

Tryptophan metabolism in experimental necrotizing acute pancreatitis

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Abstract: Pancreatic encephalopathy is a serious, often lethal complication of acute pancreatitis (AP). Its pathomechanism remains obscure. We have previously described increased blood levels of quinolinic acid (QUIN) – an endogenous neurotoxin – during edematous experimental acute pancreatitis. Several other metabolites of tryptophan (TRP) are also known to be neuroactive. The aim of the present study was to assess tryptophan and its main metabolites: kynurenine (KYN), 3-hydroxykynurenine, quinolinic acid (QUIN), kynurenic acid (KYNA), serotonin (5HT) during experimental acute necrotizing acute pancreatitis. Experimental necrotizing acute pancreatitis was induced in rats by intraductal injection of 5% sodium taurocholate. Control groups consisted of sham-operated and not operated rats. The animals were sacrificed 5 and 24 hours after the operation. We evaluated α -amylase, pancreas weight and histology as parameters of pancreatitis. A simplified neurological scoring system was applied. To assess TRP and its metabolites in plasma, we used high performance liquid chromatography. Five hours after the onset of AP we found significant increase in TRP metabolites: QUIN, KYNA, KYN, and 3HKYN in the plasma of animals with AP, as compared to the control group. When assessed 24 hours after induction of AP, those changes were no longer observed in blood. Instead, a decrease in TRP level appeared. Increase in plasma QUIN was associated with neurologic disturbances. In the present study we demonstrated transient activation of kynurenine pathway during early stages of experimental necrotizing AP, with increased blood levels of QUIN, KYNA, KYN, and 3HKYN and subsequent depletion of TRP. As some kynurenine derivatives, e.g. quinolinic acid, are endogenous toxins, they might contribute to neurologic and other organs disturbances during AP.

Key words: acute pancreatitis, tryptophan, kynurenine, 3-hydroxykynurenine, kynurenic acid, quinolinic acid, serotonin

Introduction

The hallmark of severe necrotizing acute pancreatitis (AP), in addition to local lesions, is multi-organ involvement. Extremely high mortality was observed when pancreatic encephalopathy developed [1]. This serious condition, in recent years increasingly described in literature, occurs with neuropsychiatric signs in the early course (usually within two weeks) of severe AP [2,3]. It can be suspected on the basis of clinical findings but the

final diagnosis is generally provided by post mortem examination [4]. The prevalence of encephalopathy during AP was reported from 2% [5] to 35% in one of prospective studies analyzing electroencephalographic and CSF changes [6]. In a recent study pancreatic encephalopathy was diagnosed in 18.2% of patients with severe AP [2]. The pathomechanism of the condition is unclear and there is no specific treatment [3].

We observed previously an increase in systemic quinolinic acid (QUIN) concentration during experimental edematous acute pancreatitis [7]. QUIN is a product of tryptophan (TRP) metabolism via the kynurenine (KYN) pathway (Fig. 1). QUIN is an endogenous specific agonist of excitatory N-methyl-D-aspartate (NMDA) receptor. In excess QUIN causes

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Table 1. Study design: groups specification, surgical procedures, time of sacrifice and number of animals. In the bottom panel we show the results of amylase concentration in plasma (mean ± SD), wet/dry ratio of pancreata (mean ± SD) and neurological score (median (full range)). Significant differences as compared to the control group are shown as: * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$; as compared to sham operated animals: # – $p < 0.05$; ### – $p < 0.001$. AP – acute pancreatitis; ST – sodium taurocholate (5%)

Group	Control (n = 6)	AP-5h (n = 8)	AP-24h (n = 8)	Sham-5h (n = 7)	Sham-25h (n = 8)
Procedure	no procedure	AP induction	AP induction	sham operation	sham operation
Technique	no procedure	intraductal ST infusion	intraductal ST infusion	cannulation of the duct	cannulation of the duct
Sacrifice	0 hours	5 hours	24 hours	5 hours	24 hours
Plasma amylase [IU/L]	232 ± 43	2752 ± 878 *** ###	2520 ± 1450 * #	394 ± 110	253 ± 33
Pancreas wet/dry ratio	2.1 ± 0.1	7.3 ± 0.53 * #	6.9 ± 0.53 * #	3.6 ± 0.15	3.1 ± 0.19
Neurological score	1 (1-1)	3 (2-4) ** #	3 (3-4) ** #	2 (1-3) *	1 (1-2)

Table 2. The neurological evaluation system. Based on [13], modified.

