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*Original Research*

# Can Kynurenine Pathway Tryptophan Metabolites Be Used to Monitor Cadmium Exposure?

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**Abstract**

We evaluated the possibility of using the urinary concentrations of tryptophan metabolites such as kynurenine (KYN) and kynurenic acid (KYNA) for monitoring cadmium (Cd) exposure and detecting early effects of its action in the kidney. For this purpose, we analyzed correlations between urinary excretion of both metabolites, Cd concentration and the activity of isoenzyme B of N-acetyl-β-D-glucosaminidase (NAG-B), recognized as one of the most sensitive markers of Cd nephrotoxicity. The study was conducted on rats using an experimental model, corresponding to human environmental and occupational exposure to Cd. The rats were administered 5 and 50 mg Cd/l of drinking water for 24 weeks. The administration of Cd resulted in a marked dose-dependent increase in KYN and KYNA elimination. Regression analysis revealed a linear correlation between urinary Cd concentration and KYN or KYNA excretion as well as between urinary NAG-B activity and KYN or KYNA elimination. We hypothesize that metabolites of tryptophan via kynurenine pathway such as KYN and especially KYNA can be used to monitor chronic exposure to Cd.

**Keywords:** cadmium, kynurenine, kynurenic acid, N-acetyl-β-D-glucosaminidase, rats

## Introduction

One of the first health effects of chronic environmental as well as occupational exposure to cadmium (Cd) is an injury of renal proximal tubules, which usually starts insidiously and is irreversible [1, 2]. The kidney has been considered the critical organ for Cd toxicity following long-term exposure in humans and experimental animals [3]. The kidney is characterized by its ability to compensate for renal damage and for this reason classical tests (e.g. creatinine or urea concentration in serum) are insensitive, since they only deviate late in the cascade of damage-events when already a large part of the nephron mass is lost. It is thus of paramount importance that tools for detecting Cd nephrotoxicity be sensitive enough to detect early events and, ideally, they should reflect subclinical reversible changes [4].

Previously we noted disturbances in tryptophan metabolism via kynurenine pathway under Cd exposure [5]. Moreover, literature data and our observations indicate a relationship between kidney function and tryptophan (TRP) metabolism [6, 7, 8, 9].

The kidneys have double influence on TRP conversion. They constitute the major way to eliminate its derivatives, mostly in the form of kynurenine (KYN), 3-hydroxykynurenine (3HKYN), kynurenic acid (KYNA), and xanthurenic acid (XA) [10, 11, 12]. On the other hand, they have a very rich enzymatic system, taking part in the kynurenine pathway (Fig. 1). Possessing indoleamine 2,3-dioxygenase, kynurenine 3-hydroxylase, kynureninase and kynurenine aminotransferase, the kidney is practically the second most important organ for kynurenine pathway besides the liver [13, 14, 15].

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It inspired us to evaluate the possibility of using urinary concentration of kynurenine metabolites for the monitoring of chronic exposure to Cd. Therefore, we created an experimental model using rats, corresponding to human environmental and occupational exposure to Cd, and estimated (biochemically, histopathologically and histochemically) the degree of damage to the kidney structure and function (data considered for publication). In the present paper, we evaluated the usefulness of chosen kynurenine metabolites for the monitoring of early effects of Cd exposure. For this purpose, we assessed the effect of Cd on the urinary excretion of KYN and KYNA and compared the urinary concentrations of both kynurenine metabolites with urinary Cd excretion and activity of NAG-B recognized as one of the most sensitive indicators of Cd-induced proximal tubular injury [4, 16].

## Materials and Methods

### Animals and Experimental Design

Inbred adult (2-month-old) male albino rats (Wistar strain) of initial body weight of about 180-200 g were used. The animals were housed in conventional conditions, at 22 ± 1°C, with a relative humidity of 50 ± 10% and a 12-hr light/dark cycle. They were allowed free access to drinking water (redistilled water or water solutions of $CdCl_2$) and rat chow (LSM dry diet, Fodder Manufactures, Motycz, Poland). Cd concentration in the diet was assessed in our laboratory to be 0.211 mg/kg.

