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Investigation of the kynurenine metabolite profile of tryptophan degradation in the cuprizone toxin-induced demyelination animal model of multiple sclerosis

Summary of Ph.D. Thesis

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Original Publication directly related to the Ph.D. thesis:

I. **Polyák H.**, Cseh E.K., Bohár Zs., Rajda C., Zádori D., Klivényi P., Toldi J., Vécsei L. *Cuprizone markedly decreases kynurenic acid levels in the rodent brain tissue and plasma.* **Heliyon** 2021; 7:e06124. doi: 10.1016/j.heliyon.2021.e06124 (original paper**, IF: 3.776 (2021); Q1**)

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II. Cseh E.K., Veres G., Körtési T., **Polyák H.**, Nánási N., Tajti J., Párdutz Á., Klivényi P., Vécsei L., Zádori D. *Neurotransmitter and tryptophan metabolite concentration changes in the complete Freund's adjuvant model of orofacial pain*. **J. Headache Pain. 2020**, 21: 35. doi: 10.1186/s10194-020-01105-6 (original paper, **IF: 7.277 (2020); Q1)**

III. Pukoli D., **Polyák H.**, Rajda C., Vécsei L. *Kynurenines and neurofilament light chain in multiple sclerosis*. **Front. Neurosci. 2021**, 15: 658202. doi: 10.3389/fnins.2021.658202 **(IF: 5.152 (2021); Q2)**

IV. Tanaka M., Tóth F., **Polyák H.**, Szabó Á., Mándi Y., Vécsei L. *Immune influencers in action: metabolites and enzymes of the tryptophan-kynurenine metabolic pathway*. **Biomedicines 2021**, 9: 734. doi: 10.3390/biomedicines9070734 **(IF: 4.757 (2021); Q1)**

V. Tanaka M., Spekker E., Szabó Á., **Polyák H.**, Vécsei L. *Modelling the neurodevelopmental pathogenesis in neuropsychiatric disorders. Bioactive kynurenines and their analogues as neuroprotective agents—in celebration of 80th birthday of Professor Peter Riederer*, **J. Neural Transmi. 2022**, 129:627-642. doi: 10.1007/s00702-022-02513-5 **(IF: 3.3 (2022) ; Q3)**

VI. Tanaka M., Szabó Á., Spekker E., **Polyák H.**, Tóth F., Vécsei L. *Mitochondrial Impairment: A common motif in neuropsychiatric presentation? The link to the tryptophan–kynurenine metabolic system*. **Cells 2022,** 11: 2607. doi: 10.3390/cells11162607 **(IF: 6.0 (2022); Q2)**

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List of abbreviation

ANA – anthranilic acid

CNS – central nervous system

CPZ – cuprizone

CO – control

GFAP – glial fibrillary acidic protein

3-HK – 3-hydroxy-L-kynurenine

HPLC – high-performance liquid chromatography

KP – kynurenine pathway

KYNA – kynurenic acid

LFB/CV – luxol fast blue - cresyl violet

MBP – myelin basic protein

MS – multiple sclerosis

TRP – tryptophan

UHPLCMS/MS – ultra-high performance liquid chromatography with tandem mass spectrometry

XA – xanthurenic acid

Introduction

Multiple sclerosis (MS) is an immune-mediated, chronic inflammatory, demyelinating and progressive disease of the central nervous system (CNS), which can be characterized by the lesions caused by demyelination, loss of axons and oligodendrocytes, gliosis, axonal damage, and loss of neurons in the brain and spinal cord. Worldwide, approximately 2.8 million people suffer from this disease. MS usually appears in young adulthood, placing a heavy burden on those affected by the disease, society and the economy. The MS can be typified by gender differences, which is affecting the severity and occurrence of the disease. The specific root cause of the disease is not yet completely clear however, various environmental, genetic and epigenetic factors may contribute to the development of the MS.

