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# Significant Changes in the Activity of L-glutamic Acid Decarboxylase of Mouse Hypothalamus after Peripheral Injection of Cholecystokinin-8 and Caerulein

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## ABSTRACT

**The activity of one of the metabolizing enzymes of ¥- aminobutyric acid, (GABA), was determined in mouse hypothalamus after peripheral injections of cholecystokinin-8 (CCK-8) and caerulein (CLN). The activity of this rate-limiting enzyme, L-glutamic acid decarboxylase, (GAD), did not change thirty minutes after peripheral injections of either CCK-8 or CLN in doses of 50«g/kg body weight. However, the activity of GAD started to increase 24 hours after injection and continued to increase by 15 to 17%, five days after injections. Based on the *in vitro* models, it has been shown that there are no direct interactions between CCK-8 and CLN and the enzyme GAD. These data probably support indirect but specific interactions between CCK-8, CLN and the enzyme GAD.** *Iran. Biomed. J. 3(1 & 2): 47-51, 1999*

*Keywords:* Caerulein; Cholecystokinin-8; GABA; L-Glutamic acid decarboxylase.



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## INTRODUCTION

Agents that can modulate GABA level in the central nervous system are of special therapeutic interest in disorders associated with changes in GABA content. Some of these agents are the inhibitors of GABA degradative enzymes or of GABA uptake systems, [1-6] and some act by blocking the biosynthesis of GABA or acting as antagonists of GABA receptors. The latter group of compounds may be helpful in treatment of disorders characterized by high GABA levels, and GABA-T deficiency [7].

Recently, two structurally related peptides have been the subject of interest as two potent neuromodulators. Cholecystokinin octapeptide (CCK-8), purified from the gastrointestinal and central nervous tissues [8] and caerulein (CLN), a decapeptide closely related to the C-terminal octapeptide of CCK and isolated from the skin of the frog *Hylacaerulea* [9]. These peptides were shown to have various and similar central activities [10].

Using animal models, the CCK-8 and CLN have been classified as amla analgesic [11], antinociceptic [12], antistereotypic [13], sateitic [14], sedative[15], and hypothermic [16]. Based on the enhanced release of GABA from rat cerebral cortex slices, after CCK-8 or CLN treatment [17], it has been concluded that CCK-8 and CLN may act upon the central GABA ergic system. Additionally, it has been reported that peripheral injection of CLN to rats has altered the dopamine turnover in striatum [18]. Since the regulation of the activity of dopaminergic system is under the influence of GABA ergic and glutaminergic system [19], it is possible that CLN and CCK-8 may affect the central GABA ergic system. In a study conducted by Nagahama [20], it has become evident that peripheral injection of CCK-8 and CLN to mouse reduced the GABA content of striatum while the GABA content increased in hypothalamus, and frontal cortex 60 minutes after injection. However, with CLN and CCK-8, the GABA accumulation decreased in striatum and hypothalamus after AOAA treatment. Although the effect of CCK-8 and CLN on the GABA ergic system is evident, however the mechanism of action of these peptides

*Corresponding Author. [1] Former student of Tarbiat Modarres University, present address: Dept. Of Biochemistry & Biophysics, Babol University of Medical Sciences, Babol, Iran. **Abbreviations:** AOAA, Aminooxyacetic acid; AET,2-Aminoethyl isothiouronium bromide; CCK-8, Cholecystokinin; CLN, Caerulein; GABA, γ -aminobutyric acid; GABA-T, GABA - transaminase; GAD, Glutamic acid decarboxylase; $NAD^+$, Nicotinamide adenine dinucleotide; NADH, reduced NAD;+ SA, succinic acid; SSA, succinic acid semialdehyde; SSADH, succinic acid semialdehyde dehydrogenase.

on the GABA ergic system is not clear and further investigations are needed.

In our previous investigation we reported the acute effects of peripheral injection of CLN and CCK-8 on the activities of two of the catabolizing enzymes of GABA, namely GABA-T and SSADH in the mouse hypothalamus [21]. Our data indicated that the activities of each of the enzymes decreased by almost 50% 30 minutes after peripheral injection of either CCK-8 or CLN. We also observed that there are specific, direct, and reversible interaction between the enzymes and each of the peptides, CCK-8 and CLN.

