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Inhibition of indoleamine 2,3-dioxygenase activity in IFN-γ stimulated astroglioma cells decreases intracellular NAD levels

Ross Grant[a,*], Vimal Kapoor[b]

[a]*Biomedical Mass Spectrometry Facility, Faculty of Medicine, University of NSW, Sydney 2000, Australia*
[b]*Department of Physiology and Pharmacology, School of Medical Sciences, University of NSW, Sydney 2000, Australia*

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Abstract

Astroglia provide essential metabolic and neurotropic support to cells within the CNS and participate in the cellular immune response with microglia/macrophages following activation by the pro-inflammatory cytokine IFN-γ. Activation of glial cells results in local oxidative stress and induction of a number of proteins including the enzyme indoleamine 2,3-dioxygenase (IDO). As a rate-limiting enzyme, IDO regulates tryptophan catabolism via the kynurenine pathway producing a series of metabolic precursors (some of which are neurotoxic) before complete oxidation to the essential pyridine nucleotide NAD. Inhibition of this pathway may therefore prove therapeutic in neuroinflammatory disease by reducing production of cell toxins. However, kynurenine metabolism may also be cytoprotective through *de novo* synthesis of cellular NAD levels. We investigated the hypothesis that IDO activity is directly involved in maintenance of intracellular [NAD] in activated astroglial cells through control of *de novo* synthesis. Exposure to IFN-γ increased IDO activity from 7 ± 1 nmol to 129 ± 11 nmol kynurenine/hr/mg protein. Inhibition of IDO activity with either 6-chloro-D-tryptophan (competitive inhibition), or 3-ethoxy β-carboline (non-competitive inhibition) resulted in a dose-dependent decrease in IDO activity that correlated directly with decreasing [NAD] ($R^2 = 0.92$ and 0.81, respectively). These results support the hypothesis that one important consequence of increasing IDO activity in astroglial cells during inflammation is to maintain NAD levels through *de novo* synthesis from tryptophan. Inhibition of kynurenine pathway metabolism under these conditions may significantly decrease cell viability and CNS functions unless alternate precursors for NAD synthesis are available.
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1. Introduction

Normal astrocytic cell function is essential to CNS homeostasis providing metabolic and neurotrophic support to neurones and other cell types [1–3]. Loss or reduced astroglial activity has been postulated as a significant contributor to the neurological impairment evident in some neuroinflammatory disorders [4,5]. Inflammation of the CNS results in recruitment of activated T-cells and macrophages from the periphery into the neural parenchyma [6]. These activated T-cells produce a number of cytokine mediators of the inflammatory response including the macrophage activating cytokine IFN-γ [7].

IFN-γ enhances free radical generation in macrophages [4], and induces the production of a number of proteins including IDO (EC 1.13.11.7), the rate limiting enzyme for oxidative tryptophan catabolism, in both astrocytes and macrophages [8].

As a rate limiting enzyme, IDO regulates the catabolism of tryptophan via the kynurenine pathway [9,10], a process involving the formation of a number of stable metabolites before finally producing the essential pyridine nucleotide, NAD.

In recent years, the kynurenine pathway has generated considerable interest among neuroscientists due to the observation that some kynurenine pathway metabolites have neuroactive properties [10]. This has been followed by a number of reports linking increased kynurenine metabolism with neuronal injury, particularly during neuroinflammatory disease [11]. As a consequence, the kynurenine pathway has now been identified as a potential target site for

* Corresponding author. Tel.: +61-2-9385-1609; fax: +61-2-9662-4469.
E-mail address: r.grant@unsw.edu.au (R. Grant).

Abbreviations: NAD, nicotinamide adenine dinucleotide; IDO, indoleamine 2,3-dioxygenase; IFN-γ, interferon gamma; CNS, central nervous system; 6CDT, 6-chloro-D-tryptophan; 3EBC, 3-ethoxy-β-carboline.

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pharmacological intervention in order to alleviate or prevent neuronal damage during inflammatory disease [12].

This strategy presumes that toxic kynurenine metabolites are the primary product of this increased activity. However, the effect of IDO inhibition on NAD synthesis particularly during inflammation also needs to be considered. Any pharmacological modulation of kynurenine metabolism will affect *de novo* NAD synthesis. NAD is involved in many metabolic processes; it is an essential cofactor for enzyme catalyzed reactions, an important contributor to energy (ATP) production and is an exclusive substrate for the putative nuclear repair enzyme PARP.

In light of the central role played by NAD in cellular metabolism and the fact that oxidative stress, which increases significantly during inflammation [13,14], can rapidly reduce NAD levels in glia and other cell types [15] the relationship between IDO induction and *de novo* NAD synthesis has only recently begun to be explored [16].

