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Systemic metabolism of tryptophan and its catabolites, kynurenine and 3-HAA, in mice with inflammatory arthritis

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ABSTRACT

Tryptophan is an essential amino acid. The liver is primary organ involved the oxidative catabolism of tryptophan. However, in the immune system, tryptophan and its catabolites, kynurenine and 3-hydroxyanthranilic acid (3-HAA), play an anti-inflammatory role. Rheumatoid arthritis (RA) is an autoimmune disease. Collagen induced arthritis (CIA) is an animal model of RA. Therefore, it was of interest to measure concentration of tryptophan, kynurenine and 3-HAA in mice with CIA. Concentration of tryptophan and 3-HAA was measured with HPLC methods. Concentration of kynurenine was measured with colorimetric test. mRNA expression for the kynurenine pathway genes was assessed using qRT-PCR. It has been found that in sera from diseased mice concentration of tryptophan was not changed. Concentration of kynurenine and 3-HAA was decreased. Moreover, in the livers from mice with CIA, concentration of tryptophan and kynurenine was decreased. These observations coincided with decreased mRNA expression for *Ido2* and *Afm* and increased mRNA expression for *Kynureninase* in the liver. It has been also shown that in CIA the concentration of 3-HAA was increased in the kidneys.

1. Introduction

Tryptophan is an exogenous amino acid, therefore it needs to be provided in the diet (Willcock, 1906). In addition, tryptophan and its catabolites via the kynurenine pathway (Fig. 1) including kynurenine and 3-HAA are involved in the multiple of physiological processes including immunity (Mellor et al., 2001), mood (Kanai et al., 2009), and blood pressure (Fallarino et al., 2002; Hayashi et al., 2007). In the immune system, it has been shown that an equimolar mixture of tryptophan catabolites: L-kynurenine, 3-hydroxykynurenine (3-HK), 3-HAA, and QA was able to inhibit the production of interleukin 17 (IL-17), a pro-inflammatory cytokine, in a dose dependent manner (Desvignes and Ernst, 2009). Catabolities of tryptophan can also abrogate the development of Th17 cells (Desvignes and Ernst, 2009). These cells can exacerbate inflammation (Bettelli et al., 2006). Conversely, a low concentration of tryptophan together with an equimolar mixture of L-kynurenine, AA, 3-HK, 3-HAA, and QA promoted conversion of naive $CD4^+$ T cells into $CD4+CD25^+Foxp3^+$ regulatory T cells (Treg) (Fallarino et al., 2006). In contrast to Th17 cells, Treg(s) can dampen inflammation (Bettelli et al., 2006).

Rheumatoid arthritis (RA) is an autoimmune systemic disease predominantly affecting diarthrodial joints (Smolen and Aletaha, 2009). It is estimated that around 1% of western population suffers from RA (Miller et al., in press). However, patients with RA are also more susceptible for major depression (Covic et al., 2012) and cardiovascular diseases (Kerola et al., 2012; Paccou et al., 2012). Nonetheless, the major symptoms of RA like e.g. bone erosions and degradation of cartilage are primarily driven by the exacerbated immune responses (Schett, 2011). Therefore, damping inflammation has been proven to be an effective way of preventing joint damage in RA (Smolen and Steiner, 2003). It has been also demonstrated that successful therapy of RA leads to the restoration of a functional balance between Treg(s) and Th17 cells (Wright et al., 2011). Collagen induced arthritis (CIA) is an animal model of RA (Inglis et al., 2008). However, unlike RA, CIA is a self remitting disease with three, relatively well defined stages (Inglis et al., 2008; Stuart et al., 1982). A pre-arthritic phase usually lasts 14 days after the induction of the disease. In contrast, CIA is considered to be established 10 days after the first symptoms of CIA (e.g. joint oedema, swelling, and redness) are spotted (Stuart et al., 1982). In the third stage, the disease enters a self remitting phase which manifests in the reduction of the symptoms of inflammation (Stuart et al., 1982). Interestingly, it has been proven that inhibition of INDOLEAMINE 2, 3 DIOXYGENASE 1 (IDO1), one out of three enzymes involved in the initiation of tryptophan catabolism via the kynurenine pathway (Kolodziej et al., 2011), resulted in the exacerbated symptoms of CIA (Criado et al., 2009). This observation coincided with increased mRNA expression for *Ido1* in the inguinal lymph nodes (iLN) taken form mice with established CIA (Criado et al., 2009). However, upon physiological conditions not iLN

