitoSox. Under basal conditions, glucose-cultured cells produced a significantly greater amount of mitochondrial \( O_2^{\cdot-} \) (\( \sim 18\% \) greater) than galactose cells (Fig. 7B). Cells with an increased capacity for oxidative phosphorylation are also susceptible to increased mitochondrial \( O_2^{\cdot-} \) production through increased leakage of electrons from the electron transport chain [4]. The function of proton leak is therefore to provide cells with a greater degree of protection from \( O_2^{\cdot-} \) production via increased ‘mild uncoupling’ to prevent mitochondrial \( O_2^{\cdot-} \) production without deleteriously lowering ATP synthesis [8]. As such, mitochondrial \( O_2^{\cdot-} \) production is steeply dependent on \( \Delta \psi \). The increased proton leak and subsequent decrease in \( \Delta \psi_m \) of galactose cultured cells compared to glucose cultured cells therefore explains why galactose cells had a lower basal level of mitochondrial \( O_2^{\cdot-} \) production than glucose cells. A significant increase in proton leak might be explained by a difference in membrane composition or as the consequence of an increase in the expression of proteins involved in matrix return of protons. Basal proton conductance is primarily attributable to the abundance of adenine nucleotide translocase (ANT), although inducible proton conductance is catalysed by mitochondrial uncoupling proteins (UCPs) [8]. Notably, the mitochondrial anion carriers UCP2 and UCP3 have been shown to protect against oxidative damage by uncoupling when activated, thereby lowering \( \Delta \psi \) to reduce the \( O_2^{\cdot-} \) production [3].

Sensitivity of L6 cells cultured in glucose and galactose media to antimycin A inhibition of OCR

The sensitivity of L6 cells cultured in glucose and galactose media to complex III inhibition was investigated in a dose and time dependent manner. In these experiments, a mitochondrial function assay was performed on cells cultured in either glucose or galactose with a range of AA concentrations to assess the OCR and ECAR levels with each media type. The results are presented as a percentage of baseline and since glucose cultured cells were able to maintain an increased OCR following FCCP treatment, the glucose control (DMSO) value is greater than galactose cultured control when compared to baseline values (Fig. 8A and C). At all concentrations tested, the effects of AA on OCR were identical in each media type, suggesting that the threshold for complex III inhibition was not different between L6 cells cultured in glucose (Fig. 8A) and galactose (Fig. 8C) media. This is in line with research in HeLa, fibroblast and primary muscle cells that have shown no difference in the level of complex III in glucose and galactose cultured cells [33,25,1]. Rossignol et al. [33] suggest that high amounts of complex III in glucose cells are required even when there is little or no OXPHOS for other electron transport reactions or biosynthetic needs. For example, a study by Ma et al. [21] showed that complex III plays an important role in mediating autophagy induction in a human glioblastoma H4 cell. Thus, if the levels of complex III were equivalent in glucose and galactose-grown L6 cells, this would explain why the rate of AA-induced OCR decrease was identical in both cell types. In contrast to the effects on OCR, the effects of AA on ECAR are different with each media type (Fig. 8B and D). At the two highest concentrations of AA (40 and 80 nM), there is a \( \sim 50\% \) increase in ECAR with glucose cultured cells, but a \( \sim 50\% \) reduction in ECAR with galactose cultured cells. Therefore, these results confirm what was found in Fig. 5C and D, that L6 cells cultured in galactose are unable to maintain glycolysis to support ATP production. Overall, these results indicate that the increased sensitivity of galactose cultured cells to AA is not due to a greater decrease in OCR, but likely a
difference in the response of ECAR and the subsequent levels of ATP produced by glycolysis.

Conclusions

In this study, we have demonstrated that L6 cells, like HepG2 and H9c2 cells, are able to adapt to growth in a galactose media model and are consequently more susceptible to mitochondrial toxicants. Investigation into cellular bioenergetics showed that galactose cultured L6 cells have a significantly increased OXPHOS capacity compared to glucose cultured cells. Unlike glucose cultured cells, however, cells cultured in galactose were unable to up regulate glycolysis (ECAR) and maintain sufficient ATP levels following the inhibition of OCR by mitochondrial toxicants. Further analysis into mitochondrial function revealed that galactose cultured L6 cells operated closer to state 3 respiration and consequently had a lower mitochondrial membrane potential and basal mitochondrial O$_2$ level compared to glucose cultured cells. In addition, an AA dose response revealed that there was no difference in the sensitivity of OCR to AA inhibition between glucose and galactose cultured cells. In contrast, glucose cultured cells were able to up regulate ECAR, while galactose cultured cells could not. Thus, we confirm the current understanding that galactose cell sensitivity is governed by its inability to up regulate glycolysis to produce requisite ATP for survival. Overall, these results confirm the potential utility of the L6 galactose media model to more sensitively detect mitochondrial toxicants that induce myotoxicity. However, more research is still needed with known mitochondrial toxicants to validate the importance of using tissue-specific cell lines when investigating potential target-organ toxicities using the galactose media model.

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