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ARAŞTIRMA MAKALESİ / RESEARCH ARTICLE

Gender-Dependent Cholinergic System Alterations in a Phenylketonuria Model

Fenilketonüri Modelinde Cinsiyete Bağlı Kolinerjik Sistem Değişiklikleri

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ABSTRACT

Introduction: Phenylketonuria (PKU) is a rare inherited metabolic disorder characterized by a deficiency of the enzyme phenylalanine hydroxylase. The absence of this enzyme leads to elevated levels of phenylalanine in the blood, causing accumulation in the brain and resulting in permanent brain damage. To investigate the neurological effects of PKU, an experimental PKU model was developed, and cholinergic parameters were analyzed in brain tissue and serum from both female and male rats. Serum AChE, BChE, and total ChE activities were examined, while AChE activity was specifically analyzed in hippocampal tissue.

Material and Methods: Brain tissue and serum samples were collected from female and male rats from an induced phenylketonuria model for analysis. AChE, BChE, and total ChE activities were measured in serum samples, while AChE activity was examined in hippocampal tissue. Enzyme activities were measured according to Ellman assay. The data analysis and graphical presentations were done by GraphPad prism.

Results: Hippocampal AChE activity was significantly increased in both PKU groups compared to the control groups (Male ***p=0.0001; female *p=0.046). Serum analysis revealed decreased total serum cholinergic activity in both the female and male PKU groups compared with the control groups (**p≤0.002 and ***p≤0.0003, respectively). These results were consistent with findings from total serum cholinergic activity analysis. Additionally, the AChE activity in both the female and male PKU groups was decreased compared to their respective control groups (***p≤0.002 and ***p≤0.0006, respectively). It was also found that BChE activity in the serum of male rats in the PKU group was decreased compared to male rats in the control group (*p=0.038).

Conclusion: These findings indicate that the cholinergic system in the phenylketonuria model may vary according to gender. The observed changes in both brain tissue and serum provide new insights into the gender-dependent neurological effects of PKU.

Keywords: Phenylketonuria, cholinergic system, enzyme activity, hippocampal tissue, gender differences

ÖZ

Giriş: Fenilketonüri (PKU), fenilalanin hidroksilaz enziminin eksikliği ile karakterize nadir bir kalıtsal metabolik bozukluktur. Bu enzimin yokluğu, kandaki fenilalanin düzeyinin artmasına ve beyinde birikmesine neden olarak kalıcı beyin hasarına yol açar. PKU'nun nörolojik etkilerini incelemek amacıyla geliştirilen deneysel PKU modeli, hem dişi hem de erkek sıçanlarda kolinerjik parametrelerin beyin dokusu ve serumda analiz edilmesini sağlamıştır. Bu çalışmada serumda AChE, BChE ve toplam ChE aktiviteleri ile birlikte, hipokampal dokuda AChE enzim aktivitesi incelenmiştir.

Materyal ve Metotlar: Fenilketonüri modeli oluşturulan dişi ve erkek sıçanlardan beyin dokusu ve serum örnekleri alınarak analizler gerçekleştirilmiştir. Serum örneklerinde BChE, AChE ve toplam ChE aktiviteleri ölçülürken, hipokampal dokuda yalnızca AChE aktivitesi incelenmiştir. Enzim aktiviteleri Ellman yöntemine göre ölçülmüştür. Tüm verilerin istatistiksel incelenmesi ve grafiklenmesi GraphPad prism programı kullanılarak gerçekleştirilmiştir.

