The kynurenine metabolic pathway in the eye: studies on 3-hydroxykynurenine, a putative cataractogenic compound

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Abstract The rabbit lens has an elevated content of 3-hydroxykynurenine (3OHKYN) in spite of a very low activity of the enzymes leading to its synthesis. The iris/ciliary body, on the contrary, has very high activity of 3OHKYN synthesizing enzymes but a content of 3OHKYN lower than that of the lens. These observations suggest that 3OHKYN is formed in the iris/ciliary body, released into the aqueous humor and then taken up into the lens where it may be used for the synthesis of UV filtering products. An excessive accumulation of 3OHKYN in the lens has been associated with cataract formation. We found that available selective inhibitors of kynurenine hydroxylase reduced 3OHKYN synthesis in both the lens and the iris/ciliary body.

Key words: Tryptophan metabolism; Kynurenine; Lens; Iris/ciliary body; Cataract; 3-Hydroxykynurenine; Kynurenine hydroxylase

1. Introduction

It has been proposed that 3-hydroxykynurenine (3OHKYN), a tryptophan (TRP) metabolite formed along the 'kynurenine metabolic pathway', may play a role in visual function because it is present in elevated concentrations in the lens and is able to absorb UV radiation [1]. Indeed, 3OHKYN itself, its glucoside derivative and the glucoside derivatives of 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid, one of the numerous 3OHKYN metabolites, are effective filters of sunlight and their presence in the lens of diurnal animals may be useful in protecting the retina from UV radiation [2–4]. However, a key role for 3OHKYN and its metabolites has also been proposed in the processes leading to lens opacification and senile cataract formation because they may undergo complex autoxidative processes which may lead to the formation of radical species able to react with crystallins [5–9]. The localization and properties of the enzymes of the kynurenine pathway which catalyze the reactions leading from TRP to pyridine nucleotides (NAD and NADP) have been widely studied in the past several years (see [10,11] for reviews) and it has also been shown that lens epithelial cells synthesize 3OHKYN when incubated with labeled TRP [3]. However, quantitative studies aimed at understanding the rate of 3OHKYN synthesis and catabolism in different eye structures are not available. In this study we measured the content of 3OHKYN and related metabolites together with the activity of the kynurenine pathway enzymes in different tissues of the rabbit eye, in order to study their role in visual function. The results we obtained showed that the enzymes leading to 3OHKYN synthesis are mostly present in the iris/ciliary body while the lens, in spite of an elevated content of this compound, has a very low activity of the enzymes responsible for its synthesis.

Several selective inhibitors of kynurenine hydroxylase (EC 1.14.13.9) (KH), the key enzyme in the synthesis of 3OHKYN, have previously been described utilizing brain or liver enzymatic preparations [12–15]. We report here that these inhibitors are also effective in the iris/ciliary body and the lens and therefore seem useful tools to study the role of 3OHKYN in the eye.

2. Materials and methods

2.1. Materials

(*m*-Nitrobenzoyl)-alanine (mNBA), 3,4-dimethoxy-[*N*-4-(3-nitrophenyl) thiazol-2-yl]-benzenesulfonamide (Ro 61-8048) [15] and L-[3-^{3}H]kynurenine (specific activity 14.4–16.1 Ci/mmol; 1 Ci = 37 GBq) were kindly provided by Drs. S. Roever and A. Cesura (Hoffman-La Roche, Basel, Switzerland). [$^{13}_{7}$C]QUIN was provided by Dr. J.F. Reinhard Jr. (Glaxo-Wellcome, Research Triangle Park, NC, USA). Kynurenine (KYN), kynurenic acid (KYNA), anthranilic acid (ANA), 3-hydroxyanthranilic acid (3OHANA), 3OHKYN, xanthurenic acid (XANT) and quinolinic acid (QUIN) were from Sigma (St. Louis, MO, USA). Hexafluor-2-propanol and trifluoroacetyl-imidazole were from Aldrich (Milan, Italy). All remaining compounds were from Merck (Darmstadt, Germany).