The exact pathomechanism of the disease, as well as the various molecular and metabolic processes behind the neuro-axonal damage are not completely clear, however oxidative stress largely contributes to the progression of MS, causing neuronal and axonal injury. Furthermore, the malfunctioning and regulation of the immune system, the disturbances in the balance of pro- and anti-inflammatory cytokines, the disruption of the blood-brain barrier, and the activation of glial cells are also important influencing factors. CNS infiltration of peripheral T and B immune cells, and macrophages, as well as the CNS reactivity of astrocytes and microglia, cause excessive production of inflammatory cytokines and reactive oxygen species (ROS), which ultimately results in oxidative stress in addition to reduced antioxidant activity. Based on research, in addition to oxidative stress and inflammatory responses mediated by the immune system, other neurodegenerative processes also contribute to the pathogenesis and progression of MS. Current treatments concentrate on reducing disease activity, treating attack and alleviating symptoms with disease modifying therapies. Treatments of the MS can be immunomodulatory and immunosuppressant, as well as immune reconstitution therapies.

The cuprizone (CPZ) toxin-induced demyelination animal model is excellent for studying the pathomechanism of MS. The CPZ toxin is a copper chelating agent that selectively induces apoptosis of mature oligodendrocytes. CPZ affects the function of the mitochondrial respiratory chain by inhibiting certain mitochondrial complexes. However, poisoning-induced mature oligodendrocyte cell death is not evenly distributed in the CNS, with extensive demyelination observed in the corpus callosum, cortex, striatum, hippocampus and cerebellum, as well as to a limit extent in the brainstem and spinal cord. In the CPZ treatment,

oligodendrocytosis begins, followed by microglia, macrophage and astrocyte activation. However, if the CPZ treatment is discontinued, rapid regeneration occurs, when mature oligodendrocytes are formed from the oligodendrocyte progenitor cells during remyelination and the gliosis gradually ceases.

The CPZ model can be suitable for the examination and full exploration the underlying mechanisms involved in the progressive stages of MS, which processes are independent of the infiltration of adaptive immune cells into the CNS. These similarities during the demyelination process, as well as the joint study of the remyelination phase, which can be easily influenced by the length of the CPZ toxin treatment, make the CPZ model advantageous and relevant in the investigation of the pathomechanism of MS.

The essential amino acid tryptophan (TRP) is metabolized to a significant extent, approximately 95%, though the kynurenine pathway (KP), and a lesser extent via the serotonin pathway. The metabolism of TRP can be associated with the functioning of the nervous system, inflammation and immune system processes. Various neuroactive metabolites are formed during TRP degradation. In the KP, several abnormalities have been observed in various neurodegenerative disorders, including MS.

Aims

After the successful establishment of the CPZ toxin-induced MS rodent model, our plans included the examination of the concentration of TRP, serotonin, L- kynurenine and kynurenic acid (KYNA) metabolites in the serotonin and kynurenine pathways of TRP metabolism.
Based on our previous results, the further goal of our examination was the detailed mapping of the metabolites involved in the kynurenine pathway of the TRP degradation during the CPZ toxin treatment and recovery phase.

Materials and methods

Animals

In our investigations, eight-week old male C57Bl/6J mice were used (n = 224). The animals were bred and maintained under standard laboratory conditions with 12 h–12 h light/dark cycle at 24 ± 1 ºC and 45–55% relative humidity in the Animal House of the Department of Neurology, University of Szeged. The investigations were in accordance with the Ethical Codex of Animal Experiments and were approved by the Ethics Committee of the Faculty of

Medicine, University of Szeged, and the National Food Chain Safety Office with a permission number of XI/1101/2018. The animals were housed in polycarbonate cages (530 cm^3 floor space) in groups of 4-5. Prior to the start of the experiments, all animals were acclimated to grounded standard rodent chow for 2 weeks, and animal's weight was measured every other day, thus monitoring the health status of the mice during acclimatization. Furthermore, during the entire duration of our experiments, in both the CPZ treatment (demyelination) and the recovery (remyelination) phase, we measured the animal's body weight every other day.

Treatment

In our investigations, half of the experimental animals, as the CPZ group, were given a diet containing 0.2% CPZ toxin for 5 weeks mixed into a grounded standard rodent chow with free access to water. For control group (CO), age and weight-matched animals were used, which had rodent chow and free access to water. At the end of 5-week CPZ treatment (demyelination period), half of the animals in the CPZ treated and control groups were randomly chosen and sacrificed. The surviving animals participated in a 4-week recovery phase (remyelination period). At the end of 4^{th} week, the remaining animals were sacrificed too, sample collection purpose. During the examination, at the end of demyelination and remyelination phase, behavioral tests were performed.