Bulgular: PKU gruplarında hipokampal AChE aktivitesi, kontrol gruplarına kıyasla anlamlı derecede artış göstermiştir (Erkek ***p=0.0001; dişi *p=0.046). Serum analizlerinde ise dişi ve erkek PKU gruplarında toplam serum kolinerjik aktivitesinin kontrol gruplarına göre azaldığı belirlenmiştir (**p≤0.002 ve ***p≤0.0003, sırasıyla). Toplam serum kolinerjik aktivitesi bulguları, bu sonuçlarla uyumlu olarak değerlendirilmiştir. Dişi ve erkek PKU gruplarının AChE e aktivitesi, kontrol gruplarına göre azalmış (**p≤0.002 ve ***p≤0.0006, sırasıyla) olup, erkek sıçanlarda BChE aktivitesinin de PKU grubunda kontrol grubuna göre azaldığı bulunmuştur (*p=0.038).

Sonuç: Bu bulgular, fenilketonüri modelinde kolinerjik sistem unsurlarının cinsiyete göre farklılık gösterebileceğini göstermektedir. Hem beyin dokusunda hem de serumda gözlemlenen bu değişiklikler, PKU'nun cinsiyete bağlı nörolojik etkileri üzerine yeni bakış açıları sunmaktadır.

Anahtar Sözcükler: Fenilketonüri, kolinerjik sistem, enzim aktivitesi, hipokampal doku, cinsiyet farklılıkları

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Introduction

Phenylketonuria (PKU) is a disorder of amino acid metabolism caused by an inherited deficiency of the enzyme phenylalanine hydroxylase (PAH) and dihydropteridine reductase deficiency (1, 2). Phenylketonuria is a disease caused by a congenital mutation in amino acid metabolism that, if left untreated, leads to severe mental retardation, microcephaly, seizures, and behavioral problems (2). The impaired metabolism leads to accumulation of phenylalanine (Phe) in the blood and toxic concentrations in the brain. Most studies of early-treated adult patients with PKU reported rapid deterioration of motor functions and learning and memory mechanisms, but only a very limited number of cognitive functions were examined in each study (3-6).

Acetylcholinesterase enzyme (AChE) (EC 3.1.1.7) hydrolyzes the neurotransmitter acetylcholine (ACh) and is found in high concentrations mainly in red blood cells, as well as at neuromuscular junctions and cholinergic brain synapses in the brain. Butyrylcholinesterase (BChE), also known as "pseudo" cholinesterase (EC 3.1.1.8) is a non-specific cholinesterase enzyme that hydrolyzes different types of choline esters, ubiquitous in the body it is especially found in human liver, blood serum, pancreas and central nervous system. In the brain, BChE is primarily associated with glial cells and endothelial cells (7, 8). In the brain, AChE activity is higher than BChE activity (9).

Disruption of the cholinergic system directly affects the metabolism of ACh, thereby impairing neurotransmission in the brain. As a result, emotion, behavior, learning and memory can inevitably be impaired. Therefore, identifying these changes in cholinergic activity is necessary to better understand the molecular mechanism underlying PKU. However, it remains unclear how changes in AChE activity and acetylcholine dynamics involved in PKU are affected. To solve this puzzle, this study aimed to investigate changes in AChE and BChE in both serum and hippocampus tissue samples in an in vivo model of PKU in male and female rats. Our results display a preference of Cholinergic expression with bias to gender.

Material and Methods

Creating a Phenylketonuria Model

For the animal model of PKU, 16 female and 16 male Sprague-Dawley rats, 6 days old and weighing 5 ± 2 grams, were included in the study. The animals were supplied from the Kobay Ltd laboratory and housed in the animal room of the laboratory of the Institute of Neurological Sciences and Psychiatry, Hacettepe University. Water and food intake were allowed before, during and after the experiment as ad libitum. Experimental studies began after approval by the Experimental Animal Ethics Committee (Ethics Approval: 2022 / 07-16).

4-Cl-phenylalanine (PCP) (Sigma catalog number: C8655), inhibitor of phenylalanine hydroxylase enzyme, was used to create a phenylketonuria model. 26 µmol/ml PCP stock solution and 152 µmol/ml phenylalanine stock solution were prepared by heating in 0.9% pH:7.2 saline. Rat pups were injected subcutaneously with 0.9 mg/g PCP twice a day and 5.2 mg/g Phe every day (10, 11). The control groups received an injection of saline.