The rats were allocated randomly to three experimental groups. Two groups received as the only drinking water solution of $CdCl_2$ (POCH, Poland) at a concentration of 5 or 50 mg Cd/l. Control rats drank redistilled water (uncontaminated with Cd). To assess Cd intake, 24-hour consumption of drinking water was measured for the whole course of the experiment. To collect 24-hour urine, after 1, 2, 3, 4, 5, 6, 12, 18 and 24 weeks of the exposure, the rats were placed in individual metabolic cages. Cd, NAG-B, KYN and KYNA were determined in the urine.

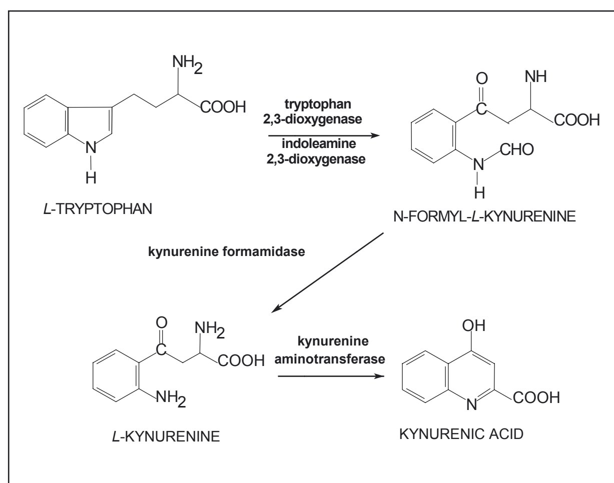


Fig. 1 Scheme of kynurenine pathway.

The study was approved by the Local Ethics Committee for animal experiments in Bialystok. Procedures involving the animals and their care conformed to the institutional guidelines, in compliance with national and international Laws and Guidelines for the Use of Animals in Biomedical Research [17].

### Chemicals

For all determinations ultra pure water received from water purification Milli-Q system (Millipore Corporation, USA) was used. All reagents and chemicals were of analytical grade or highest purity.

### Cadmium Analysis

Cd concentration in urine, after appropriate dilution with ultra pure water, was determined using flameless atomic absorption spectrometry (Atomic Absorption Spectrophotometer Z-5000, Hitachi, Japan) with electrothermal atomization in a graphite cuvette. The cathode lamp of Cd (Photron) was operated under standard conditions using its respective resonance line of 228.8 nm. The detection limit was 0.08 µg Cd/l. For Cd calibration a stock atomic absorption standard solution (Sigma, USA) was used. Internal quality control was employed to keep the measurement process reliable.

### N-Acetyl-β-D-Glucosaminidase Determination

The urinary activity of NAG-B was determined colorimetrically (Hitachi U-3010 spectrophotometer, Japan) according to Zwierz et al. [18], using p-nitrophenyl-N-acetyl-β-D-glucosamide as substrate (Sigma, USA).

### Kynurenine Determination

KYN concentration was determined by high-performance liquid chromatography (HPLC) according to Holmes [19]. The chromatographic system (Hewlett-Packard, Germany) was composed of a HP1050 pump and Rheodyne injection valve fitted with a sample loop (20 µl). Guard column - LiChrospher 100 RP-18. 5µm, 4x4 mm (Germany) was placed before the $C_{18}$ reversed - phase column (LiChrospher 100 RP – 18.5µm, 125x4 mm). The column effluent was monitored with HP 1050 series UV detector (365 nm). The output of the detector was connected to a single instrument LC-2D ChemStation (Germany). The mobile phase was composed of 0.1 M acetic acid, 0.1M ammonium acetate (pH 4.65) containing 2% of acetonitrile and it was pumped at a flow-rate of 1.5 ml/min. Chromatography was carried out at 25°C.

All reagents used in HPLC analysis of KYN and KYNA were obtained from Merck (Germany).

