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Tryptophan Loading Induces Oxidative Stress

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In previous studies tryptophan loads have been administered to human subjects in order to raise central levels of 5-hydroxytryptamine (5HT) and assess the effects of 5HT on behaviour and mood. However, tryptophan is metabolised primarily along the oxidative kynurenine pathway. In this study a 6 g oral tryptophan load was administered to 15 healthy volunteers and the levels of kynurenines and lipid peroxidation products (indicative of oxidative stress) were measured. The results demonstrate that tryptophan loading produces a highly significant increase in lipid peroxidation products in parallel with increased kynurenines. The oxidative stress may result from the generation of quinolinic acid, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid, all of which are known to have the ability to generate free radicals. The results may have implications for the use of tryptophan loading in psychiatric practice, and for the chronic use of diets high in tryptophan.

Keywords: Tryptophan; Kynurenines; Oxidative stress; Lipid peroxidation; Hydroxynonenal; Malondialdehyde

INTRODUCTION

The products of tryptophan metabolism, especially 5-hydroxytryptamine (5HT) have been implicated in a range of psychiatric disorders such as depression and schizophrenia. As a result, a large number of tests have been performed on animals and human subjects in which tryptophan levels are either depleted by the administration of an amino acid-rich cocktail lacking tryptophan, (which promotes general protein synthesis and thus leads to tryptophan depletion)[1] or increased by the administration of a tryptophan load.[2–4] However, in most tissues the overwhelming proportion of administered tryptophan is metabolised along the oxidative, or kynurenine, pathway.

The kynurenine pathway leads to the formation of neuroactive metabolites such as quinolinic acid, which is an endogenous agonist at glutamate N-methyl-D-aspartate (NMDA) receptors,[5] and kynurenic acid, a glutamate antagonist.[6] The pathway also includes compounds such as 3-hydroxykynurenine and 3-hydroxyanthranilic acid, which can generate free radicals even under physiological conditions, as can quinolinic acid itself.[7–9] We have, therefore, measured the metabolism of a tryptophan load along the kynurenine pathway in healthy subjects in parallel with the measurement of two of the major end products of lipid peroxidation—4-hydroxynonenal and malondialdehyde—as a measure of oxidative stress.[10]

METHODS

Subjects

Fifteen healthy subjects (4 males and 11 females) between 21 and 56 years of age were recruited for this study after obtaining ethical permission from the Local Ethical Committee of the Epsom and St. Helier University Hospitals NHS Trust. Informed written consent was obtained from all subjects. These numbers exclude three subjects who consumed less than 80% of the tryptophan loading mixture or who vomited within 2 h of its consumption.

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Tryptophan Loading and Blood Sampling

A resting blood sample was collected through a venous cannula, and a 6 g oral tryptophan loading dose was given as a fruit flavoured solution. Blood samples were taken 5 and 7 h after administration of the tryptophan load. Dietary intake before the study was quantitatively and qualitatively similar to normal for each subject. All subjects were closely monitored by two physicians throughout the study. Blood samples, protected from light, were centrifuged and plasma and serum were removed and frozen within 2 h of venesection. Samples were maintained at −80°C until required for analysis.

Analysis of Kynurenines

HPLC methods have been described previously in Ref. [11]. Sample preparation for HPLC used 480 μl plasma, kept on ice throughout procedure. 20 μl of 1.2 mM internal standard, 3-nitro-L-tyrosine, were added to the plasma, followed by 50 μl 4 M perchloric acid. Samples were vortexed for 30 s, centrifuged at 5000*g* for 10 min, and the supernatant collected. The precipitated proteins were resuspended in 150 μl water and 50 μl 4 M perchloric acid, and vortexed and centrifuged as before. Again the supernatant was collected. This washing and centrifugation step was repeated and the three supernatants combined. Combined supernatants were filtered and centrifuged using 0.2 μm Whatman Vectaspin Micro Anopore tubes. A volume of 100 μl was normally injected on to the HPLC.

