Responsiveness of mouse submaxillary gland arginine aminopeptidase to Aconiti Tuber extract

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Abstract

A marked increase in enzyme activity was exhibited in the submaxillary gland of male mice which were given Aconiti Tuber extract (1.0−1.2 g/kg B.W./ day) for two weeks. Also, a relative increase in enzyme activity was noticed in females as well as a somewhat smaller increase in castrated males. Moreover, an increase in the enzyme activity was exhibited in the submaxillary gland of mice which had been given aconitine for 2 weeks. The enzyme activity was enhanced in a manner similar to that of Hatimi-ziô-gan. However, the increase caused by treatment with aconitine was not observed in the adrenalectomized female mice. On the other hand, oral administration of heat-treated aconiti or Cinnamomi Cortex extracts did not produce any effect on arginine aminopeptidase activity of the mouse submaxillary gland. These results suggest that the effect of Aconiti Tuber extract for modulation of arginine aminopeptidase may proceed through affecting hormones of the testis and/or by some mechanisms through organs other than testes.

Key words Aconiti Tuber, Cinnamomi Cortex, Hatimi-ziô-gan, Rokumi-gan, submaxillary gland, arginine aminopeptidase isozymes

Abbreviations BAPNA, αN-Benzoyl-DL-arginine-*p*-nitroanilide HCl ; Hatimi-ziô-gan (Ba-Wei-Di-Huang-Wan), 八味地黄丸 ; Rokumi-gan (Liu-Wei-Wan), 六味丸

Introduction

Enzymes which accumulate in the submaxillary glands tissue provide a useful model for studying some mechanisms involved in hormonal action.[1-3] Arginine aminopeptidase in mouse submaxillary gland is a useful biochemical marker for estimation of androgenic action of certain Oriental medicines.[4] Previously, we have shown an increase in the activity of arginine aminopeptidase in submaxillary gland of intact female, male and castrated male mice by feeding a diet containing Hatimi-ziô-gan (Ba-Wei-Di-Huang-Wan) Oriental medicine. It was suggested that this effect on arginine aminopeptidase activity may proceed through the testis and/or by some unknown mechanisms through organs other than testes.[5] We also found that testes are involved in the increase of arginine aminopeptidase activity in mouse submaxillary gland after Rokumi-gan (Liu-Wei-Wan) treatment.[6] Rokumi-gan is an Oriental medicine which contains two less herbs than Hatimi-ziô-gan, Aconiti Tuber, and Cinnamomi Cortex, but otherwise identical to Hatimi-ziô-gan. Since, Hatimi-ziô-

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gan treatment resulted in a relative increase in the activity of arginine aminopeptidase in female and castrated male mice submaxillary glands, either the two herbs of Hatimi-ziô-gan, Aconiti Tuber and Cinnamomi Cortex, which are not present in Rokumi-gan, or the combination of additional herbs in Hatimi-ziô-gan act on the submaxillary gland to stimulate activity of arginine aminopeptidase. In order to examine the hypothesis that the two herbs, Cinnamomi Cortex and Aconiti Tuber, are responsible for the other mechanisms involved in stimulation of arginine aminopeptidase in the absence of testicular hormones, a dose of each of Aconiti Tuber, Cinnamomi Cortex, and heat-treated aconiti extracts was given to intact or castrated male and female mice of HR-A substrain. Moreover, the effect of alkaloid aconitine which is one of the constituents of Hatimi-ziô-gan was examined. In addition, similar studies using adrenalectomized female mice have been carried out to examine the possible relationships between the effect of aconitine in the induction of arginine aminopeptidase and in the role of adrenal gland hormones. Now, through the present study, we obtained the results suggesting that the active component that enhanced the activity of arginine aminopeptidase is most probably Aconiti Tuber substances. However other substances could not be excluded.

