

Fluoxetine Affects Glycolytic Pathway of C6 Rat Glioma Cells

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Abstract

Antidepressants (ADs) such as fluoxetine is known to target and inhibit the electron transport chain of mitochondrial, effects on rat C6 glioma cell function are expected. Also, it has been shown that fluoxetine can inhibit mitochondrial respiration in pancreatic beta cells; however whether this drug can also directly affect glycolytic metabolism is unknown. To address this idea, I have investigated effect of fluoxetine on the bioenergetics of C6 glioma. MTS assay was conducted as an indicator of the metabolic activity of C6 cell lines. Oxidative respiration was measured via oxygen consumption rate (OCR), anaerobic glycolysis was determined with lactate electrodes, where mitochondrial redox state was monitored via NAD (P) H autofluorescence. The metabolic assays showed glucose stimulated MTS reduction cells in a time and concentration dependent manner whereas, the other three mitochondrial fuels did not stimulated it in C6 glioma cells. Methylpyruvate substrate stimulated OCR, neither both glucose and α -ketoisocaproate. Glucose inhibited OCR and increased lactate production compared to the control. Glucose failed to affect NADPH levels. Fluoxetine inhibited OCR at 10 mM glucose. However, it did not affect OCR at 10 mM α -ketoisocaproate. The above results showed that C6 glioma cells are mainly depending on the glycolytic pathway and that was inhibited by Fluoxetine. In conclusion, since fluoxetine was inactive in the presence of α -ketoisocaproate our data suggest that the point of major action for these ADs in C6 glioma cells is glycolysis.

Keywords: Fluoxetine, OCR, Lactate production, NAD(P)H, MTS , C6 glioma rat cells

Introduction

Glia cells compromise over half of the brain volume. They are located in the central and peripheral nervous system where they form Schwann cells in the periphery and neuroglia cells in the central nervous system. Neuroglia

cells are considered as essential cells in the brain for its survival, their number is much more than for neurons.

Gliocytes have a major role in the energy supply, homeostasis, neurogenesis and synaptogenesis in the brain (Alvarez-Maubecin *et al.*, 2000); (Srebro and Dziobek, 2001);(Kreutzberg, 1996); (Tardy *et al.*, 1991). Early studies by Gee and colleagues pointed out that astrocytes are one of the most important type of glial cells that comprises 90% of brain and several studies have shown their benefits in the modulation of brain function (Gee and Keller, 2005).They have the important role to control the extracellular such as, dopamine, glutamate, norepinephrine, serotonin, Acetylcholine and GABA (gamma-amino butyric acid) level of some ions such as K⁺ ions and neurotransmitters in the extracellular space. For instance, astrocytes express receptors for wide range of neurotransmitters and can liberate various kinds of neuroactive substrates and trophic factors. On the basis of th-7896+ese, they probably assist features development and function of synapses.

Astrocytes

Astrocytes are the most abundant cell component in glial cells and have various supporting functions (Hu *et al.*, 2008). They have very important role in metabolic interaction between astrocytes and neurons (Magistretti, 2008a). In addition, it has been suggested that astrocytes are a valuable store of glycogen, such that as, when hypoglycaemia occurs, this may maintain the energy supply of neurones (Dienel and Cruz, 2006; Brown *et al.*, 2004; Magistretti, 2008b; Vilchez *et al.*, 2007). Glycogen may be quickly transformed into pyruvate or/and lactate and also metabolized via the tricarboxylic acid (TCA) cycle, or it could be used for biosynthesis of glutamate and glucose (Hamprecht *et al.*, 2005; Ghosh *et al.*, 2005). It has been thought that the glucose and lactate produced by glia might be transmitted to neurones for fuel (Brown *et al.*, 2004; Dringen *et al.*, 1993), the so called Astrocytes, Neuron Lactate Shuttle Hypothesis [ANLSH](Magistretti, 2008a).

