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Abstract

Parkinson's disease (PD) is a neurodegenerative disease linked to multiple causes with both genetic and environmental components. Among the environmental components proposed to act as risk factors for PD are herbicides, such as paraquat (PQ), which is used to treat crops in rural communities. PQ has been linked to PD by both epidemiological studies of humans and experimental research using animal models. Evidence linking PQ exposure to PD remains controversial to date. Here, we used an unbiased approach to establish the metabolic consequences of the exposure of differentiated foetal human mesencephalic (LUHMES) cells in culture to PQ. We have found that upon differentiation, LUHMES cells showed metabolic changes associated with decreased glycolysis and increased membrane remodelling. Further, exposure of differentiated LUHMES cells to PQ led to changes in metabolic pathways linked to energy generation, oxidative stress and excitotoxicity.

Introduction

Parkinson's disease (PD) is an age-associated neurodegenerative disorder characterised by the specific loss of dopaminergic neurons in the substantia nigra pars compacta of the brain. Most cases of PD are sporadic, but a small percentage is inherited through PD-related genes [1] [2]. It is generally accepted that the aetiology of PD likely involves both genetic and environmental factors [3]. Many epidemiological studies have linked pesticide exposure to the risk of developing PD (reviewed in [4]). Notably, illicit drug users inadvertently injected with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a by product of the production of a synthetic opioid, rapidly developed a Parkinsonian syndrome. Many researchers have indicated that the pesticide PQ (N,N'-dimethyl-4,4'-bipyridinium dichloride) is structurally similar to the active metabolite of MPTP. All of these findings led to a series of studies focusing on the role of PQ exposure in neuronal toxicity (reviewed in [5]). Here, we explored the metabolic impact of the herbicide PQ on differentiated human neurons. We report that exposure to a range of concentrations of PQ affects energy-generating metabolic networks and promotes an excitatory state in cultured human neurons.

Objective

Explore the metabolic consequences of exposing human neurons to PQ.