Behavioral tests

During the investigation, the open field test was performed to examine the movement patterns of the animals, while to determine the effects of CPZ treatment on motor function we applied the rotarod test each animal in CPZ treated and CO groups.

Behavioral measurements were carried out in the 3^{rd}, 4^{th} and 5^{th} week of CPZ poisoning and in the 3^{rd} and 4^{th} week of the remyelination phase, once a week on the same day.

Immunohistochemical and histological analysis

During immunohistochemical analysis, anti-glial fibrillary acidic protein (GFAP) for astrocyte visualization and anti-myelin basic protein (MBP) to detect myelin were applied. Furthermore, myelin damage was evaluated with luxol fast blue - cresyl violet (LFB/CV) staining too. Quantification of astrogliosis was performed by manual cell counting of the GFAP-immunopositive cells in the corpus callosum. The myelin content was determined by intensity measurement after LFB/CV and MBP staining.

Bioanalytical measurements

For high-performance liquid chromatography (HPLC) measurement, plasma and brain samples were deproteinized by precipitation, as described before. The mobile phase, in each case was a 200 mM zinc acetate solution, at final pH of 6.2 for plasma, and 5.8 for brain tissue samples, with a final concentration of 5 % of acetonitrile.

For ultra-high-performance liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS) measurement, plasma samples were mixed with aqueous FA and ice-cold acetone–MeOH containing SIL-IS mix, and supernatant was processed further. The brain samples, after measuring the weight of the five different brain regions, we homogenized them in 3x amount of ice-cooled LC-MS water. After that, the same steps as for the plasma samples were performed, with the difference that the precipitation was carried out with 100% acetonitrile. Then, plasma samples and brain regions were measured according to the previously published methodology using UHPLC-MS/MS.

Statistical Analysis

For the statistical analysis of body weight, two-way repeated-measures ANOVA was applied. For the histological analysis were used one-way analysis of variance (ANOVA), then depending on the variances of data, Sidak or Tamhane's T2 post hoc test was used. Pairwise comparisons of group means were based on the estimated marginal means with Sidak or Tamhane's T2 post hoc test with adjustment for multiple comparisons. Group values were given as means ± SEM, analyses were performed in SPSS Statistics software. Regarding the HPLC measurements, if the distribution (Shapiro–Wilk test) was proven to be Gaussian and the variances were equal (Levene test) ANOVA was used with Tukey HSD post hoc test for pairwise comparison, otherwise Kruskal-Wallis, with the Wilcoxon post hoc test was applied. Data were plotted as median (1st–3rd quartile). Regarding the UHPLC-MS/MS measurements, after checking for its assumptions (checking for outliers, Shapiro and Levene tests), we performed two-way ANOVA with estimated marginal means post hoc tests to determine significance between treatment groups, measurement times, and their interaction. In case of the assumptions were not met, we used the Sheirer–Ray–Hare test with Dunn test as post hoc. Type I errors from multiple comparisons were controlled with the Bonferroni method. We rejected null hypotheses when the corrected p level was < 0.05, and in such cases, the differences were considered significant.

Results

I. Analysis of TRP metabolism following the setting of the CPZ toxin-induced demyelination rodent model

During our investigation, already on the third day of the CPZ treatment, a significant decrease was detected in the body weight of the CPZ treated group compared to the CO group **(***p < 0.001)**. However, this disparity disappeared in the recovery phase.

The 5-week CPZ treatment caused an extensive myelin damage within the corpus callosum at the end of the demyelination period, which were analyzed by MBP (****p < 0.01**) and LFB/CV stainings (***p < 0.05**). The GFAP immunostaining showed a significant astrogliosis in the area of corpus callosum in CPZ-treated animals compared to the control group, both in the demyelination and remyelination phases (*****p < 0.001**).

