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Effects of Kynurenine Pathway Inhibition on NAD⁺ Metabolism and Cell Viability in Human Primary Astrocytes and Neurons

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Abstract: The kynurenine pathway (KP) is the principle route of L-Tryptophan (TRP) metabolism, producing several neurotoxic and neuroprotective metabolic precursors before complete oxidation to the essential pyridine nucleotide nicotinamide adenine dinucleotide (NAD⁺). KP inhibition may prove therapeutic in central nervous system (CNS) inflammation by reducing the production of excitotoxins such as quinolinic acid (QUIN). However, KP metabolism may also be cytoprotective through the de novo synthesis of intracellular NAD⁺. We tested the hypothesis that the KP is directly involved in the maintenance of intracellular NAD⁺ levels and SIRT1 function in primary astrocytes and neurons through regulation of NAD⁺ synthesis. Competitive inhibition of indoleamine 2,3 dioxygenase (IDO), and quinolinic acid phosphoribosyltransferase (QPRT) activities with 1-methyl-L-Tryptophan (1-MT), and phthalic acid (PA) respectively, resulted in a dose-dependent decrease in intracellular NAD⁺ levels and sirtuin deacetylase-1 (SIRT1) activity, and correlated directly with reduced cell viability. These results support the hypothesis that the primary role of KP activation during neuroinflammation is to maintain NAD⁺ levels through de novo synthesis from TRP. Inhibition of KP metabolism under these conditions can compromise cell viability, NAD-dependent SIRT1 activity and CNS function, unless alternative precursors for NAD⁺ synthesis are made available.

Keywords: IDO, NAD⁺, sirtuins, astrocytes neurons 1-MT

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Introduction

In recent years, the kynurenine pathway (KP) has generated considerable interest following the observation that KP metabolites may have significant and opposing actions on central neurons.¹⁻³ One consistent finding in all neuroinflammatory diseases is a dramatic, immune-mediated increase in tryptophan (TRP) catabolism via the KP.⁴⁻¹⁰ In an effort to determine whether this biochemical phenomenon is related to the development of neuropathology, a number of recent studies have investigated the neurotoxic potential of the TRP metabolite quinolinic acid (QUIN) in inflammatory neurological disease such as Alzheimer's disease (AD),^{11,12} Huntington's disease (HD),^{13,14} Amyotrophic Lateral Sclerosis (ALS),¹⁵ AIDS Dementia Complex (ADC)^{16,17} and multiple sclerosis (MS).¹⁸ As a result, the KP has been identified as a likely target for pharmaceutical intervention to perhaps slow down or prevent neuronal dysfunction associated with neuroinflammatory disease.¹⁹⁻²³

A major aim is to attempt to rectify the balance of the KP with available inhibitors. However, the effect of KP inhibition on the biosynthesis of the ubiquitous pyridine nucleotide, nicotinamide adenine dinucleotide (NAD⁺) needs also to be evaluated. The KP has been established as the *de novo* pathway for NAD⁺ synthesis in the liver and kidney.²² We have recently shown that some KP metabolites, including 3-hydroxyanthranilic acid (3-HAA), 3-hydroxykynurenine (3-HK), and QUIN can promote NAD⁺ synthesis at nanomolar concentrations in human primary astrocytes and neurons.^{23,24} Moreover, NAD⁺ concentrations can be regenerated in rat-derived astrocytes using nicotinic acid (NA), nicotinamide (NM) or QUIN following H₂O₂ mediated NAD⁺ depletion.²⁵ Given the importance for the KP for NAD⁺ synthesis, pharmacological modulation of the KP will significantly affect intracellular NAD⁺ levels.

