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Variable toxicological response to the loss of OXPHOS through 1-methyl-4-phenylpyridinium-induced mitochondrial damage and anoxia in diverse neural immortal cell lines

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Abstract

Immortal cell lines are used to investigate various aspects of neurodegeneration. These cells display high glycolytic turnover rate and produce an abundant amounts of lactate. Our previous studies indicate that these cells survive the loss of mitochondrial oxidative phosphorylation (OXPHOS) with ample glucose supply. In the current study, we investigate if cell type (w/variation in basal metabolic rate (MR)), can alter glucose utilization patterns which in turn may affect LC₅₀ for the mitochondrial toxin 1-methyl-4-phenylpyridinium (MPP⁺) in various cell lines. The data obtained indicate that cell lines MRs examined were generally consistent with the average of species adult body weight where mouse N-2A > rat-PC-12 > human SH-SY5Y. A higher MR was associated with accelerated utilization of glucose and earlier cell death with MPP⁺: LC₅₀ mouse = 294 μM, rat = 695 μM, and human = 5.25 mM at 24 h. Cell death appears to be a function of the velocity by which glucose disappears, leading to the failure of glycolysis and subsequent halt of energy production. Similar effects were also observed at higher plating densities where the demand for glucose is amplified. A time-lapse study of MPP⁺ toxicity (0–36 h) in N-2A cells indicates that an anaerobic shift occurs as early as 2 h (evidenced by a rise in lactate), followed by a descent in glucose concentrations at 4 h and exhaustion of glucose supplies at 22 h which was associated with the first detectable sign of cell death. It was also noted that MPP⁺ toxicity was not associated with the generation of reactive oxygen species (O₂⁻, H₂O₂, and NO₂) and was not attenuated by adding catalase or superoxide dismutase to the media. On the other hand, MPP⁺ toxicity was reversed by providing additional supply of glucose, pyruvate ± mitochondrial monocarboxylate transporter blocker (α-cyano-4-HCA), or pyruvate ± pyruvate dehydrogenase inhibitor (octanoyl-CoA), suggesting that the exclusive anaerobic survival compensates for the loss of OXPHOS by MPP⁺. To examine if neuroblastoma were capable of surviving the deprivation of O₂ for 24 h, a range of hypoxia to anoxia was established with various concentrations of dithionite. The data suggest that cell lines examined continue to thrive when incubated with high-glucose media (25 mM). In summary, vulnerability of immortal neuroblastoma cell lines to MPP⁺ toxicity is dependent upon glucose concentrations within the media and cell MR, which indirectly dominates the velocity of glucose use and its end point disappearance, leading to cell death by ergogenic failure.

Keywords

Glucose; Hypoxia; Glycolysis; MPP⁺; Tumor; PC-12; Neuroblastoma 2-A; SH-SY5Y; OXPHOS; Anaerobic

Introduction

Rat pheochromocytoma PC-12 cells, human SH-SY5Y, and mouse N-2A neuroblastoma cells are commonly used for in vitro studies pertaining to neurological disease. These three cell lines are of malignant phenotype, which are believed to differ from normal cells given the characteristic of glycolytic dependence (Warburg effect), an inherent mitochondrial mutation (Sun et al. 2009; Pan and Mak 2007), and a propensity to thrive under hypoxic microenvironments (Gatenby and Gawlinski 2003; Godinot et al. 2007; Griffiths et al. 2001; Liu et al. 2002). When exposed to hypoxia, tumor cells will undergo genetic adaptation leading to aggressive malignancy, metastasis, and a resistance to chemotherapy (Airley and Mobasher 2007; Sullivan et al. 2008; Tóth 2007). Tumor cells are equipped to carry out high-volume anaerobic metabolism due to over-expression of glucose transporters (GLUT1; Airley and Mobasher 2007) and glycolytic enzymes (hexokinase, phosphofructokinase, and pyruvate kinase), all of which enhance the glycolytic function (Hennipman et al. 1988; Ikezaki et al. 1992; Koukourakis et al. 2006). The accelerated pace at which anaerobic metabolism proceeds in tumor cells is the cause of the development of positron emission tomography ^{18}F -fluorodeoxyglucose which is commonly used in cancer diagnosis (Maublant et al. 1998; Burstein et al. 1998).

