Original Research

The activation of the kynurenine pathway in a rat model with renovascular hypertension

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Impact statement

As hypertension is a major health problem, our research has focused on the connection between the kynurenine pathway and hypertension. We assessed the levels of the main metabolites of dietary tryptophan and analyzed its levels in terms of high blood pressure. The results of our work indicated that in the renovascular rat's model of hypertension, an alteration of the kynurenine pathway occurred. According to our knowledge, this is the first study that has **investigated** in a comprehensive manner the alteration of the kynurenine pathway under the condition of elevated blood pressure. On the one hand, the work supports a better understanding of pathophysiological basics of the occurrence of hypertension, and on the other hand it provides potential opportunities to treat this disease.

Abstract

Hypertension is a serious condition that can lead to many health problems. The mechanisms underlying this process are still not fully understood. The kynurenine pathway may be involved in the occurrence and progression of hypertension. The purpose of this study was to examine the activity of peripheral kynurenine pathway in rats with renovascular hypertension in Goldblatt 2K1C model. Hypertension was induced in the experimental groups by constricting the renal artery of the left kidney of the rats. Determination of tryptophan (Trp) and kynurenine pathway metabolites was assessed by high-performance liquid chromatography in plasma and tissues obtained at 4, 8, and 16 weeks after the surgical intervention or sham surgery. Levels of Ang II were evaluated using commercial immunoenzymatic ELISA kits. Surgical treatment led to increased values of mean blood pressure and systolic blood pressure, whereas Trp concentrations were decreased in experimental animals compared to appropriate controls. Simultaneously, the considerable increment of kynurenine pathway components and a significant increase in the activity of tryptophan 2,3-dioxygenase were observed in rats with developed hypertension in comparison with controls. There were no differences between Ang II levels in controls and experimental groups. The inverse relationship was between plasma Trp and both SBP and Ang II values, and Trp independently affected Ang II concentrations in hypertensive rats. In contrast, tryptophan 2,3-dioxygenase activity and plasma kynurenine metabolites positively correlated with blood pressure values as well as with Ang II levels in these animals. Moreover, kynurenine was independently connected with MBP. Renovascular hypertension influences kynurenine pathway and leads to an imbalance in Trp and its metabolite levels. Tryptophan 2,3-dioxygenase and part of the kynurenine metabolites in plasma and tissues positively correlated with blood pressure values and Ang II levels. Although the mechanisms underlying this phenomenon are unclear, our experiment showed a link between renovascular hypertension and activation of kynurenine pathway.

Keywords: Renovascular hypertension, 2K1C Goldblatts' model, angiotensin II, kynurenine pathway, tryptophan metabolism, rats

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Introduction

Hypertension occurs with a frequency of 20% in adults and is considered to be the main cause of premature deaths from cardiovascular diseases worldwide.[1] One of its types is the renovascular hypertension, which can be the result of disturbances in the renal microcirculation leading to low perfusion pressure. Renovascular hypertension is reflected by the two-kidney, one-clip (2K1C) model, where unilateral stenosis of a renal artery caused an augmentation of blood pressure values.[2] In this model, depending on plasma renin activity and reversibility of hypertension, three periods were distinguished: an acute (four weeks after clipping), transient (from five to eight weeks after clipping), and chronic (from nine weeks after clipping).[3] In the acute phase of this model, the main factor that determines elevated blood pressure is angiotensin II (Ang II); however, its elevated levels are observed only for the initial 20–25

days.[4] During the transient and chronic phase, due to multiple factors, the levels of renin and Ang II are normalized, but the values of blood pressure still remain high.[3,4] Despite many studies, all the mechanisms underlying renovascular hypertension are not fully understood.

Kynurenine (KYN) pathway is a major tryptophan (Trp) metabolic pathway that generates numerous active metabolites like KYN, kynurenic acid (KYNA), 3-hydroxykynurenine (3HKYN), anthranilic acid (AA), and nicotinamide adenine dinucleotide (NAD) as the final products of the pathway.[5] In recent times, the abovementioned compounds of the KYN pathway have been connected with the occurrence of many diseases, such as inflammation, dyslipidemia, neurodegenerative processes, and cardiovascular disorders.[6,7] With regard to blood pressure, researchers have provided evidence that Trp, precursor of KYNs, has the ability to lower blood pressure after oral administration.[8] Moreover, tryptophan-containing dipeptides are selective inhibitors for the C-domain of the human angiotensin-converting enzyme (ACE)—well-known target for hypertension treatment.[9] Despite many studies, the impact of KYN on blood pressure parameters is not clearly defined. Interestingly, data from some studies have shown that failures in the pharmacological treatment of elevated blood pressure occur more frequently in patients with a higher concentration of KYN in blood plasma.[10] Furthermore, hyperkynureninemia increases the risk of occurrence of hypertension in patients with chronic kidney disease (CKD).[11] In addition, Martinsons et al.[12] pointed out that hyperkynureninemia may also lead to progression of cardiovascular disease, including hypertension. In contrast to this are studies that show the selective impact of administration of exogenous KYN in animal models. Wang et al.[13] have shown that KYN administered to spontaneously hypertensive rats (SHR) decreased the blood pressure via a soluble guanylate cyclase (sGC) pathway, which contributes to relaxation of blood vessels. In turn, Sakakibara et al.[14] noticed that this compound dilates arteries via activation of voltage-dependent K^+ channel encoded by KCNQ gene family. Moreover, KYN is a potent endothelium-derived vasodilator, mediating its effect independently of nitric oxide (NO).[10] Our earlier studies[15] have proved that KYNs are involved in endothelial dysfunction and progression of atherosclerosis in patients with CKD. Importantly, KYN is also an endogenous aryl hydrocarbon receptor (AhR) ligand. Agbor et al.[16] reported that endothelial-specific AhR knockout mice were significantly hypotensive and exhibited attenuated response to Ang II. In turn, in rats with genetically determined hypertension sense mutation occurred in a gene (E61G) coding aminotransferase which converts KYN into KYNA.[16] Kwok et al.[17] in their work linked enhanced sensitivity to glutamate and nicotine with a mutation in KYN aminotrasferase-1 (KAT) and assumed that it can be one of the causes of hypertension in SHR. In the work of Wang et al.,[18] after microinjection of KYNA into the rostral ventrolateral medulla, a substantial drop in blood pressure values was observed. Likewise, Veitenheimer and Osborn[19] noticed decreased mean arterial pressure after intrathecal administration of KYNA. Moreover, derivative of AA (HMR1766) in intravenous bolus injection decreased arterial blood pressure in anesthetized pigs.[20]

