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Augmentation of endogenous GABA pool size induced by Magainin II peptide

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ABSTRACT

Background: Gamma aminobutyric acid (GABA), an inhibitory neurotransmitter, is produced via decarboxylation of L-glutamate through the glutamic acid decarboxylase (GAD) enzyme. The synchronic action of GABA-transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH) enzymes convert the GABA metabolite into succinate. Given this background, our research was aimed at probing the effect of Magainin II, on the activity of GABA shunt metabolizing enzymes.

Methods: Male NIH mice were administered peripherally by Magainin II (50 μg/kg body weight) and saline solution (%0.9 (w/v)) as the control vehicle. At different time intervals, the mice were sacrificed to evaluate the effect of Magainin II injection on the GABA shunt pathway. The activity of hypothalamic GAD, GABA-T and SSADH enzymes were determined using relevant enzyme assays.

Results: Magainin II effectively enhanced the activity of GAD, by %90, 24 h after injection, while quenching the activities of GABA-T and SSADH by %43 and %71, respectively. In vitro models also revealed the direct but reversible interaction between the peptide and each of the individual enzymes of GABA shunt pathway.

Conclusion: This study confirms the probable role of Magainin II in increasing the GABA content of the mouse hypothalamus. This property might candidate the peptide as a novel agent for improving the symptoms of many GABA dependent psychiatric disorders.

1. Introduction

High levels of Gamma aminobutyric acid (GABA), the main inhibitory neurotransmitter within the central, peripheral and enteric nervous system, indicate the critical role of GABA in many physiological activities. Changes of the GABA content in CNS is regarded as the cause of many psychiatric disorders. Cumulative literature data point to the role of GABA as an anxiolytic [1], anesthetic [2] and sedation hypnotic agent [3]. Additionally, balancing GABA content has been evaluated for the ability to improve the symptoms of neurodegenerative disorders such as temporal lobe epilepsy (TLE), Parkinson's disease (PD) and Huntington's disease (HD) [4]. In that respect, agents that could modulate GABA levels would open a field for contriving specific therapeutic modalities.

Physiological levels of GABA is mainly controlled by the activity of GABA shunt metabolizing enzymes. Glutamic acid decarboxylase (GAD) is a pyridoxal-dependent enzyme responsible for the catalysis of L-glutamate into GABA. The conversion of GABA to succinate is the result of contributing effect of GABA-transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH). The produced succinate is then oxidized via the Krebs cycle [5].

Caerulein (CLN), a decapeptide neuromodulator, originally isolated from the skin of *Hyla caerulea*, is capable of modulating the central GABAergic systems [6]. Although the mechanism of the action is not yet completely understood, it is evident that CLN causes an increase in hypothalamic GABA content. Backed on the literature, significant sequence similarities between the precursors of procaerulein and promagainins support the concept that these two peptides probably had a common ancestral gene [7]. This striking sequence homology might postulate similar physiological and biological behaviors for Magainin II (Mag II) and CLN. Magainins, primarily classified as antimicrobial peptides, were first discovered in 1987 from the skin of *Xenopus laevis,* the African clawed frog [8]. The antimicrobial function has been attributed to the membrane-crossing capability and disturbance of the normal

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membrane integrity by the peptide [9].

Regarding the structural homology between CLN and Mag II, in this investigation we were aimed to evaluate the influence of Mag II on the mouse hypothalamic GABA content via evaluating the influence on the GABA shunt pathway enzymes namely GAD, GABA-T and SSADH. Our *in vitro* and *in vivo* results confirmed a positive modulating role on the GAD enzyme, while the other two enzymes (GABA-T and SSADH) were inhibited by Mag II administration.

2. Materials and methods

2.1. Chemicals

Adenosine 5′-diphosphate, Aminoethylisothiouronium bromide (AET), 4-Aminobutyric acid (GABA), GABAse, D- Glucose 6-phosphate, D- Glucose 6-phosphate dehydrogenase, L-Glutamic dehydrogenase, α-Nicotinamide adenine dinucleotide, α-Nicotinamide adenine dinucleotide (reduced form), α-Nicotinamide adenine dinucleotide phosphate, α-Nicotinamide adenine dinucleotide phosphate (reduced form), Oxoglutaric acid, 6-Phosphogluconic dehydrogenase, Pyridoxal 5′-phosphate, Succinic semialdehyde, Triton X-100, Tris-HCl and Magainin II (dissolved in physiological saline) all were purchased from Sigma Chemical Company (Paris, France).