Cells with inhibited mitochondrial function will have a drop in oxidative phosphorylation (OXPHOS), and its energy requirements would have to be met through enhancing anaerobic glycolysis. There is no question that accelerated anaerobic metabolism is observed in both immortal and primary neurons upon mitochondrial insult. For example, administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in animals invokes accelerated anaerobic glycolysis evidenced by a rise in striatal lactate (Koga et al. 2006) and expedited glucose utilization patterns (as detected with $[2-^{14}\text{C}]$ deoxyglucose) in the SN pars compacta and locus coeruleus, areas most affected in PD (Palacios and Wiederhold 1984). The same anaerobic shifts also occur in primates where administration of MPTP leads to accelerated uptake of 2-deoxy-D- $[^{14}\text{C}]$ glucose in SN/globus pallidus areas corresponding to dopaminergic neurodegeneration (Palombo et al. 1988; Schwartzman and Alexander 1985; Schwartzman et al. 1998). These effects are also consistently observed in various models such as excised rat brain slices where MPP^+ causes a rise in striatal glucose metabolism (Maruoka et al. 2007) and in immortal neuroblastoma where MPP^+ stimulates a rise glycolysis and a spike in the production of lactate (Basma et al. 1992).

There is a discrepancy in the reported LC_{50} for MPP^+ in vitro ranging from micromolar to 10 mM sometimes in similar immortal neuroblastoma cell lines (Mazzio and Soliman 2003a; Jung et al. 2009; Seyfried et al. 2000; Pizarro et al. 2010; Tang et al. 2010). The large range variation in LC_{50} for MPP^+ is likely the result of different experimental variables that ultimately alter media glucose concentrations. Given that a heightened rate of glycolysis is required to sustain ATP production through substrate level phosphorylation during MPP^+ -mediated loss of OXPHOS, any influential factor that alters glucose concentrations could also alter vulnerability to the toxin. In this study, we attempt to elucidate if various base metabolic rates, plating densities, and media glucose concentrations govern LC_{50} in mouse N-2A, rat-PC-12, and human SH-SY5Y cells during the exposure to MPP^+ . We have also examined the extent to which different neuroblastoma cells can survive in the complete absence of O_2 when ample glucose is sustained.

Materials and methods

Cell lines were purchased from American Type Culture Collection, ATCC (Manassas, VA, USA). Dulbecco's modified Eagle medium (DMEM), L-glutamine, fetal bovine serum, heat-inactivated (FBS), phosphate-buffered saline (PBS), Hank's balanced salt solution (HBSS),

and penicillin/streptomycin were purchased from Fischer Scientific, Mediatech, (Pittsburgh, PA, USA). All other chemicals and research supplies were purchased from Sigma Chemical (St Louis, MO, USA).

Cell culture and sample preparation

Neuro-2A (N-2A) murine brain neuroblastoma cells, rat pheochromocytoma (PC-12), and human SH-S5Y5 brain neuroblastoma were used in this study. PC-12 cells were further isolated from a sub-clone of plastic adhering cells which displayed spontaneous neurite extensions and similar response to MPP⁺ toxicity as PC-12 cells that were differentiated with NGF. Furthermore, human SH-S5Y5 brain neuroblastoma cells were not differentiated as it has been reported that differentiation with *all-trans*-retinoic acid does not intensify MPP⁺ toxicity (Cheung et al. 2009). Cells were grown in DMEM with phenol red, 10% FBS (v/v), 4 mM L-glutamine and penicillin/streptomycin (100 Units/0.1 mg/ml), and 20 μ M sodium pyruvate. Cells were grown at 37°C in 5% CO₂ atmosphere, scraped, and sub-cultured every 2–5 days. Experimental plating media contained low glucose (5.5 mM), no phenol red, and supplemented with FBS (1.8%), penicillin/streptomycin (100 U/0.1 mg/ml)/4 mM L-glutamine, and for routine experiments, the cells were plated at 0.5×10⁶ cells/ml. Energy substrates, MPP⁺, and sodium dithionite were prepared freshly in sterile HBSS + 10 mM HEPES (pH 7.4). Previously, we had determined that the preparation of pyruvate should be accomplished so that it is maintained in its acidic form—to allow transport into the cell. This can be achieved by slight neutralization so that the pH is maintained with a range where acidity is not the basis for cell toxicity. For these experiments, pyruvic acid stock solutions were prepared by combining sodium pyruvate with pyruvic acid (pH 3.85) in the HEPES buffer, followed by preparation of serial dilutions which were then added to the cells at 20% (v/v).

