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Brain Asparaginase, ACE Activity and Plasma Cortisol Level in Morphine Dependent Rats: Effect of Aspartic Acid and Naloxone

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KOYUNCUOĞLU, H., M. GÜNGÖR, N. ENGINAR, I. HATIPOĞLU AND A. HIZAL. *Brain asparaginase, ACE activity and plasma cortisol level in morphine dependent rats: Effect of aspartic acid and naloxone.* PHARMACOL BIOCHEM BEHAV **25**(5) 953–957, 1986.—The activities of the brain L-asparaginase and angiotensin converting enzyme (ACE), and the plasma cortisol level were found to be decreased in the rats implanted with morphine (M) containing pellets. Even though 10 mg/kg of naloxone (N) itself showed an inhibitory effect on ACE it abolished the inhibitions seen in the M dependent rats five min following subcutaneous injection. The chronic administration of L-aspartic acid (ASP) during the development of physical dependence or just before the N injection prevented the increase of the plasma cortisol caused by N. It is concluded that in addition to the inhibition of the brain L-asparaginase activity which was previously hypothesized to be the main reason of the development of physical dependence on opiates as a result of the related experimental and clinical data, the inhibition by M of the brain ACE activity may take part in the development of physical dependence. With regard to the plasma cortisol level, the concomitant administration of ASP with M blocks, to a great extent, the development of physical dependence on opiate. The single dose of ASP administration before N injection prevents the effect of N, the manifestation of abstinence syndrome.

L-Asparaginase ACE Morphine L-Aspartic acid Naloxone Plasma cortisol

L-ASPARTIC acid (ASP) has previously been found to antagonize effects of morphine (M), such as the inhibition by acute intravenous M injection of the brain L-asparaginase activity [15], the development of physical dependence and the manifestation of abstinence syndrome signs (shown by the antagonism of the altered brain levels of monoamines and free amino acids during the development of physical dependence on M, and the normalization of the decreased spontaneous motor activity, the attenuation of body weight loss, flying, jumping, wet dog shaking during abstinence syndrome [11–14]). As a result it has been hypothesized that the development of physical dependence on and the abstinence syndrome upon withdrawal from opiates might be related to a disequilibrium between L-asparaginase and asparagine synthetase, caused by the inhibitory effect of opiates on L-asparaginase [19, 21, 34]. In addition, the similarity between the effects of M and another L-asparaginase inhibitor [24] D-aspartic acid [16–18, 21, 22] and the successful use of ASP in the treatment of opiate addicted people [19,34] were considered as supporting evidence for the hypothesis.

Among some others, the central and peripheral adrenergic systems have been emphasized to be responsible for the majority of the abstinence syndrome signs [9, 26, 27, 29]. Thus an adrenergic alpha-2 receptor agonist, clonidine was introduced in the treatment of opiate withdrawal symptoms [8] and it has been used successfully [6,38].

It has recently been reported that the increase in plasma glucocorticoid levels can be regarded as a biochemically determinable indicator of the opiate abstinence syndrome [7]. On the other hand, clear relationships between angiotensin and both central and peripheral adrenergic systems [1, 10, 25, 33, 35], and between angiotensin and ACTH release [30, 31, 36, 37] have repeatedly been shown.

In the light of the information given above, the determination of the changes in the brain L-asparaginase activity of the morphine-dependent rats administered either chronically or acutely ASP and then subjected to naloxone-precipitated abstinence was thought to provide further support for our hypothesis. Additionally the brain angiotensin converting enzyme (ACE) activity and possible associated changes in plasma cortisol level in those different experiments were determined to find out any probable role of angiotensin in the development of physical dependence on and in the abstinence syndrome upon withdrawal from morphine.