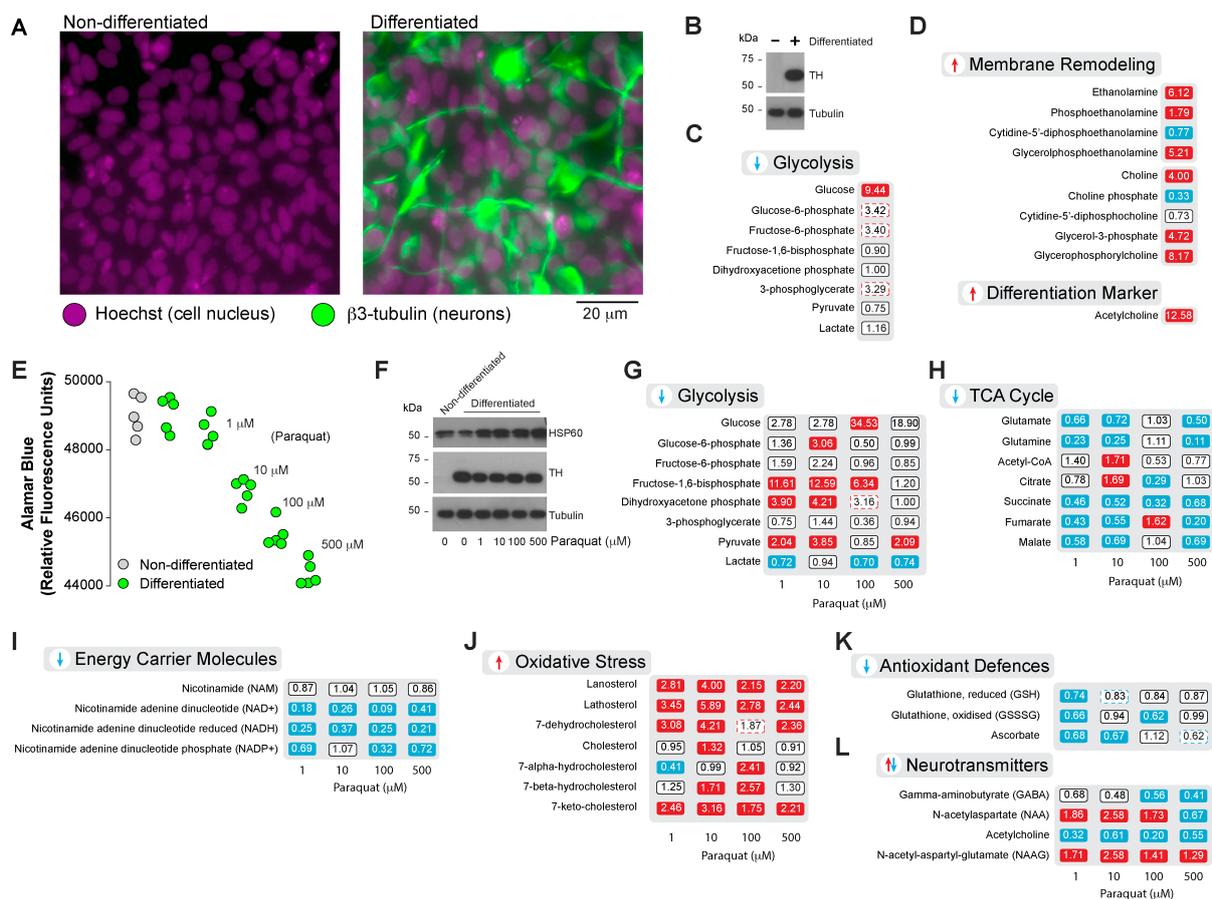


Figure Legend

Figure 1. Paraquat-induced metabolic alterations in differentiated human foetal mesencephalic cells.

(A) Representative images of LUHMES cells before differentiation and after differentiation for 7 days. Cell nuclei are labelled with Hoechst 33342 and cells differentiated into neurons are stained with anti- β 3-tubulin.

(B) Analysis of tyrosine hydroxylase levels, a dopaminergic cell marker, in LUHMES cells. Cell lysates were probed with the indicated antibodies. TH, tyrosine hydroxylase.

(C & D) Decreased glycolysis (C) and increased membrane remodelling and acetylcholine levels (D) were observed upon differentiation of LUHMES cells.

(E) Cell survival was decreased following incubation of LUHMES cells with paraquat. Cell viability is shown relative to that of untreated cells upon incubation with the indicated concentrations of paraquat for 24 h. Viability was determined by resazurin-based Alamar Blue assay.

(F) Increased levels of HSP60, a mitochondrial chaperone, following paraquat treatment in LUHMES cells. Cell lysates were probed with the indicated antibodies.

(G–L) Decreased glycolysis (G), TCA cycle activity (H), and energy carrier molecules (I), increased oxidative stress markers (J), a decreased antioxidant defence capacity (K) and alterations in neurotransmitter levels (L) were observed following treatment of differentiated LUHMES cells with increasing concentrations of paraquat. Red and blue correspond to metabolites that are significantly upregulated and downregulated ($P < 0.05$), respectively, compared with control cells (untreated cells). The dashed outlines correspond to comparisons with lower statistical significance ($0.05 < P < 0.10$). Statistical significance was determined using Welch's two-sample t-test ($n = 5$; also see Supplementary Table 1).

Cell culturing

LUHMES cells were grown in a humidified incubator at 5% (v/v) CO₂ and 37°C. Plastic cell culture vessels were pre-coated with 50 ng/mL poly-L-ornithine and 1 µg/mL fibronectin (Sigma Aldrich, UK) prepared in distilled water for 3 h. After removal of the coating solution, the culture flasks were washed 3 times with distilled water and air dried before use. Non-differentiated cells were maintained in proliferation medium, consisting of Advanced Dulbecco's modified Eagle's medium/F12 supplemented with 1× N-2 supplement (Gibco/Invitrogen, UK), 2 mM L-glutamine (Sigma, UK) and 40 ng/mL recombinant basic fibroblast growth factor (R&D Systems, UK). For differentiation, cells were cultured in proliferation medium for 24 h and were then switched to '+/+' medium for 7 days. The '+/+' medium consisted of Advanced Dulbecco's modified Eagle's medium/F12 supplemented with 1× N-2 supplement (Gibco/Invitrogen, UK), 2 mM L-glutamine (Sigma, UK), 1 mM dibutyryl-cAMP (Sigma Aldrich), 1 µg/mL tetracycline (Sigma Aldrich, UK) and 2 ng/mL recombinant human GDNF (R&D Systems, UK).

Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature and were then permeabilised and incubated for 1 h at room temperature in blocking solution (PBS, 1% BSA and 0.2% Triton X-100). Then, the cells were incubated overnight at 4°C with an anti-β₃ tubulin antibody (Abcam, UK) in blocking solution. Next, the cells were washed and incubated with Alexa Fluor 594 anti-mouse (Invitrogen, UK; 1:1000 dilution) for 1 h at room temperature, followed by incubation with anti-rabbit Alexa-488 (1:1000) as a secondary antibody for 1 h. Nuclear staining was performed by incubation of the cells with 10 µg/ml Hoechst 33342 (Molecular Probes, UK) for 10 min.

Protein extraction and western blotting

Protein extracts were prepared by scraping the cells in lysis buffer (100 mM KCl, 20 mM HEPES, pH 7.5, 5% (v/v) glycerol, 10 mM EDTA, 0.1% (v/v) Triton X-100, 10 mM DTT, 1 µg/mL leupeptin, 1 µg/mL antipain, 1 µg/mL chymostatin, and 1 µg/mL pepstatin). The cell suspensions were cleared by centrifugation at 21,000 g for 10 min at 4°C, and the protein concentrations of the supernatants were determined by Bradford assay (Bio-Rad, UK). All supernatants were mixed with 4× LDS loading buffer (Invitrogen, UK). For SDS-PAGE, equivalent amounts of proteins were resolved on 4–12% NuPAGE precast gels (Invitrogen, UK), and were transferred onto to PVDF membranes (Millipore). The membranes were blocked with TBS (0.15 M NaCl and 10 mM Tris-HCl, pH 7.5) containing 5% (w/v) dried non-fat milk for 1 h at room temperature and were then probed with the indicated primary antibody, followed by incubation with the appropriate HRP-conjugated secondary antibody. Antibody complexes were visualised using Pierce's enhanced chemiluminescence system (ECL). The primary antibodies employed for western analysis were anti-αTubulin (Cell Signalling, cat no. 2144), anti-tyrosine hydroxylase (Abcam, cat. no. ab112) and anti-HSP60 (Cell Signalling, cat. no. 4870).

Cell viability assay

Cells were plated in proliferation medium at a density of 50,000 cells per well in 96 well plates and were grown to approximately 70% confluency. The medium was then replaced with proliferation or '+/+' medium, and the cells were further grown for 7 days. Next, they were incubated in the medium in the absence or presence of PQ at several concentrations (1, 10, 100 and 500 µM) for an additional 24 h. Cell viability was analysed by Alamar Blue assay (Invitrogen, UK) according to the manufacturer's instructions.

Metabolic profiling

Global metabolic profiles were obtained from cells grown in plastic flasks (T175) and harvested with trypsin (5 mL ATV-trypsin, consisting of 138 mM NaCl, 5.4 mM KCl, 6.9 mM NaHCO₃, 5.6 mM D-glucose, 0.54 mM EDTA, and 0.5 g/L trypsin from bovine pancreas type-II-S; Sigma, UK). The cells were collected in 50 mL Falcon tubes by adding 40 mL Advanced DMEM/F12 medium and were then centrifuged at 300× g for 5 min at room temperature. Next, the supernatants were removed carefully, and the cell pellets were flash frozen on dry ice and stored at 80°C. Metabolic profiles were obtained using Metabolon Platform (Metabolon Inc., USA) as previously described [10]. Essentially, 5 replicates were analysed for each condition (n = 5).