Materials and Methods

Chemicals : αN-Benzoyl-DL-arginine-*p*-nitroanilide HCl (BAPNA) was purchased from Nakarai Chemicals, Ltd. (Tokyo, Japan) ; cellulose acetate membrane (60×220 mm) was purchased from Marcherey Nagel Co. (Germany) ; N-1-naphthylethylenediamine dihydrochloride, acrylamide, N, N-methylene bisacrylamide, and other reagents used were analytical grade products obtained from Wako Pure Chemical Industries (Tokyo, Japan). Aconitine was purchased from Sigma Chemical Co., Cinnamaldehyde was purchased from Kasei Kogyo Co., Ltd. (Tokyo, Japan). Aconiti Tuber (Hou-bushi), heat-treated aconiti (Kako-bushi) processed at 120℃ for 40 minutes and Cinnamomi Cortex (Kannan-Keihi) were obtained from Ohminedo Co., Ltd. (Nara, Japan).

Animals and treatment : Six week-old male and female HR-A substrain of mice were used throughout the experiment. The animals were maintained under control conditions with constant temperature (23±2℃), humidity (55±10%) and with a 14 hr light-10 hr darkness cycle. They had free access to food and water at all times. Castration was carried out under nembutal anesthesia (0.01 mg/g B.W. ; i.p.) with bilateral excision of testis and associated epididymides. Female mice were adrenalectomized. Two weeks after the operation the animals were used.

An alkaline solution of aconitine was prepared and administered orally to each animal by means of a metal gastric tube. Cinnamaldehyde was also administered as an aqueous solution after proper dilution.

Extracts were prepared as follows : 10% w/v mixture of each herb in 99% EtOH was heated for 4-6 hrs at 55℃. The extract obtained was evaporated by slow heating and continuous stirring until a dense syrup was obtained. The syrup was suspended in water just before administration to the mice. A dose of each-Aconiti Tuber extract (1.0-1.2 g/kg B.W./day), heat-treated Aconiti Tuber extract (1.0—1.2 g/kg B.W./day) and Cinnamomi Cortex extract (1.0 g/kg B.W./day)-was given orally by gastric tube once a day for 2 weeks, while the controls were given an equal volume of distilled water at the same intervals.

Preparation of tissue extract : Mice were killed by cervical dislocation and the submaxillary glands were removed from the surrounding tissue, washed in ice cold 0.9% NaCl solution to remove traces of blood and placed in a covered weighing flask in an ice bath. Extracts were prepared by using a single pair of glands from each animal. Tissues were weighed on a Mettler balance in a preweighed covered glass. The glands were homogenized with 9 volumes of cold 0.1M phosphate buffer pH 7.8. The homogenates were centrifuged at 20000 ×*g* for 30 minutes at 4℃ and the supernatant fluid was used for en-

zymatic assay and electrophoretic analysis.

Assay of enzyme activity : The activity of arginine aminopeptidase was measured spectrophotometrically using BAPNA as substrate. Hydrolysis of substrate yielding *p*-nitroaniline was measured using diazo-coupling by the method of Taie and Ogita.[7]

Electrophoretic analysis : Arginine aminopeptidase isozyme was analyzed by using vertical 7% polyacrylamide gels in the manner previously described by Ogita and Markert.[8] Electrophoresis was performed for 2 hrs at a constant current of 1 mA/cm of gel width.

Staining of arginine aminopeptidase isozyme : Arginine aminopeptidase activities were visualized by use of specific staining techniques employing BAPNA as substrate according to the staining method of Isobe and Ogita.[9]

Results

The effect of Cinnamomi Cortex and cinnamaldehyde

Oral administration of Cinnamomi Cortex extract (1.0 g/kg B.W./day) and cinnamaldehyde (100.0 mg/kg B.W./day) had no effect on the arginine aminopeptidase activity in the submaxillary glands of male or female mice. Moreover, a loss of the enzyme activity was observed after castration. Neither a dose of Cinnamomi Cortex extract nor cinnamaldehyde could affect the enzyme activity in castrated males, a result that was similar to that of normal ones.