Glucose analysis

Briefly, glucose was quantified as previously described (Mazzio and Soliman 2003a) using an enzymatic assay containing equal volume of glucose oxidase (20 U/ml) and a chromogenic solution containing 1 mM vanillic acid, 500 μ M of 4-aminoantipyrine, and four purpurogallin units per milliliter of horseradish peroxidase type II. Glucose was quantified at 550 nm on a UV microplate spectrophotometer (model 7600, version 5.02, Cambridge Technologies Inc., Watertown, MA, USA). Protein was determined using the Lowry protocol (Lowry et al. 1951), and the data were expressed as micromoles per milligram protein, volume equivalent or the percent of control.

Cell viability and cell death

Resazurin (almar blue) indicator dye was used as a measure of viable cell count (Evans et al. 2001; Mazzio and Soliman 2003a). Reduction of the dye by viable cells reduces the amount of oxidized form and increases the amount of fluorescent intermediate (resorufin). Quantitative analysis of dye conversion at a time point prior to full reduction of the controls was used to measure metabolic rate among the various cell lines. Metabolic rate was established by analyzing the conversion of the dye (Fluorescent Intensity (FI))/(Fixed Time Point, 4 h) under identical culture conditions and plating density. FI was measured using a microplate fluorometer (model 7620, version 5.02, Cambridge Technologies Inc.) with settings adjusted to [550/580] ([excitation/emission]) wavelengths. Fluorescein diacetate and propidium iodide staining were used to determine cell viability and cell death (Mazzio and Soliman 2003a). Photographic data were captured using an Olympus IX-70 inverted microscope equipped with an MD35 Electronic Eyepiece (Zhejiang Jincheng Sci & Tech Co., Ltd, China) and image capture using C-imaging systems PCI-Simple software (Compix Inc. Cranberry Township, PA, USA).

Lactic acid

Lactic acid was quantified by HPLC using a Shimadzu SCL-10A Controller, a LC-10AT pump (flow rate = 0.6 ml/min), an aliphatic Acclaim OA organic acid column, (5 μ m: 4 $\times$ 250 mm) where the mobile phase consisted of 10 mM Na₂SO₄, pH 2.65, adjusted with methanesulfonic acid using an SPD-M10A VP diode array detector with settings at 211 nm.

Oxygen respiration

Whole cell mitochondrial O₂ consumption was analyzed using a Clark electrode (Hansatech Instruments Ltd., Norfolk, England). The electrode quantifies concentrations of dissolved O₂ in the medium as a means to assess mitochondrial respiration. The electrode was calibrated with air-saturated deionized water (air line) and deionized water containing sodium dithionite (nitrogen line). The samples (cell media after experimental treatment) or control blanks were loaded into the chamber jacket at 25°C. After the instrument was stabilized and equilibrated, the raw data were acquired as nanomoles of O₂ per milliliter and converted to percentage of control values.

Nitrite determination

Quantification of nitrite (NO₂⁻) was determined by a spectrophotometric procedure (Park and Murphy 1994). Briefly, the Griess reagent was prepared by mixing an equal volume of 1% sulfanilamide in 0.5 N HCl and 0.1% *N*-(1-naphthyl) ethylenediamine in deionized water. The Griess reagent was added directly to the cell suspension and incubated under reduced light at room temperature for 10 min. Samples were analyzed at 550 nm on a UV micro-plate spectrophotometer (model 7600, version 5.02, Cambridge Technologies). Controls and blanks were measured simultaneously, and subtracted from the final value to eliminate interference. A standard curve was generated from a range of dilutions of sodium nitrite (1–100 μ M) prepared in the plating medium. Protein level of each sample was determined using the Lowry method (Lowry et al. 1951). The data were calculated as nanomolar NO₂⁻ per milligram protein.