NAD⁺ acts as an essential cofactor for several enzyme catalysed reactions including alcohol, lactate and amino acid metabolism. NAD⁺ also serves as an electron transporter to power oxidative phosphorylation and ATP production.²⁶ In genomic DNA, NAD⁺ is the sole substrate for the DNA nick sensor, poly-(ADP-ribose) polymerase, (PARP).²⁷ The PARP family of enzymes, particularly PARP-1, are DNA binding

enzymes activated by free-radical mediated DNA strand breaks and play a crucial role in base excision repair.^{27,28} In addition to its role in PARP activity, NAD⁺ also serves as a substrate for a new class of enzymes known as sirtuins, or silent information regulator of gene transcription.²⁹ SIRT1, the founding member of the sirtuin family of protein, has been shown to regulate gene silencing, and promote longevity.³⁰

Given the importance of KP metabolism in *de novo* NAD⁺ synthesis in human brain cells, we tested the effect of KP inhibition on intracellular NAD⁺ levels and NAD-dependent SIRT1 activity in glial and neuronal cells. More specifically, we tested the hypothesis that indoleamine 2,3 dioxygenase (IDO), and quinolinic acid phosphoribosyltransferase (QPRT) activities, two important KP enzymes, can significantly regulate intracellular NAD⁺ synthesis and NAD-dependent SIRT1 activity in human astrocytes and neurons. Results from this study indicate a strong dependence on KP metabolism through IDO and QPRT regulation for the maintenance of NAD⁺ production and SIRT1 function. Therefore, caution should be advised when administering pharmacological inhibitors to TRP metabolism during neuroinflammatory conditions.

Materials and Methods

Reagents and chemicals

Dulbecco's phosphate buffer solution (DBPS) and all other cell culture media and supplements were from Invitrogen (Melbourne, Australia) unless otherwise stated. Nicotinamide, bicine, β -nicotinamide adenine dinucleotide reduced form (β -NADH), 3-[-4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), alcohol dehydrogenase (ADH), sodium pyruvate, TRIS, γ -globulins, L-tryptophan (TRP), 1-methyl-L-TRPtophan (1-MT), phthalic acid (PA), and catalase were obtained from Sigma-Aldrich (Castle-Hill, Australia). Phenazine methosulfate (PMS) was obtained from ICN Biochemicals (Ohio, USA). Bradford reagent was obtained from BioRad, Hercules (CA, USA).

Cell cultures

Human foetal brains were obtained from 16–19 week old foetuses collected following therapeutic termination with informed consent. Mixed brain cultures were

prepared and maintained using a protocol previously described by Guillemain et al.³¹ Astrocytes and neurons were prepared from the mixed brain cell cultures, and maintained using a protocol previously described by Guillemain et al.³²

KP inhibition in astrocytes and neurons

For IDO, QPRT and LDH activities, human primary astrocytes and neurons were incubated 10 μ M, 100 μ M, and 1000 μ M 1-MT and PA, respectively (Table 1). For intracellular NAD⁺ concentrations, 1 μ M, 10 μ M, 50 μ M, 100 μ M, and 1000 μ M of inhibitors was used. SIRT1 activity was measured using 100 μ M of 1-MT and PA. Cultures were then incubated at 37 °C in 5% CO₂ for 24 hours before analysis of IDO and QPRT activities, intracellular NAD⁺ levels, and extracellular LDH and SIRT1 activities. Experiments were performed with primary cultures derived from three different human foetal brains with each individual preparation tested in triplicate.

NAD(H) microcycling assay

Intracellular NAD⁺ concentration following 24 hour incubation with the desired concentrations of KP inhibitors were measured spectrophotometrically using the thiazolyl blue microcycling assay established

by Bernofsky and Swan³³ adapted for 96 well plate format by Grant and Kapoor.²⁵

Extracellular LDH activity

LDH activity following 24 hour incubation with the desired concentrations of KP inhibitors was assayed using a standard spectrophotometric technique described by Koh and Choi (1987).³⁴

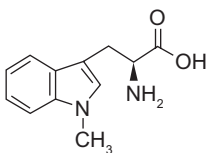
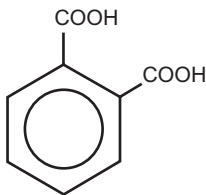
Indoleamine 2,3 dioxygenase activity

IDO activity was evaluated as previously described by.³⁵ Briefly, cellular homogenates were homogenised at 4 °C with a Teflon pestle, resuspended in 50 mM K₂HPO₄/KH₂PO₄ buffer solution (pH 6.5), and centrifuged at 12,000 g for 30 min. The reaction mixture contained 20 mM ascorbic acid, 50 μ M methylene blue, 200 μ g catalase and 2 mM TRP. After adding 20 μ g of cellular enzyme, the mixtures were incubated at 37 °C for 30 minutes. The product formed was read at 405 nm using the Model 680XR microplate reader (BioRad, Hercules, CA, USA).