METHOD

Male Wistar inbred rats with an initial body weight of

TABLE 1
THE SCHEDULE OF THE EXPERIMENTS

Groups	In the beginning	First day	Second day	Third day morning	Two hours later	15 min later	Five min later
Control	P, O	O	O	O	O	O	D
M	**P**, O	O	O	O	O	O	D
N	P, O	O	O	O	O	N	D
M+N	**P**, O	O	O	O	O	N	D
M+ASP chronic	**P, O**	**O**	**O**	**O**	O	O	D
M+ASP chronic+N	**P, O**	**O**	**O**	**O**	O	N	D
M+ASP single dose	**P**, O	O	O	O	**O**	O	D
M+ASP single dose+N	**P**, O	O	O	O	**O**	N	D

M—Morphine; ASP—L-aspartic acid; N—Naloxone.
P—Implantation of three control pellets.
P—Implantation of three M containing pellets.
O—Intraperitoneal administration of physiological saline.
O—Intraperitoneal administration of 200 mg/kg ASP in every eight hours.
N—Subcutaneous injection of 10 mg/kg N.
D—Decapitation after cervical dislocation.

about 200 g obtained from Experimental Research Centre of Istanbul Medical Faculty were used. They were kept in a room at 22–23°C on 12-hour light/dark cycle and fed with a standard regimen ad lib. They had free access to drinking water throughout the experiments. ASP, hippuryl-histidyl-leucyl (HHL) were purchased from Sigma (St. Louis); naloxone (N) was a gift from Endo Laboratories (New York)

For the estimation of brain L-asparaginase activity the method described in Worthington Enzyme Manual [41] was used (one unit is equivalent to 1.0 micromol of ammonia released from asparagine/min/g wet weight under the conditions of the assay). Brain ACE activity was determined by using a spectrophotometric method [2] based on the liberation of hippuric acid from HHL (one unit is the amount of enzyme releasing 1 n mol hippuric acid/min/g wet weight). A fluorimetric method [28] was used for the determination of plasma cortisol level (expressed as ng/ml). Two g % of ASP solution was prepared in bidistilled water. The osmolality and pH of the solution were adjusted to 290 mOsm/k and 7.4 with NaCl and 1 N.NaOH, respectively. The 0.1 g % naloxone solution was prepared in 0.9% saline.

The rats were subcutaneously (SC) implanted with either 3 pellets containing 75 mg (in toto 225 mg) morphine base [38] or 3 pellets containing only vehicle without M on the back of the animals under light ether anesthesia. According to the schedule given in Table 1, some of the rats implanted with M containing pellets were intraperitoneally (IP) given 200 mg/kg of ASP in every eight hours for two days starting just after the recovery from anesthesia. The other rats, including the control pellets implanted ones, received the same volume of physiological saline, instead. In the morning of the third day two hours after the ASP or physiological saline injections, some of the M containing pellets implanted rats (not given ASP) were IP administered 200 mg/kg of ASP, whereas the others received the same volume of physiological saline IP. Fifteen min later some of the rats implanted with pellets with or without M and some of the rats that received ASP chronically or acutely, were administered SC 10 mg/kg of N. The remaining rats were given physiological saline. The rats which had been implanted with or without M and given physiological saline throughout the experiments formed Control and M groups, respectively. N group consisted of the rats implanted with control pellets, administered physiological saline during the experiments and injected with N at the end of the experiments. The rats which had been implanted with M containing pellets and received N was called M+N group. Some of the rats implanted with M containing pellets received 200 mg/kg of ASP during the experiments and injected with either physiological saline or N at the end of the experiments constituted M+ASP chronic and M+ASP chronic+N groups, respectively. The M containing pellets implanted rats which were injected with a single dose of ASP on the last day were administered either physiological saline or 10 mg/kg of N. The rats which had been injected with saline were called M+ASP single dose group, the other ones mentioned as M+ASP single dose + N group instead.

Five min following the injections the rats were decapitated after cervical dislocation. Blood samples were collected into containers and centrifuged immediately. After the plasma had been separated they were kept at −70°C for the estimation of cortisol level for a maximum of seven days. On the other hand the brains were immediately removed and placed on containers chilled on ice. After the brains had been cleaned of extraneous tissues they were weighed. For the determination of L-asparaginase and ACE activities the different preparative procedures necessary for each determination were carried out as described elsewhere [15, 20, 21, 23].

All the results were analyzed by analysis of variance and subsequently the Dunnett's (I) test was used for statistical evaluation.