Proton- linked membrane transporters that carry lactate, pyruvate and ketone bodies through the cell membrane. Such transporters are expressed universally by astrocytes (Pierre and Pellerin, 2005; Rinholm *et al.*, 2011). There is growing evidence confirm that the rate of glycolysis in astrocytes is much higher than neurons which depends on mitochondrial respiration for their energy (Occhipinti *et al.*, 2007). Furthermore, more current studies have addressed the critical question of which biochemical steps confirm whether astrocytes is rather oxidative (oxidizing both glucose and lactate) or exhibit some glycolytic features (by exporting rather than consuming lactate)(Neves *et al.*, 2012).

The onset of depression is believed to be associated with a lack of monoamine/ catecholamine neurotransmission in the central nervous system, and as a consequence therapeutic regimes mostly focus on the use of drug classes that are able to down-regulate or upregulate central synaptic monoamine/catecholamine neurotransmission. These drugs include tricyclic anti-depressants (TCA), monoamine oxidase inhibitors (MAOI), selective serotonin reuptake inhibitors (SSRI) and non-selective serotonin reuptake inhibitors (NSRI) as well as emerging atypical classes (McIntyre *et al.*, 2006; Kiss, 2008). According to some researchers (Lustman *et al.*, 1992; Derijks *et al.*, 2008; Brown *et al.*, 2008).

Indeed, it is well established that at high doses, members of the typical class of antidepressants, such as fluoxetine can be cytotoxic human carcinoma HT29 cells (Arimochi and Morita, 2006) as well as inhibiting the proliferation in a dose dependent manner breast cancer cells (Abdul *et al.*, 1995). Several studies by (Spanová *et al.*, 1997; Levkovitz *et al.*, 2005) indicated that fluoxetine causes apoptosis in rat glioblastoma cells and human neuroblastoma cells. On the contrary, a study by (Mercier *et al.*, 2004) mainly in vivo, found that antidepressants induce neuroprotection and facilitation of neural cell survival via activation in the brain of neurotrophic factors, such as brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF). Increased BDNF was found in rat hippocampus, following reboxetine and citalopram treatment (Russo-Neustadt *et al.*, 2004). In addition, Several studies have demonstrated that fluoxetine increases the proliferation of B16F10 melanoma cells and C3 fibrosarcoma cells both in vivo and in vitro (Brandes *et al.*, 1992) as well as fluoxetine and desipramine, a tricyclic antidepressant, inhibit metastasis of B16F10 lineage in young mice, but promote it in old mice (Kubera *et al.*, 2009). The mechanisms that underlie Antidepressants neurotoxicity remain unclear. Their neurotoxicity may result from disruptive actions on mitochondria and oxidative respiration. For example, previous investigations showed that antidepressants such as fluoxetine especially at dosages at the higher end of their therapeutic window, affect the function of C6 rat glioma cells of; a phenomena that will have consequences for impairment of mitochondrial function of human glioma cells (Daley *et al.*, 2005). However, whether this drug can also directly affect glycolytic metabolism is unknown. I have sought to answer this question by studying metabolic profile of C6 rat glioma cells. MTS reduction by using MTS assay, oxidative respiration was measured via oxygen consumption rate (OCR), anaerobic glycolysis was determined with lactate electrodes, where mitochondrial redox state was monitored via NAD(P)H autofluorescence.

Experimental procedure

Cell lines used

C6 rat glioma cell lines (ATCC CRL-2199) is derived from the N-nitrosomethylurea-induced glial rat in 1989. This cell line maintained in G418 media for 14 days, cloned, and evaluated for beta-gal production. LacZ7 is a specific markers express in rat brain can be detected by histochemistry. These cells are adherent and when grown in the T-75 cm² flasks, they form stable monolayers (Benda *et al.*, 1968). Cell cultured medium was Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) fetal calf serum, 1% (w/v) Penicillin 1000 unit/ml- streptomycin 0.1mg/ml and 2 mM glutamine. Passage number used 66-100. This cell line is an astrocytic cell culture model, which is less aerobic and more dependent on glycolysis for its bioenergetics.