Quinolinic acid phosphoribosyl transferase activity

QPRTase activity was determined by measuring the formation of nicotinic acid mononucleotide (NAMN)

Table 1. Inhibitors used in these experiments.

Inhibitor	Chemical Structure	Action
1-methyl-L-Tryptophan (1-MT)		Competitive IDO inhibitor
Phthalic acid (PA)		Competitive QPRT inhibitor

using a continuous UV spectrophotometric assay as previously described by Rahman et al (2009).³⁶

SIRT1 deacetylase activity

SIRT1 deacetylase activity was evaluated on cellular homogenate using the Cyclex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit (CycLex, Nagano, Japan).

Bradford protein assay for the quantification of total protein

NAD⁺ concentration and extracellular LDH activity were adjusted for variations in cell number using the Bradford protein assay described by Bradford.³⁷

Data analysis

Results obtained are presented as the means \pm the standard error of the mean (SEM). Significant differences were verified using the two-tailed t-test with equal variance and 1-WAY ANOVA. Differences between treatment groups were considered significant if *P* was less than 0.05 (*P* < 0.05).

Results

Effect of 1-MT and PA on IDO and QPRT activities in human astrocytes and neurons

Consistent with previous studies, a dose-dependent inhibition of IDO activity was observed following treatment with 1-MT, a competitive inhibitor of IDO (Fig. 1A). A maximum reduction in IDO activity was observed in astrocytes and neurons treated with 1 mM 1-MT. Similarly, a dose-dependent inhibition of QPRT activity was reported following treatment with PA (Fig 1B). Likewise, a maximum inhibitory response was observed in human brain cells treated with 1 mM PA.

Effect of 1-MT and PA on intracellular NAD⁺ levels in human astrocytes and neurons

Importantly, the effect of decreasing IDO and QPRT activities on intracellular NAD⁺ levels in these cell types was highly correlated. NAD⁺ levels declined in a dose-dependent manner with increasing concentrations of 1-MT (Fig. 2A) and PA (Fig. 2B) respectively after 24 hours incubation.