Results & Discussion

To assess the metabolic adaptation of human neurons to paraquat, we used a foetal human mesencephalic cell line (LUHMES, Lund human mesencephalic) as a model system. These conditionally immortalised cells can be differentiated *in vitro* into post-mitotic neuronal cells [6]. The culturing of LUHMES cells in differentiation medium for 7 days resulted in the expression of the neuronal marker β 3-tubulin in a subset of cells (Fig. 1A) and of tyrosine hydroxylase (Fig. 1B), a dopaminergic neuronal marker associated with the differentiation of these cells [7]. We have next analysed the global metabolic changes in differentiated LUHMES cells. The levels of several intermediates in glycolysis were increased in the differentiated cells, suggesting that their glycolytic activity was decreased compared with that in non-differentiated cells (Fig. 1C). In addition, the level of pyruvate, a product of glycolysis, was decreased, consistent with the attenuation of glycolysis. This may reflect a decreased energy demand of these cells. Further, the levels of many building blocks of membranes and intermediates in membrane synthesis and degradation, including precursors for lipids synthesised by mitochondria, such as ethanolamine and phosphoethanolamine, as well as precursors for endoplasmic reticulum lipid synthesis, such as choline and choline phosphate, were significantly altered in the differentiated LUHMES cells (Fig. 1D). Taken together, these findings suggest that membrane remodelling was more prevalent in the differentiated LUHMES cells. This may be a consequence of the specialisation process and function of the differentiated cells. Further, the differentiation of mesencephalic cells into post-mitotic neurons resulted in increased levels of the neurotransmitter acetylcholine, consistent with the specialisation of these cells (Fig. 1D). Even though we have detected an increase in dopaminergic lineage markers in differentiated LUHMES cells (Fig. 1B), metabolic analysis failed to detect neurotransmitters such as dopamine that are present in dopaminergic cells.

We have next investigated the metabolic consequences of the exposure of differentiated LUHMES cells to paraquat. Exposure to increasing concentrations of paraquat for 24 h resulted in decreased cell viability (Fig. 1E) and an increase in the expression of heat shock protein 60 (HSP60), a mitochondrial stress marker (Fig. 1F). However, at the minimum paraquat concentration employed in our study (1 μ M), no significant effect on cell viability was observed. We have next analysed the metabolic changes associated with the exposure of differentiated cells to paraquat for 24 h. Decreased glycolytic activity was observed following treatment with 1 μ M and 10 μ M paraquat, as indicated by significantly increased levels of glucose, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, dihydroxyacetone phosphate, 3-phosphoglycerate and pyruvate. The build up of glycolytic intermediates may have reflected decreased pyruvate fuelling the TCA cycle, which appeared to be inhibited (Fig. 1H), possibly as a consequence of mitochondrial impairment. Moreover, decreased levels of NAD^+ or direct inhibition of glycolysis by paraquat may have been contributing factors to the decreased glycolytic activity. At 500 μ M paraquat, and to a lesser degree, at 100 μ M, only minor changes in glycolysis were observed, possibly indicating that the cells were metabolically compromised and had limited glycolytic capacity (Fig. 1G). The levels of many TCA cycle intermediates were significantly decreased, suggesting that the cycle was less active (Fig. 1H). The effects of PQ on both glycolysis and TCA cycle suggests a compromise in the energy-generating capacity of differentiated LUHMES. A loss of ATP, the intracellular energy currency, has also been reported following exposure of differentiated LUHMES cells to the MPP^+ , the active metabolite of MPTP [7]. Paraquat has been suggested to interfere with the electron transfer chain, resulting in the formation of O_2^- . Aconitase, an iron cluster (Fe_4S_4)-containing enzyme responsible for the conversion of citrate to iso-citrate, is readily damaged by oxidative stress [8]. Increased citrate levels were observed at 10 μ M paraquat, consistent with aconitase inhibition. In addition, the levels of glutamine and many other amino acids were decreased (also see Suppl. Table 1), suggesting an attempt by anaplerotic reactions to replenish TCA cycle intermediates. We also detected significant decreases in the levels of the redox couple NAD^+ and NADH following paraquat treatment, as well as a decrease in the level of the phosphorylated equivalent, NADP^+ (Fig. 1I). NADPH is important for paraquat toxicity by cellular redox cycling, and it drives the formation of superoxide ions (O_2^-) [9]. Oxidative stress results in DNA damage and activation of poly(ADP-ribose) polymerases (PARPs), en-