Hydrogen peroxide and superoxide assays

Hydrogen peroxide (H₂O₂) level was measured by the modification of a previously described method (Holt et al. 1997). After experimental treatment, sample supernatants were assessed for H₂O₂ using a peroxidase-linked continuous assay. The chromogenic solution contained (final working concentration) 1 mM vanillic acid, 500 μ M 4-aminoantipyrine, and horseradish peroxidase (4 U/ml) in PBS with 2 mM of HEPES (pH 7.4). The chromogenic reagent was added to each sample and incubated for 10 min at 37°C. Samples were analyzed at 550 nm on a UV Microplate Spectrophotometer (model 7600, version 5.02, Cambridge Technologies). Controls and blanks were measured simultaneously and subtracted from the final value to eliminate interference. Protein was assessed by the Lowry method (Lowry et al. 1951). Final data were expressed as nanomolar H₂O₂ per milligram protein or volume equivalent. Superoxide was determined using a nitroblue tetrazolium (NBT) assay. Briefly, a stock solution of 1 mg/ml NBT was prepared in HBSS with 10 mM HEPES buffer, pH 7.6. The reagent was added to the samples (11%, v/v), incubated for 30 min at 37°C, and quantified by measuring purple NB-diformazan at 560 nm on a UV microplate spectrophotometer.

Data analyses

Statistical analysis was performed using Graphpad Prism (version 3.0, Graphpad Software Inc., San Diego, CA, USA). The data were expressed as the mean $\pm$ SEM (*N*=4) for each group. Significance of difference between the groups was assessed using one-way analysis of variance (ANOVA), followed by a Tukey post-hoc means comparison test. The lethal concentrations (LC₅₀) were established from dose-dependent data with Origin Lab 7.5 SR6, and significance

of difference between the groups was assessed using one-way ANOVA, followed by a Tukey post-hoc means comparison test.

Results

Metabolic diversity among the various cell lines studied was determined under strictly controlled experimental conditions (Fig. 1). The data experiments were conducted at the same time, with the same media, in 96-well plates, with identical culture conditions, timeline, and procedure. The data obtained show that at equal plating densities (0.5×10^6 cells/ml) of 50,000 cells per well, differences were evidenced in metabolic rate (conversion from alamar blue→resorufin/4 h), glucose remaining (micromolar per milligram protein), and extent of O₂ consumed (nanomoles per milliliter) after 24 h (Fig. 1). The data in Fig. 1 corroborate that a higher metabolic rate corresponds to less glucose remaining in the media and greater O₂ consumed in the order of mouse > rat > human. In our study, we used rat PC-12 cells that were derived from a sub-clone plastic-adhering variant in our laboratory. The obtained results indicate that all cell lines displayed neurite extensions and adhered to plastic without the use of growth factors. In the next experiment, we show that the LC₅₀ for MPP⁺ (0–10 mM) over 24 h were lowest in cells having higher metabolic rate (Fig. 2a), which was significantly exacerbated after 48 h (Fig. 2b). A similar trend was observed for the effects of MPP⁺ on hampering mitochondrial O₂ consumption (Fig. 2c).