Score	1	2	3	4
Spontaneous activity in cage for 5 min	moves and approaches at least two sides of cage	moves but does not approach at least two sides of cage	barely moves	no movement

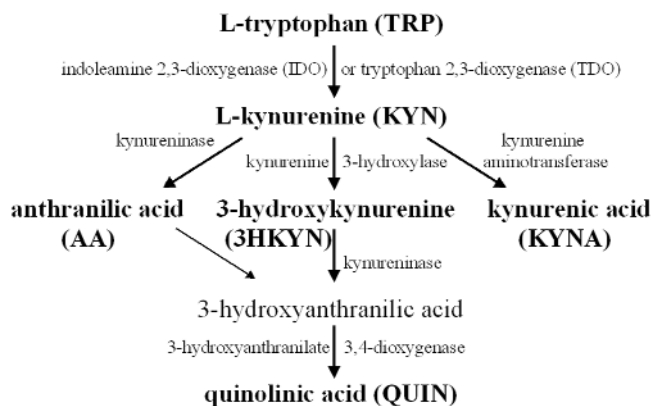


Fig. 1. The simplified scheme of kynurenine pathway (enzymes included).

seizures, as was demonstrated in animals [8]. QUIN was also shown to be implicated in hepatic and AIDS-related encephalopathy. In patients dying of hepatic coma, the concentrations of QUIN in the frontal cortex were found to be 2- to 3 times greater than in samples taken from control subjects [8].

Several other metabolites of tryptophan (TRP) are also known to be neuroactive: 3-hydroxykynurenine, kynurenic acid (KYNA), serotonin (5HT) [9-11]. Interestingly, while 3HKYN and QUIN were described for their neurotoxic properties, KYNA and 5HT have neuroprotective potential. To our knowledge there have been no reports on above-mentioned metabolites in acute pancreatitis.

The aim of the present study was to assess tryptophan and its main metabolites during acute necrotizing acute pancreatitis. Our interest in the subject is supported by the availability of therapeutic agents counteracting the toxicity of QUIN and other tryptophan metabolites [8].

Materials and methods

Animal model. All experiments were conducted with the prior approval of the Local Ethical Committee and according to international guidelines. Male Wistar rats weighing about 200g were used. They received standard chow and water *ad libitum* and were kept at the temperature of 21°C with a 12 h dark-light cycle. The animals were deprived of food starting 12 hours before the experiment, but had free access to water.

Necrotizing AP was induced according to Aho *et al.* [12]. After peritoneal anesthesia with sodium pentobarbital 40mg/kg, a mid-line laparotomy was performed. The biliopancreatic duct was cannulated transduodenally with a blunt injection needle at a depth of 5 mm. The hepatic duct at the hilum of the liver was closed with a small bulldog clamp and a 2-0 silk ligature was tightened around the needle and the wall of the duct. A 5% sodium taurocholate (0.1 ml/100g) in saline was injected under a steady manual pressure over a period of 60 sec. After removal of the needle and the ligature, the incision was closed in two layers using 8-0 Vicryl sutures. The abdomen was closed in two layers and 4 ml saline was injected in the back subcutaneously.

After the operation the animals were observed for neurological deficits. We used a simplified scoring system, based on the work of Garcia *et al.* [13] (Table 2).

Experimental animals were sacrificed at 5 or 24 hours after the operation (Table 1). Blood was taken from the right ventricle under peritoneal anesthesia with sodium pentobarbital 40 mg/kg. 7.2 ml of blood was poured into a tube containing 0.8 ml of 3.8% sodium citrate. Immediately after sampling, the blood was centrifuged at 3.500 rpm for 10 minutes at 4°C. Plasma was separated and stored at -70°C until it was assayed.

Tissue samples of pancreas head were fixed in formaldehyde for subsequent paraffin embedding and staining. A portion of pancreas was taken for wet/dry ratio assessment (as described below).

