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Effects of *Veratrum nigrum* alkaloids on central catecholaminergic neurons of renal hypertensive rats

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KEY WORDS *Veratrum nigrum*; renal hypertension; immunohistochemistry; catecholamines; tyrosine 3-monooxygenase

ABSTRACT

AIM: To study the central hypotensive mechanism of *Veratrum nigrum* L var *ussurience* Nakai alkaloids (*Vn*A) in renal hypertensive rats (RHR). **METHODS**: The quantitative method of immunocytochemistry (ICC) was used to observe and detect the effect of *Vn*A (30 $\mu g \cdot kg^{-1}$, iv) on activity of central catecholaminergic (CA) neurons of C1, C2, A1, and A5 areas in RHR. **RESULTS**: *Vn*A increased the immunoreactivity (IR) of tyrosine 3-monooxygenase (TM)-immunopositive (IP) neurons of C1, C2, and A5 areas in RHR experimental group compared with RHR control group [positive units: (1.9 ± 0.4), (1.18 ± 0.23), (1.2 ± 0.4) *vs* (0.15 ± 0.22), (0.31 ± 0.16), (0.69 ± 0.20), respectively]; IR of TM-IP neurons of C1 and C2 areas in RHR control group was decreased compared with sham-operated group [positive units: (0.15 ± 0.22), (0.31 ± 0.16) *vs* (1.45 ± 0.29), (1.36 ± 0.25), respectively]. **CONCLUSION**: *Vn*A increased the activity of central CA neurons in RHR to exert its hypotensive effect.

INTRODUCTION

Veratrum nigrum L exits in abundant amount in China. Several kinds of domestic *Veratrum nigrum* L alkaloids have been reported with apparent hypotensive effect[1-4]; *Veratrum nigrum* L var *ussurience* Nakai alkaloids (*Vn*A), extracted from the roots of *Veratrum nigrum* L var *ussurience* Nakai of Mount Qian in Liaoning Province, were the most effective among them[2]. The isolation and structure elucidation of eight alkaloids in *Vn*A have been reported[5-7]. The main data of the main alkaloids are in the following: Neogermbudine: colorless prism, mp 147 ℃ - 149 ℃, $[\alpha]_D$ - 21.4° (c = 0.078, CH_3OH), HR-MS: found 709.4048, calcd for $C_{37}H_{59}NO_{12}$ (M^+) 709.4039. Germerine: colorless prism, mp 207 ℃ - 209 ℃, $[\alpha]_D$ + 5.16° (c = 0.63, $CHCl_3$), HR-MS: found 693.4056, calcd for $C_{37}H_{59}NO_{11}$ (M^+) 693.4087. 15-*O*-(2-methylbutyroyl)germine: colorless prism, mp 221 ℃ - 223 ℃, $[\alpha]_D$ - 24.1° (c = 0.77, $CHCl_3$), HR-MS: found 593.3561, calcd for $C_{32}H_{51}NO_9$ (M^+) 593.3563. Verussurinine: colorless amorphous powder, $[\alpha]_D$ - 28.4° (c = 0.48, pyridine), HR-MS: found 593.3561, cacld for $C_{32}H_{51}NO_9$ (M^+) 593.3563. Germidine: colorless prism, mp 231 ℃ - 233 ℃, $[\alpha]_D$ + 6.85° (c = 0.62, $CHCl_3$), HR-MS: found 635.3667, cacld for $C_{34}H_{53}NO_{10}$ (M^+) 635.3668. Our previous research indicated that *Vn*A produced immediate and remarkable hypotensive effects on normotensive dogs (3 $\mu g \cdot kg^{-1}$, iv), rats (30 $\mu g \cdot kg^{-1}$, iv), and renal hypertensive rats (RHR, 30 $\mu g \cdot kg^{-1}$, iv) and central mechanism played a very important role in this process in RHR. The present paper was to study further the central hypotensive mechanism of *Vn*A on RHR and to provide clues to find efficient antihypertensive components with low toxicity from *Veratrum nigrum* L.