The data presented in Figs. 1 and 2 suggest that the higher cellular metabolic rate, the faster is glucose disappearance, where this drop exacerbates MPP⁺ toxicity as a result of a shift toward anaerobic glycolysis. In order to evaluate the mechanism of cell death as it relates to the compromise of energy as a result of MPP⁺, we chose to use N-2A cells due to its sensitivity, robust metabolic activity, ease of use, and because they are of neural origin from a spontaneous malignant tumor. Time-lapse analysis (0–36 h) of MPP⁺ toxicity in the most vulnerable cell line N-2A cells was conducted (Fig. 3). In addition, Fig. 3 shows that MPP⁺ (500 μM) caused a time-dependent depletion of glucose concentrations, also accompanied by a rise in lactate where cell death was tantamount to exhaustion of glucose supplies (Fig. 3, right panel vs. left panel, control). The data obtained indicate that MPP⁺ accelerates glucose depletion, to which a 95% drop was associated with detectable signs of cell death. These findings indicate that a loss of OXPHOS by MPP⁺ precipitates cell death by accelerating anaerobic glycolysis, leading to depletion of glucose. Given that higher cell densities should expedite a loss of glucose concentrations in vitro, one would expect to see greater cell death from MPP⁺ accordingly (Fig. 4). The data in Fig. 4 provide evidence to suggest this is the case, where 61% greater toxicity occurs from MPP⁺ (500 μM) in N-2A cells after 24 h, when density was doubled to 50,000 cells/well in 96-well plates. In order to corroborate the data from Fig. 3 (time-lapse analysis), metabolic changes over a range of MPP⁺ (0–500 μM) in N-2A cells were measured after 24 h (Fig. 5). These findings show that a decline in glucose and a rise in lactate are critical events parallel to cell death. There was no evidence to confirm the presence of a number of free radicals or its association with cell death. Thus far, the data obtained indicate that the toxic effects of MPP⁺ are highly due to the loss of glucose supplies in the media, to which metabolic rate and cell count control the former.

The results of experiments presented in Figs. 1, 2, 3, 4, and 5 were achieved using low-glucose standard experimental media: DMEM–phenol red containing 1,000 mg/l glucose, equiv 5.5 mM glucose. In the next experiment, DMEM was prepared fresh from powdered media and supplemented with 1 mM glucose (very low glucose). Here, we assess the protective effects of glucose, pyruvate, or antioxidant enzymes (Fig. 6). The toxicity of MPP⁺ in very low glucose media was reversed by 10 mM glucose or pyruvate, but not 10 U/ml of superoxide dismutase (SOD) or catalase. To further demonstrate that the mitochondria may be an expendable organelle, the protective effects of pyruvate against MPP⁺ were monitored in the presence or

absence of a mitochondrial monocarboxylic acid transport blocker cyano-4-hydroxycinnamate (α -cyano-4-HCA) in N-2A cells (Fig. 7a, left panel). These findings indicate that blocking pyruvate entry into the mitochondrial to sustain the tricarboxylic acid cycle (TCA) is not necessary to sustain the protective effects of pyruvate because anaerobic glycolysis through lactic acid dehydrogenase (LDH) alone is adequate to compensate for the effects of a mitochondrial toxin. These effects were also similar to those observed in the presence of a pyruvate dehydrogenase complex inhibitor (octanoyl-CoA; Fig. 7a, right panel). In order to examine the effects of α -cyano-4-HCA alone on glucose utilization in N-2A controls, it was found that blocking pyruvate as a substrate for the TCA cycle reduced mitochondrial O₂ consumption as would be expected and slightly augmented glucose utilization (Fig. 7b), indicating that the concentrations used were adequate for analysis in Fig. 7a.

In order to establish that the examined cells are capable of surviving in high-glucose media without mitochondrial use, the effects of O₂ deprivation by sodium dithionite addition to the plating media was evaluated (Fig. 8). The data show that cell survival continues to occur unabated in the complete absence of dissolved O₂. To confirm these findings, photographic analysis of cell viability with fluorescein diacetate and propidium iodide was obtained (Fig. 9). A cell death control was established for photographic analysis by inducing toxicity by addition of 700 μ M of H₂O₂. In summary, these findings suggest that glucose plays a foremost critical role in cell survival during loss of OXPHOS incurred by either the presence of a mitochondrial toxin or lack of O₂ in neuroblastoma where vulnerability should take into consideration cell density, metabolic rate, and concentrations of glucose within the experimental design.