Experimental design. Rats were randomly allocated to five groups according to Table 1. Control animals were not operated on. In both groups with acute pancreatitis, the disease was induced as described above. After 5 or 24 hours the animals were sacrificed. Sham-operated rats were laparotomized in the midline. The biliopancreatic duct was cannulated transduodenally with a blunt injection needle at a depth of 5 mm. A 2-0 silk ligature was tightened around the needle

and the wall of the duct, but no injection was performed. After 1 minute a needle was withdrawn and both intestine and abdominal wall were closed in two layers. The time of sacrifice of sham-operated animals was either 5 or 24 hours.

Amylase. Plasma amylase activity was measured using a quantitative colorimetric assay (Phadebas Amylase Test, Pharmacia & Upjohn, Sweden).

HPLC determination of TRP and its metabolites. Prior to analysis, the plasma was deproteinized with 2 M trichloroacetic acid, and after centrifugation at 14,000 rpm for 10 minutes (temp. 4°C), supernatant was filtered through a filter (WATERS 0.45 µm).

TRP, KYNA and anthranilic acid (AA) were assessed according to the method described by Herve *et al.* [14]. The reverse-phase system was composed of a Waters Spherisorb S3 ODS2 150 × 2.1 mm column (USA), HP 1050 pump (Germany) and Rheodyne injection valve. The effluent was monitored using a fluorescence detector HP 1046A (Germany). Excitation and emission wavelengths were set at 254/404 nm for TRP and KYNA and for 320/420 nm for AA. The mobile phase was pumped at a flow rate 0.25 ml/min and consisted of 50 mM acetic acid, 0.25 M zinc acetate (pH 4.9) and 1.2% of acetonitrile. Chromatography was carried out at 25°C.

KYN was assessed according to the method described by Holmes [15]. The chromatographic system Hewlett-Packard (Germany) was composed of a HP 1050 series pump, Rheodyne injection valve fitted with a sample loop (5 µl). A column – LiChrospher 100 RP-18. 5 µm., 125 × 4 mm (Germany). Using a HP 1050 series UV detector (Germany) monitored the column effluent (365 nm). The output of the detector was connected to a single instrument LC-2D ChemStation (Germany). The mobile phase was pumped at a flow-rate of 1.5 ml/min. The mobile phase consisted of 0.1 M acetic acid, 0.1 M ammonium acetate (pH 4.65) containing 2% of acetonitrile. Chromatography was carried out at 25°C.

3HKYN, 5HT and 5HIAA were assessed according to Heyes *et al.* [16]. The reversed-phase HPLC system Hewlett-Packard (Germany) was composed of a HP 1050 series pump, Rheodyne injection valve fitted with a sample loop (5 µl). A column – S3 ODS2 150 × 2.1 mm was purchased from Waters-Spherisorb. The column effluent was assessed using a programmable electrochemical detector HP 0149A (Germany), with the potential of working electrode – 0.6 V. The output of the detector was connected to a single instrument LC-2D ChemStation (Germany). The mobile phase was pumped at a flow-rate of 0.25 ml/min. 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. Chromatography was carried out at 25°C.

QUIN was measured using HPLC technique as described by Werner-Felmayer *et al.* [17]. The chromatographic system Hewlett-Packard (Germany) was composed of a HP 1050 series pump, Rheodyne injection valve fitted with a sample loop (20 µl). Partisil 10 SAX 250 × 4.6 mm (Phase Separations, USA) column was eluted with 50 mM potassium phosphate (pH 2.0) containing 12% methanol at a flow rate of 2 ml/min. The amount of 2 ml of plasma was concentrated on Sep-Pack cartridges (WATERS Accell™ Plus QMA), washed in 2 ml of water, and eluted with 0.2 ml 4 M H₃PO₄ (recovery of spiked quinolinic acid 92%). Using a HP 1050 series UV detector (Germany) the column effluent was monitored (272 nm). The output of the detector was connected to a single instrument LC-2D ChemStation (Germany). Chromatography was carried out at 25°C.

Histological examination. A 3-5 mm³ portion of pancreas head was fixed in 10% neutral formaldehyde solution and subsequently embedded in paraffin. Sections were cut at 5 µm of thickness and

stained with hematoxylin and eosin. Assessment comprised edema, inflammatory infiltration, fat necrosis, parenchymal necrosis and hemorrhage.