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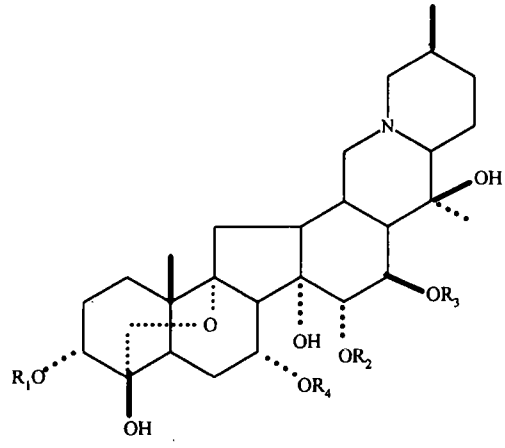

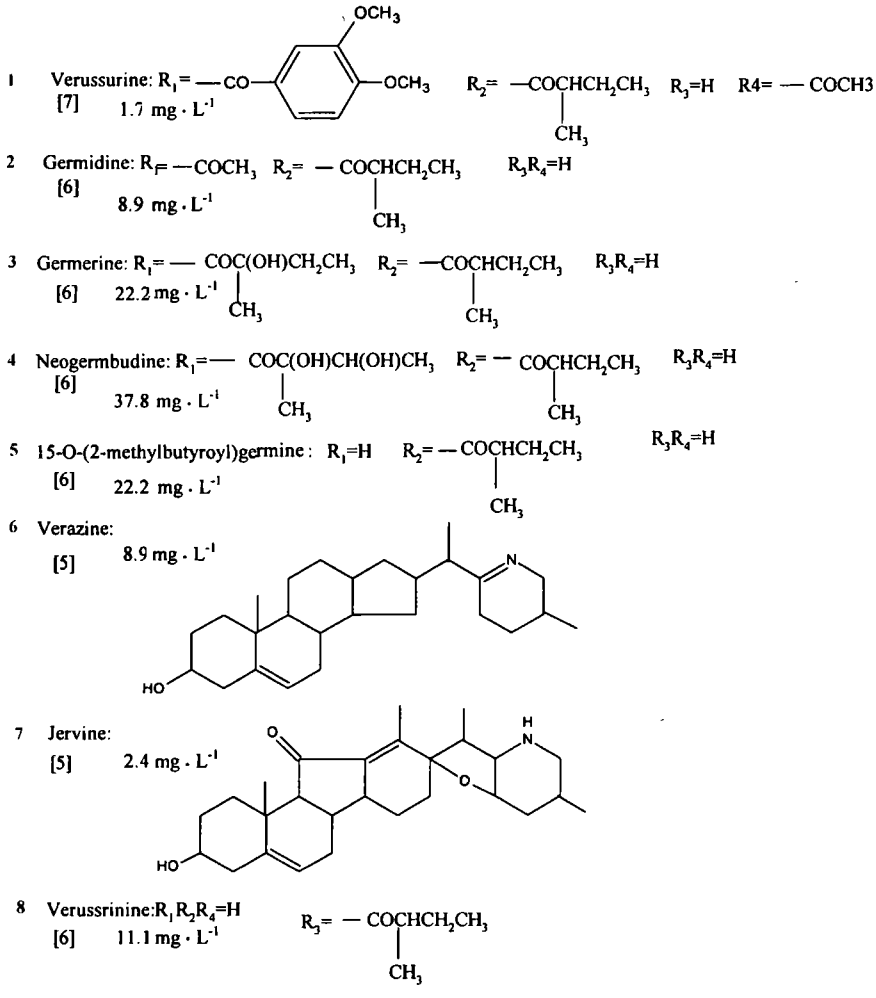

Veratrum nigrum L var *ussuriense* Nakai

MATERIALS AND METHODS

Rats Sprague-Dawley (SD, 180-220 g, female and male) rats were obtained from the Experimental Animal Center of Dalian Medical University (Grade Ⅱ, Certificate № 022).

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Drugs and Reagents *Vn*A hydrochloric injection (100 mg·L^{-1}, diluted with normal saline) was provided by Dalian Institute for Medical and Pharmaceutical Science, lot № 950613; bovine serum albumin (BSA), monoclonal antimouse tyrosine 3-monooxygenase (TM) serum and 3,3′-diaminobenzidine (DAB) were purchased from Sigma Co, USA; avidine-biotin-peroxidase complex (ABC) kit was produced by Vector Co, USA.

Preparation of brain sections Renal hypertension was produced by perirenal wrapping method[8]. Sham-operated group was made simutaneously. Before and after surgery, blood pressure (BP) was measured by tail cuff method. Systolic blood pressure (SBP) which was increased by 4 kPa and greater than 21.3 kPa after 3 wk was considered hypertensive.