Discussion

The current study investigates the extent by which metabolic patterns of immortal neural cell lines regulate glucose concentrations, which underlie altered vulnerability to mitochondrial toxicity in vitro. The data obtained in the study suggest that cell death upon exposure to MPP⁺ appears to be a direct function of a greater metabolic rate and cell density, which indirectly controls velocity of glucose utilization and ultimate exhaustion of energy supply. The metabolic rates among three neuroblastoma using rat, human, and mouse cell lines appear to be strikingly different in a manner consistent with the typical mouse–elephant curve for basal metabolic rate (BMR). In brief, while this study examines metabolism at the cellular level, whole body systemic energy metabolism is primarily required to produce energy to sustain cellular, organ, or systemic processes. In animals, BMR is typically measured using O₂ consumption per gram per hour in the whole body at rest at neutral temperature after fasting relative to body mass (gram) where $BMR = M^{2/3}$. This also means that across all species, the BMR per gram tissue increases as animals get smaller (Sadava et al. 2003). From the data in this study on variable rates of cellular O₂ consumption, it appears that species origin may play a role in varying vulnerability to the toxic effects of MPP⁺ due to variation in base metabolic rates.

In normal respiring cells with functional mitochondria, the means by which energy is produced involves the glycolytic pathway which converts 1 M of glucose to 2 M of pyruvic acid, which is then transported and converted to acetyl-CoA by pyruvate dehydrogenase complex (PDHC) in the mitochondria. This is the first step in aerobic metabolism which ultimately generates reducing equivalents (NADH²/FADH²) for entry into the electron transport to produce ATP through OXPHOS. When MPP⁺ inhibits mitochondrial function, pyruvate is re-routed through LDH to drive SLP, supported by the data in this study where we show failure of both PDHC or pyruvate mitochondrial transporter inhibitors to reverse the protective effects of pyruvate against MPP⁺. These findings are also consistent with our previous findings that the protective

affects of glucose against MPP^+ is not associated with reversal of OXPHOS function (Mazzio and Soliman 2003a).

Regardless of the differences between malignant neuroblastoma and normal neurons, it is believed that the major cause of MPP^+ toxicity and degeneration in PD is neuronal ATP depletion (Wang et al. 2007; Maruoka et al. 2007). Therefore, overcoming the loss of mitochondrial ATP production upon insult to the mitochondria could be providing neural protection. This can be achieved metabolically by augmenting the capacity of glycolysis through the administration of substrates and cofactors that drive SLP. A number of intermediates such as nicotinic acid adenine dinucleotide-reduced ($NADH$), pyruvate and phosphoenolpyruvate, as well as fructose, glucose, and magnesium have shown protection against MPP^+ in diverse neural and immortal neuroblastoma cell lines (Mazzio and Soliman 2003a, b; Jia et al. 2008; Hashimoto et al. 2008). While glucose is considered the primary energy fuel, ultimately, SLP is driven by oxidation–reduction reactions that are controlled by the availability of NAD^+ . In brief, LDH converts $NADH^+$ to NAD^+ , which, through glyceraldehyde-3-phosphate dehydrogenase activity, can regenerate $NADH^+$ and propel ATP production through the glycolytic enzymes phosphoglycerate kinase and pyruvate kinase. The toxicity of MPTP in animals is associated with a rise in striatal lactate (Koga et al. 2006); a drop in ATP/NAD^+ (Cosi and Marien 1998, 1999; Iwashita et al. 2004) and a nicotinamide deficiency exacerbate vulnerability of DAergic neurons to degenerate (Williams et al. 2005). The loss of NAD^+ could also occur through apoptosis which would indirectly render glycolysis inactive. For this reason, nicotinamide is protective against MPTP by attenuating the loss of NAD^+ incurred by pro-apoptotic poly(ADP-ribose) polymerase-I (PARP-I) activation (Cosi and Marien 1998; Schulz et al. 1995; Mukherjee et al. 1997).

In this study, it is of interest to note the absence of free radicals or antioxidant protection associated with MPP^+ toxicity. Our protocols did not include the use of 2,7-dichlorofluorescein diacetate (a common ROS probe) due to interferences previously noted with fluctuating amines in solution and experimental treatments. However, we did include standard ROS protocols having previously been used for quantifying specific free radicals generated by autooxidation of catecholamines or inflammation (Mazzio et al. 2004; Park and Murphy 1994). While clearly ROS play a role in inflammatory-mediated neurodegeneration, there are inconsistencies within the literature regarding a specific role for MPP^+ -mediated ROS neuronal damage in particular at complex I (Chinta et al. 2009). The lack of ROS effects, as mediated by MPP^+ , as found in this study has been reported in human SH-SY5Y cells (Caneda-Ferrón et al. 2008). Our studies also corroborate the work by Caneda-Ferrón et al. (2008) where we have reported earlier that ROS-mediated cell death is the predominant cause of catecholamine toxicity (but not MPP^+) in vitro (Mazzio et al. 2004).