A Waters HPLC system was used with a C18 reversed phase column (Phenomenex Kingsorb 250 × 4.6 mm, 5 μm). Samples were analysed using both a dual wavelength UV detector (250 nm for tryptophan and 365 nm for kynurenine) and a fluorescence detector (excitation wavelength 344 nm and emission wavelength 390 nm for kynurenic acid) connected in series. The mobile phase for UV and fluorescence detection was 50 mM acetic acid, 100 mM zinc acetate containing 3% acetonitrile, based on the composition described by Herve *et al.*[12] For electrochemical detection of 3-hydroxykynurenine, 3-hydroxyanthranilic acid and xanthurenic acid, the oxidation voltage was +0.65 V. The mobile phase for electrochemical detection was 50 mM phosphoric acid, 50 mM citric acid, 60 μM EDTA, 8 mM heptane sulphonic acid, 2 mM sodium chloride, with the pH adjusted to 3.1 with potassium hydroxide, and completed by the addition of 5% methanol.

Levels of quinolinic acid were measured by GC/MS of the t-butyldimethylsilyl (tBDMS) derivative, after sample cleanup using solid phase extraction. To 1 ml plasma, 100 μl of the internal standard (10 μM dipicolinic acid) and 2 ml 0.02 M acetate buffer pH 2.0–2.2 was added. A Waters SepPak Vac C18 (500 mg, 3 ml) cartridge was conditioned with 1 ml 0.5 M HC1 and successively washed with 5 ml methanol, 2 ml water and 2 ml 0.02 M acetate buffer pH 2.0–2.2. The sample was applied to the pre-treated cartridge. The cartridge was then washed with 2 ml water, followed by 2 ml hexane and dried before eluting quinolinic acid with 3 × 2 ml 2% ammonia in methanol. 50 μl of 2 μg/ml prazepam in ethanol was added to the quinolinic acid extract and the mixture dried. The dried extract was mixed with 0.5 ml methanol and again dried. 20 μl of derivatising agent, N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) + 1% tert-butyldimethylchlorosilane (TBDMCS) was added and the sample incubated for 2 h at 90°C. The derivatised extract was analysed by GC/MS using a Thermo GC8000 and MD800 quadrupole mass detector. 1 μl sample was injected using splitless injection at 190°C on to a 30 m × 0.2 mm RTX5 GC column. The GC oven temperature was increased from 120°C to 230°C over 5 min followed by a rise to 260°C over 16 min. The detector, using electron impact at 70 eV, was set to maximum voltage monitoring ions at m/z of 338.1 and 380.1 with a dwell time of 0.3 s.

Lipid Peroxidation Products

An aliquot of 100 μl of serum was used for quantifying the concentrations of the lipid peroxidation products malondialdehyde and 4-hydroxynonenal measured using a Bioxytech LPO-586 colorimetric assay (Biostat). The analysis involved the reaction of N-methyl-2-phenylindole with these peroxidation products to form a stable chromogenic indolic dimer which was estimated spectrophotometrically at 586 nm.[13,14] All samples were tested in duplicate.

Statistics

Data are expressed as mean ± 1 S.E.M. Where multiple comparisons were being made comparing a series of data points with the basal level, a repeated measures analysis of variance (ANOVA) was performed followed by Dunnett's post-test. In all cases a significance threshold of 5% ($P < 0.05$) was employed.

RESULTS

Oxidative Stress

Basal levels of the lipid peroxidation products malondialdehyde and 4-hydroxynonenal before tryptophan loading were 0.67 ± 0.08 μM ($n = 15$). There was a significant increase of peroxidation

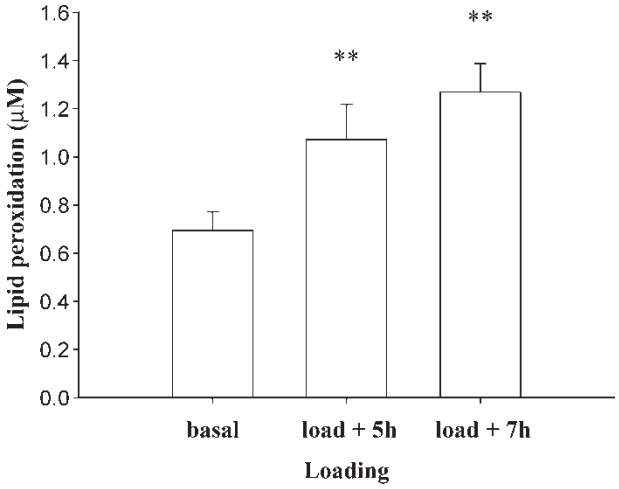

FIGURE 1 Histogram showing the levels of the lipid peroxidation products (malondialdehyde and 4-hydroxynonenal) in healthy subjects at a basal state and 5 and 7 h following an oral tryptophan load. $^{**}P < 0.01$ relative to the basal state.

products at 5 h (71.08% increase; $P < 0.01$) and a further increase at 7 h (109% increase; $P < 0.01$) after tryptophan loading (Fig. 1).