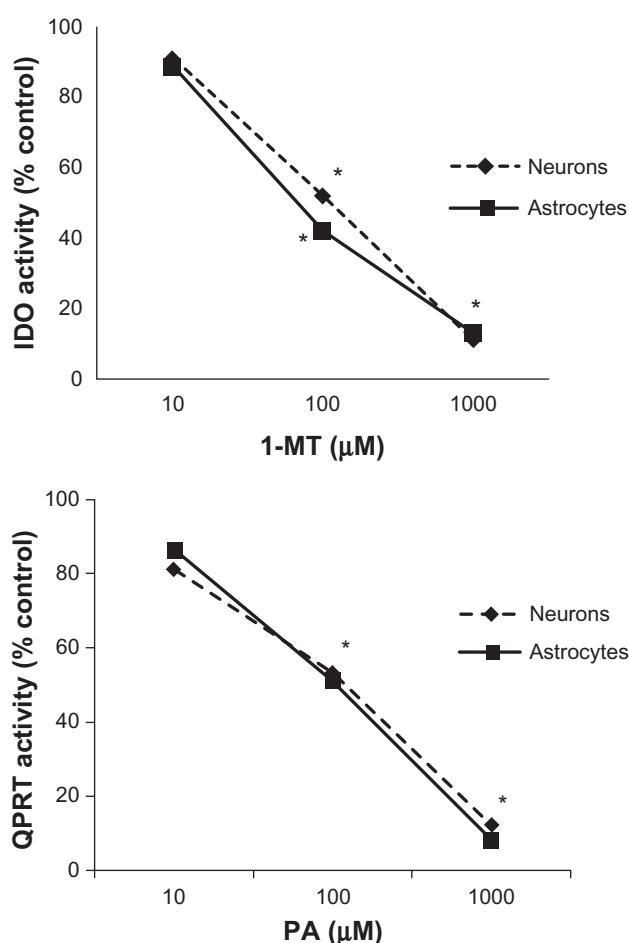


Figure 1. A) Effect of 1-MT on IDO activity in human astrocytes and neurons. A dose-dependent inhibition of IDO activity was observed following treatment with 1-MT in human astrocytes and neurons. For astrocytes, no 1-MT (control) = 35.86 nmol kynurenine/hr/mg protein; 10 μM 1-MT = 31.15 \pm 5.61 nmol kynurenine/hr/mg protein; 100 μM 1-MT = 14.70 \pm 4.85 nmol kynurenine/hr/mg protein; 1000 μM 1-MT = 4.55 \pm 1.93 nmol kynurenine/hr/mg protein; Significance **P* < 0.05 compared to previous dose (*n* = 4 for each treatment group). For neurons, no 1-MT (control) = 27.22 \pm 7.28 nmol kynurenine/hr/mg protein; 10 μM 1-MT = 24.77 \pm 6.74 nmol kynurenine/hr/mg protein; 100 μM 1-MT = 14.15 \pm 2.94 nmol kynurenine/hr/mg protein; 1000 μM 1-MT = 2.99 \pm 1.42 nmol kynurenine/hr/mg protein; Significance **P* < 0.05 compared to previous dose (*n* = 4 for each treatment group). **B)** PA on QPRT activity in human astrocytes and neurons. A dose-dependent inhibition of QPRT activity was observed following treatment with PA in human astrocytes and neurons. For astrocytes, no PA (control) = 41.33 \pm 8.32 nmol kynurenine/hr/mg protein; 10 μM PA = 35.54 \pm 3.22 nmol kynurenine/hr/mg protein; 100 μM PA = 21.08 \pm 7.39 nmol kynurenine/hr/mg protein; 1000 μM PA = 3.31 \pm 1.32 nmol kynurenine/hr/mg protein; Significance **P* < 0.05 compared to previous dose (*n* = 4 for each treatment group). For neurons, no PA (control) = 21.55 \pm 3.62 nmol kynurenine/hr/mg protein; 10 μM PA = 17.46 \pm 3.49 nmol kynurenine/hr/mg protein; 100 μM PA = 11.42 \pm 3.11 nmol kynurenine/hr/mg protein; 1000 μM PA = 2.59 \pm 0.81 nmol kynurenine/hr/mg protein; Significance **P* < 0.05 compared to previous dose (*n* = 4 for each treatment group).

Effect of 1-MT and PA on cellular viability in human astrocytes and neurons

The release of lactate dehydrogenase (LDH) into culture supernatant correlates with the amount of cell death and membrane damage, providing an accurate