The effect of Aconiti Tuber and heat-treated Aconiti Tuber extracts

The zymogram of arginine aminopeptidase in the male submaxillary gland is shown in Fig. 1a. It can be seen that there was an increase in arginine aminopeptidase activity and its number of bands by Aconiti Tuber extract treatment. In female zymograms as shown in Fig. 1b, there was an increase in arginine aminopeptidase activity as well as in the number of bands by Aconiti Tuber extract treatment. Moreover castrated male arginine aminopeptidase isozyme patterns were converted to female isozyme ones (Fig. 1c). The densitometric scans of the gels of arginine aminopeptidase isozyme using BAPNA as substrate were shown in Fig. 2. It can be seen that no change was observed in the activity of band A of each male, female, and castrated male after treatment with Aconiti Tuber extract. Band B was not apparent in female and castrated male. However, this band appeared, and bands C and D were increased in Aconiti Tuber extract treated males. In females and castrated males, band D was not detected but band C was apparent in those given Aconiti Tuber extract. Bands E and F increased in males as well as in females and castrated males after treatment

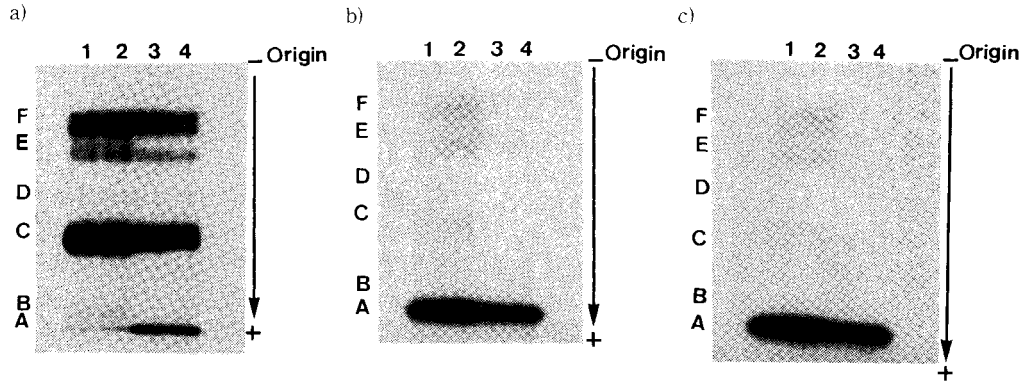

Fig. 1 Zymogroms of arginine aminopeptidase in the submaxillary glands of different sex of HR-A mice.
a) Male, b) Female, c) Castrated male. Channels 1 and 2=Mice given Aconiti Tuber extract. Channels 3 and 4=Control mice. A−F=Arginine aminopeptidase isozyme bands.

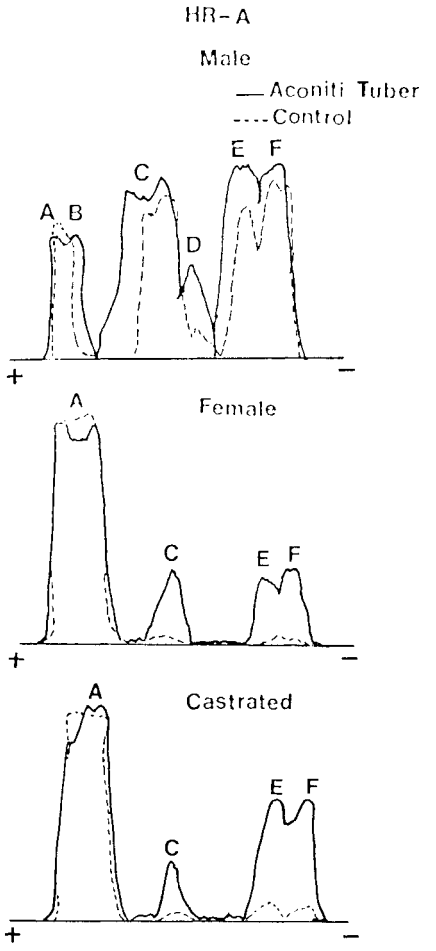

Fig. 2 Densitometric scans of arginine aminopeptidase isozyme using BAPNA as substrate.