MTS Assay

The water-soluble salt MTS (3-[4, 5dimethylthiazol-2-yl] -5-[3-carboxymethoxy-phenyl] -2-[4-sulfophenyl] -2H-tetrazolium) assay depends on reduction of the MTS substrate to produce coloured formazan. This assay does not need to solubilize the cells by using acidified isopropanol, which may disrupt the cells integrity. MTS receives electrons via an intermediate electron acceptor Phenazine methosulfate (PMS) which amplifies (fluorescence signal at 490 nM (Cory *et al.*, 1991; Janjic and Wollheim, 1992; Segu *et al.*, 1998). The MTS assay was performed according to the manufacturer's instructions (Celltiter 96 aqueous nonradioactive cell- proliferation assay, Promega, Madison, WI). The absorbance was measured at 490 nm by plate reader. Readings were corrected for the background by subtracting the blank (MTS/PMS reagent in Hanks without the cells) from the readings. Three experiments have been performed with triplicate wells in each experiment.

Oxygen Consumption Rate (OCR)

Mitochondrial respiratory rate of 1.1B4 and MIN6 cell suspensions were measured polarographically using Clark Oxygen. The partial pressure of O₂ (PO₂) was measured at a polarographic voltage of 0.6 V with electrodes previously calibrated at 100% air saturation (vigorous gassing with air, 0.25mM) and 0% (addition of Na₂S₂O₄) (Daunt *et al.*, 2006). The background of Oxygen consumption by electrode was determined by the addition of 6 mM sodium azide to block oxidative respiration at cytochrome C.

Lactate production

The rate of lactate production of both 1.1B4 and MIN6 was measured polarographically with sarissa lactate enzyme electrodes (Sarissa Biomedical Ltd Coventry, UK) as previously mentioned by Brown *et al.*, 2012. 4 mL of

cell suspension were loaded in to a chamber, where the potential of the lactate electrode was set at 500 mV relative to Ag/AgCl reference electrode. Electrical current will be generated due to the oxidation of lactate by the lactate oxidase enzyme on the surface of a carbon fiber electrode. This current is a stoichiometric measure of lactate concentration. The lactate output rate was calculated according to the following equation:

$$E_{\text{Substrate}} \text{ (nmol } 10^7 \text{ cell}^{-1} \text{ min}^{-1}) = (\text{substrate}) * 60 / (\text{cells count}) / \text{calibration value.}$$

NAD (P) H measurements

NAD(P)H experiments were carried out using epifluorescence microscope as previously described (e.g. Duchen, 1992a,b; Duchen and Biscoe, 1992a,b). NAD(P)H produced from glucose and/or amino acid metabolism in the mitochondria . Cells were excited by light at 350 nm and measured with a bandpass fliter combination between 400 and 500 nm. To normalize the NAD(P)H response experiments, 10 mM glucose was used to reach a maximal response and this was set as a 100%.

Glucose-response relationships

The glucose-response relationships were quantified by best fits of the data with the following equation using prism via best fit non-linear regression:

$$Y = \frac{Y_{\text{MAX}} - Y_{\text{MIN}}}{1 + (EC_{50}/G)^h} + Y_{\text{MIN}}$$

Where G is the concentration of glucose, EC₅₀ is the concentration that Produces the half-maximal response, Y is the response magnitude, Y_{MAX} is the maximal response, Y_{MIN} is the minimal response, and h is an index of slope. Fits were performed in PRISM 6 (GraphPad Software Inc., San Deigo, CA).

Concentration-response relationships

Concentration-response relationships were constructed from multiple experiments using solely single concentrations. The Concentration-response relationships were quantified by fitting the data (without weighting) with the following sigmoid equation:

$$Y = (100 - Y_{\text{max}}) + \frac{Y_{\text{max}}}{(1 + ([AD]/EC_{50})^h)}$$

Where Y is the magnitude of effect as a percentage of Y_{max} , the maximum effect, $[AD]$ is the concentration of antidepressant, EC_{50} is the concentration that produces half-maximal effect ($Y_{max}/2$) and h is an index of slope. Fits were performed in PRISM 6 (GraphPad Software Inc., San Deigo, CA).