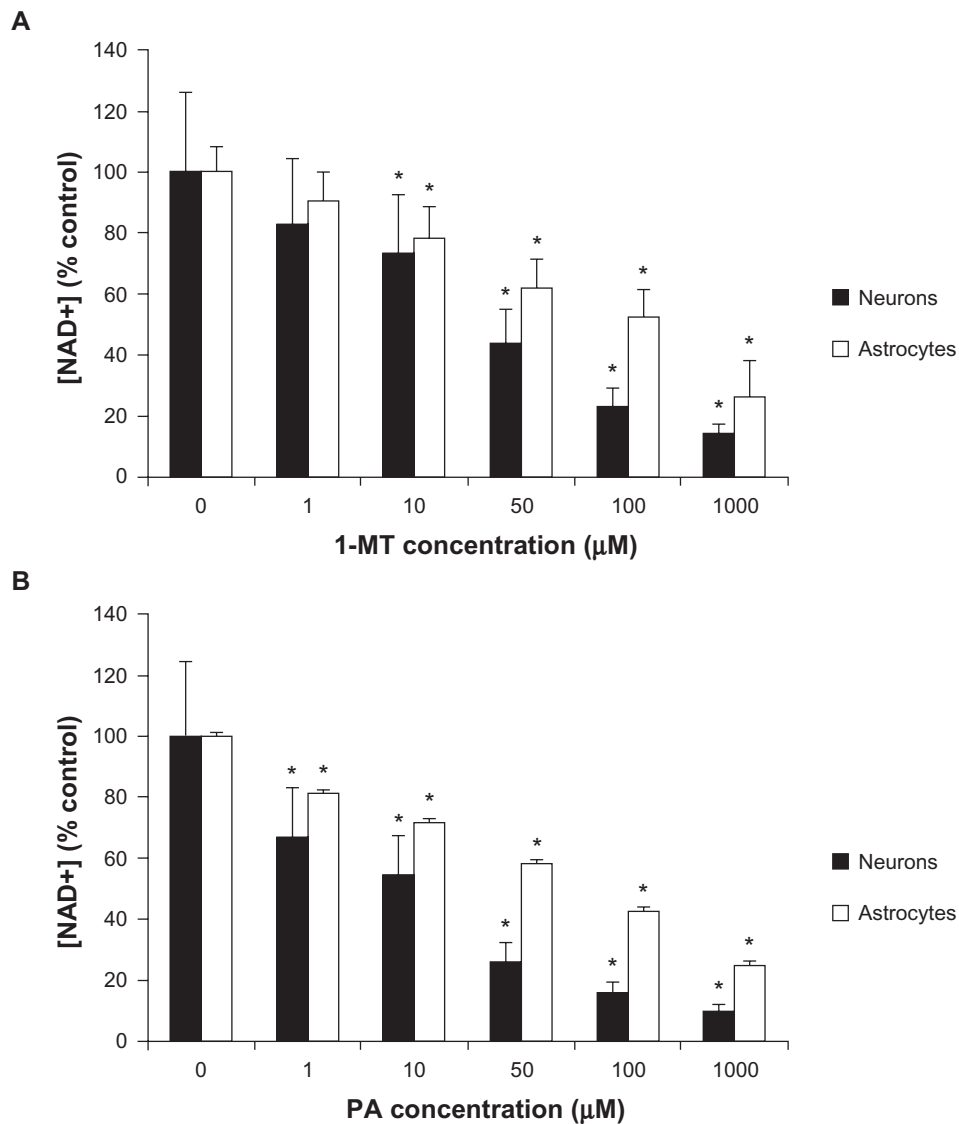


Figure 2. Effect of (A) 1-MT and (B) PA on intracellular NAD⁺ levels in human astrocytes and neurons. NAD⁺ levels significantly declined in a dose-dependent manner with increasing concentrations of (A) 1-MT and (B) PA respectively following 24 hours incubation with the selected inhibitor. Significance * $P < 0.05$ compared to previous dose ($n = 4$ for each treatment group).

measure of cellular toxicity. Showing an inverse correlation with intracellular NAD⁺ levels, extracellular LDH activity was significantly increased with increased concentrations of either 1-MT (Fig. 3A), or PA (Fig. 3B) in both astrocytes and neurons after 24 hours incubation.

Effect of 1-MT and PA on SIRT1 activity in human astrocytes and neurons

Reduced SIRT1 activity has been shown to decrease longevity in a range of organisms.

Addition of either 1-MT or PA at 100 μM significantly reduced SIRT1 activity in human astrocytes and neurons (Fig 4). 1-MT reduced SIRT1 activity by

51.4% and 56.8% in human astrocytes and neurons. In a similar fashion, PA reduced SIRT1 activity by 50.9% and 57.4% in human astrocytes and neurons. To help verify that the results obtained for enzyme activity were specific for SIRT1, we tested the effect of sirtinol, a synthetic SIRT1 inhibitor on SIRT1 activity.³⁸ Our data shows that in the presence of sirtinol SIRT1 activity declined by 90% and 84% in human astrocytes and neurons.

