

A cross-fostering analysis of bromine ion concentration in rats that inhaled 1-bromopropane vapor

Toru ISHIDAO¹, Yukiko FUETA¹, Susumu UENO², Yasuhiro YOSHIDA³, and Hajime HORI¹

¹ Department of Environmental Management, School of Health Sciences, University of Occupational and Environmental Health, Japan.

² Department of Occupational Toxicology, Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, Japan.

³ Department of Immunology and Parasitology, School of Medicine, University of Occupational and Environmental Health, Japan.

Correspondence to: Toru Ishidao, Department of Environmental Management, School of Health Sciences, University of Occupational and Environmental Health, Japan.

Iseigaoka 1-1, Yahatanishi-ku, Kitakyushu 807-8555, Japan

(e-mail: ishidao@health.uoeh-u.ac.jp)

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1 **Abstract: Objective:** Inhaled 1-bromopropane decomposes easily and releases bromine
2 ion. However, the kinetics and transfer of bromine ion into the next generation have not
3 been clarified. In this work, the kinetics of bromine ion transfer to the next generation
4 was investigated by using cross-fostering analysis and a one-compartment model.
5 **Methods:** Pregnant Wistar rats were exposed to 700 ppm of 1-bromopropane vapor for
6 6 h per day during gestation days (GDs) 1–20. After birth, cross-fostering was
7 performed between mother exposure groups and mother control groups, and the pups
8 were subdivided into the following four groups: exposure group, postnatal exposure
9 group, gestation exposure group, and control group. Bromine ion concentrations in the
10 brain were measured temporally. **Results:** Bromine ion concentrations in mother rats
11 were lower than those in virgin rats, and the concentrations in fetuses were higher than
12 those in mothers on GD20. In the postnatal period, the concentrations in the gestation
13 exposure group decreased with time, and the biological half-life was 3.1 days.
14 Conversely, bromine ion concentration in the postnatal exposure group increased until
15 postnatal day 4 and then decreased. This tendency was also observed in the exposure
16 group. A one-compartment model was applied to analyze the behavior of bromine ion
17 concentration in the brain. By taking into account the increase of body weight and
18 change in the bromine ion uptake rate in pups, the bromine ion concentrations in the
19 brains of the rats could be estimated with acceptable precision.

20

21 **Key words:** 1-Bromopropane inhalation, Cross-fostering, Bromine ion concentration,
22 One-compartment model, Animal experiment

23

24 1-Bromopropane (1-BP, CAS no. 106-94-5) is widely used as a substitute for
25 chlorofluorocarbons, which destroy the ozone layer. The toxicity of 1-BP has been

1 reviewed¹⁾, and the Japan Society for Occupational Health recommends an occupation
2 exposure limit of 0.5 ppm²⁾. Previously, we studied the effects of inhaled 1-BP vapor in
3 male rats on the nervous³⁻⁸⁾ and immune systems^{9, 10)}.

4 We also studied the effects of inhaled 1-BP vapor on metabolism in male rats and
5 reported that 1-BP rapidly decomposes and releases bromine ion in the blood¹¹⁾,
6 indicating that bromine ion is a major index of 1-BP exposure. Recently, because of the
7 health effects reported in female workers exposed to 1-BP¹²⁻¹⁴⁾, there is concern
8 regarding the health effects of 1-BP exposure on the next generation. Some researchers
9 have reported results of experiments in female animals¹⁵⁻¹⁷⁾; however, the kinetics of
10 bromine ion distribution to the next generation has not been elucidated. In this study,
11 pregnant rats were exposed to 700 ppm of 1-BP vapor, and the concentration of bromine
12 ion in the rat brain was measured. The distribution of bromine in fetuses and
13 cross-fostered pups was investigated. A one-compartment model was employed to
14 analyze the behavior of bromine ion in rats.