All injections were initiated subcutaneously on the 6th day postnatal and terminated on the 25th day postnatal. The animals were sacrificed with guillotine scissors on the same day by anesthetizing the animals with subcutaneous injection of ketamine/xylazine (90/10 mg/kg. The brain was quickly removed and the hippocampal tissue was harvested. Blood sample was collected to serum tubes. Samples were stored at -80°C until analyzed.

Sample Preparation for Enzyme Activity in the Hippocampus and Serum

Rat hippocampus was weighed and stored at -80°C until use. Hippocampal tissue was homogenized in 50 mM Tris pH 7.4 buffer containing 2 mM EDTA, 0.5% Triton X-100, and protease inhibitor cocktail for 3 x 10 seconds on ice using a model Pro-200 homogenizer (PRO scientific, CT, USA). To avoid protein denaturation, all treatments and solutions were performed on ice. The homogenates were centrifuged at 13,000g for 15 min at +4°C. The supernatant was separated and aliquoted after centrifugation. Subsequently, the aliquots were used to determine enzyme activity and protein levels by BCA Protein Assay Kit (Thermo Fisher catalog number 23225)

The blood collection procedure involved the use of serum tubes, which contain silica for promoting clot formation. This process was administered by a trained phlebotomist. Following collection, the samples were left to clot in a dark environment at room temperature for a duration of 1 hour. Subsequently, the tubes underwent centrifugation at 1300× g for 10 minutes. The resulting serum was carefully extracted and divided into polypropylene 0.5 ml tubes. Within 50 minutes post-centrifugation, the serum samples were promptly stored at -80°C to maintain their integrity for subsequent analysis.

Activity Measurement of Cholinesterase in Hippocampus and Serum

To determine the activity of AChE in hippocampus and serum, the activity medium was prepared with a final concentration of 50 mM MOPS pH 7.4 buffer, 0.25 mM DTNB (ΔA_{412}, DTNB=14.2 mM^{-1} cm^{-1}), 1.0 mM ACh, and 50 mM iso-OMPA, a selective BChE inhibitor (12). The reaction was started by adding the sample and the activity was continuously monitored at 412 nm for five minutes at 37°C using a UV-visible spectrophotometer (SpectraMax M2 microplate reader; Molecular Devices, CA, USA). The same enzyme activity

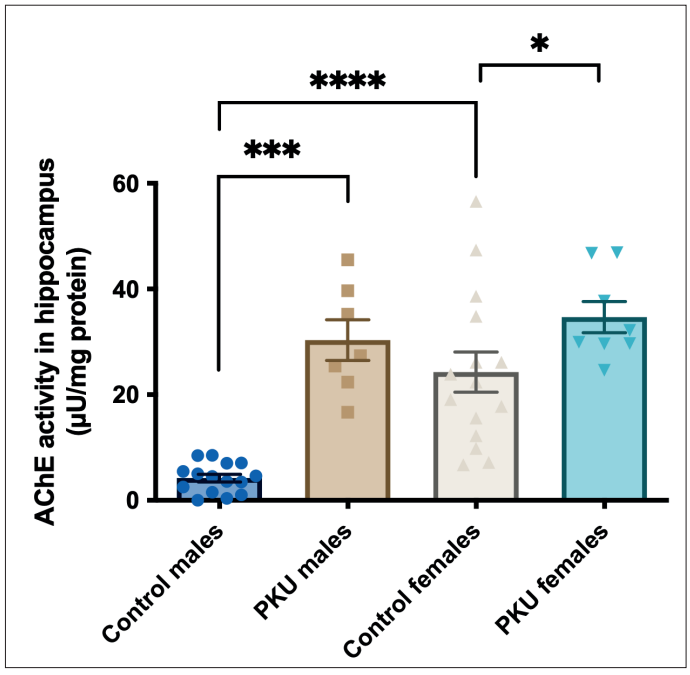

Figure 1. AChE activity in hippocampus. Statistical significance was determined by Mann Whitney-U. The data were expressed as the median and interquartile ranges (Min to max, n=8). * p≤0.05, *** p≤ 0.001, **** p≤ 0.0001.

experiment was done in the absence of iso-OMPA to measure total cholinesterase activity. Under these conditions, one unit of AChE or total cholinesterase is defined as the amount of enzyme that catalyzes the formation of 1.0 µmole of thiocholine per minute (U=µmol/min). Results were presented as the microunits per milligram of protein (µU/mg).