Discussion

Numerous studies have shown that NAD⁺ turnover is increased during chronic oxidative stress and neuroinflammation.^{25,39-41} As neuroinflammation is

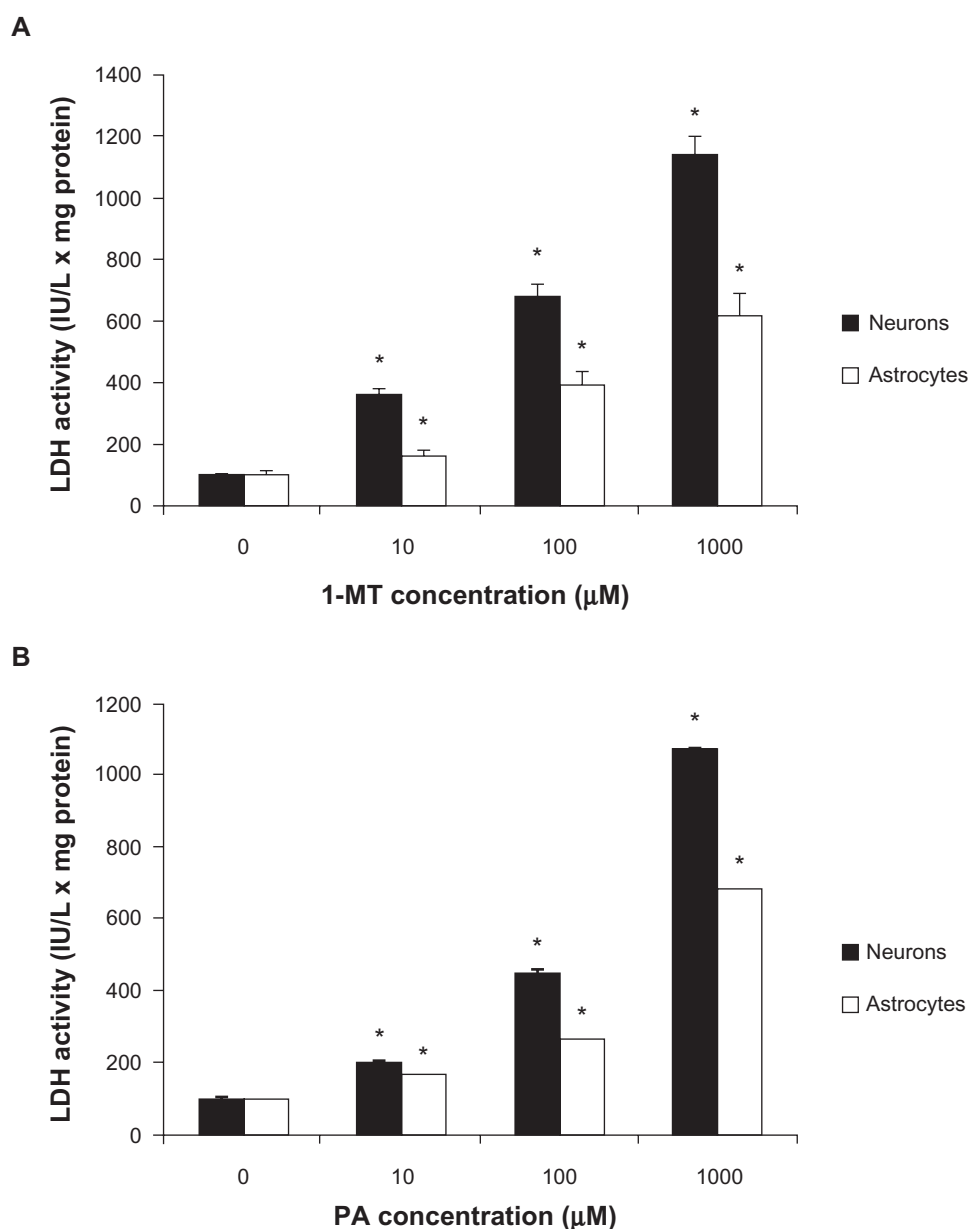


Figure 3. Effect of (A) 1-MT and (B) PA on extracellular LDH activity in human astrocyte and neuron cultures. Extracellular LDH activities were significantly elevated in a dose-dependent manner with increasing concentrations of (A) 1-MT and (B) PA respectively, following 24 hours incubation with the selected inhibitor. Significance $*P < 0.05$ compared to previous dose ($n = 4$ for each treatment group).