### Kynurenic Acid Determination

KYNA concentration was determined according to Hevere et al. [20]. The reversed-phase HPLC system consisted of a Waters Spherisorb S3 ODS2 150x2.1 mm column, HP 1050 series pump, Rheodyne injection valve fitted with a sample loop (5 µl). The column effluent was monitored using a programmable fluorescence detector HP 1046A. The optimized conditions were determined by recording fluorescence spectra with a stop-flow technique. Excitation and emission wavelengths were set to 254/404 nm for TRP and KYNA and 320/420 nm for AA. The output of the detector was connected to a single instrument LC-2D ChemStation. The mobile phase consisted of 50 mM acetic acid, 0.25 M zinc acetate (pH-4.9), containing 1.2% of acetonitrile and it was pumped at a flow-rate of 0.25 ml/min. Chromatography was carried out at 25$^{o}$C.

Recovery tests were performed in triplicate by spiking the sample before deproteinization with 0.2 µM KYN and 50 nM KYNA. Results were not significantly different from 100%, respectively: 97.4 ± 1.8 and 98.6 ± 2.0 (mean ± SD). Detection limits were determined by analysis of standard solution from 0.1 – 10 µM KYN and 0.05 – 5 µM KYNA. The detection limits, as signal-to-noise ratio of 3 with an injection volume of 5µl were: 50 nM KYN and 5 nM KYNA.

### Creatinine Analysis

To normalize Cd, KYN and KYNA concentrations, creatinine in the urine was determined colorimetrically using a commercially available diagnostic laboratory test (POCh, Poland).

### Statistical Analysis

The values are expressed as the mean ± SD; n – represents the number of results. Multiple group comparisons were performed by one-way analysis of variance, and differences between the groups were estimated with Tukey-Kramer test. P value less than 0.05 was considered statistically significant. Correlations were analyzed using the Spearman test.

## Results

### Cd Concentration in Urine

Exposure to 5 and 50 mg Cd/l resulted in a marked dose-dependent increase in urinary Cd excretion (Table 1).

### NAG-B Activity in Urine

The rats exposed to 5 mg Cd/l showed an increase in the urinary NAG-B activity after 12 weeks of the experiment (Table 2). This effect was considerably intensified in the subsequent weeks of exposure. The administration of 50 mg Cd/l resulted in an increase in the urinary NAG-B activity already after the 1st week, but the longer exposure did not cause further enhancement in this enzyme excretion. A high correlation coefficient was noted between Cd excretion and NAG-B activity in urine (Table 3).

### KYN Concentration in Urine

In the rats treated with 5 mg Cd/l a significant increase in the urinary elimination of KYN was observed only after 18 weeks of exposure (Table 4). However, the intoxication with 50 mg Cd/l increased KYN excretion from the 6th week of the treatment and in the subsequent weeks of the experiment this effect was intensified. There was a significant correlation between Cd and KYN excretion and between KYN concentration and NAG-B activity in urine (Table 3).

### KYNA Concentration in Urine

The administration of Cd resulted in a marked dose-dependent increase in KYNA urinary elimination. At 5 mg Cd/l an elevated excretion of KYNA was observed after 18 weeks of the experiment. At the higher exposure (50 mg Cd/l) an increase in KYNA concentration was noted already after 4 weeks, but the prolongation of exposure had no significant effect. Regression analysis revealed a linear correlation between urinary Cd concentration and KYNA excretion as well as between NAG-B activity and KYNA elimination (Table 3).

## Discussion

In our experimental model using rats we have noted that Cd at the levels corresponding to human environmental (5 mg Cd/l) and occupational (50 mg Cd/l) exposure damages the kidneys structurally and functionally in the dose- and time-dependent manner. Cd injures the whole kidney but the main tubule (proximal convoluted tubule and straight tubule) is the critical place for its action [3]. We evaluated the use of urinary concentrations of KYN and KYNA to monitor Cd exposure and detect early effects of its action in the kidneys. For this purpose, we analyzed correlations between urinary excretion of both kynurenine metabolites, Cd concentration and the activity of NAG-B, recognized as one of the most sensitive markers of Cd nephrotoxicity [4, 16]. In the created experimental model we have also confirmed that the urinary activities of NAG-B increase in the early stage of proximal tubular injury.