The rats were divided into three groups: sham-operated control group (NS, iv, $n=6$), RHR control group (NS, iv, $n=6$), and RHR experimental group (*Vn*A, 30 μg·kg^{-1}, iv, $n=7$). All rats were anesthetized with pentobarbital sodium (36 mg·kg^{-1}, ip) and injected with drugs via poplitea vein. Five minutes later, rats were transcardially perfused with 1 % paraformaldehyde (PF, pH 7.4) 100 mL followed by 4 % PF (pH 7.4) 200 mL to fix brain tissues and then placed at 4 ℃ overnight. The brain tissues were put in 4 % PF (pH 7.4) for post-fixation for 24 h and then transferred into 20 % sucrose solution. After the sinking of the brain tissues, successive coronal sections (50 μm thick) were cut on Vibratome (Xiangshan Equipment Factory, Zhejiang). The brain sections respectively in adrenergic neurons (C1, C2 areas), noradrenergic neurons (A1 area) of medulla oblongata, and noradrenergic neurons (A5 area) of pons were selected for the immunocytochemistry (ICC) steps.

ABC method of ICC The steps were: 1) washed with 0.01 mol·L^{-1} phosphate-buffered saline (PBS; pH 7.4) for 10 min twice; 2) preincubated with 2 % Triton solution at room temperature (RT) for 5 min; 3) washed with 0.01 mol·L^{-1} PBS for 10 min, 3 times; 4) blocked with 2 % BSA at RT for 20 min, and washed 3 times with 0.01 mol·L^{-1} PBS for 10 min after the each following step; 5) incubated at 4 ℃ with monoclonal antimouse TM serum (1∶1000) for 36 h; 6) incubated biotinylated secondary anti-serum (rabbit anti-mouse IgG, 1∶400) for 1 h; 7) incubated with ABC at RT (A∶B∶PBS = 1∶1∶400) for 2 h; 8) stained with DAB/H_2O_2 (0.05 % DAB, 0.00024 % H_2O_2). The sections were observed and photographed under a Olympus microscope (Japan).

Quantitative analysis of ICC For each animal of the three groups, six sections were taken for analysis with Q-500MC image analyzing system (LeiCa, German). In each section, single microscopic field that contained almost all the immunopositive neurons of each area was chosen respectively to be analyzed using the same illumination intensity at 200 × magnification. Quantitative assessments were performed by conversion from mean gray scale values to the positive units (PU) according to the quantitative method of ICC[9].

$$PU = |G_\alpha - G_\beta| \times 100 \div G_{max}$$

G_α: the mean gray level of the positive reaction products in the studied structure;

G_β: the mean gray level of the background;

G_{max}: the maximal gray level of the image analyzing system.

Statistical analysis The data were expressed as $\bar{x} \pm s$ and analysed with ANOVA.

RESULTS

TM-immunoreactivity (IR) was observed in neuron bodies and fibers of C1 and C2 areas in three groups. The neuron bodies were mainly double- or multi-polar. IR was also observed in larger double- or multi-polar neurons of A5 area. The brown immunopositive (IP) parcels were even-distributed through the plasma of above-mentioned TM-IR neurons and unstained round nucleus were observed in central or side part in some neurons.

In RHR control group, compared with sham-operated group: The TM-IR in C1 and C2 areas were decreased both under microscope and by statistics ($P<0.01$, $P<0.01$ respectively). IR of TM-IP neurons in A5 area was decreased apparently under microscope, but the difference was not significant ($P>0.05$). IR of TM-IP neurons in A1 area was unchanged both under microscope and by statistics ($P>0.05$).

In RHR experimental group, compared with RHR control group: IR of TM-IP neurons in C1, C2, and A5 areas were all increased ($P<0.01$, $P<0.01$, and $P<0.05$ respectively). IR of TM-IP neurons in A1

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area was unchanged both under microscope and by statistics ($P>0.05$) (Fig 1, Tab 1).

DISSCUSSION

Central catecholaminergic system plays a very important role in the control of BP. The adrenergic neurons in C1 and C2 areas may exert an important inhibitory function on cardiovascular action[10,11]. Noradrenergic neurons concentrate mainly in A1 and A5 areas, which can produce hypotensive effect when stimulated by sodium glutamate. It suggested that A1 and A5 areas were hypotensive areas[12,13].