associated with chronic increase in ROS activity, marked microglial infiltration, and subsequent PARP activation, NAD^+ turnover is likely to be significantly increased.^{25,39–41} Therefore, a better understanding of the de novo NAD^+ biosynthetic pathway in human brain cells will provide a basis for the regeneration of NAD^+ . To our knowledge, the role of the KP in NAD^+ synthesis has not been studied in the CNS. If the KP is essential for NAD^+ synthesis, then it is conceivable that inhibition will result in a decrease in NAD^+ in human brain cells.

In this study, we have shown for the first time, that competitive inhibition of IDO and QPRT, in both astrocytes and neurons results in a dose-dependent decrease in intracellular NAD^+ , highlighting the essential role of the KP in NAD^+ production in human brain cells. KP inhibition leads to a decrease in NAD^+ synthesis and a dose dependent increase in extracellular LDH activity indicating reduced cell viability and increased cell death. Importantly, while IDO and QPRT activities appear to be inhibited equally in both astrocytes and neurons, intracellular NAD^+ levels and

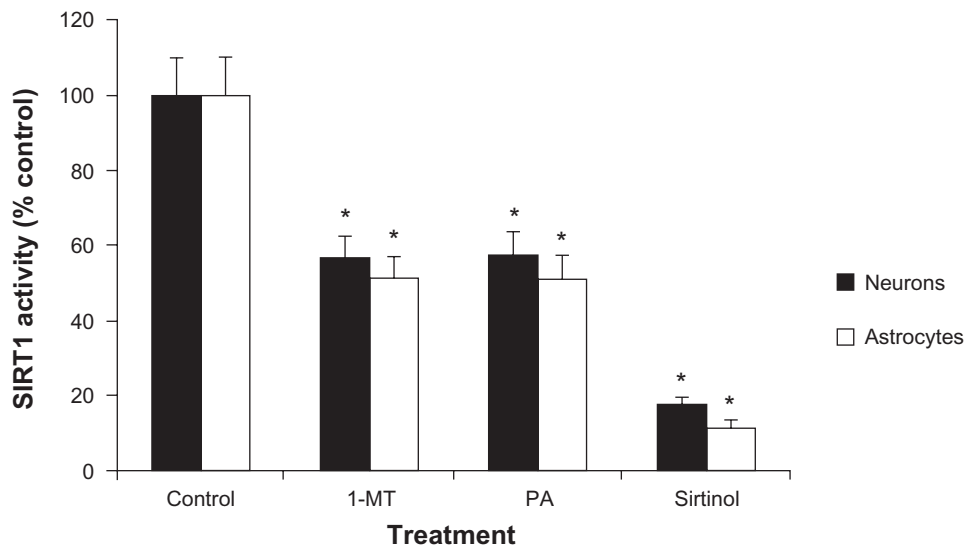


Figure 4. Effect of 1-MT and PA on SIRT1 activity in human astrocytes and neurons. Treatment with 1-MT or PA at 100 μ M significantly reduced SIRT1 activity in human astrocytes and neurons. Significance $*P < 0.05$ compared to control ($n = 4$ for each treatment group).

cell viability are significantly higher in astrocytes than neurons, suggesting that changes in KP metabolism have a greater effect on the neuronal population compared to glial cells.