Statistical analysis

Data are expressed as mean \pm S.E.M. Parametric statistical analysis was used if the data was checked to be normally distributed (D' Agostino Pearson omnibus normality test). Statistical analysis was assessed in Prism (6.0, GraphPad software, San Diego California, USA). EC_{50} and h values are quoted with 95% confidence intervals. Statistical significant was accepted at $P < 0.05$.

Results

Responses to Fuels

There was slight increase in MTS reduction with increasing glucose concentration at 1 mM glucose, which saturated at 10 mM in the presence and absence of azide (Fig 1A). The EC_{50} of glucose stimulated MTS reduction was 1.13 mM (0.9 to 1.4 95% CI) and h of 1 in the absence of azide (Fig. 1A). However, EC_{50} of glucose stimulated MTS reduction was 1.5 mM (1.4 to 2.6 95% CI) and h of 1 in the presence of azide (Fig.1A). On the contrary, 3-MG (10 mmol/L), a non metabolizable analog of glucose, failed to stimulate MTS reduction in HIT-T15 cells (not shown). However, MP which is preferentially metabolized in the mitochondria of pancreatic beta cells (Segu *et al.*, 1998) failed to stimulate MTS reduction compared to the vehicle control (H_2O). (Fig 1B) Both KIC and LEU (pure mitochondrial fuels) (Segu *et al.*, 1998) did not stimulate MTS reduction in the presence and absence of azide (Fig.1C&D). Although the increments in MTS signal which found in the presence of glucose in contrast to MP, KIC and LEU failed to affect MTS reduction, these studies suggest that the predominate path is glycolytic pathway in C6 glioma cells.

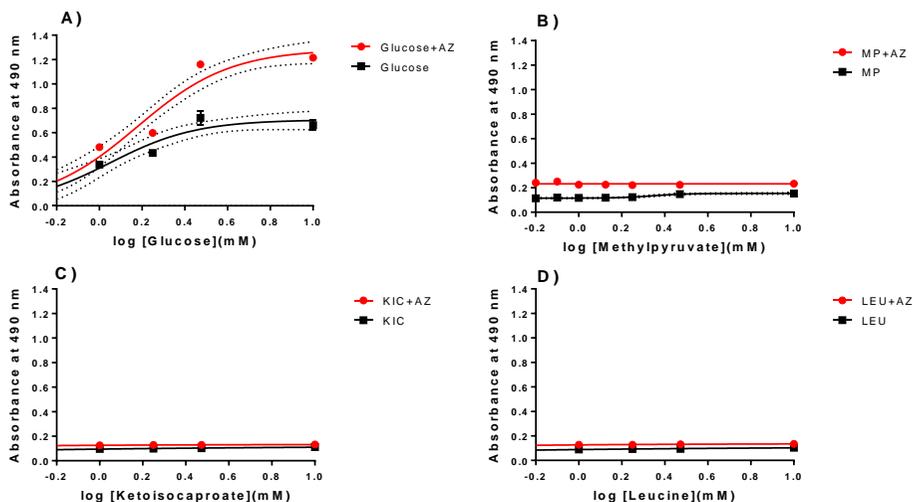


Figure 1: Effects of pure mitochondrial fuels with azide (AZ) and without AZ on MTS reduction in C6 glioma cells: effects of glucose (A), Methylpyruvate (MP) (B), Ketoisocaproate (KIC) (C) Leucine (LEU) (D). Cells were incubated for 120 minute. Data are means \pm SEM. N= 9 experiments.

Since glucose failed to increase oxygen consumption rate (OCR) in C6 glioma cells (as shown in figure 2A), it is difficult to determine if an increased metabolism causes an increase OCR. Thus, we used α -ketoisocaproate (KIC) (Duchen *et al.*, 1993; Gao *et al.*, 2003; Daunt *et al.*, 2006) and methylpyruvate (MP)(Jijakli *et al.*, 1996) (Fuels that feed directly into the mitochondrial tricarboxylic acid cycle. MP significantly stimulated ΔO_2 compared to the control (H_2O) ($P < 0.01$, Dunnett's multicomparison test) in C6 glioma cells (Fig. 2B). However, KIC failed to stimulate OCR compared to the vehicle control (H_2O) (Fig. 2C).