Kynurenines

The effects of tryptophan loading on the kynurenine pathway at the 5 and 7 h time points are summarised in Table I. Significant increases were recorded in the plasma concentrations of tryptophan itself, kynurenine, kynurenic acid, 3-hydroxykynurenine, 3-hydroxyanthranilic acid and quinolinic acid, at 5 h after tryptophan loading, with maintained increases of tryptophan, kynurenine, 3-hydroxyanthranilic acid and quinolinic at 7 h (Table I). The kynurenine/tryptophan (k/t) ratio was also increased significantly at both times ($P < 0.01$), consistent with the evidence that tryptophan can induce activity of the liver enzyme tryptophan-2,3-dioxygenase.

DISCUSSION

The results indicate increased lipid peroxidation as a result of tryptophan loading. Interest in the potential neuropathological relevance of the kynurenine pathway has centred on two primary hypotheses. The first is the ability of kynurenine metabolites to modulate activity at glutamate receptors,[6,9] especially those sensitive to NMDA. Activation of NMDA receptors is known to activate free radical formation both by the conversion of xanthine dehydrogenase into xanthine oxidase, and by the stimulation of nitric oxide synthase. The second reason for interest is in the direct generation of reactive oxygen species such as hydrogen peroxide by 3-hydroxykynurenine and 3-hydroxyanthranilic acid[15,16] as well as quinolinic acid.[7] These properties of the kynurenine pathway could lead to cellular oxidative stress and cellular damage or death. More difficult is assessing whether this increased oxidative stress has been limited by the activity of indoleamine/tryptophan—2,3-dioxygenase (IDO/TDO). This first enzyme of the kynurenine pathway converts tryptophan into kynurenine (Fig. 2) and uses superoxide as a co-substrate. Activation of the pathway should then lower superoxide and thus peroxynitrite levels, and hence reduce total oxidative stress. The results must represent a balance between these conflicting mechanisms which is sometimes injurious[8] and sometimes protective,[17] partly from the generation of the glutamate antagonist kynurenic acid[9] and partly from its using up superoxide. The increased oxidative stress suggested by the present data may also result from the activation by tryptophan of the kynurenine pathway in leucocytes:kynurenine-generated oxidative stress may make a significant contribution to the "toxic burst" generated by macrophages to act against infecting organisms.

Although the emphasis in this paper has been on the effect of tryptophan loading upon the kynurenine pathway, largely because tryptophan loading is known to induce TDO, it is likely that changes will also occur in the other tryptophan metabolic pathways leading to 5HT, melatonin and the kynurenamines. Future work should investigate these other pathways in parallel with the kynurenines in order to obtain a more complete picture of the net effects of tryptophan loading.

In summary, we propose that activation of the kynurenine pathway produces oxidative stress.

TABLE I Levels of tryptophan metabolites (mean ± 1 S.E.M.) in plasma 5 and 7 h after an oral tryptophan load ($n = 15$ in each case). Significant increases ($P < 0.05$) compared to basal: $^{**}P < 0.01$

Compound	Basal (μM)	Tryptophan loaded+5 h (μM)	Tryptophan loaded+7 h (μM)
Tryptophan (t)	65.5 ± 3.9	310.2 ± 14.4 (**)	147.3 ± 10.0 (**)
Kynurenine (k)	1.76 ± 0.36	64.12 ± 9.45 (**)	34.64 ± 4.88 (**)
k/t ratio	0.026 ± 0.004	0.214 ± 0.035 (**)	0.241 ± 0.035 (**)
Kynurenic acid	0.056 ± 0.007	5.29 ± 0.99 (**)	1.28 ± 0.29
3-hydroxy-kynurenine	0.23 ± 0.05	12.29 ± 3.75 (**)	4.74 ± 1.81
3-hydroxy-anthranilic acid	0.18 ± 0.05	1.71 ± 0.28 (**)	0.98 ± 0.24 (**)
Xanthurenic acid	0.03 ± 0.02	8.62 ± 2.74 (**)	1.60 ± 0.72
Quinolinic acid	0.85 ± 0.37	5.70 ± 1.56 (**)	6.21 ± 2.06 (**)