The existence of divergent theories and research results provides the basis for focusing on the KYN pathway in conditions of renal-induced hypertension. The aim of the present study was to evaluate the activity of peripheral KYN pathway in rats with hypertension in the Goldblatt 2K1C model. Because Ang II a is well-recognized factor that determines the elevated blood pressure in an acute phase of renovascular hypertension,[3] we focused on transient and chronic phase of the disease, in which pathobiology is multifactorial. For this reason, we determined the concentrations of Trp and its metabolites derived from KYN pathway in plasma and tissues in rats with renovascular hypertension and healthy animals. We also examined the enzymatic activity of two main enzymes of the pathway: indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO). All the obtained results were correlated with blood pressure parameters and Ang II concentrations. Finally, to make our research comprehensive, we evaluated the impact of the Trp metabolism on blood pressure parameters and Ang II levels based on a multiple regression analysis of the cross-sectional results.

Materials and methods

Wistar male rats, aged four to five weeks and weighing around 140–160 g were used. The rats were housed in individual cages in a room with 12 h light/dark cycles and with ad libitum access to chow and tap water. All experimental procedures in the study were conducted in accordance with the institutional guidelines and approved by the Local Bioethics Committee (Agreement No. 2004/36). A total of 60 rats were randomly divided into six groups, 10 animals in each (one to three groups: 4/8/16-week control groups, underwent sham surgery without harming left renal arteries and four to six groups: 4/8/16-week experimental groups, underwent surgical induction of hypertension with left renal arteries clipped).

Induction of renal hypertension in 2K1C model

The rats were anesthetized with pentobarbital (i.p., 40 mg/kg). Hypertension was induced by Goldblatt model.[2,3] Standardized surgical clips ($Ø = 0.22$ mm) were placed on the left renal artery. Animals were kept on a heated table to avoid chilling and loss of temperature. Blood and tissue samples were collected from both kidneys, lungs, and liver after 4, 8, and 16 weeks.

Blood pressure measurements

Blood pressure was measured with the apparatus Harvard Rat Trail Blood Pressure Monitor by using the bloodless tail-cuff method, according to Zatz[21] using a photo sensor on the rats' tail. This method enables the mean and systolic blood pressure (SBP) measurement.

Concentration of assays of metabolites formed in the KYN pathway

Determination of Trp and its metabolite levels in plasma, lungs, and liver was assessed by high-performance liquid chromatography (HPLC). Blood was withdrawn from the heart's right ventricle and mixed with 3.18% of sodium citrate in a proportion of 1:9. To obtain plasma, the whole blood was centrifuged at 3500 r/min for 15 min at 4°C. The obtained plasma was divided into two groups: for Ang II assays and for Trp and KYNs assays. In the next step, plasma samples were deproteinated by 2 M $HClO_4$ and centrifuged at 14,000 r/min for 30 min at 4°C. The supernatant was filtered using 0.45 μm syringe filters. Until assays, samples were stored at −80°C.

The chromatographic equipment was an Agilent Technologies 1260 series LC system composed of G1321 binary pump VL, G1379B degasser, G1329A autosampler, G1330B thermostat for autosampler, G1316A column thermostat, G1315C diode array detector, and Hewlett Packard HP1046A fluorescence and HP1046A electrochemical detectors.

Two independent samples of tissue were obtained from both the kidneys, liver, and lungs . The first one was homogenized in ice by thrice the volume of 20% trichloroacetic acid. Then the obtained homogenates were centrifuged at 14,000 r/min for 30 min at 4°C. The supernatant was filtered using 0.45 μm filters and subsequently was analyzed using HPLC. The second sample was subjected to enzymatic assays. Both the samples were homogenized using buffer from 0.14 M and 20 mM K_2HPO_4 with pH = 6.5. Before the assays, the samples were stored at −80°C.

The prepared samples (2 μl) were separated on an ODS column (Waters Spherisorb 3 μm ODS 2, 2.1 × 150 mm), KYN concentration was measured according to Holmes.[22] The column effluent was monitored with a diode array detector (KYN-365 nm, Trp-260 nm). The mobile phase was composed of 0.1 M acetic acid, 0.1 M ammonium acetate (pH 4.6) containing 1.8% of acetonitrile, and it was pumped at a flow-rate of 0.2 ml/min.

3-HKYN was measured as described by Heyes.[23] The column effluent was monitored using a programmable electrochemical detector. The potential of the working electrode was 0.6 V. The mobile phase consisted of 0.1 M triethylamine, 0.1 M phosphoric acid, 0.3 mM EDTA, 8.2 mM heptane-1-sulfonic acid sodium salt, containing 2% of acetonitrile and was pumped at a flow-rate of 0.25 ml/min; 2μl of the supernatant was injected into the HPLC system for analysis.

KYNA and AA concentrations were determined according to Herve et al.[24] The column (Phenomenex PEPTIDE 3.6 μm XB-C18 4.6 × 250 mm) effluent was monitored by using a programmable fluorescence detector. The optimized conditions were determined by recording fluorescence spectra with a stop-flow technique. Excitation and emission wavelengths were set at 254/404 nm for KYNA, 320/420 nm for AA. The mobile phase consisted of 20 mM sodium acetate, 5 mM zinc acetate, containing 8% of acetonitrile that was pumped at a flow-rate of 0.6 ml/min; 5 μl of the supernatant was injected into the HPLC system for analysis. The output of the detector was connected to a single-instrument LC ChemStation. Chromatography was carried out at 25°C.