Table 1. Urinary excretion of cadmium (ng/mg creatinine) in rats.

| Weeks of experiment | Control | 5 mg Cd/l | 50 mg Cd/l |
|---|---|---|---|
| 1 | 0.95 ± 0.15 | 5.00 ± 1.03 ** | 18.20 ± 4.68 *** $^{ooo}$ |
| 2 | 0.97 ± 0.16 | 4.92 ± 1.22 ** | 15.77 ± 4.85 *** $^{ooo}$ |
| 3 | 0.89 ± 0.09 | 5.01 ± 2.09 ** | 17.92 ± 3.87 *** $^{ooo}$ |
| 4 | 0.91 ± 0.18 | 5.27 ± 1.21 ** | 18.10 ± 3.10 *** $^{ooo}$ |
| 5 | 1.00 ± 0.10 | 5.11 ± 0.77 ** | 17.92 ± 3.44 *** $^{ooo}$ |
| 6 | 0.91 ± 0.14 | 6.22 ± 1.21 *** | 17.52 ± 2.11 *** $^{ooo}$ |
| 12 | 0.93 ± 0.13 | 5.63 ± 2.03 ** | 19.50 ± 4.22 *** $^{ooo}$ |
| 18 | 0.91 ± 0.13 | 4.12 ± 0.57 ** | 15.67 ± 1.19 *** $^{ooo}$ |
| 24 | 0.93 ± 0.14 | 7.52 ± 2.99 ** | 20.43 ± 4.20 *** $^{ooo}$ |

Values are means ± SD of 6 animals. Multiple group comparisons were performed by one-way analysis of variance – ANOVA. Bartelett's test was used for homogeneity of variances and significant intergroup differences were assessed by Tukey-Kramer test. Comparison with the control: **$p < 0.01$, ***$p < 0.001$; comparison with the rats exposed to 5 mg Cd/l: $^{OOO}p < 0.001$.

Table 2. Effect of cadmium on NAG-B activity (IU/l) in urine of rats.

| Weeks of experiment | Control | 5 mg Cd/l | 50 mg Cd/l |
|---|---|---|---|
| 1 | 1.92 ± 1.01 | 2.10 ± 1.04 | 5.09 ± 2.10 *** $^{oo}$ |
| 2 | 1.74 ± 0.40 | 2.36 ± 0.71 | 8.33 ± 2.11 *** $^{ooo}$ |
| 3 | 1.49 ± 0.50 | 2.63 ± 0.78 | 6.43 ± 1.50 *** $^{ooo}$ |
| 4 | 1.82 ± 0.65 | 2.72 ± 0.59 | 5.32 ± 1.22 *** $^{ooo}$ |
| 5 | 2.43 ± 0.77 | 2.33 ± 0.94 | 6.11 ± 1.36 *** $^{ooo}$ |
| 6 | 2.01 ± 0.47 | 2.32 ± 0.78 | 6.74 ± 1.31 *** $^{ooo}$ |
| 12 | 1.50 ± 0.42 | 3.40 ± 1.02 * | 6.42 ± 2.33 *** $^{oo}$ |
| 18 | 2.02 ± 0.55 | 4.36 ± 1.11 ** | 8.33 ± 1.98 *** $^{ooo}$ |
| 24 | 1.82 ± 0.38 | 4.10 ± 1.21 *** | 6.03 ± 1.44 *** $^{oo}$ |

Values are means ± SD of 6 animals. Multiple group comparisons were performed by one-way analysis of variance – ANOVA. Bartelett's test was used for homogeneity of variances and significant intergroup differences were assessed by Tukey-Kramer test. Comparison with the control: **$p < 0.01$, ***$p < 0.001$; comparison with the rats exposed to 5 mg Cd/l: $^{OOO}p < 0.001$.