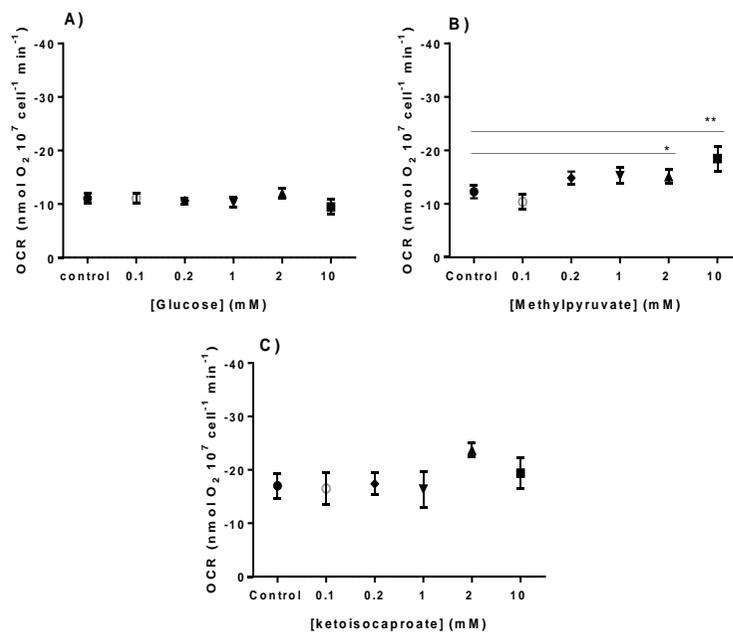


Figure 2: Relationship between glucose (A), methylpyruvate (B) and α -ketoisocaproate (C) concentration with O₂ consumption in C6 glioma cells. Data are mean and \pm SEM. N= 6-10 experiments.

Oxygen consumption rate and Lactate output

Polarographic detection of O₂ consumption and lactate output was used to measure the rate of respiration (Δ O₂) and anaerobic glycolysis respectively. The aim of this experiment was to investigate the effect of 10mM glucose on the OCR and lactate production of C6 glioma cells. In the absence of exogenous metabolic substrate, suspensions of C6 cells consumed O₂ (Δ O₂) at linear rate commensurate with cell density at 12 ± 1.14 nmoles 10^7 cells⁻¹ min⁻¹. Addition of 10mM glucose inhibited Δ O₂ by $\sim 48\%$ ($p < 0.05$, t test) compared to the basal (Fig. 3A). However, in the absence of exogenous metabolic substrate, lactate out of C6 cells was linear rate commensurate with cell density at -3 ± 6.8 nmoles 10^7 cells⁻¹ min⁻¹. 10 mM glucose caused an increase in the lactate output by $\sim 122\%$ ($p < 0.05$, t test) compared to basal condition ($p < 0.05$, t test; Fig. 3B).

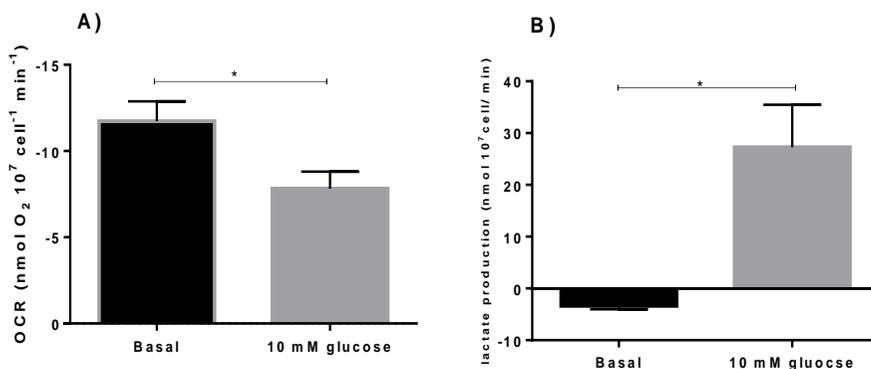


Figure 3: The effect of glucose 10mM on (A) Oxygen consumption rate (OCR) and (B) lactate production of C6 glioma. Data are mean ± SEM. N=7 experiments.