Evaluation of enzyme activities

The activity of IDO in both kidneys and lungs was assessed by the modified method previously described by Heyes et al.[25] To 50 μL of tissue homogenate were added 50 μL of 100 mM K_2HPO_4 (pH = 7.0), 20 μL (50 μM) of methylene blue, 20 μL (0.5 mg/ml) of catalase, 10 μL (50 mM) of ascorbic acid, and 50 μL (4 mM) of Trp as substrate for IDO. After 60 min of incubation of reaction mixture at 37°C, the reaction was stopped by the addition of 0.1 ml of 20% trichloroacetic acid. Then, incubation was started again for 30 min at 50°C in order to hydrolyze N-formyl-L-kynurenine to KYN. Afterward, the samples were centrifuged at 14,000 r/min for 30 min at 4°C. The supernatant was filtered using 0.45 μm syringe filter and then was analyzed by chromatography for the assessment of KYN levels. The sensitivity of this method was 5 pmol/mg/h.

The activity of TDO in the liver was analyzed by the modified method previously described by Salter et al.[26] To 100 μL of tissue homogenate (in dilution of 1 volume of homogenate and 5 volumes of buffer: 0.14 M KCl, 20 mM K_2HPO_4 with pH = 7.0) were added 50 μL of 200 mM K_2HPO_4/ KH_2PO_4 (pH = 7.0), 20 μL (1.4 mg/ml) of methemoglobin and Trp as a substrate for the enzyme. After 60 min of incubation of reaction mixture at 37°C, the reaction was stopped by the addition of 0.1 ml of 20% trichloroacetic acid. Then, the mixture was incubated at 50°C for 30 min in an attempt to hydrolyze N-formyl-L-kynurenine to KYN. Subsequently, samples were centrifuged at 14,000 r/min for 30 min at 4°C. The supernatant was filtered using 0.45 μm syringe filter and then was analyzed using HPLC.

Determination of Ang II concentrations in plasma

The concentration of Ang II in plasma was measured with commercial immune-enzymatic kits (Phoenix Pharmaceuticals EIA, Burlingame, CA 94010, USA). Reading extinction was performed using ELISA Microplate Reader with the wavelength of 450 nm. Results were evaluated using the standard curve and were presented in ng/ml.

Statistical analysis

The normally distributed data were presented as mean ± 1SD, while the non-Gaussian data as median (full range). Normality of distribution was tested using Shapiro–Wilk W test. Multiple group comparisons were performed by one-way analysis of variance, and significant differences between groups were assessed by Tukey–Kramer test or non-parametric Mann–Whitney's U-test. The correlations between study variables were calculated by Spearman's rank correlation. Multiple regression analysis was performed using a stepwise model to determine the combined influence of tryptophan metabolites on Ang II and blood pressure values. A two-tailed p value <0.05 was considered statistically significant. Computations were performed

using GraphPad 6 Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Changes in arterial blood pressure parameter and concentration of angiotensin II in rats with induced renal hypertension

According to the results of mean and SBP shown in Figure 1, 2K1C model resulted in the induction of hypertension in all the experimental groups compared with the corresponding controls. Ang II concentrations in rats' plasma are shown in Table 1. No significant differences between the levels of Ang II in controls and experimental groups were observed.

Evaluation of enzymatic activity of TDO and IDO in rats with induced renal hypertension

Enzymatic activity of TDO was significantly higher in the experimental groups in comparison to controls. However, its activity in rats with renal-induced hypertension mildly decreased with time. No changes in IDO activity were observed in lungs and both kidneys, apart from a slight increase in its activity in the right kidney at four weeks after the induction of hypertension (Table 1). The mild negative correlation between IDO activity in the left kidney and MBP was noticed (Table 2). In turn, TDO positively correlated with Ang II (Figure 3).

Changes in tryptophan concentrations in rats with renal-induced hypertension

In controls, no statistically significant changes in the concentration of Trp in plasma (Figure 2) and its levels in liver, both kidneys, and lungs were observed (Table 1). However, in the experimental groups, we noticed a decreased concentration of Trp in plasma (Figure 2) and its levels in the above-mentioned tissues except the right kidney (Table 1). Moreover, a negative correlation occurred between the mean blood pressure (MBP) and Trp levels in the liver and the left kidney (Table 2), as well as between the concentration of Trp in plasma and Ang II (Figure 3). Analysis of total Trp degradation through the KYN pathway in plasma demonstrated that in 2K1C animals, the concentration of this amino acid decreased by 34.2%, 21.6%, and 19.8% in comparison with control rats in 4, 8, and 16 weeks since surgery (Figure 4). Moreover, multiple regression analysis shows that Trp in plasma is independently associated with Ang II (Table 3).

Changes in concentrations of KYN in rats with renal-induced hypertension

As expected, no significant changes of KYN concentration in plasma (Figure 2) and its levels in lungs, liver, and kidneys were found in control groups (Table 1). In turn, rats with renal-induced hypertension had higher concentrations of KYN in plasma (Figure 2) and tissues (Table 1). Interestingly, KYN levels were balanced in the oldest animals after 16 weeks of the experiment. Furthermore, levels of this compound in all mentioned tissues except lung and left kidney correlated with MBP (Table 2). According to the ratio, the highest increase in plasma KYN concentration of 82.6% was observed in the first 4 weeks of experience, while in 8 and 16 weeks, the levels were increased by 68.8% and 27.4%, respectively (Figure 4). In addition, the plasma concentration of KYN was independently connected with values of MBP (Table 3).

Changes in concentrations of kynurenic acid in rats with induced hypertension

Concentrations of KYNA in plasma were significantly elevated in groups with hypertension in comparison to controls (Figure 2) and positively correlated with MBP (Table 2), as well as Ang II (Figure 3). Moreover, statistically significant increases of KYNA levels were observed in all tissues. In this case, we also observed balance in KYNA levels in lungs, liver, and the right kidney in the oldest animals (Table 1). Besides, KYNA levels in liver negatively correlated with MBP (Table 2). The concentration of KYNA in plasma at four and eight weeks remained at the same high level of 126.3% and 128.4%, respectively. At 16 weeks, the concentration was maintained at about 100.2% higher compared with the control. Despite a reduction of the increase in KYNA concentration ratio, KYNA/KYN