Table 3. Correlation coefficients between estimated biochemical parameters after cadmium treatment in rats[a].

| Parameters | Cd | NAG-B | KYN | KYNA |
|---|---|---|---|---|
| Cd | - | $y = 2.316 + 0.183x$<br>$r = 0.6622, p < 0.001$ | $y = 8.914 + 0.270x$<br>$r = 0.3317, p < 0.001$ | $y = 12.855 + 0.493x$<br>$r = 0.5365, p < 0.001$ |
| NAG–B | $y = - 0.870 + 2.400x$<br>$r = 0.6622, p < 0.001$ | - | $y = 6.495 + 1.210x$<br>$r = 0.4103, p < 0.001$ | $y = 10.133 + 1.715x$<br>$r = 0.5341, p < 0.001$ |
| KYN | $y = 4.049 + 0.408x$<br>$r = 0.3317, p < 0.001$ | $y = 2.355 + 0.139x$<br>$r = 0.4103, p < 0.001$ | - | $y = 10.022 + 0.631x$<br>$r = 0.5579, p < 0.001$ |
| KYNA | $y = - 1.349 + 0.583x$<br>$r = 0.5365, p < 0.001$ | $y = 1.179 + 0.160x$<br>$r = 0.5341, p < 0.001$ | $y = 2.800 + 0.495x$<br>$r = 0.5579, p < 0.001$ | - |

[A]Correlations were analysed using Spearman test. $P < 0.05$ was considered statistically significant

KYN is the main product of TRP degradation in peripheral tissues, which is further converted to a series of metabolites, among others to KYNA. Renal excretion is the main route of KYN and KYNA elimination [21]. In addition, the kidney is able to uptake KYN from the blood and excrete its metabolite - KYNA [10, 19]. Thus, the impairment of kidney function is likely to be associated with changes in the urinary excretion of both substances. Abnormalities in KYN and KYNA excretion have been reported recently in humans and rats with chronic renal insufficiency [12]. The disturbances in kynurenine pathway of TRP degradation in renal failure might be of clinical relevance. It has been demonstrated that in the central nervous system kynurenines (quinolinic acid (QA) or 3-hydroxykynurenine (3-HKYN)) may favor the effects of excitotoxins by generating reactive oxygen species [22]. Niwa et al. [23] showed that KYN methabolites are able to penetrate into the brain and evoke seizures, convulsions and muscle cramps. Apart from their actions in the central nervous system, KYN metabolites exert a number of disadvantageous peripheral effects. For example, QA has been shown to inhibit gluconeogenesis, erythropoiesis and lymphocyte blast formation [9, 24]; therefore, accumulation might be related to cellular metabolism disturbances, anemia and immunosuppression observed in uremia. Garacia et al. [25] have proposed that also xanthurenic acid (XA), due to its hydrophilic properties

Table 4. Effect of cadmium on urinary excretion of kynurenine (μmol/mmol creatinine) in rats.

| Weeks of experiment | Control | 5 mg Cd/l | 50 mg Cd/l |
|---|---|---|---|
| 1 | 4.52 ± 2.23 | 5.52 ± 3.36 | 6.06 ± 2.01 |
| 2 | 5.21 ± 3.42 | 7.31 ± 5.62 | 8.55 ± 4.72 |
| 3 | 5.36 ± 3.34 | 7.36 ± 3.67 | 8.02 ± 4.87 |
| 4 | 6.03 ± 4.31 | 10.89 ± 7.74 | 11.51 ± 4.15 |
| 5 | 7.38 ± 3.16 | 13.34 ± 6.11 | 12.83 ± 5.33 |
| 6 | 6.02 ± 2.61 | 11.07 ± 3.02 | 14.84 ± 6.04 * |
| 12 | 9.34 ± 4.53 | 15.72 ± 5.54 | 23.74 ± 10.18 * |
| 18 | 6.78 ± 2.74 | 17.23 ± 6.41 * | 17.72 ± 6.12 * |
| 24 | 8.09 ± 2.91 | 16.01 ± 7.47 | 18.01 ± 8.27 * |

Values are means ± SD of 6 animals. Multiple group comparisons were performed by one-way analysis of variance – ANOVA. Bartelett's test was used for homogeneity of variances and significant intergroup differences were assessed by Tukey-Kramer test. Comparison with the control: *$p < 0.05$.