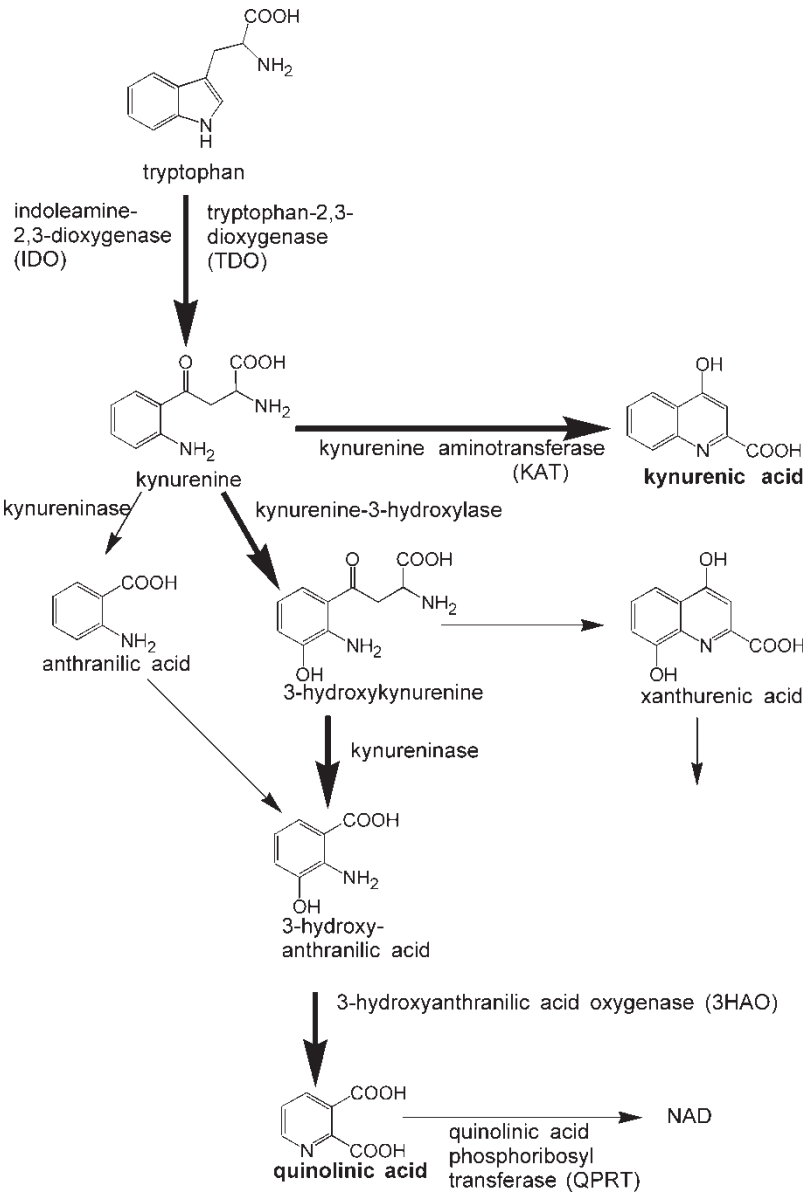

FIGURE 2 A simplified diagram of the kynurenine pathway.

Caution should be exercised in the use of tryptophan loading in subjects in whom the induction of oxidative stress would be contra-indicated. The present results could also have implications for dietary regimens which include a chronically raised intake of tryptophan, such as the Atkins Diet,[18] which has been implicated in the production of cardiovascular disease.