2.2. Tissue preparation

Tissues were obtained from male New Zealand rabbits (2.5–3 kg) killed with an i.v. injection of 100 mg sodium thiopental. The eyes were rapidly collected, the cornea was removed and the bulbs dissected into iris/ciliary body, lens, vitreous and humor aqueous. A portion of the liver, lung and blood of each animal was also collected and all tissues were stored at −80°C until processed. 0.5 g of each tissue was homogenized with an equal volume of 20% trichloroacetic acid and centrifuged at 14 000 rpm for 10 min. Aliquots of supernatants were injected in the HPLC apparatus for measurements of the contents of KYN, 3OHKYN, ANA, 3OHANA, XANT and KYNA. A portion of the supernatant was extracted with chloroform for QUIN measurement (see below). Another portion of each tissue was homogenized in eight volumes (w/v) of ice-cold 0.1 M potassium phosphate buffer pH 8 containing EDTA (1 mM), dithiothreitol (1 mM), leupeptin (2 μg/ml), aprotinin (2 μg/ml) and phenylmethylsulfonyl fluoride (100 μg/ml) for the evaluation of the enzymatic activities. The homogenates were centrifuged at 15 000 rpm for 10 min to obtain a crude preparation of mitochondria which was re-suspended in 8 volumes (w/v) of the homogenization buffer.

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Abbreviations: ANA, anthranilic acid; 3OHANA, 3-hydroxyanthranilic acid; KYN, kynurenine; KYNA, kynurenic acid; 3OHKYN, 3-hydroxykynurenine; XANT, xanthurenic acid; QUIN, quinolinic acid; TRP, tryptophan; IDO, indoleamine 2,3-dioxygenase; KH, kynurenine hydroxylase; KASE, kynureninase; KAT, kynurenine aminotransferase; mNBA, (*m*-nitrobenzoyl)-alanine; Ro 61-8048, 3,4-dimethoxy-[*N*-4-(3-nitrophenyl) thiazol-2-yl]-benzenesulfonamide

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2.3. Evaluation of enzyme activities

Indoleamine 2,3-dioxygenase (IDO) (EC 1.13.11.42) activity was evaluated according to Takikawa et al. [16]. The reaction mixture consisted of 50 μl of the cytosol preparation of tissue homogenates and 50 μl of 0.1 M phosphate buffer pH 6.5 containing 20 mM ascorbate, 50 μM methylene blue, 200 μg catalase and 2 mM TRP. After 30 min incubation at 37°C, the reaction was terminated with 100 μl of 20% (w/v) trichloroacetic acid, and the mixtures centrifuged at 14 000 rpm for 5 min. Aliquots of the supernatants were injected into the HPLC apparatus for measurements of KYN synthesis.

KH activity was evaluated in some experiments by monitoring the synthesis of 3OHKYN using HPLC and electrochemical detection [12] and in other experiments by using the radiometric method described by Erickson et al. [17]. In the first approach the reaction mixture was: 50 μl of crude mitochondria preparation and 50 μl of 0.1 M phosphate buffer pH 8 containing NADPH 8 mM and KYN 2 mM. After 30 min incubation at 37°C, the reaction was terminated with 100 μl of 20% (w/v) trichloroacetic acid, and the samples centrifuged at 14 000 rpm for 5 min. Aliquots of the supernatant were injected into the HPLC apparatus. In the second approach, 0.2 μCi of L-[3-^{3}H]KYN was added to the reaction mixture. In this case the reaction was terminated by the addition of 500 μl of a 5% suspension of activated charcoal (Norit A) and after shaking for 1 min, the samples were centrifuged at 14 000 rpm for 10 min. 250 μl of the supernatants were added to vials containing 4 ml of scintillation fluid (Insta-Gel Plus, Packard) for the measurement of radioactivity. These methods gave similar results, and the use of radioactivity was preferred for tissues with low KH activity.