Table 5. Effect of cadmium on urinary excretion of kynurenic acid (μmol/mmol creatinine) in rats.

| Weeks of experiment | Control | 5 mg Cd/l | 50 mg Cd/l |
|---|---|---|---|
| 1 | 10.35 ± 4.92 | 9.21 ± 3.67 | 11.64 ± 3.31 |
| 2 | 9.21 ± 2.85 | 10.84 ± 5.15 | 16.21 ± 5.11 |
| 3 | 12.12 ± 4.37 | 11.25 ± 7.08 | 19.42 ± 7.87 |
| 4 | 13.43 ± 3.12 | 19.17 ± 2.27 | 23.86 ± 5.67 * |
| 5 | 10.86 ± 6.13 | 16.81 ± 3.85 | 20.94 ± 6.35 * |
| 6 | 11.17 ± 3.78 | 16.08 ± 3.01 | 22.86 ± 5.17 ** O |
| 12 | 13.62 ± 6.45 | 19.83 ± 6.57 | 28.31 ± 7.75 * |
| 18 | 12.29 ± 3.90 | 19.74 ± 4.45 * | 30.44 ± 11.34 ** O |
| 24 | 14.84 ± 4.81 | 22.04 ± 6.45 * | 25.91 ± 5.23 ** O |

Values are means ± SD of 6 animals. Multiple groups comparisons were performed by one-way analysis of variance – ANOVA. Bartelett's test was used for homogeneity of variances and significant intergroup differences were assessed by Tukey-Kramer test. Comparison with the control: *$p < 0.05$, **$p < 0.01$; comparison with the rats exposed to 5 mg Cd/l: O $p < 0.05$.

and binding to erythrocyte membrane, could be involved in the pathogenesis of anemia.

The increase in NAG-B activity in urine and in the urinary excretion of TRP metabolites such as KYN and KYNA observed in Cd-treated rats depended on the level and duration of exposure. However, at both levels of exposure the changes in the urinary excretion of NAG-B occurred earlier than those in KYN and KYNA excretion. At 5 mg Cd/l the increase in NAG-B activity was noted after 12 weeks of the experiment whereas urinary concentration of KYNA was increased after 18 weeks and KYN – only after 18 weeks. On this basis, we speculate that at a low intoxication with Cd, comparable with human environmental exposure, the urinary concentration of KYNA, but not KYN, can be used, together with Cd concentration and NAG-B activity, for monitoring kidney status. At exposure to 50 mg Cd/l the urinary activity of NAG-B was increased already after the first week of the experiment, whereas the concentration of KYNA was elevated after 4 weeks and that of KYN after 6 weeks. Thus, it can be concluded that at higher intoxication, comparable with human occupational exposure, both metabolites of tryptophan via kynurenine pathway are useful for the detection of early Cd action in the kidneys. The usefulness of KYN and especially KYNA for the monitoring of chronic exposure to Cd is confirmed by positive correlation coefficients between the urinary excretion of Cd and KYN or KYNA as well as between NAG-B activity and KYN or KYNA concentration. Statistical analysis of all the data clearly indicates that KYNA may be more useful than KYN for the monitoring of Cd exposure. It is important to note that these parameters should be measured at least with Cd concentrations, as KYN and KYNA are unspecific markers of the kidney status. Disturbances in the kynurenine pathway have been reported in many pathological states other than renal failure, including bacterial or mycotic infections and neurodegenerative diseases [26, 27, 28, 29].

In summary, our present and previous studies allow the hypothesis that metabolites of tryptophan via kynurenine pathway such as KYN and especially KYNA can be used to monitor chronic exposure to Cd. However, further studies involving human subjects exposed to Cd environmentally and occupationally are required to assess the usefulness of these metabolites.

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