with Aconiti Tuber extract. However, these bands were detected in control. The submaxillary gland of each mouse was assayed as a single sample and results were pooled for each of three groups : male, female, and castrated male. Precise induction of arginine aminopeptidase activity in males was observed. As shown in Fig. 3a, enzyme activity was increased about 2.3 times in Aconiti Tuber extract treated males as compared with that of control mice. Furthermore, a relative increase in the enzyme activity resulted in females that had been given Aconiti extract (Fig. 3b). The activity of arginine aminopeptidase in the submaxillary gland after castration was influenced by Aconiti Tuber extract administration (Fig. 3c). A somewhat smaller increase was noticed in the activity of the enzyme in castrated males after treatment with Aconiti Tuber extract. As shown in Fig. 3a, b and c, however, enzyme activity was not affected in males, in females, or in castrated males after the oral administration of heat-treated Aconiti Tuber extract for two weeks.

The effect of aconitine on arginine aminopeptidase activity

The effect of orally administered aconitine on arginine aminopeptidase isozymes in the submaxillary glands of both sexes of HR-A mice was observed. Arginine aminopeptidase activity of aconitine treated mice varied in accord with sex. It was found that a dose of aconitine (0.8 mg/kg

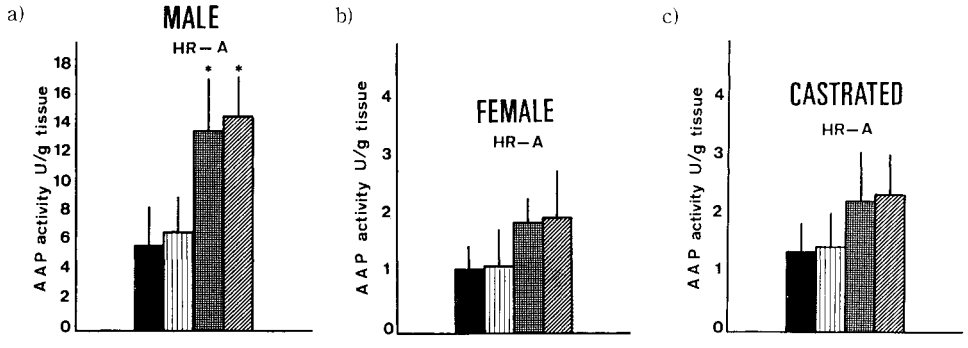

Fig. 3 Arginine aminopeptidase in the submaxilary galnd of different sex of HR-A mice. a) Male, b) Female, c) Castrated male. ■, Control mice ; ▥, Mice given heat-treated Aconiti extract ; ▦, Mice given Aconiti Tuber extract ; ▨, Mice given aconitine. Bars represent means of two experiments, 7−8 mice each. Vertical lines indicate S.E. $p<0.05$.

Table I The effect of aconitine on arginine aminopeptidase in adrenalectomized mice.

Treatment	Arginine aminopeptidase activity Unit/g tissue
None	1.5±0.6
None + Aconitine	2.5±0.4
Adrenalectomy	0.8±0.3
Adrenalectomy + Aconitine	1.0±0.4

Six-week old HR-A female mice were adrenalectomized and from 15 days after the operation aconitine (0.8 mg/kg B.W.) were given daily for 2 weeks. Enzyme activity was measured in mice of the same age. Results are means±S.E. of values in five separate determinations.

B.W./day) given for two weeks resulted in an increase of the enzyme. Fig. 3a shows that the activity was elevated about 2.5 times in aconitine treated males as compared to controls. In females and castrated males, the activity of arginine aminopeptidase was somewhat increased by administration of aconitine (Figs. 3b and c).

The data in Table I indicated that a dose of aconitine (0.8 mg / kg B.W./ day) given for 2 weeks caused a relative increase in arginine aminopeptidase activity in the submaxillary gland of intact female mice, however, this increase caused by aconitine was not observed in adrenalectomized HR-A female mice.