In the present study we report the acute and long-lasting effects of CCK-8 and CLN on the activity of L-glutamic acid decarboxylase of the mouse hypothalamus which is considered to be the rate-limiting enzyme of the metabolizing system of GABA[22].

## MATERIALS AND METHODS

***Materials.*** GABAase, glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, pyridoxal phosphate, bovine serum albumin, GABA, glutamate, $NADP^+$, NADPH, ADP, Triton X-100, and Tris-HCl were purchased from Sigma Chemical Company (Paris, France). CCK-8 (sulfated form) and CLN were obtained from Peninsula Laboratories Europe Ltd. (England). CCK-8 and CLN were dissolved in physiological saline for injections. Chemicals were used without further purification. Male NIH mice, weighing about 25-30 gr, purchased from Razi institute (Karaj, Iran) and kept in groups of 15, in cages in animal house with 10-hour light cycle from 8 a.m. to 6 p.m., with free access to standard laboratory food and water.

***Tissue extract.*** Mice were injected intraperitoneally with CCK-8 or CLN, 50 μg/kg body weight, and the control groups were injected with equal volume of the physiological saline. At different time intervals, the mice were sacrificed by decapitation and their brains were removed from the skulls in less than one minute. Using an iced chilled glass plate, the hypothalamuses were removed quickly from the brains and weighed. Each tissue sample was homogenized in 9 ml/g of chilled 0.1M sodium phosphate buffer, pH 7.0, containing 20 μM pyridoxal phosphate, 1 mm AET, and 0.1% Triton X-100 (w/v).

***GAD assay.*** Twenty eight μl of each of the homogenated samples were placed in 1.5-ml Eppendorf tubes and warmed to 37°C for 2 minutes. To each of the tubes was added 1.7 μl of GAD assay reagent [0.1 M Sodium phosphate, pH 6.8; 5 mM glutamate; 250 μM pyridoxal phosphate; 0.4% mercaptoethanol (w/v)]. The reaction mixtures were incubated at 38°C for 60 min. The reactions were stopped by adding 0.6 μl of 0.25 N HCl solution to each Eppendorf tubes and kept at 60°C for 10 min. The amount of GABA produced in each samples were measured as described below according to the method described by Oliver H. Lowry [23] and David L. Martin [24].

***GABA assay.*** The amount of GABA produced by GAD was determined by measuring pyridine nucleotide, NADPH, by an enzymatic cycling method [23]. The GAD assay mixture was cooled to 37°C and 4.3 μl of GABA assay reagent, containing: 0.3 M Tris-HCl, pH 8.9; 8 mM α-keto-glutarate; 0.5 mM $NADP^+$, 0.01% mercaptoethanol W/V; and 0.1 mg GBA ase/ml was added. The mixture was incubated at 37°C for 30 minutes and the reaction was stopped by adding 0.6 μl of 0.25 N NaOH and keeping at 60°C for 10 minutes. The control sample was treated in the same way.

***Enzymatic cycling process.*** Four μl of the GABA assay mixture was transferred to 50 μl of NADPH cycling reagent. The cycling reagent consists of: 0.1 M Tris-HCl, pH 8., 4 mM α-ketoglut-arate; 1.5 mM glucose -6-phosphate; 0.1 mM ADP; 0.02% bovine serum albumin; 20 mM $NH_4Cl$; 11 μg/ml glucose-6-phosphate dehydrogenase; and 25 μg/ml glutamate dehydrogenase. The mixture was kept at 38°C for 30 minutes and then to a 100° C bath for 2 minutes to stop the enzymatic reactions. The control sample has been subjected to the same procedure. The mixture was cooled to room temperature and 10 μl of 6-phosphogluconate assay reagent consisting of: 0.1 M Tris-HCl, pH 8.0; 0.1 mM EDTA; and 0.1 mM $NADP^+$, was added. The mixture was kept 30 minutes at room temperature and the fluorescence was measured at 455 nm. The control sample has been subjected to the same procedure and the fluorescence has been measured at 455 nm. Standards and blanks have been carried out through the entire process, including any procedure before cycling. Additionally, the endogenous GABA content has been determined along each experiment by incubating aliquots of each sample at 60°C for 10 minutes to inactivate primarily GAD and to





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prevent conversion of L-glutamate to GABA. The results obtained have been subtracted from total GABA measured in those samples which had not been subjected to heat inactivation. By using the GABA standard graph, GAD activity, in each experiment, was expressed in the no. of nanomole of GABA (in terms of NADPH) synthesized by GAD per milligram of hypothalamus per hour.