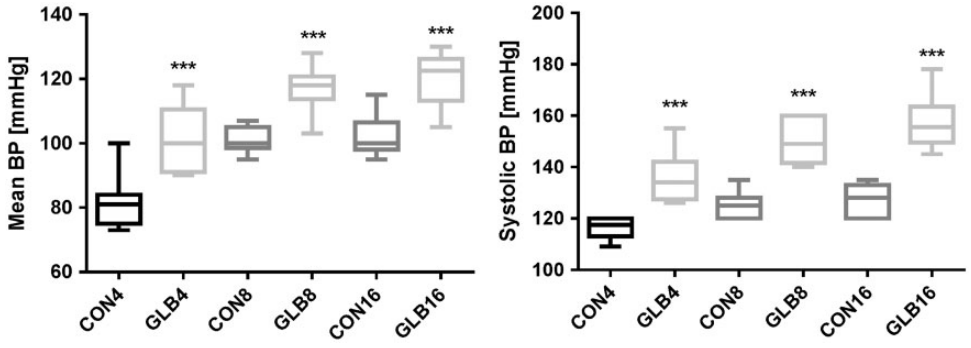

Figure 1 The values of mean and systolic blood pressure (BP) in rats with renovascular hypertension (GLB) and controls (CON). CON4, CON8, and CON16—rats from control group after 4, 8, and 16 weeks of sham, respectively, GLB4, GLB8, and GLB 16—rats from experimental group after 4, 8, and 16 weeks of surgical induction of hypertension, respectively (***$p < 0.001$)

Table 1 Changes in tissue kynurenine pathway metabolism and plasma angiotensin II (Ang II) levels in rats with renovascular hypertension (GLB) and appropriate controls (CON)

		CON4	GLB4	CON8	GLB8	CON16	GLB16
Trp (nmol/g)	Lung	27.9 ± 4.4	18.8 ± 4.7**	30.1 ± 4.9	23.3 ± 3.4**	31.7 ± 4.6	23.3 ± 5.9**
	Liver	37.7 ± 7.2	25.6 ± 4.9***	34.4 ± 6.5	17.9 ± 9.9***	35.0 ± 7.0	24.4 ± 6.6**
	Left kidney	91.1 ± 9.6	74.6 ± 8.3**	88.3 ± 10.7	61.9 ± 9.8***	85.7 ± 21.5	66.3 ± 15.0*
	Right kidney	83.9 ± 5.7	83.7 ± 10.1	84.9 ± 11.9	79.2 ± 12.6	86.8 ± 21.9	72.9 ± 24.2
KYN (nmol/g)	Lung	1.1 ± 0.4	1.9 ± 0.8*	1.3 ± 0.5	1.4 ± 0.8	1.2 ± 0.4	0.9 ± 0.6
	Liver	1.6 ± 0.7	3.1 ± 1.0**	1.8 ± 0.9	3.0 ± 1.2*	2.5 ± 0.3	2.9 ± 0.6
	Left kidney	1.9 ± 0.3	2.9 ± 1.0*	1.6 ± 0.3	2.8 ± 1.4*	1.042 ± 0.613	2.1 ± 1.2*
	Right kidney	1.7 ± 0.5	2.3 ± 0.3*	1.4 ± 0.4	1.6 ± 0.5	0.9 ± 0.5	1.4 ± 0.3*
KYNA (pmol/g)	Lung	25.6 ± 3.3	37.4 ± 8.4**	25.1 ± 4.3	32.4 ± 6.5*	24.6 ± 3.5	29.2 ± 8.7
	Liver	19.3 ± 7.3	27.6 ± 4.1*	17.9 ± 6.3	28.3 ± 8.8*	19.4 ± 5.9	20.7 ± 6.9
	Left kidney	185.7 ± 21.3	171.6 ± 17.5	188.1 ± 21.7	162.3 ± 22.3*	177.1 ± 9.8	192.4 ± 14.4*
	Right kidney	187.9 ± 29.2	232.0 ± 39.2*	167.7 ± 18.9	183.9 ± 14.5	182.9 ± 20.4	194.2 ± 11.6
3HKYN (pmol/g)	Lung	120.0 ± 25.4	142.1 ± 26.4	108.7 ± 24.1	139.4 ± 29.7*	86.3 ± 22.6	126.3 ± 27.8**
	Liver	456.2 ± 44.3	552.2 ± 72.9*	435.0 ± 45.6	535.9 ± 83.4*	517.9 ± 82.8	502.1 ± 86.2
	Left kidney	393.3 ± 48.2	303.1 ± 53.1**	414.5 ± 28.3	346.8 ± 44.2**	371.3 ± 57.8	380.2 ± 67.2
	Right kidney	377.3 ± 23.8	467.4 ± 42.9***	360.4 ± 31.8	458.6 ± 66.5**	372.7 ± 25.9	437.3 ± 41.8**
Ang II (ng/ml)	Plasma	1.749 (0.63–6.00)	2.090 (0.49–10.00)	2.857 (0.57–7.00)	2.815 (0.55–10.00)	2.000 (0.41–7.00)	2.135 (0.30–10.00)
TDO activity (pmol/mg/h)	Liver	382.1 ± 48.8	500.9 ± 64.7***	348.8 ± 58.5	462.2 ± 75.5**	315.9 ± 73.5	443.9 ± 60.4***
IDO activity (pmol/mg/h)	Left kidney	58.5 ± 16.7	57.5 ± 16.5	62.4 ± 15.3	57.9 ± 15.1	45.5 ± 13.9	43.2 ± 14.6
	Right kidney	43.9 ± 9.6	58.5 ± 15.5*	50.3 ± 10.4	52.4 ± 14.5	44.0 ± 14.2	57.0 ± 15.0
	Lung	51.2 ± 20.7	54.6 ± 20.4	55.4 ± 15.1	52.8 ± 12.8	50.9 ± 10.6	67.1 ± 18.6

Trp: tryptophan; KYN: kynurenine; KYNA: kynurenic acid; 3HKYN: 3-hydroxykynurenine; TDO: tryptophan 2,3-dioxygenase; IDO: indoleamine 2,3-dioxygenase.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table 2 The correlations between mean and systolic blood pressure and activity of TDO, IDO, as well as levels of Trp, and its metabolites: KYN, KYNA, 3HKYN, and AA in plasma, lung, liver, and kidneys of rats with renovascular hypertension.