TM is the synthetic enzyme of CA and it is also a mark enzyme of CA. This paper used ABC method with monoclonal anti-TM serum to observe the influence

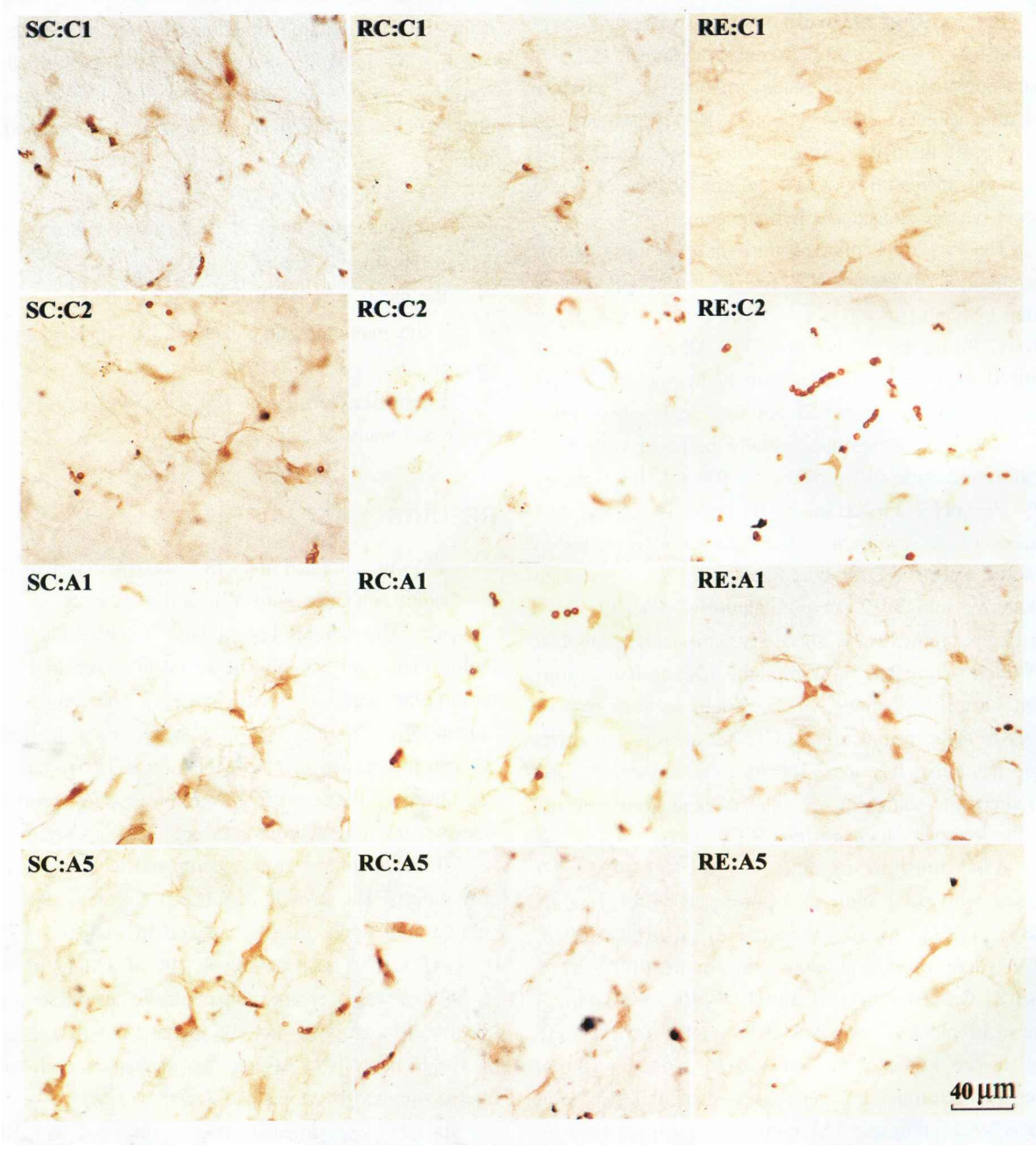

Fig 1. Effects of *Vn*A (30 μg·kg^{-1}, iv) on immunoreactivity of catecholaminergic neurons in C1, C2, A1, and A5 areas with immunocytochemistry method. SC: sham-control group; RC: RHR control group; RE: RHR experimental group. Magnification × 200.