2.2. Laboratory animals

Male NIH mice, weighing about 25–30 g, were purchased from Razi institute (Karaj, Iran) and kept under standard conditions in the animal house of the institute of Biochemistry and Biophysics, University of Tehran. Ad libitum access to food and water at a controlled temperature of 20 ± 2 C under a 10 h light cycle was provided for the mice. All animal experiments and methods were conducted according to the guidelines of animal ethics committee of University of Tehran.

2.3. Experimental design and procedures

Mice were randomly assigned into groups of six. Freshly prepared Magainin II (50 μg/kg body weight) injected intraperitonealy to the treated groups. An equal volume of physiological saline was used for control group administration. At different time intervals, the mice decapitation was followed by rapid brain dissection from the skull and removal of hypothalamus. Finally, the collected tissues were weighed and subjected to homogenization using a Teflon glass homogenizer.

2.4. Homogenate preparation for GAD assay

Each sample was minced and homogenized in 100 mM sodium phosphate buffer (pH = 7.0), containing 20 μM pyridoxal phosphate, 0.1% Triton X-100 (W/V), and 1 mM AET.

2.5. Homogenate preparation for GABA-T and SSADH assay

A 10% (W/V) tissue homogenate was obtained using an ice-cold solution containing 0.32 M sucrose and 4.5 mM 2-mercaptoethanol. The mixture was ready by adding 3 vol of the ice-cold Triton medium (%0.67 W/V Triton X-100, 50 mM Tris-HCl, pH = 8.5 and 4.5 mM 2-mercaptoethanol) to 1 volume of the homogenate and allowed to stand for an hour before use.

2.6. GAD assay

An aliquot (170 μL) of the GAD assay solution (0.1 M sodium phosphate, pH = 6.8, 5 mM glutamate, 250 μM pyridoxal phosphate, 0.4% mercapthoethanol) was added to 100 μL of each of the homogenated sample. After incubation at 38 C for 60 min, the reaction was stopped by adding 60 μL of 0.25 N HCl and the tubes were kept at 60 C for 10 min. The GABA content of each sample was then determined by measuring the NADPH content, using an enzymatic cycling method [10]. Briefly, 430 μL of GABA assay reagent (0.3 M Tris-HCl, pH = 8.9, 8 mM α-ketoglutarate, 0.5 mM $NADP^+$, 0.01% W/V mercaptoethanol, and 0.1 mg GABAse) was appended to the GAD assay solution followed by 30 min incubation at 37 C. The reaction was stopped by adding 60 μL of 0.25 N NaOH, incubating at 60 C for 10 min. Then, 40 μL of the GABA assay mixture was transferred to 500 μL of NADPH cycling reagent (0.1 M Tris-HCl, pH = 8.0, 4 mM α-ketoglutarate, 1.5 mM glucose 6- phosphate, 0.1 mM ADP, 0.02% bovine serum albumin, 20 mM ammonium chloride, 11 μg/mL glucose 6-phosphate dehydrogenase and 25 μg/mL glutamate dehydrogenase). The mixture was incubated for half an hour at 38 C and heated at 100 C for 2 min afterward. At the end, 1 mL of 6-phosphogluconate assay reagent (0.1 M Tris-HCl, pH 8.0, 0.1 mM EDTA and 0.1 mM $NADP^+$) was subjoined to the droplets. After keeping the mixture at room temperature for 30 min, the fluorescence was measured at λ = 455 nm. The control sample has been subjected to the same procedure. It is important to note that the degradation probability of the produced GABA is negligible as the optimum pH for the GABA degrading enzyme is far from pH = 6.8 of the GAD assay mixture. Finally, the GAD activity was expressed in μmol/g hypothalamus/h by using NADPH calibration graph in the range of 0.001–0.005 mg/mL.