Abbreviations: 3-HAA, 3-hydroxyanthranilic acid; 3-HK, 3-hydroxykynurenine; AA, anthranilic acid; AFM, arylformamidase; CIA, collagen induced arthritis; HAAO, 3-hydroxyanthranilate 3,4 dioxygenase; HPLC, high pressure liquid chromatography; IDO, indoleamine 2,3 dioxygenase; iLN, inguinal lymph nodes; KMO, kynurenine 3-monooxygenase; KYNU, Kynureninease; QA, Quinolinic acid; RA, rheumatoid arthritis; TDO, tryptophan 2,3 dioxygenase.

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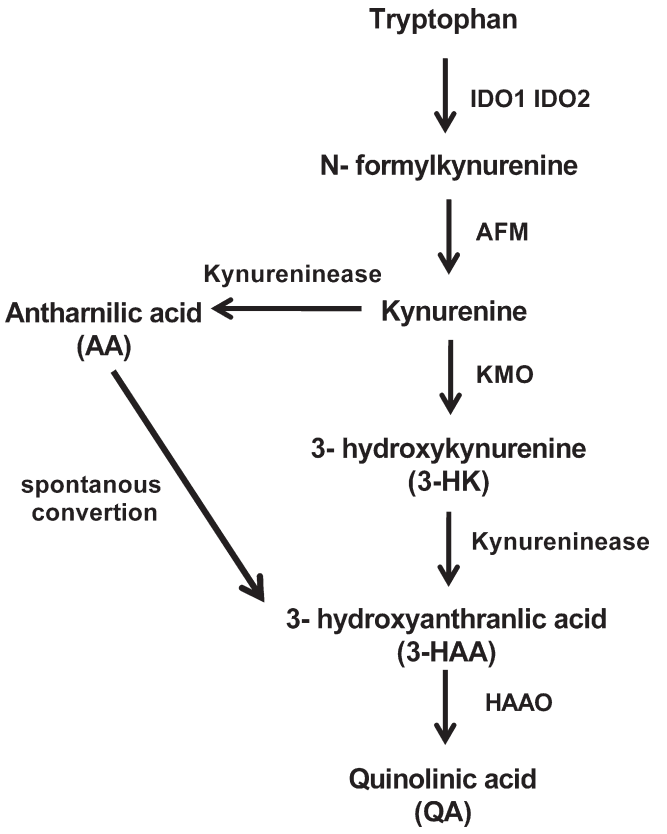

Fig. 1. Simplified schematic representation of the kynurenine pathway. Enzymes involved in tryptophan metabolism via the kynurenine pathway: indoleamine 2,3 dioxygenase 1 (EC 1.13.11.17) **IDO1**, indoleamine 2,3 dioxygenase 2 (EC 1.13.11.52) **IDO2**, arylformamidase (EC 3.5.1.9) **AFM**, kynurenine 3-monooxygenase (EC 2.6.1.7) **KMO**, Kynureninease (EC 3.7.1.3), 3-hydroxyanthranilate 3,4 dioxygenase (EC 1.13.11.6) **HAAO.**

but the liver is a primary organ involved in the regulation of tryptophan concentration in the blood (Kolodziej et al., 2011; Saito et al., 1993; Takikawa et al., 1986). Moreover, during gynaecological cancers (de Jong et al., 2011) and in mice with CIA (Criado et al., 2009; Szanto et al., 2007) kynurenine to tryptophan ratio has been reported to be altered suggesting that during certain diseases concentration of tryptophan and its catabolites in serum could be regulated by the activity of the kynurenine pathway acting locally e.g. iLN.

The end products of metabolism are excreted in urine. Interestingly, it has been reported that urine from patients with RA contained an increased concentration of 3-HAA (Beetham et al., 1964; Labadarios et al., 1978; Spiera, 1963). Hence, an anti-inflammatory action of 3-HAA could be hampered by its excessive disposal in urine resulting in the reduction of the concentration of 3-HAA in the blood.