15

16 **Methods**

17 *Animals*

18 Female (9-week-old) and male (10-week-old) Wistar rats were purchased from
19 Kyudo Co., Ltd. (Saga, Japan). After acclimation in polycarbonate cages with dry chips,
20 they were housed in pairs in animal rooms under 12-h light–dark cycle conditions at 22
21 $\pm 1^\circ\text{C}$ and $55 \pm 5\%$ relative humidity, with free access to food and water. The presence
22 of sperm in the vaginal smear was defined as day 0 of gestation (GD0; female rats were
23 11 weeks old). In the inhalation study, the female rats were divided into three groups:
24 1-BP-exposed virgin female group (n = 5), 1-BP-exposed mother group (n = 11), and
25 the control mother group (n = 5). After the final exposure of mother rats on GD20, they

1 were housed in an animal room for the onset of birth. Postnatal day (PND) i.e., the day
2 after birth, was defined as day 0 (PND0 = GD21). On PND1, a litter size of eight pups
3 was assembled and cross-fostering^{17, 18)} of pups was performed between mother
4 exposure groups (n = 3) and mother control groups (n = 3). The pups were subdivided
5 into four groups: (1) exposure group (1-BP-exposed pups were raised by their birth
6 mother exposed to 1-BP), (2) postnatal exposure group (control pups were raised by
7 1-BP-exposed mother), (3) gestation exposure group (1-BP-exposed pups were raised
8 by control mother), and (4) control group (control pups were raised by their control
9 mother). The experimental groups are summarized in Table 1. Body weight was
10 measured periodically. The experiments were conducted per the guidance of the Ethics
11 Committee of Animal Care and Experimentation in accordance with The Guiding
12 Principle for Animal Care Experimentation, University of Occupational and
13 Environmental Health, Japan (AE03-065), which conforms to the National Institutes of
14 Health Guide for the Care and Use of Laboratory Animals and the Japanese Law for
15 Animal Welfare and Care.

16

17 *Exposure*

18 Reagent-grade 1-BP was obtained from Kanto Chemical Co., Ltd. (Tokyo, Japan).
19 1-BP vapor was introduced into a 400-*l* stainless-steel exposure chamber. Details of this
20 apparatus and procedure have been given elsewhere¹¹⁾. In order to study change in
21 bromine ion in blood and brain when the condition of dysfunction of feedback
22 inhibition (i.e., disinhibition) was confirmed, exposure concentration was designed to be
23 700 ppm, which was higher than LOAEL (400 ppm) for disinhibition⁷⁾. The actual
24 concentration of 1-BP vapor in the chamber was 701.3 ± 5.2 ppm. In the control group,
25 only clean air was introduced into the chamber. The exposure period was 6 h per day

1 between 9 a.m. and 3 p.m. throughout gestation or GD1-20 (virgin female group was
2 exposed until GD21). Table 1 displays the age of the rats on sampling day. They were
3 deeply anesthetized with diethyl ether and then decapitated. The brains and the
4 stomachs with milk from only the exposure group on PND1 were gently removed and
5 stored in a freezer.

6

7 *Measurement of bromine ion concentration*

8 The brains (cerebrum and diencephalon) and stomachs (0.25 g) were homogenized
9 with water (1.5 ml) at 0°C. The sample (1 ml) was dispensed into a vial, and 0.1 ml of
10 dimethyl sulfate was added to convert bromine ion to methyl bromide. Then, 0.1 ml of
11 an aqueous solution of isopropyl alcohol (0.5 volume percent) was added as an internal
12 standard. The vial was heated at 50°C for 1 h. The bromine ion concentration was
13 determined by measuring peak area of methyl bromide vapor in the headspace by using
14 a gas chromatograph mass spectrometer (GC/MS, QP-5050; Shimadzu, Kyoto,
15 Japan)¹¹.