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References

[1] Reilly, J.G., McTavish, S.F.B. and Young, A.H. (1997) "Rapid depletion of plasma tryptophan: a review of studies and experimental methodology", *J. Psychopharmacol.* **11**, 381–392.
[2] Cook, E.H., Anderson, G.M., Heninger, G.R., Fletcher, K.E., Freedman, D.X. and Leventhal, B.L. (1992) "Tryptophan loading in hyperserotonaemic and normoserotonaemic adults", *Biol. Psychiat.* **31**, 525–528.
[3] Huether, G., Hajak, G., Reimer, A., Poeggeler, B., Blomer, M., Rodenbecj, A. and Ruther, E. (1992) "The metabolic fate of infused L-tryptophan in men: possible clinical implications of the accumulation of circulating tryptophan and tryptophan metabolites", *Psychopharmacology* **109**, 422–432.
[4] Luciana, M., Burgun, E.D., Berman, M. and Hanson, K.L. (2001) "Effects of tryptophan loading on verbal, spatial and effective working memory functions in healthy adults", *J. Psychopharmacol.* **15**, 219–230.
[5] Stone, T.W. and Perkins, M.N. (1981) "Quinolinic acid: a potent endogenous excitant at amino acid receptors in the CNS", *Eur. J. Pharmacol.* **72**, 411–412.
[6] Perkins, M.N. and Stone, T.W. (1982) "An iontophoretic investigation of the action of convulsant kynurenines and

their interaction with the endogenous excitant quinolinic acid", *Brain Res.* **247**, 184–187.
[7] Behan, W.M.H., McDonald, M., Darlington, L.G. and Stone, T.W. (1999) "Oxidative stress as a mechanism for quinolinic acid—induced hippocampal damage: protection by melatonin and deprenyl", *Br. J. Pharmacol.* **128**, 1754–1760.
[8] Stone, T.W. (2001) "Kynurenines in the CNS: from endogenous obscurity to therapeutic importance", *Prog. Neurobiol.* **64**, 185–218.
[9] Stone, T.W. and Darlington, L.G. (2002) "Endogenous kynurenines as targets for drug discovery and development", *Nature Revs. Drug Discovery* **1**, 609–620.
[10] Esterbauer, H., Schaur, R.J. and Zollner, H. (1991) "Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes", *Free Radic. Biol. Med.* **11**, 81–128.
[11] Stone, T.W., Mackay, G.M., Forrest, C.M., Clark, C.J. and Darlington, L.G. (2003) "Tryptophan metabolites and brain disorders", *Clin. Chem. Lab. Med.* **41**, 852–859.
[12] Herve, C., Beyne, P., Jamault, H. and Delacoux, E. (1996) "Determination of tryptophan and its kynurenine pathway metabolites in human serum by high-performance liquid chromatography with simultaneous ultraviolet and fluorimetric detection", *J. Chromatogr. B* **675**, 157–161.
[13] Gerard-Monnier, D., Erdelmeier, I., Regnard, K., Moze-Henry, N., Yadan, J.C. and Chaudiere, J. (1998) "Reactions of 1-methyl-2-phenylindole with malondialdehyde and 4-hydroxyalkenals. Analytical applications to a colorimetric assay of lipid peroxidation", *Chem. Res. Toxicol.* **11**, 1176–1183.
[14] Erdelmeier, I., Gerard-Monnier, D., Yadan, J.C. and Chaudiere, J. (1998) "TI Reactions of N-methyl-2-phenylindole with malondialdehyde and 4-hydroxyalkenals. Mechanistic aspects of the colorimetric assay of lipid peroxidation", *Chem. Res. Toxicol.* **11**, 1184–1194.
[15] Eastman, C.L. and Guilarte, T.R. (1990) "The role of hydrogen peroxide in the *in vitro* cytotoxicity of 3-hydroxykynurenine", *Neurochem. Res.* **15**, 1101–1107.
[16] Okuda, S., Nishiyama, N., Saito, H. and Katsuki, H. (1996) "Hydrogen peroxide-mediated neuronal cell death induced by an endogenous neurotoxin, 3-hydroxykynurenine", *Proc. Nat. Acad. Sci. USA* **93**, 12553–12558.
[17] Munn, D.H., Zhou, M., Attwood, J.T., Bondarev, I., Conway, S.J., Marshall, B., Brown, C. and Mellor, A.L. (1998) "Prevention of allogeneic fetal rejection by tryptophan catabolism", *Science* **281**, 1191–1193.
[18] Foster, G.D., Wyatt, H.R., Hill, J.O., McGuckin, B.G., Brill, C., Mohammed, B.S., Szapary, P.O., Rader, D.J., Edman, J.S. and Klein, S. (2003) "A randomized trial of a low-carbohydrate diet for obesity", *N. Engl. J. Med.* **348**, 2082–2090.