Pancreatic edema. Pancreas water content was assessed by the tissue wet/dry ratio. Pieces of pancreas were weighed immediately after harvesting (wet weight) and again after desiccation (dry weight). Desiccation was achieved in an oven at 120°C for 24 hours.

Neurological evaluation. We adapted a simplified scheme of Garcia *et al.* [13] (Table 2). Spontaneous activity of experimental animals was observed for 5 min. in their normal environment (in a cage).

Statistical analysis. All the groups were tested for normality. Gaussian results were further evaluated using ANOVA, t-Student and Pearson tests. Alternatively, non-parametric tests, such as: Kruskal-Wallis, Mann-Whitney and Spearman's test were used to assess population homogeneity, inter-group differences and correlations between chosen metabolites, accordingly. The threshold of significance was p<0.05.

Results

Histologic assessment (Fig. 2), amylase activity measurement in plasma and wet/dry weight ratio (Table 1) have confirmed the presence of necrotizing acute pancreatitis in studied animals. Marked increase in plasma amylase activity was seen in the rats with necrotizing acute pancreatitis, both 5 hours and 24 hours after the onset of AP, as compared to the control group and to sham-operated rats (Table 1). The increase exceeded 10-fold in both cases. Sham operated animals did not differ significantly from the control group, despite the cannulation of biliopancreatic duct. Also the wet/dry ratio of pancreata was substantially increased in animals with experimental AP, both after 5 and 24 hours, as compared to the control animals, as well as sham operated rats (Table 1). The wet/dry ratio in the rodents was apparently greater after sham operation than in not operated animals, but no significant difference was found.

In animals with AP we observed a significant reduction in neurological functions defined by applied scoring system (Table 2), as compared to the control (p<0.01) and sham-operated animals (p<0.05) (Table 1). We observed a transient impairment in neurological function of rats 5 hours after sham-operation, which was no longer seen after 24 hours.

We observed substantial irregularities in TRP metabolism during necrotizing acute pancreatitis in rats. TRP (Table 3) showed a significant decrease after 24 hours from the onset of AP (p<0.05 as compared to the control group and AP after 5 hours). In contrast to TRP, we observed during AP early changes in such metabolites' levels as: KYN, 3HKYN, KYNA and QUIN. The greatest increases were found in QUIN concentration (2^{1/2}-fold, p<0.05); lesser for KYNA (about 2-fold), 3HKYN and AA (about 1^{1/2}-fold).

Table 3. Plasma concentrations of L-tryptophan (TRP), kynurenine (KYN), 3-hydroxykynurenine (3HKYN), kynurenic acid (KYNA), anthranilic acid (AA), quinolinic acid, serotonin (5HT) and 5-hydroxyindolacetic acid (5HIAA) in plasma. Data represent the mean \pm SD for AA and QA or median (full range) for the rest. Significant differences as compared to the control group are shown as: * – $p < 0.05$; ** – $p < 0.01$; as compared to sham operated animals: # – $p < 0.05$; ### – $p < 0.001$; as compared to AP-5h: ^ – $p < 0.05$; ^^ – $p < 0.01$; ^^ – $p < 0.001$. AP – acute pancreatitis.

Group	Control (n = 6)	AP-5h (n = 8)	AP-24h (n = 8)	Sham-5h (n = 7)	Sham-25h (n = 8)
TRP [μ M]	31.2 (26.7-33.1)	31.9 (21.4-36.8)	19.5 (8.1-34.2)*^	32.7 (12.6-34.4)	27.2 (17.5-30.8)
KYN [μ M]	2.58 (2.19-2.87)	4.2 (2.4-6.7)*	1.72 (0.99-3.95)^^	3.3 (2.2-4.8)	2 (1.8-3.1)
3HKYN [nM]	55.2 (28.6-55.9)	81.1 (55.2-110)*#	51.7 (24.1-121)	65.5 (48.3-82.8)	36.2 (24.1-79.3)
KYNA [nM]	42.9 (42.4-53.7)	86.6 (35.7-123)*	25.6 (11.3-114)^	42.1 (33.2-119)	28.1 (7.1-42.1)**
AA [nM]	135.7 \pm 46.1	224 \pm 47***	89.4 \pm 44.5^^^	156.6 \pm 45	87.5 \pm 40.2
QUIN [nM]	149.3 \pm 19.1	371 \pm 48****	113 \pm 65^^^	93.4 \pm 27.2*	120 \pm 51.2
5HT [nM]	100 (57-124)	48.6 (21-200)	63.8 (40.2-174)	53.2 (34.2-81.6)*	109 (48.5-118)
5HIAA [nM]	28.8 (27.8-35.8)	27.8 (10.9-39.8)	36.2 (21.9-117)	31.8 (24.9-48.7)	13.9 (2.9-22.9)**