***In vitro assay of intermolecular interactions.*** Aliquots of CCK and CLN solutions in Tris buffer (0.1M, pH 8.0) were incubated with 8 mmole of GAD in ice bath. The concentration of the peptides varied between 0 to 0.03 mmole/ml of the test solution. The control samples did not contain CCK-8 or CLN. After two hours of incubation, 250µl of each sample, along with its corresponding control sample, was subjected to determination of GAD activity using methods described previously.

***GABA standard graph.*** Different concentrations of GABA were incubated in reagents for assaying GABA in terms of NADPH, as described before. The GABA concentrations ranged between zero to 0.6 mmole per liter.

## RESULTS

Figure 1 shows that there is a linear relationship between the GABA, in the range of zero to 0.6 mmolar, and the fluorescence emission of NADPH (at 455 nm). The fluorescence is produced as a result of conversion of GABA into SSA and subsequent oxidation of SSA to SA and NADPH by GABAase. This graph has been frequently applied through our investigation to obtain the GAD activity in terms of GABA produced by the enzyme GAD.

Table 1 indicates that there is no acute effect on the GAD activity after peripheral injection of CCK-8 and CLN in doses of 50 µg/kg body weight, as compared with the control group of mice receiving the same volume of normal saline injections. However, the activity of GAD started to increase almost 24 hours after CCK-8 and CLN injections. The enhancement of the activity reached its maximum by 72 to 96 hours after treatments. Regardless of the data in the Table 1, our data in Table 2 clearly indicates that there is no direct intermolecular interactions between the enzyme GAD and each of the peptides, CCK-8 or CLN, when used at different concentrations (0.005 to 0.03 mmole per milliliter of the test solution).

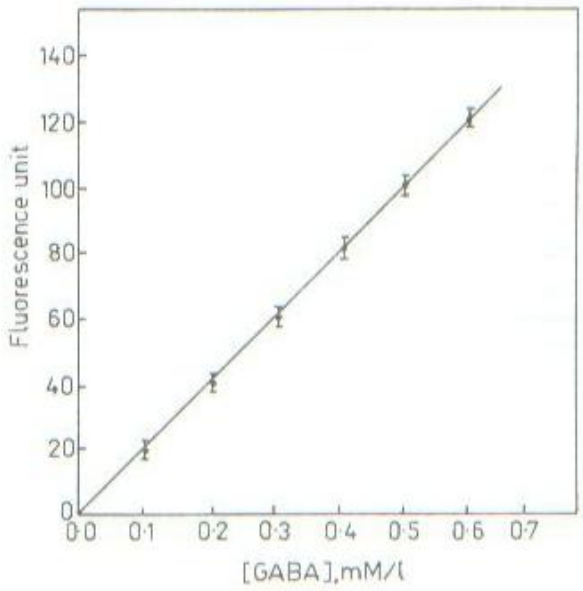


**Fig. 1.** Calibration of GABA. Different GABA concentrations have been subjected to GABA assay test and NADPH enzymatic cycling method. The fluorescence emission has been measured at 455 nm. Each point is the average of two determinations. For experimental details see the material and method section.

## DISCUSSION

Despite the accumulated data (11-17) in the literature concerning the behavioral and physiological changes induced in the test animals by CCK-8 or CLN administrations, the exact mechanism of action and the exact site(s) of action for these two peptides in the central nervous system have not been reported.

In our previous report [21], it was indicated that there are direct and reversible interaction between CCK-8 and CLN and two catabolizing enzymes of GABA, namely, GABA-T and SSADH. Our *in vivo* and *in vitro* studies indicated that the activities of GABA-T and SSADH were quenched by more than 50 percent, 30 minutes after peripheral injections of either CCK-8 or CLN (50 µg/kg body weight).