NADPH autofluorescence

An increase in the autofluorescence signals an increase in the reduced state of the pyridine nucleotide NAD(P)H. Increased activity of the tricarboxylic acid cycle may increase substrate supply and lead to an increase in NAD(P)H (Duchen et al., 1993). The aim of this experiment is to support that the lack of an OCR with glucose is indeed due to an abnormal mitochondrial oxidation. Figure 4 A shows a sample trace of the action of 10 mM glucose on the NAD(P)H levels of a single C6 glioma cell. Exposure of C6 glioma cells to glucose had no effect on the NAD(P)H levels compared to the basal (Fig.4B).

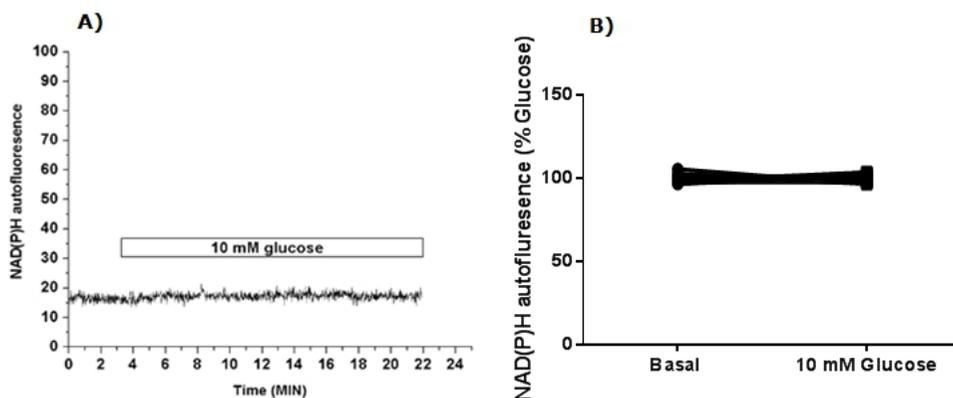


Figure 4: Effect of 10 mM glucose on the NAD(P)H levels in the C6 glioma cells. (A) representative time course of 10 mM glucose on the NAD(P)H levels of a single C6 glioma cells monitored by the change in NAD(P)H fluorescence. (B) Before and after plot of NADPH fluorescence for single C6 glioma cells in response to the glucose 10 mM, the sugar had no effect on NAD(P)H levels compared to the basal.). N= 19 cells collected from 3 experiments where the minimum number of cells is 2 in each one.

To further explore the metabolic pathways possibly affected by fluoxetine in C6 glioma cells, other metabolic substrate that stimulates mitochondrial respiration via entry into different steps of the oxidation pathway (Duchen *et al.*, 1993; Gao *et al.*, 2003; Daunt *et al.*, 2006) for glucose was used. 30 μM fluoxetine had no effect in the presence α -ketoisocaproate (Fig. 5A). However, at glucose concentration of 10 mM, 30 μM fluoxetine inhibited ΔO_2 by $\sim 21 \pm 12.36\%$ ($P < 0.05$, Dunn's multiple comparisons test) (Fig. 5B).