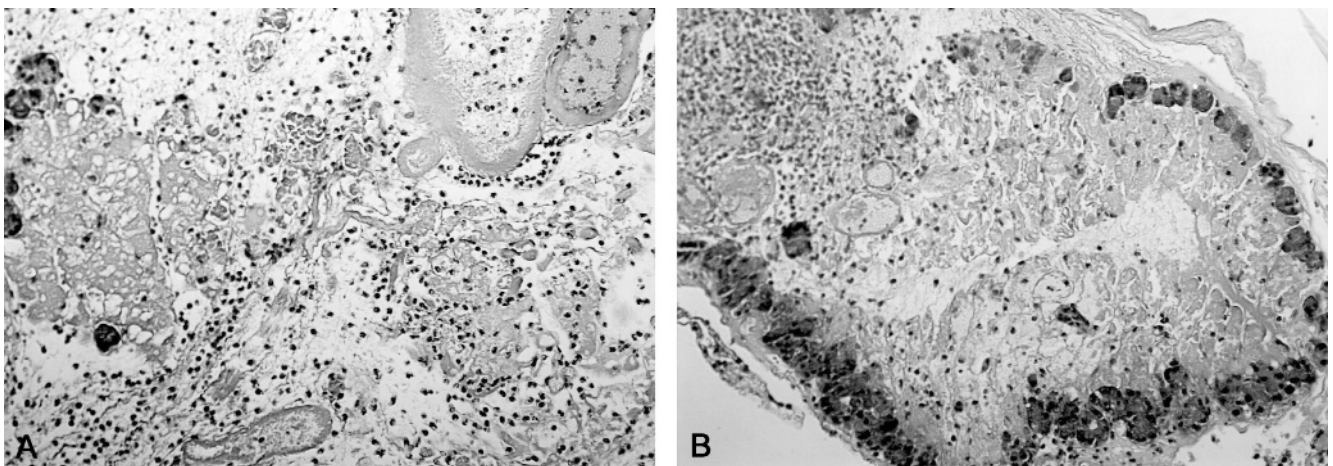


Fig. 2. Histology of pancreata of rats with necrotizing acute pancreatitis: A – 5 hours after the induction of AP (H&E, original magnification $\times 200$); B – 24 hours after the induction of AP (H&E, original magnification $\times 200$).

After 24 hours a drop could be observed in KYN concentration in AP group ($p < 0.01$ as compared to the AP-5h group). Also other metabolites underwent normalization after 24 hours: QUIN, KYNA, 3HKYN and AA ($p < 0.001$ for QUIN and AA as compared to AP-5 hours) (Table 3).

Neither 5HT nor 5HIAA changed significantly after induction of AP. In sham-operated animals 5HT had lower values 5 hours after the operation than in the control group ($p < 0.05$). There was also a decrease in QUIN level 5 hours after sham operation.

There were no significant correlations between plasma amylase activity or wet/dry ratio of pancreata and any of the studied metabolites. However, using Spearman's test, we found a high association between neurological score and QUIN ($p < 0.05$; $R = 0.918$), as assessed 5 hours after induction of AP. Similar correlation was found for AA ($p < 0.05$; $R = 0.848$).

Discussion

In the present study we demonstrated for the first time an early increase in several TRP metabolites after induction of experimental necrotizing AP. In descending order this was observed for: QUIN (2 $\frac{1}{2}$ -fold), KYNA (2-fold), KYN, AA and 3HKYN (1 $\frac{1}{2}$ -fold). Those changes were of short duration and after 24 hours were no longer observed. By that time, however, a significant decrease in TRP was noted (by about $\frac{1}{3}$). This might suggest increased turnover of TRP via kynurenine pathway in the early stage of AP.