2.7. GABA-T and SSADH assay

A coupled enzyme system was used for assaying the activity of GABA-T enzyme. In this system, the endogenous SSADH is utilized to convert the preformed succinic semialdehyde (SSA) to succinate (SA) in the presence of NAD^+. The concurrently produced NADH was used as a measure of GABA-T activity. The incubation mixture was composed of 50 mM Tris-HCl, pH = 8.5, 3.0 mM GABA, 2 mM 2-oxoglutaric acid, 20 mM 2-mercaptoethanol, 1.1 mM NAD^+ and 0.2 mL of Triton-treated homogenate in a total volume of 1.125 as described by Th. De Boer [11]. After a preincubation period of 30 min at 22 C, 0.1 mL of 33.75 mM GABA was added to the ice-cold incubation solution and the reaction was started by incubating the sample tubes at 22 C. Finally, the formation of NADH was measured spectrophotometrically at λ = 340 nm based on the NADH calibration graph. The enzyme activity was expressed in μmol/g hypothalamus/h.

By an almost similar approach, the activity of SSADH was determined for each sample. The enzyme (SSADH) in the hypothalamic homogenate would convert SSA to SA in the presence of NAD^+. The incubation solution contained 50 mM Tris-HCl, pH 8.5, 0.25 mM NAD^+, 0.3 mM SSA, 8.2 mM 2-mercaptoethanol, and 0.2 mL of each homogenate. The mixture was subjected to the same preincubation condition as mentioned for the assay of GABA-T. Finally, 0.1 mL of SSA solution (3.375 mM solution) was added to the reaction mixture and the same steps were followed for measuring the SSADH enzyme activity, as mentioned for GABA-T assay.

2.8. In vitro enzymatic assays

Aliquotes of each of the homogenated samples were exposed to different concentrations of Mag II (0–0.03 mmol/mL), and incubated for 2 h on ice. The enzymes' activities were determined (before and after dialysis) by the relevant methods which were previously described for each enzyme.

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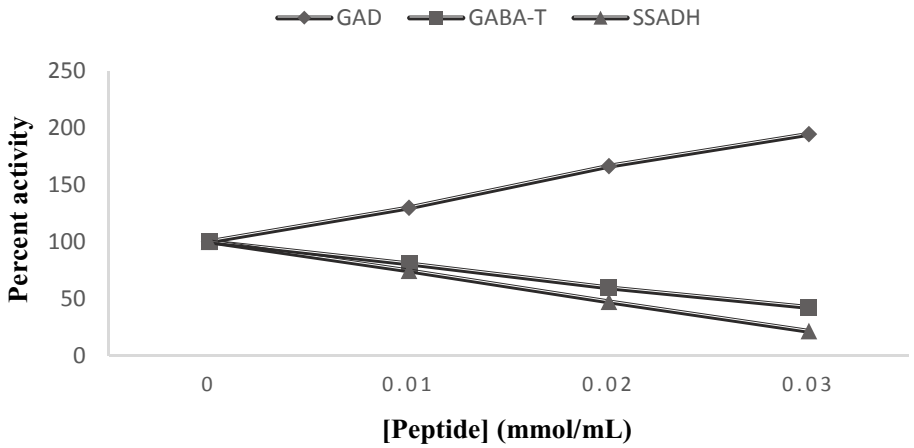

Fig. 1. The direct interaction between Mag II peptide and each of the enzymes; glutamic acid decarboxylase (GAD), GABA-transaminase (GABA-T), succinic semialdehyde dehydrogenase (SSADH). The enhancement in the peptide concentration caused a depression in GABA-T and SSADH activity, while the GAD activity was increased throughout the increment in peptide concentration.

Table 1
Direct interaction between Mag II peptide and each of the individual enzymes; GAD, GABA-T and SSADH, before and after dialysis against Tris-HCl buffer (50 mM, pH = 8.5). The values represent the mean ± S.D. N = 6 for each determination and P < 0.01 vs. control (student's t-test).