In this study we investigated the hypothesis that IDO activity in IFN-γ stimulated astroglioma cells significantly influences intracellular NAD levels. The results presented indicate that IDO activity is predictive of intracellular NAD concentration suggesting a role for increased kynurenine metabolism through IDO regulation in the maintenance of cellular energy metabolism during inflammation and therefore caution when applying pharmacological inhibitors to kynurenine metabolism under these conditions.

2. Materials and methods

2.1. Materials

3-Amino benzamide (3ABA), phenazine methosulfate, alcohol dehydrogenase (bakers yeast), bicine, 3-[4,5-dimethylthiozol-2-*y*]-2,5-diphenyltetrazolium bromide (MTT), catalase (3200 U/mg solid, bovine liver), tryptophan, *p*-dimethylamino-benzaldehyde (Ehrlich's reagent), were purchased from Sigma–Aldrich. Phoenix, human recombinant IFN-γ was purchased from Australian Laboratory Services.

2.2. Cell culture

Human astroglioma cells (HTB-138) were maintained in culture in 75 cm^3 flasks (Costar, EK medical) in DMEM + 10% foetal bovine serum (FBS). The day before the experiment, cells were trypsinised washed and transferred to 24-well tissue culture plates (Greiner labortechnik).

2.3. IDO inhibition in IFN-γ-treated astroglioma cells

10^5 or 10^6 cells/well were seeded into 24-well culture plates. Each well contained 1 mL of DMEM supplemented with 10% FBS. Cultures were left to equilibrate for 24 hr before treatment with any one or combination of the following drugs: the competitive IDO inhibitor, 6CDT (0–1 mM) [17], or the non-competitive IDO inhibitor, 3EBC (0–200 μM) [18].

Following the addition of drug, cultures were incubated for 60 min at 37° in 5% CO_2 before the addition of IFN-γ (600 U/mL) to selected wells. Cultures were then incubated at 37° in 5% CO_2 for 40 hr before analysis of cellular NAD, IDO activity and supernatant kynurenine.

2.4. Biochemical analysis

The cellular pyridine nucleotide content (NAD^+ + NADH) was measured by the Thiazolyl blue microcycling assay of Bernofsky and Swan [19] adapted to 96-well plate format as described previously [20].

IDO activity was determined in the cell homogenate by the method of Takikawa *et al.* [21].

The change in kynurenine concentration in the supernatant was measured spectrophotometrically as previously described [22].

2.5. Total protein assay

Cultured medium was aspirated from each culture well and the cells washed twice with Tris buffered saline (pH 7.5), before being homogenised by sonication in 500 μL of PBS. The total protein per culture well was measured using the commercially available Bradford assay method and reagents (BIORAD), adapted to 96-well microtitre plate format.

2.6. Statistical analysis

Significant differences between treatment groups, at $P < 0.05$ level of significance, were determined using Student's *t*-test. Correlation coefficients were calculated by the method of least squares.

3. Results and discussion

IDO activity increased significantly ($P < 0.0001$) following 40 hr exposure to IFN-γ (7 ± 1 nmol to 129 ± 11 nmol kynurenine/hr/mg protein, N = 8) with a corresponding increase in extracellular kynurenine concentration from 0 to 28 ± 1 μM, demonstrating functional tryptophan catabolism via the kynurenine pathway. We investigated the relationship between IDO activity and intracellular NAD by measuring the concentration of intracellular NAD at different levels of IDO inhibition.

Consistent with previous studies [17] a dose-dependent inhibition of IDO activity was achieved in IFN-γ activated cells following treatment with either 6CDT or 3EBC (competitive and non-competitive inhibitors of IDO, respectively). A maximum reduction in IDO activity of

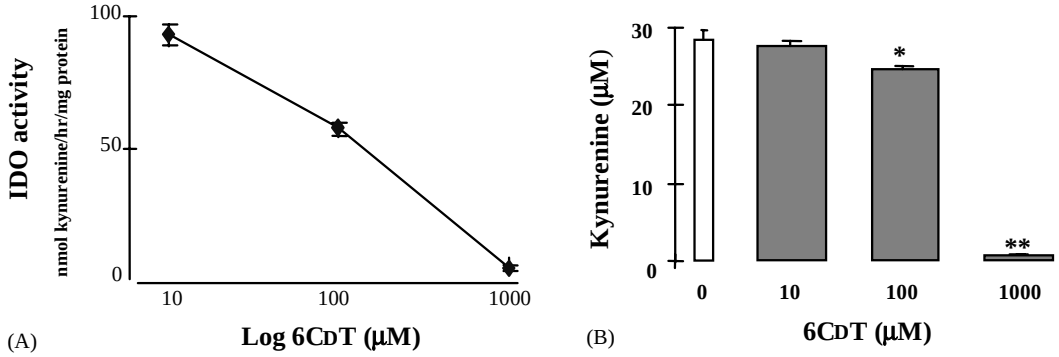

Fig. 1. The effect of 6CDT on IDO activity and kynurenine production in cultured astroglioma cells. (A) A near logarithmic decrease in IDO activity was observed in the presence of increasing concentrations of 6CDT. (B) Culture supernatant kynurenine concentration decreased with increasing [6CDT]. 0 μM 6CDT (control) = 28.5 ± 1.2 μM, N = 8, 100 μM 6CDT = 24.7 ± 0.4 μM, N = 4 ($^{*}P < 0.05$ compared to 10 μM), 1000 μM 6CDT = 0.7 μM, N = 4 ($^{**}P < 0.001$ compared to 100 μM).