The activity of the enzyme able to cleave 3OHKYN and KYN (kynureninase, KASE) (EC 3.7.1.3) was evaluated by measuring the formation of ANA. The enzymatic mixture consisted of 50 μl of the cytosol preparation and 50 μl of 0.1 M phosphate buffer pH 8 containing 200 μM pyridoxal phosphate and 2 mM KYN. After 30 min of incubation at 37°C, the reaction was terminated with 100 μl of 20% (w/v) trichloroacetic acid, and the samples centrifuged at 14 000 rpm for 5 min. Aliquots of the supernatant were injected in the HPLC apparatus for ANA quantitation (see below).

Kynurenine aminotransferase (KAT) is the second enzyme able to metabolize KYN and 3OHKYN with the synthesis of KYNA and XANT, respectively. This activity was evaluated by measuring XANT formation. The reaction mixture consisted of: 50 μl cytosol preparation, 50 μl 0.1 M phosphate buffer pH 8, 200 μM pyridoxal phosphate, 20 mM α-ketoglutaric acid and 2 mM 3OHKYN. After 30 min incubation at 37°C, the reaction was terminated with 100 μl of 20% (w/v) trichloroacetic acid. The samples were centrifuged at 14 000 rpm for 5 min and aliquots of the supernatant were injected in the HPLC apparatus for the measurement of XANT (see below).

2.4. Measurements of KYN, KYNA and XANT

KYN, KYNA and XANT were measured using HPLC and UV detection as described by Holmes [18]. Briefly, a reverse phase column (Spherisorb S5 ODS2, 10 cm) and a mobile phase containing 0.1 mM ammonium acetate, 0.1 M acetic acid and 2% acetonitrile, 1 ml/min flow rate were used. KYN was detected at 365 nm while KYNA and XANT were detected at 338 nm (UV detector: Perkin Elmer model LC 90).

2.5. Measurements of ANA and 3OHANA

ANA and 3OHANA were measured using HPLC and fluorimetric detection as previously described [14]. Separation was obtained using a reverse phase column (S5 ODS2, 15 cm) and a mobile phase (flow rate of 0.8 ml/min) composed of 20 mM sodium acetate buffer pH 4.5 and 5% methanol. Detection was obtained with a Perkin Elmer model LC 240 fluorimeter: excitation and emission wavelengths were 313 and 420 nm respectively.

2.6. Measurements of 3OHKYN

3OHKYN was measured using HPLC and electrochemical detection [19]. Separation was obtained with a reverse phase column (Spherisorb S5 ODS2, 25 cm) and a mobile phase (flow rate 1.5 ml/min) composed of 2% acetonitrile, 0.9% triethylamine, 0.59% phosphoric acid, 9 mM heptane sulfonic acid and 0.25 mM sodium EDTA. Detection was obtained with a coulometric detector (ESA model 5100 A) operating at a preoxidation voltage of 0.03 V and an oxidation voltage of 0.23 V.

2.7. Measurements of QUIN

QUIN was measured using GC/MS and a modification of procedures previously reported [20,21]. Briefly, after the addition of [$^{13}_{7}$C]quinolinate as an internal standard, each sample was dried and derivatized at 80°C for 1 h with 100 μl of hexafluoro-2-propanol and 100 μl of trifluoroacetyl-imidazole. 100 μl of water and 100 μl of heptane were added to the derivatized samples which were vigorously mixed and then frozen at −80°C. The heptane fraction was collected and injected into a GC/MS system (HP 6890/5973 MSD, HP5973MSD) equipped with an automatic injector. The chromatographic column used was a HP 5MS 30 m×0.25 mm×0.25 μm. The carrier gas was helium at a constant flow of 1.2 ml/min. The oven temperature was: 1 min at 80°C, raised at a rate of 10°C/min to 135°C and then at a rate of 25°C/min to 300°C. Injector and transfer line temperatures were 230°C and 270°C respectively. The mass spectrometry detector operated in negative ion chemical ionization mode using methane as a negative gas. The recorded ions were *m/z* 467 for quinolinate and *m/z* 474 for [$^{13}_{7}$C]quinolinate. The dwell time was 70 ms for each ion.

2.8. Protein determination

Protein content was determined by the Pierce bicinchoninic acid assay kit according to the manufacturer's instructions.