RESULTS

The mean values (±SD) of the brain L-asparaginase and

TABLE 2

THE MEAN VALUES (± SD) AND THEIR STATISTICAL EVALUATIONS OF THE BRAIN L-ASPARAGINASE AND ANGIOTENSIN CONVERTING ENZYME (ACE) ACTIVITIES AND PLASMA CORTISOL LEVEL

Groups	L-Asparaginase activity U/g wet weight	ACE activity U/g wet weight	Plasma cortisol level mg/ml
Control	0.225 ± 0.015 (14)	126.93 ± 8.03 (10)	529 ± 48 (12)
M	0.197 ± 0.013* (14)	108.09 ± 5.28* (10)	468 ± 46† (12)
N	0.221 ± 0.014 (10)	111.23 ± 6.07* (10)	509 ± 47 (10)
M+N	0.219 ± 0.018§ (14)	129.72 ± 13.75 (10)	582 ± 46†§ (12)
M+ASP chronic	0.187 ± 0.017† (14)	108.06 ± 7.33† (11)	522 ± 63§ (12)
M+ASP chronic +N	0.202 ± 0.022† (14)	113.85 ± 9.09† (10)	532 ± 60§ (12)
M+ASP single dose	0.191 ± 0.016 (14)	112.46 ± 7.21† (11)	477 ± 68† (11)
M+ASP single dose+N	0.197 ± 0.017 (14)	115.44 ± 9.09 (10)	506 ± 46 (11)
F Values	10, 60	8, 81	5, 45

Control—Vehicle containing pellet implanted group.
N—Naloxone injected "Control" group.
M—Morphine containing pellet implanted group.
M+N—Naloxone injected "M" group.
M+ASP chronic—Chronically L-aspartic acid administered "M" group.
M+ASP chronic+N—Naloxone injected "M+ASP chronic" group.
M+ASP single dose—Single dose L-aspartic acid administered "M" group.
M+ASP single dose+N—Naloxone injected "M+ASP single" group.
The figures in the parentheses indicate the number of animals.
Statistical significance: *0.01; †0.05; referring to Control; ‡0.01; §0.05; referring to the M group.
When the values of the M+ASP chronic and M+ASP single dose groups were compared with the values of the M+ASP chronic+N and M+ASP single dose+N groups respectively no significant differences were found.

ACE activities, and the plasma cortisol level with their statistical evaluations are shown in Table 2.

F values of the brain L-asparaginase and ACE activities, and the plasma cortisol level were found to be 10.60, 8.81 and 5.45, respectively. F value of these three parameters appeared 3.29.

The brain L-asparaginase and ACE activities, and the plasma cortisol level of the rats implanted with M pellets (the M group) were significantly lower than control. The administration of N to the rats implanted with control pellets (the N group) caused a significant decrease in the ACE activity only. The injection of N to the rats belonging to the M group normalized the decrease by M in the levels of the L-asparaginase and ACE activities. The increase in plasma cortisol level was so great that the significantly decreased level of plasma cortisol reached a significantly higher level than control. The brain L-asparaginase and ACE activities of the M pellets implanted and chronically ASP administered rats (the M+ASP chronic group) appeared to be significantly lower than control. No significant change was observed in the cortisol level when compared to control, but it was significantly higher than that of the M group. The administration of N to the rats of the M+ASP chronic group for precipitating abstinence syndrome resulted in a rise in the brain L-asparaginase activity (the level of significance rose from 0.01 to 0.05). In spite of this rise the activity of L-asparaginase still remained significantly lower than control. No significant change was found in the ACE activity and in the cortisol level. After single dose ASP administration to the rats implanted with M containing pellets (the M+ASP single dose group), the activities of the brain L-asparaginase and ACE enzymes, and the plasma cortisol level were found to be significantly lower than control. The statistical evaluation between the values of the M group and the M+ASP single dose group did not show any significant difference. After N administration, the L-asparaginase and ACE activities had no significant change, whereas the plasma cortisol level which had been found to be significantly decreased in the M+ASP single dose group returned towards control value. No significant change was observed between

the values of the M+ASP single dose group and those of the M+ASP single dose + N group.