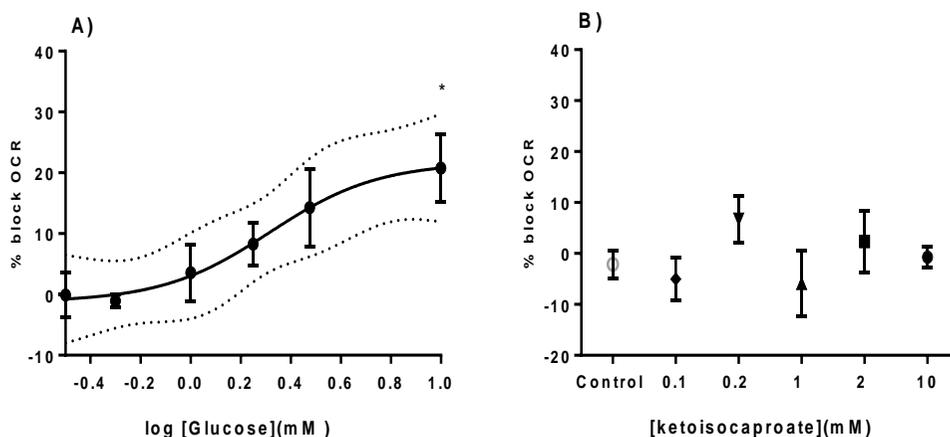


Figure 5: The percentage effect of fluoxetine in 10 mM glucose (A) and 10 mM α -ketoisocaproate (B) on the OCR in C6 glioma cells. Statistical significance is relative to the control (Cells+MTS reagent) determined via Dunnett's multiple comparisons test. Data is corrected for vehicle and background. The lines are all best fits of equation 1 to the data with IC_{50} given in chapter two sections 2.8. Data are the means \pm S.E.M. N=5 individual experiments.

I repeated the same experiment by using MTS reduction in 1.1B4. Fig. 3.8 A & B shows the curve of best fit for the fluoxetine concentration-effect graph in 10 mM α -ketoisocaproate and 10 mM glucose. In 10 mM α -ketoisocaproate fluoxetine failed to affect MTS reduction (Fig. 6). In 10 mM glucose, there was a concentration-dependent decrease in MTS reduction with increasing fluoxetine concentration. The IC_{50} of fluoxetine inhibited MTS reduction was 4.4 μM (1.70 to 0.17 95% CI) and h (slope of index) of 1 ($p < 0.0001$, Dunn's multiple comparisons test) (Fig. 6). These data are similar to the previous findings in MIN6 cells. This proposed that fluoxetine may inhibit metabolism at a step/s before the TCA cycle. In summary, these results explains that, a predominantly mitochondrial effect of these drugs explains these drugs target mitochondria which decrease redox status, as monitored by

the MTS assay (Elmorsy *et al.*, 2014), appears to be the primary cause of impairment of bioenergetics of C6 glioma cells.

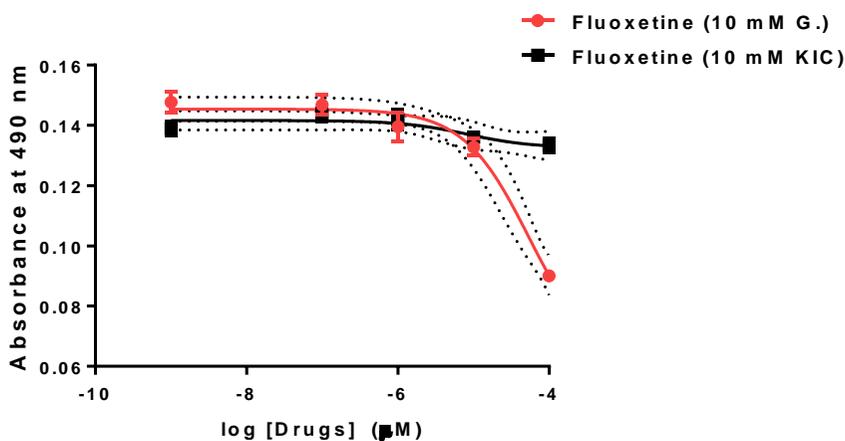


Figure 6: Concentration–effect relationships for the effect of fluoxetine in 10 mM glucose and 10 mM α -ketoisocaproate on the reduction of MTS in C6 glioma cells. Statistical significance is relative to the control (Cells+MTS reagent) determined via Dunnett's multiple comparisons test. Data is corrected for vehicle and background. The lines are all best fits of equation 1 to the data with IC_{50} given in chapter two sections 2.8. Data are the means \pm S.E.M. N=4 individual experiments.