Thus, taken together, it was of interest to test if CIA could affect metabolism of tryptophan, kynurenine and 3-HAA in the liver. In addition, changes in the mRNA expression for the subsequent enzymes on the kynurenine pathway were assessed in the liver. The concentration of 3-HAA has been also measured in the kidneys taken from naive and the diseased mice. The concentration of tryptophan, kynurenine, and 3-HAA in the sera was measured in the diseased mice as well as in the healthy animals.

2. Materials and methods

2.1. Experimental animals

In this study, male DBA/1 mice of age between 8 and 12 weeks were purchased from Harlan (Bicester), UK. Mice were housed in groups of 6–8, and maintained at 21 °C ± 2 °C on a 12 hour light/dark cycle (7 am–7 pm) with food and water *ad libitum*. All experimental procedures were approved by the UK Home Office.

2.2. Development of CIA and tissue harvesting

Male DBA/1 mice (8–12 weeks old) were immunized subcutaneously at the base of the tail with bovine type II collagen (200 μg) emulsified in complete Freund's adjuvant (CFA 3.3 mg *Mycobacterium tuberculosis* per ml of oil; Difco, West Molesley, UK). Mice were sacrificed 14 days after immunisation (pre-arthritic animals) or 10 days after onset of disease (established arthritis) (Inglis et al., 2008). Mice were killed at the appropriate time points and tissues (liver and kidneys) were immediately frozen on dry ice and kept at −80 °C.

2.3. Isolation of tryptophan, AA, and 3-HAA from serum, livers and the kidneys

Tissues were homogenised in buffer containing: 50 mM Tris–HCl pH = 7.7, 150 mM NaCl (Sigma), 5 mM $CaCl_2$ (Sigma) using the PRECELLYS^R 24 lyser/homogeniser (Bertin Technologies, France) with two cycles of shaking separated by 20 seconds apart, each lasting for 45 seconds in the room temperature. After homogenisation, samples (including sera) were centrifuged at 14,000*g* and the supernatant was collected and mixed with 150 μl of a solution containing: 2 mM ascorbic acid (Sigma), 240 μM 3-nitrotyrosine (3-NT); (Sigma), 4 M perchloric acid (PA); (AnalaR) (Forrest et al., 2010). The samples were then vigorously vortexed for 30 seconds and centrifuged at 5000 g for 15 min. The supernatant was collected and the pellets were resuspended in 150 μl of 4 M PA, vortexed for 30 seconds and centrifuged at 5000 rpm. This procedure was repeated three times in order to ensure efficient recovery of metabolites. Finally, pooled supernatants were transferred into the filtration tubes (0.22 mm cellulose acetate, Spin-X® Costar®) and centrifuged at 3000 *g*.

2.4. HPLC analysis

The concentration of tryptophan, AA, and 3-HAA was determined with HPLC method. The HPLC system (UltiMate 3000) was provided by Dionex, UK. All chromatographic procedures were performed in 37 °C, with C18 column (Acclaim 120, Dionex, UK) 3 μm, 120 Å; 4.6 × 150 mm, and injection volume of 10 μl. Tryptophan concentration was determined by HPLC with fluorescence detection (excitation λ = 284 nm; emission λ = 365 nm). The mobile phase (1 ml/min flow rate) consisted of 50 mM acetic acid, 100 mM zinc acetate, and 3% acetonitrile).

The concentration of AA and 3-HAA was determined by HPLC with fluorescence detection (excitation λ = 320 nm; emission λ = 420 nm). The mobile phase (1 ml/min of flow rate) consisted of 25 mM sodium acetate (Sigma), 1 mM acetic acid (pH 5.5).

Tryptophan, AA, and 3-HAA concentration in tissues were calculated based on the standard curves derived from analysis of pure substances provided by Sigma and adjusted to the tissues mass. In order to increase precision and reduce errors due to the differences in small molecule recovery 3-NT (present in the homogenisation solution) was used as an internal/external standard.

2.5. Kynurenine measurements

Kynurenine concentration was assessed by colorimetric assay (Hara et al., 2008). An equal volume (100 μl) of Ehrlich reagent (0.4% *p*-dimethylaminobenzaldehyde, Sigma, in acetic acid) was mixed with the tissue homogenate (100 μl). Absorbance was determined at 490 nm. Kynurenine concentration in the tissues was calculated from the standard curve prepared from kynurenine (Sigma) and adjusted to the tissues mass.