The present *in vitro* investigations show that there is no direct interaction between each of the peptides (CCK-8 and CLN) and the enzyme GAD. However, our *in vivo* investigations have shown that the activity of GAD increased by 15 to 17 percent almost 80 hours after injections of either CCK-8 or CLN in the treated mice brains. These observations indicate that there is indirect interaction (s) between CCK-8 and CLN and the GABA ergic system in the mouse brain. Obviously, the enhanced level of





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**Table 1**. Effect of Peripheral injection of CCK-8 and CLN, (50 μg per Kg body weight) on GAD activity at different time intervals. The values are expressed as the means (± SD) of six determinations. *Significantly different from control (P<0.01). The activity of GAD is expressed in nmole/mg hypothalamus/h.

| Delay time (hr) | Control (0.9% saline) | + CCK-8 (50 μg/kg) | + CLN (50 μg/kg) |
|---|---|---|---|
| 0.5 | 38.15 ± 0.93 | 38.16 ± 0.83 | 38.19 ± 0.73 |
| 1 | 38.17 ± 1.11 | 38.14 ± 0.89 | 38.12 ± 1.12 |
| 2 | 38.12 ± 0.84 | 38.17 ± 1.11 | 38.16 ± 1.01 |
| 5 | 38.13 ± 0.72 | 38.18 ± 0.84 | 38.16 ± 0.73 |
| 10 | 38.16 ± 1/12 | 38.15 ± 0.93 | 38.17 ± 0.64 |
| 24 | 38.12 ± 0.74 | 38.18 ± 1.75 | 39.02 ± 1.17 |
| 48 | 38.32 ± 0.99 | 39.64 ± 0.49 | 40.28 ± 0.38* |
| 72 | 37.97 ± 0.84 | 43.46 ± 0.63* | 43.99 ± 0.87* |
| 96 | 37.40 ± 0.75 | 42.91 ± 0.57* | 43.31 ± 0.70* |
| (Day 7) 168 | 37.54 ± 0.52 | 41.34 ± 0.78 | 41.68 ± 0.84 |
| (Day 14) 336 | 37.53 ± 0.85 | 38.08 ± 0.58 | 37.92 ± 0.96 |

**Table 2**. The *in vitro* effect of CCK-8 and CLN on the GAD activity at different concentrations of each peptide. The enzyme concentrations have been kept constant throughout the measurements at 8.15 mmole/ml. For experimental details see the material and method section. The activities are expressed in nmole/mg hypothalamus/h and each value represents the mean ± SD.

| (mmole/ml) | Control | + CCK-8 | + CLN |
|---|---|---|---|
| 0.005 | 37.98 ± 0.13 | 37.83 ± 0.50 | 37.52 ± 0.73 |
| 0.010 | 37.68 ± 0.43 | 37.47 ± 0.79 | 37.32 ± 0.57 |
| 0.015 | 37.93 ± 0.53 | 37.81 ± 0.47 | 38.03 ± 0.69 |
| 0.020 | 38.23 ± 0.69 | 38.14 ± 0.53 | 38.26 ± 0.37 |
| 0.025 | 37.63 ± 0.36 | 38.23 ± 0.42 | 37.40 ± 0.74 |
| 0.030 | 38.17 ± 0.39 | 37.91 ± 0.63 | 38.13 ± 0.46 |

GAD and the simultaneous inhibitions of GABA-T and SSADH will elevate the GABA content of the central nervous system. Our previous and the present findings are in agreement with Nagahama's findings which states that CCK-8 and CLN effect the GABA content and accumulation induced by AOAA treatment in mouse striatum, hypothalamus, and frontal cortex [20]. In addition, it has been reported that CLN and CCK-8 enhanced GABA release from rat cerebral cortex slices *in vitro.*

From our accumulated data it may be concluded that the interaction(s) between CCK-8 and CLN and the enzymes GABA-T and SSADH are specific. Secondly, it may be concluded that there is no direct intermolecular interaction(s) between GAD and the peptides CCK-8 or CLN. However, significant and indirect interaction(s) may exist between GAD and the peptides at the transcriptional or translational levels. These investigations are in progress and will be published soon.

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