3. Results

3.1. Content and distribution of the 'kynurenines' in the eye

Table 1 reports the content of the 'kynurenines' in the iris/ciliary body, lens, aqueous humor, vitreous and blood. The concentration of 3OHKYN in the lens was seven times higher and in the iris/ciliary body two times higher than in the blood. The concentration of XANT, a direct 3OHKYN metabolite, was also significantly higher in the lens and in the iris/ciliary body than in the blood. The concentration of 3OHANA,

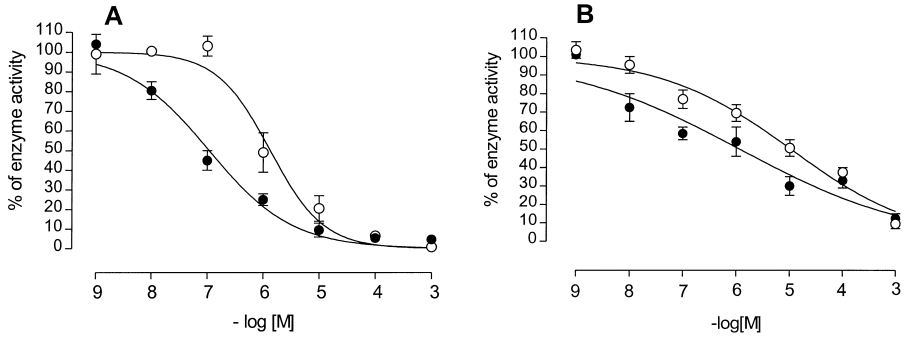

Fig. 1. Inhibition of iris/ciliary body (A) and lens (B) kynurenine hydroxylase activity by Ro 61-8048 (filled circles) and mNBA (open circles). The concentration of KYN used was 100 μM (see Section 2). Each point is the mean ± S.E.M. of three experiments conducted in duplicate.

Table 1
Contents of the 'kynurenines' in rabbit blood and eye structures

	KYN	KYNA	ANA	3OHKYN	XANT	3OHANA	QUIN
Iris/ciliary body	6.7 ± 0.43	0.9 ± 0.5	0.12 ± 0.01	1.85 ± 0.5*	1.72 ± 0.4*	0.04 ± 0.01*	1.04 ± 0.4**
Aqueous humor	1 ± 0.2**	0.16 ± 0.03*	0.02 ± 0.01*	0.6 ± 0.2	0.04 ± 0.01	0.05 ± 0.01*	0.16 ± 0.05
Lens	2.4 ± 0.2*	0.02 ± 0.01**	0.08 ± 0.03	5 ± 1.3**	0.25 ± 0.06*	0.06 ± 0.01*	0.02 ± 0.01*
Vitreous	0.46 ± 0.11**	0.02 ± 0.01**	0.2 ± 0.1*	0.14 ± 0.05*	0.05 ± 0.01	0.05 ± 0.02*	0.02 ± 0.01*
Blood	7.3 ± 1.8	0.64 ± 0.3	0.11 ± 0.01	0.73 ± 0.16	0.07 ± 0.02	0.19 ± 0.05	0.22 ± 0.08

Values are nmol/g or nmol/ml (mean ± S.E.M. of at least five samples). *$P < 0.05$; **$P < 0.01$ vs. blood, ANOVA and Tukey *t*-test.

another 3OHKYN metabolite, was, on the contrary, three times lower in the lens and iris/ciliary body than in the blood.

3.2. Distribution of the kynurenine pathway enzymes in the eye

In order to study why the iris/ciliary body and lens had 3OHKYN concentrations higher than the blood, we measured in these eye structures the activity of the following enzymes: IDO, KH, KASE and KAT, which are involved in the synthesis and catabolism of 3OHKYN. Table 2 shows that IDO activity was particularly elevated in the iris/ciliary body where it reached a level similar to that found in the lung, an organ with very high enzymatic activity [22]. KH activity was also notably elevated in the iris/ciliary body where it reached a level 10-fold higher than that observed in the liver, an organ particularly rich in this enzyme [23] (Table 2). The enzymatic activities responsible for 3OHKYN catabolism (namely, KASE and KAT) were several times lower in the iris/ciliary body than in the liver (Table 2). Unexpectedly, the activity of the enzymes responsible for 3OHKYN formation was quite low in the lens, a structure having a high 3OHKYN content (see Tables 1 and 2).