		Mean blood pressure		Systolic blood pressure	
		r_s	*p*	r_s	*p*
Trp	Plasma	−0.1139	NS	**−0.3227**	**0.047**
	Lung	−0.2791	NS	**−0.3485**	**0.0233**
	Liver	**−0.5919**	**0.0001**	**−0.6442**	**0.0001**
	Left kidney	**−0.6149**	**0.0001**	**−0.6687**	**0.0001**
	Right kidney	−0.2122	NS	−0.1977	NS
TDO	Activity	0.1789	NS	**0.3988**	**0.0054**
	Lung	0.1808	NS	**0.3435**	**0.0181**
IDO	Left kidney	**−0.337**	**0.0205**	−0.1882	NS
	Right kidney	0.2302	NS	0.1128	NS
	Plasma	**0.3030**	**0.0383**	**0.4421**	**0.0028**
	Lung	0.0031	NS	0.06331	NS
KYN	Liver	**0.4607**	**0.0011**	**0.4685**	**0.0009**
	Left kidney	0.0081	NS	**0.3034**	**0.0382**
	Right kidney	**−0.3323**	**0.0286**	−0.2006	NS
	Plasma	**0.5197**	**0.0002**	**0.5936**	**0.0001**
	Left kidney	0.0721	NS	0.1322	NS
KYNA	Right kidney	0.2127	NS	−0.0451	NS
	Lung	0.2057	NS	0.2231	NS
	Liver	**−0.2913**	**0.047**	−0.1782	NS
	Plasma	**0.3498**	**0.0016**	**0.5095**	**0.0003**
	Left kidney	−0.2020	NS	−0.2761	NS
3HKYN	Right kidney	**0.3633**	**0.0131**	**0.4235**	**0.0027**
	Lung	**0.2954**	**0.0382**	**0.4178**	**0.0032**
	Liver	0.1250	NS	0.1650	NS
AA	Plasma	**0.3633**	**0.0131**	**0.4234**	**0.0030**

Trp: tryptophan; IDO: indoleamine 2,3-dioxygenase; TDO: tryptophan 2,3-dioxygenase; KYN: kynurenine; KYNA: kynurenic acid; HKYN: 3-hydroxykynurenine; AA: anthranilic acid.
Results are shown as Spearman's rank correlation coefficients (r_s), and its statistical significance value (p).

raised from the value of 34.4–43.5 with an increase in blood pressure (Figure 4).

Changes in concentrations of 3-hydroxykynurenine in rats with induced hypertension

Like other metabolites, the levels of 3HKYN did not change in the control groups (Table 1). However, increases of 3HKYN concentrations were observed in plasma (Figure 2) and in tissues like lungs, liver, and the right kidney of the experimental groups. Furthermore, changes of level of 3HKYN in the left kidney were also statistically significant; but in this case, the drop was observed (Table 1). Positive correlation was found between the plasma 3HKYN concentration and MBP (Table 2), and Ang II (Figure 3). The levels of 3HKYN in lungs and the right kidney positively correlated only with the mean and SBP (Table 2). The most pronounced changes in the 3HKYN concentration were observed in four weeks. In 16 weeks, the increase of plasma 3HKYN concentration amounted 48.5% when compared with the control. 3HKYN/KYN ratio increased with hypertension development and reached 37.5%, 36.1%, and 48.7% in 4, 8, and 16 weeks, respectively (Figure 4).

Changes in concentrations of anthranilic acid in rats with induced hypertension

Similarly, as in previously described metabolites, we did not observe any significant changes in AA concentrations in plasma of the control groups, but in hypertensive rats, its concentrations were increased (Figure 2). We did not find any statistically significant correlations between AA plasma concentrations and MBP. The augmentation of plasma AA concentration was 78.5%, 66.7%, and 39.2% in 4, 8, and 16 weeks, respectively, in comparison with control. The AA/KYN ratio was the highest and increased by 82.9%, 83.9%, and 92.7% (Figure 4).

Discussion

Disturbances of renin–angiotensin–aldosterone (RAA) system seem to be the main cause of renovascular hypertension, which is still an unresolved medical problem that leads to increased mortality and morbidity.[1] The results of the present study shed new light on the pathophysiology underlying that abnormality and indicate that the activation of the peripheral KYN pathway could be one of the mechanisms involved in the renovascular hypertension.