Treatments		Enzyme activity (μmol/g/h)		
Before dialysis	Target	GAD	GABA-T	SSADH
	Control	7.80 ± 1.83	6.90 ± 1.66	24.90 ± 1.23
	+Magainin II	15.10 ± 1.43	3.23 ± 1.02	5.93 ± 1.12
After dialysis	Control	6.90 ± 1.62	6.60 ± 1.43	23.80 ± 1.19
	+Magainin II	7.10 ± 1.79	6.30 ± 1.26	22.60 ± 1.12

3. Results and discussion

Population-based studies have demonstrated the increasing prevalence of common psychiatric disorders [12]. The inhibitory neurotransmitter, GABA, is associated with the etiology of many psychiatric disorders. As a key molecule, GABA is involved in the regulation of neuronal excitation and inhibition balance (indicated as E/I balance) leading to healthy behavior and cognition [13]. Fluctuated E/I ratio due to the GABA decline has been linked to many mental health problems such as sleeping disorders, attention deficit, hyperactivity and in extreme cases seizer [14]. In that respect, compounds capable of modulating the brain GABA content are of special interest to the pharmaceutical industry.

The present study evaluated the mechanism of GABA metabolism under the effect of Magainin II. As shown in Fig. 1, our *in vitro* evaluation clearly confirmed the direct interaction between Mag II and each of the three enzymes. The reversibility of the interactions was assessed via dialysis which restored the full activity of each of the enzymes (Table 1).

Peripheral administration of Mag II exhibited the augmented activity of the GABA biosynthesis enzyme, GAD. In contrast, GABA-T and SSADH assay displayed the inhibitory role of the peptide on these two enzymes, implying that the Mag II peptide treatment enhances the GABA level in the mouse hypothalamus. Based on the results shown in Fig. 2, Mag II administration (50 μg/kg body weight) elevated the activity of GAD by almost %63 in about 30 min after injection. The maximum activity enhancement, by almost %90, was recorded after 24 h. However, the activity of the enzyme started to decline within 48–72 h after injection and returned to the basal level. The enzyme, GAD, is considered as the rate-limiting enzyme of GABA metabolizing system, thus, the role of Mag II peptide would become more significant knowing the fact that Mag

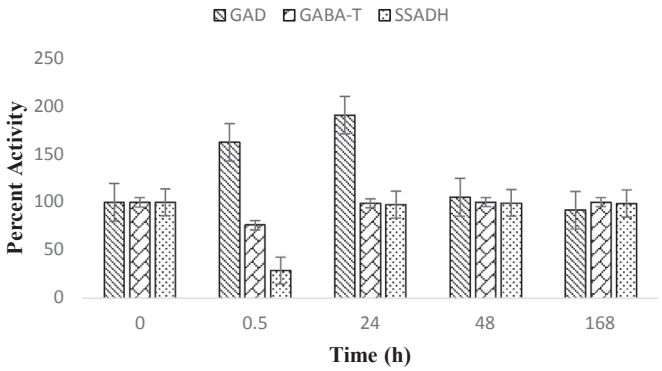

Fig. 2. Effects of peripheral injection of Mag II (50 μg/kg B.W.) on glutamic acid decarboxylase (GAD), GABA-transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH) activities. N = 6 for each determination. P < 0.01 vs. control (student's t-test). The vertical bars represent standard deviation.

II preferably targets the GAD activity in comparison to the two catabolizing enzymes, GABA-T and SSADH. Peripheral injection of Mag II (50 μg/kg body weight) inhibited the GABA-T activity by almost %43 one hour after injection (data not shown). However, the enzyme activity is gradually restored to the basal level, 24 h after injection (Fig. 2). Moreover, Mag II administration diminished the activity of SSADH. Our data clearly indicated that the activity of SSADH catabolizing enzyme was quenched by almost %71 in about 30 min after injection. This extent of inhibition would certainly leads to higher-than-normal level of the substrate, SSA, which in turn would probably block the function of GABA-T. Overall, it might be concluded that Mag II would block the activity of GABA-T both through direct interaction and also via induced substrate-level inhibition. The final consequence would be higher endogenous level

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of GABA. The interacting biological significance and the probable medical application of this new approach, endogenous augmentation of GABA level, is under our present investigation and the results would be published in due date.

Conflict of interest

The authors declare that they have no competing interests.

Declaration of interest

None.

Acknowledgment

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