During the HPLC bioanalytical measurements, at the end of the demyelination phase, a significant decrease in KYNA concentration was observed in the plasma (*****p < 0.001**), hippocampus (***p < 0.05**) and cortex (****p < 0.01**), which differences disappeared by the 4th week of remyelination period.

II. Mapping of metabolites concentration involved in the kynurenine pathway TRP metabolism in the CPZ-induced rodent model

During CPZ treatment, a significant decrease was observed in the body weight of the intoxicated animals compared to the CO (*****p < 0.001**). However, during the remyelination period, this difference showed a decreasing trend and at the end of the investigation disappeared.

During immunohistochemical analysis by LFB/CV staining, the CPZ toxin poisoning caused a significant reduction of myelin in the corpus callosum area was visible in the 3rd week of treatment (****p < 0.01**), and the demyelination was even more extensive in the 5th week of CPZ intoxication (*****p < 0.001**). However, in the 2nd week of the recovery phase, there were no more signs of myelin damage on the brain slices.

During UHPLC-MS/MS bioanalytical measurements, in the 1st week of the CPZ treatment we observed significant differences in the concentration of KYNA (*****p < 0.001**), 3-hydroxy-L-kynurenine (3-HK) (***p < 0.05**), and xanthurenic acid (XA) (*****p < 0.001**) of the plasma samples and differences continued until the end of CPZ treatment, because in the 5th week of

CPZ treatment, we also observed differences in plasma TRP (****p < 0.01**) and anthranilic acid (ANA) (*****p < 0.001**) concentrations, in addition to KYNA (****p < 0.01**), 3-HK (*****p < 0.001**) and XA (*****p < 0.001**) between CPZ and CO groups. However, in the 2nd week of the remyelination phase, these concentration distinctions vanished while the concentration of the mentioned metabolites were in the same range in both groups in the recovery period.

During the examination of the brain regions, CPZ poisoning caused significant decrease in the level of 3-HK (*****p < 0.001**) and ANA (*****p < 0.001; **p < 0.01**) of the striatum, cortex, hippocampus and brainstem in the toxin treated group. Furthermore, we found an elevated TRP levels in the striatum (*****p < 0.001**), cortex (*****p < 0.001**) and hippocampus (****p < 0.01**) in the CPZ group at the end of the demyelination period.

Discussion

In the recent decades, MS and its CPZ toxin-induced demyelination animal model have been widely researched. However, TRP metabolism and its KP have not been the focus of research in the CPZ induced model until now.

Our research group was the first to report the KP deviations in the CPZ mouse model. The model is suitable for modeling the progressive form of MS, as the main histopathological appearance of damage caused by poisoning is very similar to the changes in the lesion pattern of MS types III and IV, which characterized by demyelination, oligodendrocyte apoptosis, as well as microglia and macrophage activation.

In the CPZ toxin-induced demyelination animal model, we examined in detail the breakdown of tryptophan, the distribution of the various metabolites produced during its metabolism with bioanalytical measurements. Thereby we mapped the distribution of TRP, serotonin and some kynurenine metabolites in plasma samples and in different brain regions, including the areas most affected by damage caused by intoxication; as well as the differences in response to CPZ treatment in the demyelination phase, as well as then in the recovery period during remyelination. During our studies in the CPZ model, we already experienced a significant difference in body weight between CO and CPZ groups at the beginning of the treatment, which exist until the end of the demyelination period, then disappeared during the remyelination phase, and the body weight of the two groups was the same at the end of investigation. In the analysis of the open-field and rotarod behavior tests did not show differences between groups. However, our immunohistological analyzes showed a significant

degree of demyelination and astrocyte activation in the CPZ-treated group compared to the CO in the 5th week of poisoning. During the analysis of the brain regions and plasma samples using the HLPC technique, we observed a significant decrease in KYNA concentration in the plasma, hippocampus and cortex of the CPZ group, as a result of the treatment, which differences completely disappeared in the recovery phase and the metabolite level of CO and treated animals moved in the same range.

Our histological results, which are consistent with the literature data, as well as our results obtained with the HPLC technique motivated us to investigate how the metabolic pathway of TRP changes during CPZ treatment and after its withdraw. The samples collected at different times of the CPZ treatment enabled the monitoring of the degree of myelin damage and the change in the concentration of metabolites involved in TRP breakdown.