96% was observed in activated astroglioma cells when treated with 1 mM 6CDT (Fig. 1A), while a maximum dose of 200 μM 3EBC resulted in a 52% reduction in IDO activity.

Extracellular kynurenine levels also decreased with decreasing IDO activity (Fig. 1B), indicating that tryptophan catabolism, and therefore *de novo* NAD synthesis, via the kynurenine pathway was proportionately reduced under these conditions.

Addition of either 6CDT (a tryptophan analogue) or 3EBC (a tryptophan derivative) up to 1000 and 200 μM, respectively, to the culture medium did not significantly increase the efflux of the cytoplasmic enzyme lactate dehydrogenase (LDH) over the experimental period. This indicates that the observed changes in kynurenine pathway metabolism are likely due to the action of each inhibitor on IDO directly rather than a generalized disruption of cellular activity.

Importantly, the effect of decreasing IDO activity on intracellular NAD in these cells was highly correlated. NAD levels declined in a dose-dependent fashion with decreasing IDO activity. The decrease in cellular NAD levels observed following treatment with either 6CDT or 3EBC correlated directly with the percent inhibition of IDO, $R^2 = 0.92$, and 0.81, respectively (Fig. 2A and B).

The close correlation between IDO activity and cellular NAD levels observed in this study indicates that *de novo* synthesis via the kynurenine pathway is an important source of NAD in these IFN-γ activated cells. This observation is both significant and consistent with previous work in this area [16,20]. The effect of inflammatory cytokines such as IFN-γ on tryptophan catabolism and subsequent secretion of various kynurenine pathway intermediates into the CNS during inflammation has been well documented [23]. It has been proposed that due to the apparent detrimental effects some kynurenine metabolites have on CNS cells, neuronal pathology may be prevented by inhibiting IDO activity during inflammation [24,25]. However, the results presented in this report, supporting a role for IDO induction in NAD synthesis, suggest that inhibition of the kynurenine pathway during inflammation may significantly reduce NAD levels in astroglial cells. In light of the importance of glial cell function to CNS homeostasis and neuronal activity, reducing astroglial viability by preventing *de novo* NAD biosynthesis is likely to have a negative impact on CNS function unless other precursors

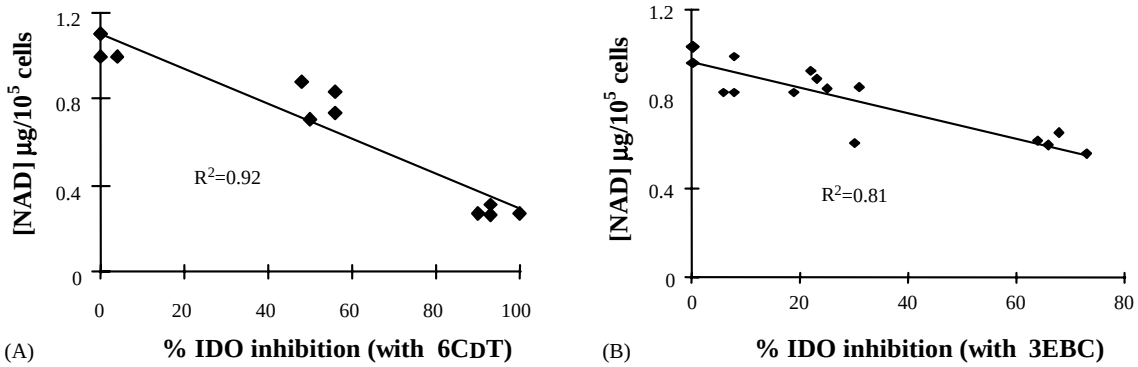

Fig. 2. The effect of IDO inhibition on intracellular NAD concentration in astroglioma cells. Astroglial cells were exposed to 600 U/mL of IFN-γ for 40 hr in the presence of either (A) 0–1000 μM of 6-chloro-D-tryptophan (6CDT) (N = 4 per treatment group) or (B) 0–200 μM of 3-ethoxy-β-carboline (3EBC) (N = 4–6 per treatment group). Decreased NAD levels correlated directly ($R^2 = 0.92$ and 0.81, respectively) with decreasing IDO activity. Results are presented as a percentage of the maximum activity of IDO measured in cells exposed to IFN-γ for 40 hr without drug.

of NAD, such as nicotinamide or nicotinic acid are available [15]. However, the efficacy of these molecules to act as NAD substrates under inflammatory conditions has yet to be demonstrated.

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