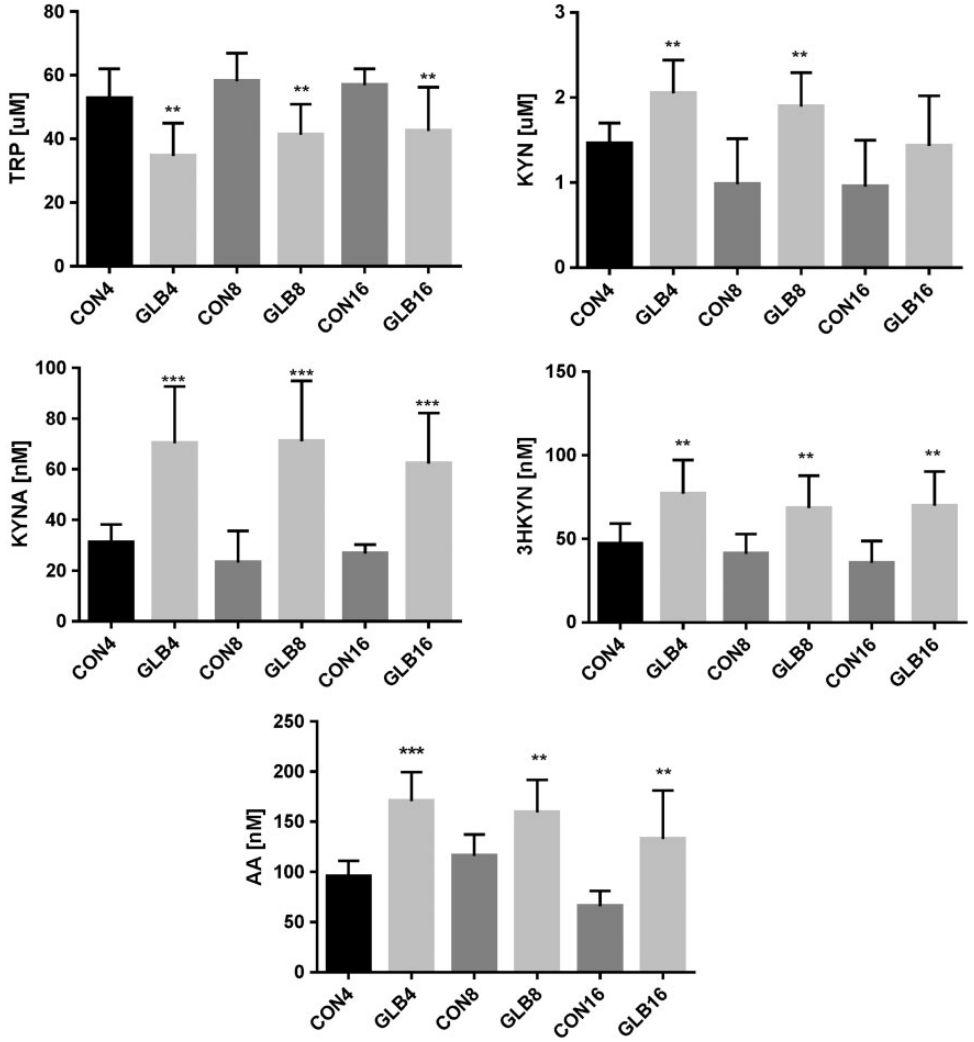

Figure 2 Changes in concentrations of tryptophan (Trp) and its metabolites: kynurenine (KYN), kynurenic acid (KYNA), 3-hydroxykynurenine (3HKYN) and anthranilic acid (AA) in plasma of rats with renovascular hypertension (GLB) and controls (CON). CON4, CON8, and CON16—rats from control group after 4, 8, and 16 weeks of sham surgery, respectively, GLB4, GLB8, and GLB 16—rats from experimental group after 4, 8, and 16 weeks of surgical induction of hypertension, respectively (**$p < 0.01$; ***$p < 0.001$)

We used Goldblatt model (2K1C) to induce renovascular hypertension. The mentioned model is characterized by changes in the activity of local RAA system, which persists despite the normalization of plasma renin activity and systemic levels of Ang II.[4] Comparing the obtained values of blood pressure as well as Ang II with literature data, it can be assumed that the measurements were carried out during transient (4–8 weeks) and chronic (8–16 weeks after unilateral renal artery clipping) phases of the disease. It was also reflected by the initial increase and subsequent stabilization of MBP and SBP values. The obtained blood pressure parameters (Figure 1) confirmed that 2K1C is the validated model of hypertension, which enabled assessment of other parameters in this model.

TDO and IDO are the main enzymes of the first step of KYN pathway, which convert Trp into KYN.[5] During our experiments, we observed increased activity of TDO in conditions of higher blood pressure, whereas the activity of IDO did not change significantly (Table 1). Importantly, our study shows almost 10-fold higher activity of TDO compared to IDO (Table 1). It is worth pointing out that this enzyme has a much higher capacity for the regulation of Trp metabolism through KYN pathway in comparison to IDO.[27] Simultaneously, we noticed the decrease in Trp and increase in KYN concentrations in plasma and some tissues of hypertensive rats (Table 1). Taking into account the activity of the enzymes, this suggests that in the used model of renovascular hypertension, the activation of peripheral KYN pathway was partly TDO-dependent. Increased activity of TDO can be caused by a possible occurrence of transient hypoxia in the first phase of 2K1C model, and this finding is in line with the data of Yoshino et al.[28] showing that hypoxia increased hepatic TDO activity in rats. On the other hand, the changes in the activity of enzymes initiating KYN pathway activation can be connected with increased concentration of superoxide dismutase (SOD) in the hypertensive animals.[29] In SHR, the significant positive correlation was observed between SBP

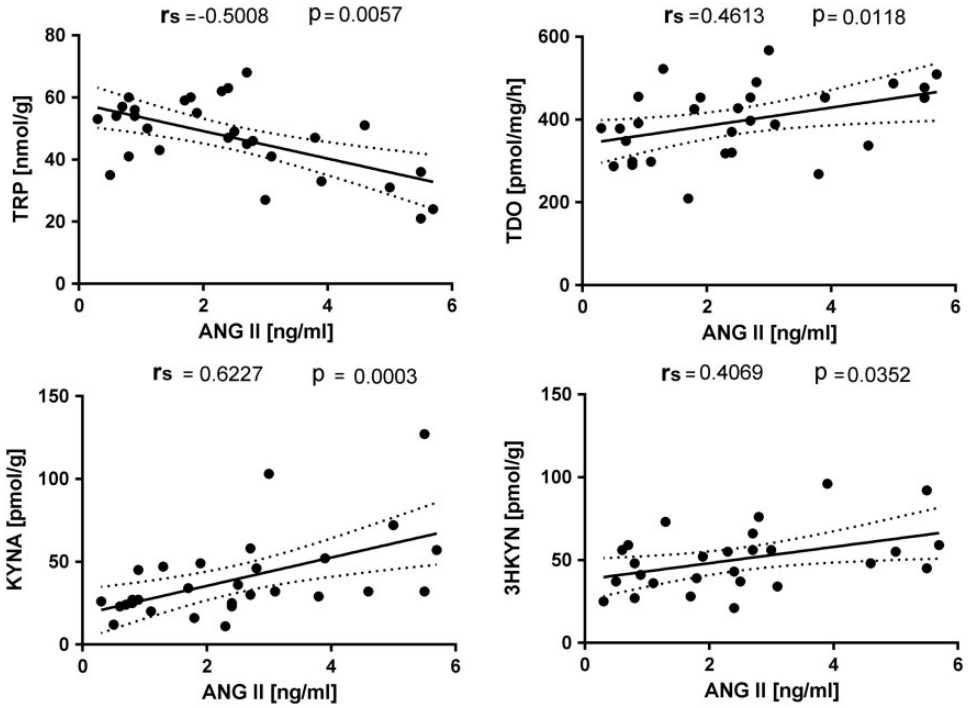

Figure 3 Spearman's correlations between angiotensin II (Ang II) concentrations and tryptophan (Trp), tryptophan 2,3-dioxygenase (TDO) activity, and kynurenine pathway metabolites: kynurenic acid (KYNA), and 3-hydroxykynurenine (3HKYN) in plasma of rats with renovascular hypertension