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Tab 1. Influence of *Vn*A (30 μ/kg, iv) on the immunoreactivity of catecholaminergic neurons in medulla oblongata and pons of RHR. $\bar{x} \pm s$. $^{a}P > 0.05$, $^{b}P < 0.05$, $^{c}P < 0.01$ *vs* **sham-operated group.** $^{d}P > 0.05$, $^{e}P < 0.05$, $^{f}P < 0.01$ *vs* **RHR control group.**

	SBP (kPa)	Positive units			
		C1	C2	A1	A5
Sham-operated control group ($n = 6$)	18.1 ± 2.3	1.45 ± 0.29	1.36 ± 0.25	0.86 ± 0.29	1.0 ± 0.3
RHR control group ($n = 6$)	27.9 ± 1.9	0.15 ± 0.22^{c}	0.31 ± 0.16^{c}	1.09 ± 0.48^{a}	0.69 ± 0.20^{a}
RHR experimental Group ($n = 7$)	27.6 ± 2.7	1.9 ± 0.4^{f}	1.18 ± 0.23^{f}	1.18 ± 0.28^{d}	1.2 ± 0.4^{e}

of *Vn*A (iv) on CA neurons in medulla oblongata and pons of RHR.

In RHR control group (NS, iv), compared with sham-operated group (NS, iv): IR of TM-IP neurons in A1 area of RHR was unchanged, which indicated that the synthetic function of CA neurons in A1 area had not been changed. IR of TM-IP neurons in C1 and C2 areas were decreased, which indicated that the synthetic function of CA neurons in such areas had been insufficient. Although IR of TM-IP neurons in A5 area was not changed statistically, it had a tendency to be decreased under microscope, which indicated that the synthetic function of CA neurons in A5 area may be insufficient. Because the RHR rats in the experiment had formed hypertension, above-mentioned changes can not be judged as cause or results of hypertension of model rats, and we can conjecture that insufficient function of CA neurons in C1 and C2 areas may play a critical role in the development and maintaining of hypertension in RHR and that CA neurons in A5 area may take part in this process.

In RHR experimental group (*Vn*A, iv) compared with RHR control group (NS, iv): IR of TM-IP neurons in C1, C2, and A5 areas was increased, which indicated that synthetic function of CA neurons in C1, C2, and A5 areas had been increased, ie, that *Vn*A exert its central hypotensive action by increasing the activity of CA neurons in C1, C2, and A5 areas. IR of TM-IP neurons in A1 area of RHR was unchanged, which indicated that CA neurons in A1 area had not participated in the hypotensive process of *Vn*A on RHR. Whether *Vn*A promoted the relieve of CA by increasing activity of neurons in C1, C2, and A5 areas and whether CA exerted a hypotensive effect by interacting with α-receptor of synoptic post-membrane are needed to be investigated.

Our research of the hypotensive mechanism of *Vn*A provided the theoretical evidence for its exploitation as new drug and future clinical use; in addition, it is first reported that ICC method combined with image analyzing system was used to study the central hypotensive mechanism of drugs locally, qualitatively, and quantitatively.

In summary, *Vn*A can strengthen the activity of catecholaminergic neurons in C1, C2, and A5 areas of RHR to exert its hypotensive effect. Insufficient function of catecholaminergic neurons in C1 and C2 areas may play a very important role in the development and maintaining of hypertension in RHR.

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乌苏里藜芦碱对肾性高血压大鼠中枢儿茶酚胺能神经元的作用

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关键词 藜芦; 肾性高血压; 免疫组织化学; 儿茶酚胺类; 酪氨酸 3-单加氧酶

目的: 进一步研究乌苏里藜芦碱(*Vn*A)对肾性高血压大鼠(RHR)的中枢降压机制. **方法**: 采用免疫细胞化学定量方法观察并测定 *Vn*A (30 μg·kg^{-1}, iv)对 RHR 脑内 C1、C2、A1、A5 区儿茶酚胺(CA)能神经元活性的影响. **结果**: *Vn*A 可明显增强 RHR C1、C2、A5 区酪氨酸 3-单加氧酶(TM)-免疫反应阳性(IP)神经元的免疫反应活性(IR); 与假手术对照组相比, RHR 对照组 C1、C2 区 TM-IP 神经元的 IR 明显减弱. **结论**: *Vn*A 可通过增强 RHR C1、C2、A5 区 CA 能神经元的活性发挥其降压作用.

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