Data Analysis

All measurements were repeated at least twice. Results are presented as mean standard error of the mean (SEM) (standard error of the mean). Mann Whitney-U test was used for statistical analysis enzyme activity. GraphPad Prism 9.0 was used for statistical analysis and graphical plots. Normality and conformance check of the data were performed using the GraphPad Prism 9.0 program prior to statistical analysis.

Results

Cholinergic Activity in the Hippocampus

Hippocampus AChE activity in PKU male group was 30.32± 3.85 µU/mg; while the AChE activity in the male control group was 4.21±0.72 µU/mg (p***≤0.0001). (Figure 1A). Analysis of the basal hippocampal AChE activity revealed a significant difference with bias to gender. In the female PKU group and the male PKU group was statistically higher than the control groups (Figure 1A). Likewise, AChE activity was 34.67±2.94 µU/mg in the PKU female group; while it was 24.28± 3.78 µU/mg in the female control group (*p≤ 0.046) (Figure 1).

Cholinergic Activity in the Serum

Utilizing the BChE inhibitor iso-ompa, we directly measured AChE activity in serum. We found that the PKU groups had lower AChE activity than the control groups (Figure 1B) according to gender. In female groups, AChE activity was decreased in the PKU group compared to the control group; (p***≤0.002; 7.69±0.10 µU/mg , 10.86±0.53 µU/mg). As in the female groups, a corresponding trend was observed in male groups; AChE activity was decreased in the PKU group compared to the control group (p***≤0.0006; 8.40± 0.39 µU/mg, 11.21± 0.40 µU/mg) (Figure 2A).

BChE activity was also effected through this intervention and displayed gender based difference. BChE activity was found to be statistically lower only in the male PKU group than in the male control group (Figure 2A). BChE activity in the male PKU group; 4.36±0.31 µU/mg, male control BChE activity 5.29±0.21 µU/mg (*p≤0.038). Such a difference could not be observed in female groups. BChE activity in female groups was not statistically significant (p= 0.130; female PKU: 4.49±0.14 µU/mg; female control: 5.29±0.49 µU/mg) (Figure 2B).

Evaluation of total serum cholinesterase activity revealed that, serum cholinesterase activity in both genders in the PKU group was statistically lower than the control groups. (Figure 2C) Total cholinesterase activity in the female PKU group was 12.18±0.20 µU/mg whereas in the female control group it was 16.16±0.77 µU/mg. Hence, total cholinesterase activity of the female PKU group was statistically lower than the control group (p**≤0.002). Similarly, while the total cholinesterase activity of the male PKU group was 12.75±0.66 µU/mg, it was 16.50±0.49 µU/mg in the male control group. There was a statistically significant decrease in total cholinesterase activity among the male groups (p***≤ 0.0003) (Figure 2C).

Discussion

Phenylketonuria is a congenital disorder of phenylalanine metabolism caused by a deficiency of phenylalanine hydroxylase. It is a rare disease that occurs in about 1 in 10,000 people. A mutation of the phenylalanine hydroxylase enzyme and a mutation of the dihydrobiopterin enzyme cause an accumulation of Phe and its metabolites in the tissues and body fluids of PKU patients. The main signs and symptoms are found in the brain, but the pathophysiology of this disease is not well understood. (2).