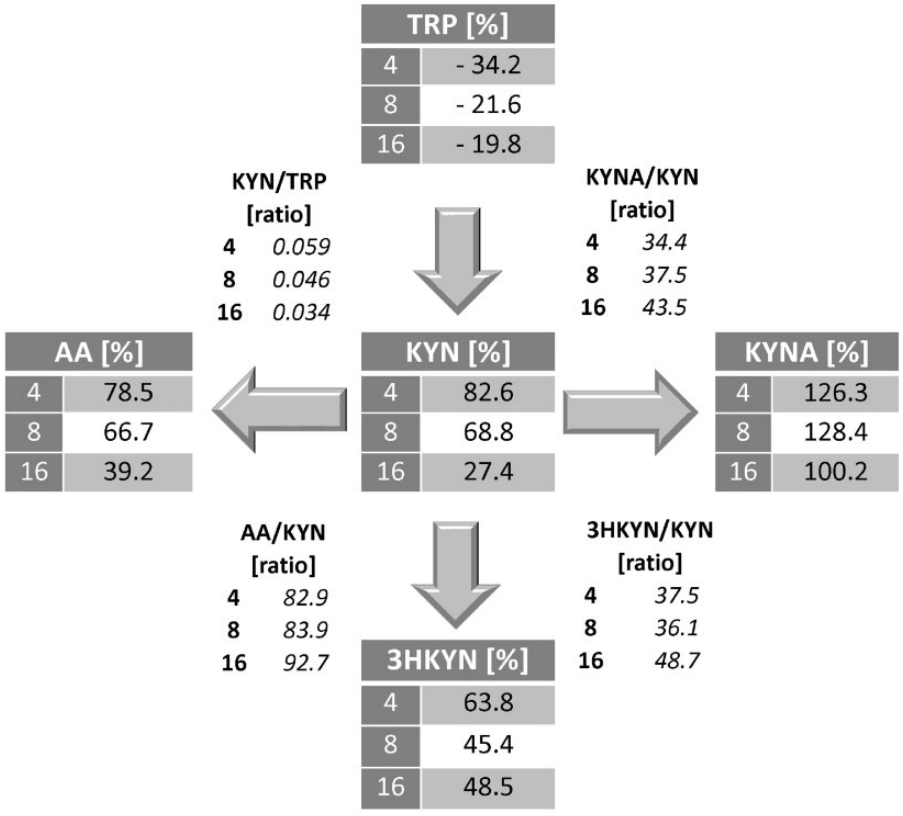

Figure 4 The plasma metabolism of tryptophan (Trp) via kynurenine (KYN) pathway in rats with renovascular hypertension during 4, 8, and 16 weeks of experiment. The results are presented as % of increase of compounds of kynurenine pathway or ratios—the quantitative relation between specific compounds of kynurenine pathway from experimental groups with renal hypertension. KYNA: kynurenic acid; 3HKYN: 3-hydroxykynurenine; AA: anthranilic acid

Table 3 Variables independently associated with the levels of angiotensin II and mean blood pressure in multiple regression analysis

	Independent variables	Regression coefficient	Standard error	*p*
Angiotensin II	Tryptophan	−0.426	0.189	0.033
Mean blood pressure*	Kynurenine	0.395	0.185	0.042

Multiple *r* for variables in the model: 0.660 (*0.611), $r^2 = 0.436$ (*0.373), adjusted $r^2 = 0.313$ (*0.325), $p < 0.016$ ($^*p < 0.002$), standard error of estimate: 1.355 (*11.014).

and the activity of the antioxidant enzyme Cu/Zn-SOD in erythrocytes.[30] As increased superoxide production was also documented in 2K1C model,[31] and it is possible that it was observed in our study, the lack of increase in IDO activity in experimental animals may be caused by the increased activity of antioxidant enzyme—SOD, which is an inhibitor of IDO.[32] At this point, we must mention that IDO exists in two isozymes—IDO-1 and IDO-2.[33] In this study, due to procedure restrictions, only total activity of IDO was evaluated. Thus, our results suggest that among the three Trp catabolic enzymes (e.g., IDO-1, IDO-2, and TDO), only dysregulation of TDO expression is responsible for the blood Trp and KYN concentration imbalance in rats with hypertension. However, there is also a possibility that increase in one isoform of IDO and decrease in the second resulted in unchanged overall IDO activity. Moreover, the observed Trp decrease can also be a result of increased uptake of free Trp and rapid equilibration between free and albumin-bound Trp. Badawy et al.[34] suggest that flux of Trp through KYN pathway is determined mainly by plasma-free Trp. Bearing in mind that hypertension is a condition associated with the increased level of catecholamines[35] stimulating lipolysis and subsequent displacement of albumin-bound Trp by non-esterified fatty acids,[36] plasma-free Trp can be markedly increased, although total Trp tends to decrease. Furthermore, Guicheney et al.[37] demonstrated that plasma free-Trp is elevated in SHR. In case of non-esterified fatty acids, Lu et al.[38] showed its close correlation with hypertension. Although we did not measure it, free Trp is most likely to have been the most important factor in activation of the KYN pathway.

The drop in total Trp content in rats with 2K1C linking with the simultaneous increase in KYN concentrations indicates the activation of KYN pathway during hypertension progression, which was most evident in the transient phase of the disease (Table 1; Figure 4). At the beginning of this phase, the decrease in plasma Trp concentrations reached approximately 34%, which is a substantial result in the case of exogenous amino acid. Although there were no differences in Ang II concentrations between hypertensive and control animals, the strong inverse relationship was observed between plasma Ang II and Trp concentrations in experimental groups. Furthermore, Trp was independently associated with Ang II levels, supporting the hypothesis that metabolism of this amino acid through KYN pathway may play a role in renovascular hypertension. At the same time, the highest increase in plasma KYN concentration of approximately 83% was observed, and its concentrations in most of the examined organs correlated positively with the values of blood pressure (Table 2). These results suggest that the initial increase of blood pressure was associated with the increased concentrations of KYN, that in the current study was a factor independently affected MBP values. This finding highlights the possible role of KYN as one of the factors that play a role in blood pressure increment during the early phase of renovascular hypertension. At the later phase of the disease, KYN concentrations in experimental groups reached approximately 70% after 8 weeks and 27% after 16 weeks compared to controls (Figure 4). This gradual drop in KYN levels was probably due to its metabolism into other components of KYN pathway, such as KYNA, AA, and 3HKYN. Alternatively, the depletion of substrate—Trp may be a cause of a decline in KYN concentrations, as the low levels of this amino acid were detected in plasma and majority of tissues in experimental rats. Simultaneously, we observed the stabilization of blood pressure in the experimental animals. Thus, it is possible that in transient and chronic phase of experiment, KYN can play a pivotal role in the stabilization of BP parameters. The results of multiple regression analysis showing KYN as an independent variable associated with MBP confirmed this hypothesis.