Acute pancreatitis triggers a cascade of cytokine synthesis. The main molecules involved seem to be interleukines (IL-1 β , IL-6, IL-8) and TNF- α [18]. Also a rise in interferon- γ (INF- γ) during AP has been recently reported, especially in severe form of the disease [19]. INF- γ typically induces a depletion in TRP

and an increase in KYN, which in turn is further metabolised to other compounds, including QUIN (Fig. 1). There is evidence that $\text{INF-}\gamma$ and $\text{TNF-}\alpha$, when present together, stimulate QUIN production from TRP to levels exceeding neurotoxic threshold [8]. In this study we found an increase in KYN as assessed 5 hours after induction of the disease (Fig. 3). Interestingly, TRP level decreased only after 24 hours after induction of AP. Our results stand in favor for early kynurenine pathway activation by IDO induction in AP, although to obtain a direct evidence, a measurement of enzymes activity would be of value.

At least two of the above-mentioned TRP metabolites have neurotoxic properties. This was first discovered for QUIN, which caused seizures, when injected to the brain of rodents [20]. Similar effects were demonstrated by intravenous administration of QUIN in rats with immature blood-brain barrier [10]. QUIN is an endogenous agonist at the N-methyl-D-aspartate sensitive subtype of glutamate receptors in the brain [8]. It has also been suggested, that QUIN caused brain damage via lipid peroxidation [21].

QUIN, when increased, has a potential of producing multiple organ impairment. The direct way leads to excitotoxicity through N-methyl-D-aspartate (NMDA) receptor activation in the nervous system. Additionally, QUIN was reported to act via production of reactive oxygen species [22]. QUIN provokes seizures when injected into brain, but also after intravenous administration when blood-brain barrier is not competent. Under physiologic conditions in rats only 0.3% of intravenously administered QUIN penetrates the blood brain-barrier in a single passage, however, even then brain concentration of QUIN may increase up to 10 times [8]. It has recently been shown that blood-brain barrier permeability markedly increases in the early course of AP [23]. It is also known that interferon- γ activation increases local synthesis of QUIN in brain, independently of changes in blood-brain barrier permeability [24]. Thus both increased blood-brain barrier permeability and enhanced local synthesis might contribute to potential QUIN neurotoxicity in AP.

NMDA receptors activation does not explain all QUIN actions. It has been established that QUIN induces the formation of reactive oxygen species and raises lipid peroxidation by up to 256% [8]. This might aggravate the detrimental effect of free oxygen species reported in AP [18]. Other actions of QUIN include cardiac contractility impairment, probably by modulation of heart calcium channel [25] and the inhibition of erythropoiesis [26].

3HKYN is another potent neurotoxin, with receptor-independent mode of action. It is one of most potent known generators of reactive oxygen species [27]. When injected into rat brain, 3HKYN caused tissue damage around the injection site. QUIN needed

4 times greater doses for the same effect [28]. Moreover, 3HKYN was reported to potentiate the neurotoxicity of QUIN [29].

Another of TRP metabolites – KYNA is known for its neuroprotective action and prevention of damage induced by excess of QUIN [10]. Also KYN can play a protective role against quinolinic acid-induced neurotoxicity [30]. The addition of KYNA inhibited lipid peroxidation induced by QUIN in brain tissue [21]. Similar properties were attributed to 5HT [9].

An intriguing question is why QUIN rises only temporarily. One of possible explanations is the passage of QUIN outside blood vessels. The main compartment, where edema develops during AP is abdominal cavity, especially peripancreatic space [31]. However, blood vessel wall permeability is markedly increased also in brain during AP, as early as a few hours after its onset [23]. This, however, is merely a speculation and potential QUIN increase in the tissues during AP needs further studies.

To sum up, we have found a transient, albeit marked increase in plasma concentrations of several TRP metabolites: QUIN, KYNA, KYN, AA, 3HKYN in rats with experimental necrotizing AP. Despite short duration of QUIN increase in blood, it might contribute to neurological and possibly other organ disturbances during AP. This is supported by the association between QUIN level in plasma and neurological deficits in the animal model of taurocholate-induced AP. Studies on tissue concentrations of QUIN and other kynurenines during AP might bring new insights into this intriguing problem.

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