During the analysis of the histological damage caused by intoxication, there was no difference in the myelin content between the groups in the 1st week of treatment. However, in the 3rd weeks of intoxication, we already experienced significant myelin damage in the group treated with CPZ, which became even more pronounced of the 5th week of poisoning and developed into severe demyelination in the area of the corpus callosum, which condition characterized by significant demyelination, extensive astrogliosis and microgliosis and severe axonal damage.

For bioanalytical measurements, UHPLCMS/MS analysis was used to map the metabolites of TRP degradation. Already at the beginning of the CPZ treatment, we experienced differences in the concentration of the KYNA, XA and 3-HK in the plasma samples, which remained until the end of the intoxication, supplemented by the difference in TRP and ANA levels in the CPZ-treated group, which differences between groups disappeared during remyelination. In our studies, as CPZ poisoning progressed, in addition to more pronounced demyelination, we observed differences in body weight and kynurenine metabolite concentration, which quickly disappeared during recovery period, this suggests the effectiveness of the remyelination ability. Not to mention the differences in ANA, 3-HK and TRP levels during the demyelination phase in the examination of the brain regions, and the normalization of the concentration in the remyelination period.

During our investigation, we observed a significant decrease in the levels of 3-HK and ANA, as well as an increase in the concentration of TRP metabolite in certain brain regions of the toxin-treated group, including the striatum, hippocampus, cortex and brainstem, which areas

are referred to in the literature as severely demyelinated brain regions during CPZ intoxication.

In the present study, we confirmed the involvement of the metabolic pathway of TRP degradation in the CPZ rodent model. Based on the differences in the KP and the changes in some metabolite concentrations, further questions appear as to the mechanisms behind these shifts. It may happen that CPZ poisoning affects the functions of certain enzymes in the KP, thereby affecting the metabolite concentrations; or some neuroprotective metabolites are used up due to their beneficial effect. Thus reducing the degree of damage and helping remyelination, but a compensatory mechanism may also arise in the background; or perhaps completely different processes from these. Nevertheless, the exact role and effect of the metabolic breakdown of the TRP in the CPZ demyelination model may be clarified by conducting further research.

Overall, in our investigation, during HPLC and UHPLC-MS/MS bioanalytical measurements, we experienced a significant decrease in the plasma KYNA, 3-HK, XA and ANA concentrations of the CPZ-treated group, as well as we observed a notable increase in the TRP level as a result of the CPZ poisoning. However, we were unable to reproduce the reduced KYNA concentration obtained with the HPLC method in the mentioned regions by UHPLC-MS/MS analysis. One of the possible reasons for this is the different bioanalytical measurement methods applied during our studies. Nevertheless, as a result of CPZ intoxication, a remarkable decrease in the levels of 3-HK and ANA was observed in several brain regions, including striatum, cortex, hippocampus and brainstem, while the concentration of TRP increased in the striatum, cortex and hippocampus of CPZ-treated animals compared to the control group.

Conclusion

Our study, conducted in the CPZ toxin-induced demyelination animal model, was the first to report in full detail the kynurenine metabolite profile of TRP breakdown during progressive demyelination.

In addition to the basic histological analyses, behavioral and physical state assessment, we revealed the involvement of kynurenine metabolites in the processes of damage caused by CPZ poisoning on both sides of the blood-brain barrier and then highlighted the effectiveness of the remyelination ability in the field of TRP metabolism as well.

Our research results can serve as a starting point for further studies, with the help of which we can get even closer to understanding the processes that cause damage and the mechanisms connecting TRP degradation, what role certain metabolites can play in the creation of damage, how they influence the demyelination or remyelination processes. Furthermore, by detecting these processes, we can get a more transparent picture and get closer to discover the pathomechanism of MS.

In the near future, the regulation of the amount of certain metabolites may serve as a possible therapeutic tool in the treatment of MS, as well as may hold potential targets for drug research. Therefore, the research can serve as a basis for the creation of artificially synthesized compounds with new attack points, which may present a new therapeutic option in the treatment of MS.

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