While a large number of studies report that MPP^+ induces oxidative stress in N-2A, PC-12, and mesencephalic cells, data supporting this are often evidenced by a reduction in intracellular GSH, expression or activity of SOD, catalase and glutathione peroxidase enzymes, and lipid peroxidation to which protective plant polyphenolics can attenuate toxicity (Tsai and Yin 2008; Amazzal et al. 2007; Tian et al. 2007). However, two questions emerge. (1) To what extent does exhaustion of glucose supplies within the media play a role in decline in protein synthesis or endogenous antioxidant enzyme expressions and (2) to what extent do protective antioxidants mediate effects through anti-apoptotic cell signaling systems that ultimately control supplies of NAD^+ and therefore glycolysis. It has been proven that a number of plant-derived polyphenolics (which are also antioxidants) protect against MPP^+ as a result of anti-apoptotic properties (i.e., resveratrol, quercetin, and luteolin) which may modulate mRNA and protein expression of Bax and Bcl-2 (Alvira et al. 2007; Wruck et al. 2007; Bournival et al. 2009). If this is the case, these antioxidants would also prevent PARP-1-induced depletion of cellular NAD^+ , which could otherwise halt essential redox reactions required to drive anaerobic

glycolysis to sustain ATP (Tatton et al. 1999). Both additional supplies of glucose and anti-apoptotic polyphenolics could mediate the same effects through different pathways where glucose serves to sustain ATP concentration to prevent mitochondrial transition pore (MTP) opening and polyphenolics prevent downstream associated pro-apoptotic pathways, which spare PARP-1 depletion of NAD⁺, thereby indirectly preventing halt of glycolysis. Both glucose and NAD⁺ are required to drive glycolysis. For this reason, substances which prevent MPP⁺-mediated MPT opening, cytochrome c release, reduction in Bcl-2, and caspase-3 or PARP1 activation all seem to provide protection (Pu et al. 2003; Han and Lee 2009). Ultimately, future research will be required to assess if it is the loss of ATP initiated by MPP⁺ that initiates apoptosis which exacerbates depletion of NAD⁺ in addition to glucose through heightened glycolysis or through ROS-mediated MTP opening and oxidative DNA damage as previously believed.

In summary, the findings in this study demonstrate that immortal cell line vulnerability to the toxicity of MPP⁺ or hypoxia in vitro are a direct function of metabolic rate and glucose concentrations within the experimental design. These factors should be considered when interpreting toxicological findings of mitochondrial toxins or hypoxia in vitro.

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Fig. 1. Relative metabolic rate in neuroblastoma of diverse species origin (mouse N-2A, rat PC-12, human SH-SY5Y) after 24-h incubation at 37°C. The data represent glucose utilization (μM glucose/cell number), dissolved O_2 in the media (nmol/ml), and metabolic rate as determined by fluorescence intensity of almar blue after 4-h incubation. The data are expressed as the mean $\pm$ SEM ($N=4$), and differences from mouse N-2A cells were determined by a one-way ANOVA followed by a Tukey post hoc test. * $P<0.05$

Fig. 2.