Previous studies have demonstrated that KYN, but not Trp or other KYN metabolites, was able to relax pre-constricted porcine coronary arteries and mouse or rabbit aorta in a dose-dependent manner.[13] Wang et al.[13] presented KYN as a vasoactive compound acting via sGC-cGMP-dependent protein kinase pathway. In turn, Sakakibara et al.[14] observed that KYN dilates arteries via activation of Kv7 channels. These results indicated that KYN can be taken into consideration as a possible target for antihypertensive drug development.[39] Despite contrary data regarding the role of KYN in the progression of hypertension, we demonstrated that KYN and its metabolites are connected with this disease development. Our results do not confirm the hypotensive effects of KYN both in transient and chronic phase of observations. On the other hand, KYN may be a part of the commissioning of compensatory mechanisms. Furthermore, we speculated that the impact of KYN on blood vessels may vary according to in vitro or in vivo conditions. The existence of the persistent inflammation in studies performed on patients with sepsis may explain the vasodilatory action of KYN[40] that may be distinct in the conditions present in the current study.

One of the further metabolites of Trp that was elevated in this study was KYNA, whose concentration was increased in plasma and all the examined tissues, with the highest level present in the kidneys. After the initial drop (in left kidney in Goldblatt group after eight weeks), KYNA levels had increased and remained at a high level in plasma, lungs, and liver. In our study, plasma KYNA concentration positively correlated with MBP and SBP, and we noticed inverse relationship only between liver KYNA and MBP values (Tables 1 and 2). Moreover, the strong correlation between Ang II and KYNA was shown (Figure 3). These associations documented the link between increased

KYNA and elevated Ang II and blood pressure parameters. However, in the present study, we are not able to designate the precise vasoactive role of this KYN metabolite. It is not excluded that the hypotensive effect of KYNA is limited only by the central action. KYNA, as an antagonist of a7 nicotinic receptor in the central nervous system (CNS), has been implicated in the autonomic regulation of blood pressure.[41] It has been proven that the injection of KYNA in the rostral ventrolateral medulla—an area in the brainstem that controls autonomic functions—reduced blood pressure in SHR.[42] An increase in KYNA concentration in peripheral tissues does not lead to elevation of its concentrations in the CNS, due to inadequate penetration through the blood–brain barrier.[43] Thus, it is possible that KYNA is related to renovascular hypertension in the periphery, but its central effect on blood vessel wall may be distinct. On the other hand, KYNA can act by different receptors; for example, it is an endogenous ligand for AhR,[44] whose activation by industrial dioxins can impair endothelium-dependent vasodilatation leading to hypertension.[45] KYNA is also the inhibitor of NMDA receptor, whose activation results in increased NO production and vasodilatation.[46] Because KYNA/KYN ratio increases with the pressure, we presume that this compound plays an important role in the intensification of changes occurring in the blood vessels during the development of renovascular hypertension.

Similarly, as in the case of KYNA, the concentration of AA was increased in the plasma of the animals with hypertension by nearly 61% (Table 1). As with KYNA, AA/KYN ratio also rose simultaneously with the development of hypertension (Figure 4). Unfortunately, our technical limitations did not allow us to determine the levels of AA in obtained tissues. However, plasma AA concentration did correlate neither with blood pressure parameters, nor with Ang II levels. Currently, there are no available studies showing changes in the concentration of AA during high blood pressure, and its role in this abnormality is still not determined. Data from studies of Schindler et al.[20] indicate that HMR1766 and S3448—derivatives of AA are agonists of a ubiquitous NO receptor (precisely, sGC—the heme-enzyme soluble guanylyl cyclase), which may result in increased concentration of cGMP and vasorelaxation. In a recent clinical observation, AA/KYN ratio was decreased in patients with pulmonary arterial hypertension compared with controls.[47] These data suggest that AA can represent the adaptive mechanisms to counteract the changes occurring during hypertension.

The current study also provided evidence for the accumulation of 3-HKYN in plasma and tissues (excluding left kidney) of rats in experimental groups (Table 1). Furthermore, while levels of KYN in chronic phase gradually normalized, 3HKYN/KYN ratio was significantly increased, and the accumulation of 3HKYN in plasma and tissues was associated with higher values of MBP, SBP as well as with Ang II concentrations (Table 2; Figure 3). These results suggest that 3HKYN may rather exert a hypertensive effect in 2K1C model, although its compensatory effect cannot be excluded. 3HKYN is the compound with the ability to generate free radicals,[48] which are one of the well-recognized factors in hypertension development.[49] Our previous findings showed that oxidative stress generated by 3HKYN may be partially responsible for increased endothelial dysfunction in patients with chronic kidney disease.[50] Although our study showed the connection between 3HKYN, blood pressure parameters and Ang II, its role in renovascular hypertension still remains unknown.

Summing up, the results of our study showed for the first time that renovascular hypertension is associated with the activation of the KYN pathway—a pivotal metabolic pathway of dietary Trp. The alterations of these pathway components were in close relationship with blood pressure values and Ang II concentration, suggesting the connection between them. An important factor leading to the activation of the KYN pathway seems to be the free form of Trp. Although the impact of Trp metabolites on blood pressure still remains not fully understood, our experiment suggests that particular metabolites of KYN pathway may play previously unknown contradictory role in the field of vascular tension. All of these mentioned components need further examination, which allows us to take a step further toward the understanding of the complicated mechanism of renovascular hypertension resulting in new effective therapeutic methods.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. BJ, PK, TKA, and PD have conducted the experiment. KT, KM, and DP wrote the manuscript. PD was the head of the conducted project.

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