3.3. Effects of Ro 61-8048 and mNBA.

Ro 61-8048 and mNBA inhibited KH activity in the iris/ciliary body with IC_{50}s of 0.1 ± 0.06 and 1.3 ± 0.2 μM, respectively (Fig. 1A). In the lens, the IC_{50} was 1 ± 0.2 for Ro 61-8048 and 7 ± 2 μM for mNBA (Fig. 1B).

4. Discussion

The present data show that: (1) the iris/ciliary body has a very high level of 3OHKYN-forming and a low level of 3OHKYN-catabolizing enzyme activities; (2) the lens contains elevated 3OHKYN concentrations in spite of low activities of 3OHKYN-forming enzymes (see Tables 1 and 2). It is therefore reasonable to propose that 3OHKYN is mostly formed in the iris/ciliary body, released into the aqueous humor and then taken up into the lens where it may be used for the synthesis of UV-filtering compounds. In support of this hypothesis is the demonstration that 3OHKYN is more concentrated in the anterior portion of the lens [3] and that the lens epithelial cells express the neutral amino acid carrier which is able to take up KYN and 3OHKYN [24–26]. An excessive accumulation of 3OHKYN or of its metabolites, however, may participate in the processes leading to the formation of free radical species which are responsible for a number of tissue injuries [27,28] including lens opacification and cataract formation [9,29,30]. In this respect XANT seems particularly important, as it is present in the lens at a concentration approximately four times that of the blood (see Table 1) and it may be metabolized into oxo- and dioxo-XANT, both of which have been involved in the cataractogenic process [31,32]. Another 3OHKYN metabolite previously studied in the process of cataract formation is 3OHANA, which may easily undergo autoxidation and be responsible for the synthesis of reactive compounds (cinnabarinic acid and *p*-quinone dimer) which seem to participate in the process of lens opacification [7]. However, in the lens, the activity of the 3OHANA-forming enzyme (KASE) is relatively low (Table 2) and 3OHANA content is significantly lower than in blood (Table 1). These observations tend to rule out the possibility that 3OHANA has a basic role in cataract formation processes. Our proposal that the iris/ciliary body releases 3OHKYN into the aqueous humor could explain the elevated content of this compound in the lens. However, we cannot completely rule out the possibility that the low activity of 3OHKYN-forming enzymes in the whole lens is the result of a high activity in the epithelium diluted by the protein of the whole lens.

The extremely elevated activity of 3OHKYN-forming enzymes in the iris/ciliary body may suggest that 3OHKYN plays other important roles in this eye structure. In insects, these enzymes are involved in the synthesis of eye pigments [33] and it is reasonable to extend these observations to mammals.

We have previously studied the pharmacological effects of Ro 61-8048 and mNBA, two KH inhibitors [12,13,34], and the present studies show that these compounds are not only active in the liver and the brain, but also in the iris/ciliary body and lens (Fig. 1). Differences in IC_{50} values can probably be explained by unspecific binding to proteins. It can therefore be proposed that the above mentioned KH inhibitors may be useful tools for reducing 3OHKYN content in the eye and for studying the role of this TRP metabolite in eye physiology and pathology.

Table 2
IDO, KH, KASE and KAT activity in rabbit iris/ciliary body, lens, liver and lung

	IDO	KH	KASE	KAT
Iris/ciliary body	326 ± 44	130 ± 20	0.46 ± 0.17	5.8 ± 0.6
Lens	8 ± 1.2	2.3 ± 0.7	0.13 ± 0.02	0.06 ± 0.01
Liver	62.5 ± 11	17.2 ± 5	247 ± 7	82.5 ± 12
Lung	425 ± 53	1.15 ± 0.4	4.4 ± 0.6	2.32 ± 0.7

Values are pmol/mg protein/min (mean ± S.E.M. of five experiments).

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