a Relative cell death in various neuroblastoma cell lines (mouse N-2A, rat PC-12, and human SH-S5Y5) after 24 h $\pm$ MPP⁺ (0–10 mM) at 37°C. The data represent cell viability as percent untreated controls and are expressed as the mean $\pm$ SEM ($N=4$). Statistical differences from untreated controls were determined by a one-way ANOVA followed by a Tukey post hoc test. * $P<0.05$. **b** Relative cell death in various neuroblastoma cell lines (mouse N-2A, rat PC-12, and human SH-S5Y5) after 48 h $\pm$ MPP⁺ (0–10 mM) at 37°C. The data represent cell viability as percent untreated controls and are expressed as the mean $\pm$ SEM ($N=4$). Statistical differences from untreated controls were determined by a one-way ANOVA followed by a Tukey post hoc test. * $P<0.05$. **c** Relative loss of mitochondrial O₂ consumption in various neuroblastoma cell lines (mouse N-2A, rat PC-12, and human SH-S5Y5) after 24-h incubation $\pm$ MPP⁺ (0–10 mM). The data represent dissolved O₂ concentration in the media as percent untreated control and are expressed as the mean $\pm$ SEM ($N=4$). A statistical difference from the control group was determined by a one-way ANOVA followed by a Tukey post hoc test. * $P<0.05$

Fig. 3.

Metabolic events during time-lapse analysis of MPP⁺ (500 μM) toxicity in N2-A cells over 36 h of incubation at 37°C. The data represent metabolic parameters (glucose concentration, lactic acid concentration, and cell viability) as percent untreated controls and are expressed as the mean ± SEM (*N*=4). Statistical differences from untreated controls were determined by a one way ANOVA followed by a Tukey post hoc test. **P*<0.05

Fig. 4.

Effects of cell density on MPP⁺ toxicity (500 µM) in N-2A cells after 24 h of incubation. The data represent cell viability as percent untreated controls and are expressed as the mean $\pm$ SEM ($N=4$). Statistical differences between the two groups were determined by a two-way ANOVA where $P<0.05$

Fig. 5.

Metabolic events associated with low dose MPP⁺ (0– 500 μM) toxicity in N2-A cells after 24 h of incubation at 37° C. The data represent metabolic parameters (viability, dissolved O₂, glucose remaining, lactic acid, and ROS) as percent untreated controls and are expressed as the mean ± SEM (N= 4). Statistical differences from the untreated controls were determined by a one-way ANOVA followed by a Tukey post hoc test. *P<0.05

Fig. 6. Protective effects of energy substrates or antioxidant enzymes against MPP⁺ (0–250 μM) toxicity in very low glucose media (1 mM). The data represent percent control and are expressed as the mean ± SEM (*N*=4). Statistical differences from the untreated controls were determined by a one-way ANOVA followed by a Tukey post hoc test. **P*<0.05

Fig. 7.

a The protective effects of pyruvate in the presence or absence of α -cyano-4-HCA (pyruvate mitochondrial transport blocker) and octanoyl-CoA (pyruvate dehydrogenase inhibitor) against MPP⁺ toxicity (500 μ M) in N2A cells after 24 h of incubation at 37°C. The data represent cell viability as percent untreated controls and are expressed as the mean $\pm$ SEM ($N=4$). A statistical difference from the control group was determined by a one-way ANOVA followed by a Tukey post hoc test. * $P<0.05$. **b** The effects of α -cyano-4-HCA (pyruvate mitochondrial transport blocker) and Octanoyl-CoA (Pyruvate dehydrogenase inhibitor) on O₂ consumption and glucose utilization in N2A cells after 24-h incubation at 37°C. The data represent glucose utilization (μ M glucose remaining/mg protein, *left panel*) and O₂ dissolved O₂ remaining (nmol/ml) and are expressed as the mean $\pm$ SEM ($N=4$). A statistical difference from the control group was determined by a one-way ANOVA followed by a Tukey post hoc test. * $P<0.05$

Fig. 8.

Metabolic effects of hypoxia in high glucose media (25 mM) in the presence of varying concentration of dithionite after 24 h of incubation at 37°C in N-2A cells. The data represent dissolved O₂ (nmol/ml) and metabolic rate as percent untreated controls. The data are expressed as the mean ± SEM (*N*=4), and differences from the controls were determined by a one-way ANOVA followed by a Tukey post hoc test. **P*<0.05

Fig. 9.

Photographic analysis of morphology (phase contrast), cell death (propidium iodide), and cell viability (fluorescein diacetate) in N2A cells after 24-h incubation at 37°C with various concentrations of dithionite in high-glucose media (25 mM). A method validation control was established by inducing cell death in N2A cell controls with 700 μM of H₂O₂