DISCUSSION

The previous experimental findings clearly showed that most of the precipitated abstinence syndrome signs, especially by N administration, steeply decline ten min after N injection [3]. This means that central biochemical changes and processes responsible for the manifestations of abstinence syndrome signs probably reach their maximum levels in the first five min. For this reason the rats used in this study were sacrificed five min following N injection to better demonstrate changes in the biochemically determined parameters. As the animals were killed five min following N administration, which falls in the middle of the generally accepted observation period for precipitated abstinence syndrome signs, it would not be correct to compare the symptoms of the different groups in order to show the difference in the extent of physical dependence. For these kinds of comparisons and the evaluation of therapeutic efficacy of ASP, which had been done and shown previously [12, 14, 19, 34], parallel experiments would have to be performed.

Concerning the dose of N used in the present study it could be said that it was suitable for the aim of the study since "dominant" withdrawal symptoms become more pronounced when the dose of the opioid antagonists used for the precipitated abstinence syndrome is increased [3]. Therefore rather high doses of opioid antagonists would cause a more potent precipitated abstinence syndrome, which could better reflect biochemical changes and behavioural differences [3, 12, 14].

A single dose of M has been shown to have an inhibitory effect on the brain L-asparaginase [15,21] and ACE [19] activities. Consistent with the results obtained by using a single dose of M, the chronic M administration by implanting M-containing pellets also had an inhibitory effect on both brain L-asparaginase and ACE (Table 2). Ten mg/kg single dose of N resulted in a significant decrease in the ACE activity as expected [20]. In spite of its inhibitory effect on the brain ACE activity, N antagonized the inhibition by chronic M administration of the brain ACE activity (Table 2). The sudden normalization by N administration of the brain L-asparaginase and ACE activities to the inhibited states and associated circumstances of which the M dependent rats had been adapted, resulted in the increase of plasma cortisol level, possibly together with some other sudden drastic changes in L-asparaginase and angiotensin II regulated and/or modulated hormonal and neurotransmitter systems, probably including cholinergic and serotonergic ones [3, 4, 30, 39]. The mechanisms underlying abstinence syndrome symptoms would include all these very complicated and interrelated sudden changes.

Even though the chronic ASP administration to the rats implanted with M-containing pellets seemed not to antagonize the inhibitory effect on M on the brain L-asparaginase and ACE activities, it was able to provide the way(s) to keep the plasma cortisol level very close to control. This can be related to the restored equilibrium between L-asparaginase and asparagine synthetase due to the inhibition of asparagine synthetase by asparagine as the end-product of asparagine synthetase which catalyzes the biosynthesis of asparagine from ASP and ammonia [8, 18, 20]. After the administration of N, which precipitates abstinence syndrome in opiate-dependent organisms, the activities of the brain L-asparaginase and ACE remained significantly lower than control and the plasma cortisol level did not show any further increase (Table 2). Thus, the chronic administration of ASP during the concomitant administration of M provided by the implantation of M-containing pellets can prevent the organism from manifesting abstinence syndrome [12–14], in which the sudden normalization of the inhibited activities of brain L-asparaginase and ACE, and the associated sudden increase in the basal level of plasma cortisol seem to be the main reasons for the manifestation of abstinence syndrome, as seen in the M+N group (Table 2).

The basis of the single dose ASP prevention from N precipitated abstinence syndrome appears to be different from those of the chronic ASP administration. Since the single dose ASP administration did not cause any significant changes in the brain L-asparaginase and ACE activities, and in the plasma cortisol level the preventive effects of the single dose ASP can, to a great extent, be explained firstly, by its own inhibitory effect on L-asparaginase and ACE, and secondly, by serving as the precursor of asparagine synthesized by asparagine synthetase according to the hypothesis [19, 21, 34].

On the basis of the results obtained in the experiments it is possible to reach the following conclusions: (1) In addition to the inhibition of the brain L-asparaginase activity the inhibition by M of the brain ACE activity appeared to take part in the development of physical dependence on opiates; (2) The manifestation of the abstinence syndrome is related, at least to a great extent, to the sudden normalization of the M inhibited brain L-asparaginase and ACE activities, and associated sudden changes in hormonal and neurotransmitter systems; (3) Whereas the administration of ASP together with opiates can antagonize the development of physical dependence, the administration of ASP before the onset of abstinence syndrome does not allow the abstinence syndrome to manifest.

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