Although intellectual retardation can be prevented by lowering blood phenylalanine concentrations, the neurocognitive and psychosocial outcomes of patients with phenylketonuria still remain below those of healthy individuals. (2, 13).

Cholinergic neurotransmission plays an important role in the normal human central nervous system and has been associated with cognitive function (14-16). One of the main enzymes

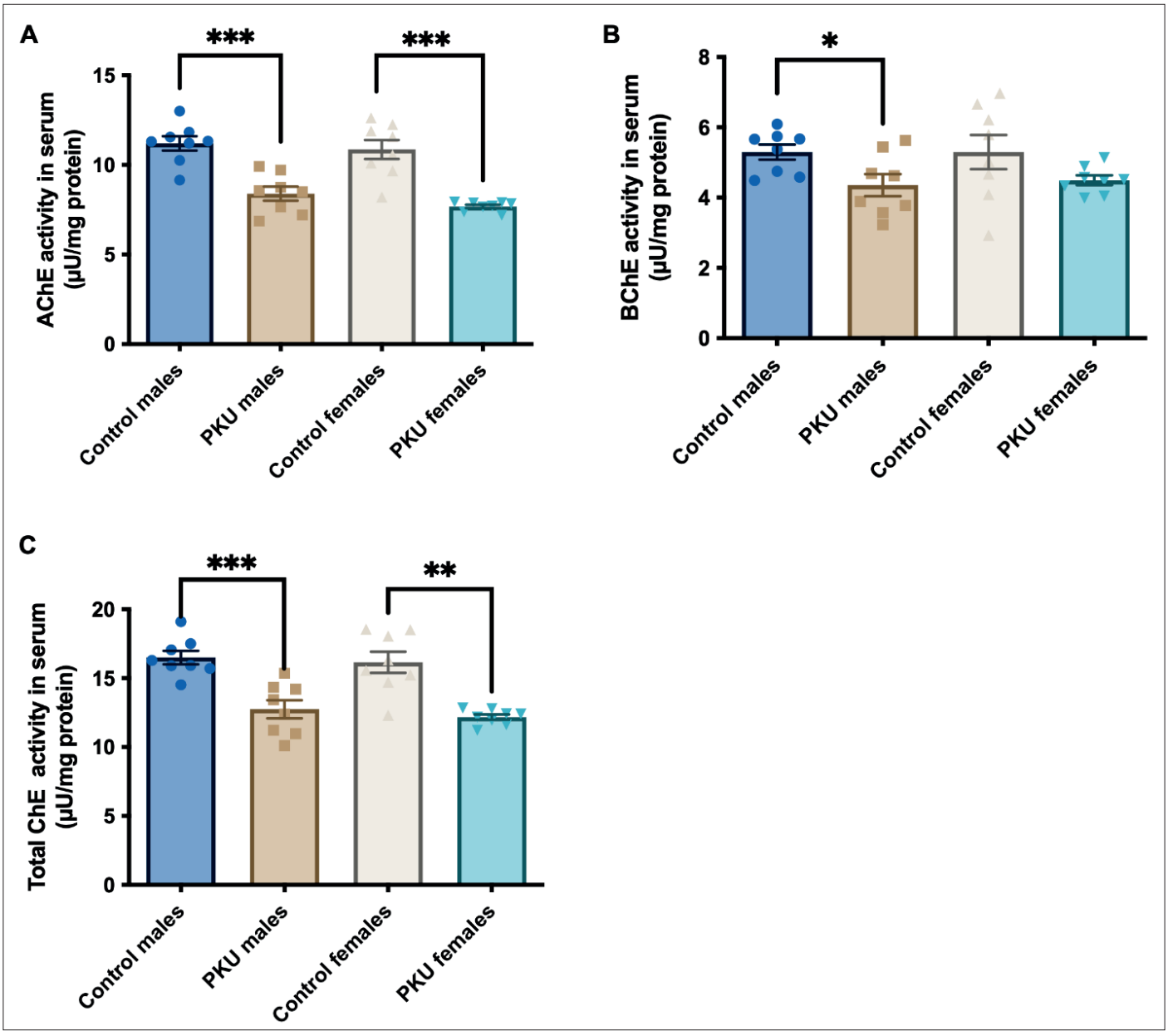

Figure 2. AChE, BChE and total serum cholinesterase activity in serum. A) AChE activity in serum. B) BChE activity in serum. C) Total serum cholinesterase in serum. Statistical significance was determined by Mann Whitney-U. The data were expressed as the median and interquartile ranges (Min to max, n=8). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