As NAD^+ serves as a substrate for SIRT1 activity, we have also shown herein that inhibition of KP metabolism can lead to a significant decline in SIRT1 activity in the astrocytes and neurons. This data supports an earlier study in which we showed that primary human astrocytes cultured in media deficient in TRP, NA and NM resulted in a 50% decrease in intracellular NAD^+ levels after 24 hours.⁴³ The decrease in NAD^+ was partially ameliorated following supplementation of the culture media with TRP or NAD^+ salvage pathway precursors, NA or NM.⁴³

In mammalian cells, IDO represents the primary enzyme for oxidative TRP catabolism to kynurenine via the KP in both astrocytes, and neurons.⁴⁴ Interferon- β 1, an activator of IDO is currently used for the treatment of relapse-remitting MS due to the importance of the KP in promoting adaptive immunity through IDO-mediated down-regulation of T-cell proliferation. However, overactivation of the KP may lead to increased levels of QUIN and other neurotoxic metabolites generated by perivascular macrophages. Indeed, IDO over-expression and the accumulation of the neurotoxic TRP metabolite, QUIN has been implicated not only in the pathogenesis of MS, but also in the neurological deficits observed in later stages of the disease.¹⁸ Therefore, inhibition of IDO

has been suggested as a potential therapeutic target to reduce QUIN toxicity in the MS brain.⁴⁵ However, in a mouse model for MS, daily application of the IDO inhibitor, 1-MT aggravated disease progression indicating that IDO inhibition exacerbates disease.⁴⁶ This is supported by our results showing that IDO inhibition reduces NAD^+ synthesis and SIRT1 function, and therefore promoting cell death.

QPRT converts QUIN to NAMN and carbon dioxide in the presence of Mg^{2+} and 5-phosphoribosyl-1-pyrophosphate (PRPP).⁴⁴ In the brain, QPRT is one of the rate-limiting enzymes of NAD^+ synthesis from TRP, and therefore likely to influence QUIN levels in the CNS.⁴⁷ We have previously shown that human cerebral neurons can take up exogenous QUIN but can only catabolise a small amount.⁴⁸ This may be likely due to the rapid saturation of QPRT. Indeed we have shown that neuronal QPRT activity is saturated when QUIN concentration exceeds 500 nM.³⁶ Thus, QPRT activity is essential for the maintenance of cellular energy metabolism and DNA repair. A reduction in QPRT activity can be envisioned to lead to an accumulation of QUIN, and likely to induce a cytotoxic cascade within astrocytes and neurons.³¹

As previously mentioned, SIRT1 belongs to a highly conserved gene family known as sirtuins, which encode NAD^+ -dependent histone and non-histone deacetylases promoting DNA stability and improved lifespan in yeasts and small mammals.²⁹ SIRT1 also regulates the acetylation of a number



of transcription factors, including the peroxisome proliferator-activated receptor- γ (PPAR γ), p53, and the FOXO family of transcription factors, all of which represent key metabolic regulators.⁴⁹ Our data shows that SIRT1 activity goes hand in hand with those of NAD⁺ metabolism, suggesting a causal relationship between SIRT1 function and NAD⁺ synthesis. Other NAD⁺-dependent targets present in human brain cells include the cytosolic SIRT2, which is known to deacetylate tubulin, and the mitochondrial sirtuins (SIRT3, SIRT4, and SIRT5).⁵⁰ Additional work is needed to determine the effect of KP inhibition on the activity of these proteins. However, as NAD⁺ is the substrate for sirtuins, it is likely that inhibition of IDO and QPRT may also negatively impact on the function of these proteins.

Given the importance of the KP for de novo synthesis of NAD⁺, the current study suggests that KP inhibition should be carried out with caution. While we have shown that IDO and QPRT inhibition can deplete intracellular NAD⁺ levels and reduce cell viability under normal physiological conditions, another study has shown a similar affect on NAD⁺ levels following IDO inhibition in primary murine astrocytes stimulated with IFN- γ , leading to a similar effect on cell viability.⁴² In the light of the growing importance of glial cell function and neuronal activity, inhibiting KP metabolism may be deleterious to de novo NAD⁺ synthesis and CNS function unless alternative precursors are made available.

Disclosure

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

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