zymes that deplete intracellular NAD⁺ stores, resulting in the formation of nicotinamide. Nicotinamide levels were not altered, suggesting that either PARP activity or the rate of NAD⁺ synthesis from nicotinamide was low. It is possible that paraquat directly inhibits NAD⁺ formation from either tryptophan or nicotinamide. However, there was no indication that either pathway was inhibited. Alternatively, the decreased NAD⁺ level may have been a consequence of adaptation to an altered energy status (described further below). Depletion of NAD⁺ was strongly associated with decreased energy metabolism. We next focused on the induction of oxidative stress markers following paraquat treatment. Cholesterol is an important lipid component of cellular membranes, and it is readily oxidised by the reactive oxygen species (ROS) to 7- α / β -hydrocholesterol or 7-ketocholesterol. We observed increases in the levels of oxidised cholesterol derivatives, consistent with paraquat-induced oxidative stress to cell membranes. As a consequence of cholesterol degradation by ROS, the levels of markers for cholesterol synthesis, such as lanosterol, lathosterol and 7-dehydrocholesterol, were significantly increased; these changes may represent a cellular response designed to replenish the cholesterol pool (Fig. 1J). In addition, levels of the abundant small molecular weight antioxidants glutathione (GSH/reduced) and ascorbate were decreased following paraquat treatment, suggesting a reduction in the antioxidant buffering capacity of the paraquat-treated cells (Fig. 1K). Next, we examined the effects of paraquat on neurotransmitter levels in LUHMES cells. The levels of several neurotransmitters were altered following paraquat treatment, with the strongest effect observed at the 10 μ M concentration (Fig. 1L). NAA, NAAG and acetylcholine are excitatory, while GABA is inhibitory. The results of this study suggests that paraquat elicits complex changes favouring an excitatory state. Interestingly, synaptic activity has been shown to promote resistance to oxidative stress, suggesting that these changes may be the part of the antioxidant response to paraquat.

Conclusions

Here, we explored the metabolic profile of human cells differentiated *in vitro* into post-mitotic neurons. Using this approach, we identified the main metabolic changes associated with our differentiation protocol. Furthermore, by treating differentiated cells with the toxin paraquat, we identified metabolic alterations associated with exposure to this herbicide. We have shown that differentiation of LUHMES cells causes changes in glycolysis and increases in the levels of markers of membrane remodelling and differentiation. We have also shown that exposure of differentiated cells to paraquat affects cellular pathways involved in energy generation and oxidative stress and neurotransmitters associated with an excitotoxic state.

Limitations

This is a cell culture based experimental system in which conditionally immortalised cells were induced to differentiate *in vitro*. We employed a human cell line that can be easily expanded and differentiated into neurons. LUHMES cells are amenable to global metabolic profiling using our analysis platform, as a large number of cells are easily obtainable. Previous reports took a dopaminergic phenotype of differentiated LUHMES cells for granted and used them for mechanistic studies on neurodegeneration. In our study, we failed to detect any metabolic markers of dopaminergic cell differentiation. Therefore, it would be interesting to compare the metabolic profile of LUHMES cells to that of primary mid-brain dopaminergic neurons (mDAs) and to determine if PQ leads to similar metabolic alterations in mDAs.

Interestingly, PQ induced decreased levels of NAD⁺. It is unclear whether PQ directly inhibits NAD⁺ formation or whether the decreased NAD⁺ was a consequence of the altered energy status. NAD⁺ is an important co-factor in more than 200 reactions. Hence, many of the metabolic changes may have been associated with NAD⁺ depletion. The role of NAD⁺ could be assessed by supplementing the cell culture medium with the precursor niacin. Moreover, a time course experiment would be appropriate to delineate the progression of the global metabolic changes. PQ-treated cells showed a reduction in their antioxidant buffering capacity, suggesting that a pre-treatment of cells with an-

tioxidants could affect some of the metabolic alterations caused by this toxin.

Additional Information

Methods

Cell culturing

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room temperature. Next, the supernatants were removed carefully, and the cell pellets were flash frozen on dry ice and stored at 80°C. Metabolic profiles were obtained using Metabolon Platform (Metabolon Inc., USA) as previously described [10]. Essentially, 5 replicates were analysed for each condition (n = 5).

Supplementary Material

Please see <https://sciencematters.io/articles/201611000025>.

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Ethics Statement

Not applicable.

Citations

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