regulating cholinergic neurotransmission is AChE, which catalyzes the hydrolysis of the neurotransmitter acetylcholine (17). AChE also has non-classical effects (18-22), which suggests that it has a wide range of activity in the nervous system. Interestingly, there is evidence that the related enzyme BChE also plays an important role in the nervous system, as a co-regulator of the action of acetylcholine and as an enzyme with AChE-independent functions. (23-27).

In a reported study, the AChE activity in the erythrocyte membrane of PKU patients was measured. Although it was reported that AChE activity in the PKU group decreased compared to the control group, the gender distribution in the PKU group was not specified (28). Untreated PKU patients often exhibit psychomotor problems, memory deficits, and epileptic seizures (29)

Due to the involvement of cholinergic and adrenergic interactions in controlling memory processes (17). the low AChE activities associated with low dopamine levels, as detected by Stylianos Tsakiris and colleagues, may explain some of the clinical symptoms mentioned above. Additionally, the observed decrease in Phe AChE activity may lead to an increase in acetylcholine concentration in the synaptic cleft, which could explain the tremors commonly observed in PKU patients "off-diet." The detection of AChE in erythrocyte membranes of PKU patients may serve as a useful marker for the neurotoxic effects of Phe (28). Parallel to the results of this study, our findings also

show that serum AChE activity levels decreased in the PKU group. Along with AChE activity, BChE and total cholinesterase activities also decreased.

In PKU groups, AChE activity increased in the hippocampus depending on gender. It was shown that BChE enzyme activity in the brain is much lower compared to AChE enzyme activity (9). Therefore, BChE activity could not be detected in the hippocampus in this study. It is known that there is counter-regulation between AChE and BChE activities (30). A homeostatic level of ChE activity is necessary to appropriately regulate ACh levels in both neural and non-neural tissues. Consistent with our findings, the balance of AChE and BChE regulation continues in the PKU model.

In another study reported by Tsakiris and colleagues, AChE activity decreased by up to 18% in adult rat brain homogenates following preincubation with 0.48-1.8 mM Phe (31). This suggests that high doses of Phe reduce AChE activity. However, the question arises whether this study reflects a true PKU model. In our study, inhibitors from the literature were used to create an animal model of PKU. Compared to the control groups, both male and female PKU groups had higher AChE activity in the rat hippocampus. There are no studies in the literature on phenylketonuria and BChE enzyme activity. While this makes it difficult to discuss our results, it also makes these results unique in the literature. In the PKU groups, serum BChE, ChE, and AChE activities decreased. However, only the male group was affected by the changes in BChE findings. In a reported study (11) it was found that the PKU male group performed worse in behavioral tests compared to the female group. Additionally, negative changes were observed in the cAMP/CREB/BDNF pathway in the male group. In another reported study (32) lipid peroxidation and IL1β levels increased in the PKU male group. Based on all this evidence, the oxidative damage observed in the PKU group may have led to cholinergic alterations. Measuring serum ChE and BChE activity in phenylketonuria patients is the first study in the literature. When evaluating the results obtained, PKU and cholinergic parameters vary according to gender. In addition to the limited number of studies on this topic in the literature, there are no gender-based studies available. Hence as a first report of its kind we hope that these findings will lead the way to more detailed studies.

Ethics Committee Approval: Ethical approval was obtained from Hacettepe University Animal Experiments Local Ethics Committee (numbered 2022/07-16).

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