Discussion

Proteins that accumulate in submaxillary gland tissue such as epidermal growth factor, nerve growth factor, kallikrein, and some enzymes including arginine aminopeptidase proved a useful model for studying some aspects of hormonal action.[10-15] Sex differences have been reported in mouse submaxillary gland arginine aminopeptidase which is considered a marker for determination of androgenic action.[3,4,10] Previously, we have suggested that the effect of active components of Hatimi-ziô-gan for modulation of arginine aminopeptidase activity in the mouse submaxillary gland may proceed through the testis and/or through organs other than testes.[5] We also, found that testes are involved in the increase of arginine aminopeptidase in mouse submaxillary gland with Rokumi-gan treatment.[6] Based on these results, we suggest that the effect of Hatimi-ziô-gan on arginine aminopeptidase might be through substances concentrated in Cinnamomi Cortex or Aconiti Tuber. The rise in arginine aminopeptidase activity in submaxillary gland of HR-A (Hatimi-ziô-gan responder A) mice after Aconiti Tuber extract given orally indicated that the active principles which induced the activity of the enzyme are most probably Aconiti Tuber constituent. The increase of arginine aminopeptidase enzyme produced by the herb must be due to the content of aconitine because Aconiti (kako bushi) processed at 120℃ for 40 minutes was ineffective, and a great part of aconitines was decomposed by the processing.[16] Hatimi-ziô-gan contains a variety of substances, but we concluded that aconitine is responsible for the effects of the medicines on the increase of arginine aminopeptidase synthesis. The pharmacological evaluation of aconitine on the muscle, brain, blood pressure and heart rate has been reported.[17-19] Also, aconitine has been shown to produce a persistent increase in sodium conductance, leading to repetitive depolarization.[20] Other substances of Hatimi-ziô-gan may also act on the enzyme by affecting testis hormones. This conclusion was suggested by the effects of Rokumi-gan on enhancing arginine aminopeptidase in intact males, in spite of the fact that Rokumi-gan contains no aconitine. In addition, studies using adrenalectomized female mice revealed that administration of aconitine did not show changes in arginine aminopeptidase activity of submaxillary glands. It has been repor-

ted that aconitine affected adrenal gland hormones.[18,20] Thus, it is possible that adrenal gland hormones might contribute to the enhancement of arginine aminopeptidase activity in mouse submaxillary gland. Adrenergic agents have been reported to stimulate the release of the enzyme from submaxillary glands.[22-24] Cortisol has been shown to increase the submaxillary gland kallikrein-like activity and kallikrein content in adrenalectomized female rats.[14] Moreover, it was reported that glucocorticoids may be involved in a compensatory way when androgens are absent.[23,25,26] Thus, based upon these findings and the present data, it appears that the adrenal gland, perhaps through its production of various steroids, was affected by aconitine and the activity of arginine aminopeptidase of the submaxillary gland was increased.

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和文抄録

マウスに附子エキス（1.0～1.2 g/kg B.W./day）あるいは桂枝エキス（1.0 g/kg B.W./day）を経口投与し，それらエキスの顎下腺に対する影響をアルギニンアミノペプチダーゼ活性を指標として検討した。

附子エキスを2週間，HR-A系統マウスに経口投与すると，マウス顎下腺アルギニンアミノペプチダーゼ活性は雄において著しく上昇した。また，雌でも幾分上昇した。しかし去勢した雄では附子エキス投与による活性の上昇は雌のそれに等しかった。また，副腎を摘出したマウスに投与したところ，顎下腺アルギニンアミノペプチダーゼ活性の誘導上昇は認められなかった。

一方，桂枝エキスを2週間経口投与したマウス群では，雄，雌ならびに去勢した雄のいずれの顎下腺に対してもアニギニンアミノペプチダーゼ活性の上昇は認められなかった。

このことは，アルギニンアミノペプチダーゼ活性を指標酵素として観察する限り，精巣を介さずにマウス顎下腺に及ぼす八味地黄丸効果は附子の成分によって副腎を介して発現されることを暗示している。

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