Discussion

Effect of mitochondrial fuels on the MTS reduction

Assessments of the metabolic activity of C6 glioma rat cells are very important; during the process of fuel metabolism, which are created reducing equivalents in the cytosolic and mitochondrial compartments C6 cells, which produce a measureable signal. Therefore, during static incubations of these cells with glucose may increase the signal derived via the reduction of MTS in a time and dose-dependent manner. There was a progressive increase in the MTS reduction with an increase in glucose concentration. The EC_{50} for glucose-stimulated MTS reduction was 1.2 mM. However, Pure mitochondrial fuels (KIC, MR or LEU) failed to affect MTS signal. These data suggest that the central importance of extra-mitochondrial, glycolytically derived reducing equivalents NAD(P)H for generation of the MTS signal (Segu *et al.*, 1998).

Metabolism

This is the first study to investigate the catabolic metabolism on oxygen consumption rate (OCR) and lactate production of C6 glioma cells. Basal O_2 consumption of C6 glioma cells showed a linear pattern which varied between experiments. Figure 3A showed that basal OCR at 12 nmoles 10^7 cells⁻¹ min⁻¹ of C6 glioma cells. Earlier studies confirm that basal levels of

Oxygen consumption rate were roughly $20 \text{ nmol } 10^7 \text{ cells}^{-1} \text{ min}^{-1}$ in pancreatic beta cell lines (MIN6) (Daunt *et al.*, 2006). Difference in the basal respiration may be due to the presence of fatty acids in the cells suspensions (Lopaschuk *et al.*, 2010) . Figure 3B showed that basal lactate production at $-3 \text{ nmoles } 10^7 \text{ cells}^{-1} \text{ min}^{-1}$ C6 glioma cells. This suggests that the differences may be due to drift in the lactate electrode. No previous data had been reported for lactate output in C6 glioma cells. The 1-fold inhibition in the rate of OCR produced by 10 mM glucose for C6 glioma cells accompanied by 6-fold stimulation in the lactate production for C6 glioma cells. This suggests the adding of glucose was either metabolized solely by glycolysis and apparently not by the TCA, or the cells have marginal oxidative capacity (WARBURG, 1956). This may lead to inhibit of oxidative respiration by glycolytic products Crabtree effect (Diaz-Ruiz *et al.*, 2011).

NAD(P)H levels

The measurement of the endogenous fluorescent compound NAD(P)H serves as an indicator of glucose-induced changes of the energy metabolism (Panten *et al.*, 1973; Duchen *et al.*, 1993; Martens *et al.*, 2005; Ghaly *et al.*, 2014). As NAD(P)H autofluorescence results mainly from the mitochondria (Pralong *et al.*, 1990; Patterson *et al.*, 2000; Luciani *et al.*, 2006; De Marchi *et al.*, 2014; Ghaly *et al.*, 2014). An increase in the fluorescence signals an increase in the reduced state of the pyridine nucleotide NAD(P)H. Thus, an increased activity of the tricarboxylic acid cycle in response to increased substrate supply should lead to an increase in NAD(P)H (Duchen *et al.*, 1993). The results denote that 10 mM glucose did not stimulate NAD(P)H levels. This suggests that the glucose was metabolized solely by glycolysis, and not by the TCA, glucose significantly increased the lactate output with decrease of NAD(P)H levels.

Fluoxetine effect the glycolytic pathway of C6 glioma cells

In order to investigate whether the fluoxetine acts on the glycolytic pathway, my findings have reported that fluoxetine does not cause any effect on the OCR and MTS reduction whereas, it inhibited the OCR in 10 mM glucose in C6 glioma cells. This suggests that fluoxetine may inhibit metabolism at a step/s before the TCA cycle. How does the concentration of glucose affect fluoxetine's action as shown in the Fig. 5,6. Addition of glucose may affect the pH of the cytosol. For example, the presence of glucose may change the cytosolic pH to be more acidic as a result of high rate of glycolysis in 1.1B4 cells; this in turn will attract the antidepressants such as fluoxetine which may have ionisation constants (pK_a 's) >7.4 leading to accumulate this drug in the cytosol (Lombardo *et al.*, 2004).

Conclusion

Our results suggest that the glycolytic pathway is the predominate path for glucose utilization in the C6 glioma cell model. Since fluoxetine was inactive in the presence of α -ketoisocaproate our data suggest that the point of major action for these ADs in glioma cells is glycolysis.

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