16

17 *Estimation method of bromine ion concentration*

18 As previously described, inhaled 1-BP was metabolized and bromine ions were
19 released. In this study, the behavior of released bromine ion concentration in the brain
20 was analyzed by using a one-compartment model¹⁹. We assumed the bromine ion
21 uptake rate, i.e., the generation rate of bromine ion, is equal to the 1-BP uptake rate
22 because 1-BP is decomposed quickly¹¹ and releases bromine ion. Under this assumption,
23 mass balance equations of bromine ion during exposure and clearance periods
24 respectively were as follows:

25

1
$$\frac{dx}{dt} = R - kx \quad (1)$$

2

3
$$\frac{dx}{dt} = -kx \quad (2)$$

4

5 where x is the amount of bromine ion (μg); t is time (h); R is the generation rate of
 6 bromine ion ($\mu\text{g/h}$), which corresponds to the 1-BP uptake rate; and k is the excretion
 7 rate constant (1/h). From equations (1) and (2), the bromine ion concentrations C ($\mu\text{g/g}$)
 8 during exposure and clearance respectively were obtained as follows:

9

10
$$C = \frac{R}{\rho V k} (1 - e^{-kt}) \quad (3)$$

11

12
$$C = C_0 e^{-kt} \quad (4)$$

13

14 where V is the volume of the compartment (ml), ρ is the density of the compartment
 15 (g/ml), and C_0 is the initial concentration during clearance ($\mu\text{g/g}$). The excretion rate
 16 constant k is given by the biological half-life, $t_{1/2}$ (h) or $T_{1/2}$ (days).

17

18
$$k = \frac{\ln 2}{t_{1/2}(\text{h})} = \frac{0.693}{T_{1/2}(\text{days}) \times 24} \quad (5)$$

19

20 **Experimental Results**

21 Fig. 1 shows the change in the average body weight of mother rats exposed to 700
 22 ppm of 1-BP up to GD20 and that of the pups after the exposure. The time, T (on the

1 horizontal axis), includes the GDs and PNDs. Litter sizes of exposed mothers and
2 control mothers were 15.0 ± 2.8 and 14.9 ± 2.5 pups, respectively. The body weight of
3 both mothers and pups increased rapidly. This tendency was also observed in the control
4 group, and there was no significant difference between the exposure group and the
5 control group. For the virgin female group, body weight did not change significantly
6 (271.1 ± 17.0 g) during GD1-20.

7 Bromine ion concentration in the rat brain ($\mu\text{g/g-brain}$) exposed to 700 ppm of 1-BP
8 on GDs is presented as symbols in Fig. 2. The bromine ion concentration in mother rats
9 was lower than that in virgin rats, and the concentration in fetuses was higher than that
10 in mothers. Fig. 3 shows changes in bromine ion concentration in pup brain for PNDs.
11 The concentration in the gestation exposure group decreased between PND4 and PND8,
12 whereas that in the postnatal exposure group increased from PND2 to PND4 and then
13 decreased. This tendency was also observed in the exposure group, although the
14 concentration on PND1 was lower than that on GD20 (fetus in Fig. 2). Specifically, the
15 concentration in the exposure group was the highest just after birth, but decreased at
16 PND1. The concentration then increased from PND1 to PND3, but decreased again with
17 time. In the control pups, the bromine ion concentration was $11.2 \pm 7.7 \mu\text{g/g-brain}$ on
18 PND3.

19 The bromine ion concentration in pup stomachs with milk from the exposure group
20 on PND1 was $830.6 \pm 188.8 \mu\text{g/g-stomach}$, which was about twice as much as that in
21 the mother brain at GD20 (Fig. 2).

22

23 **Discussion**

24 The one-compartment model was applied to analyze the bromine ion concentration
25 in the brains of virgin females, mothers, fetuses, and pups. Equations (3) and (4) have

1 two parameters, the excretion rate constant k and the 1-BP uptake rate R . The excretion
 2 rate constant, k , can be easily calculated from equation (5) by using the biological
 3 half-life $T_{1/2}$ (days). In our previous work¹¹⁾, $T_{1/2}$ for male rats was 4.7–15.0 days in
 4 blood and 5.0–7.5 days in urine. Therefore, $T_{1/2} = 7.0$ days was used for mothers and
 5 virgin females in this study. $T_{1/2}$ in pups was 3.1 days, obtained by experimental data.
 6 Equation (4) was applied to the data from PND1 for the exposure group and from PND4
 7 and PND8 for the gestation exposure group as shown in Fig. 3. $T_{1/2} = 3.1$ days was also
 8 used for fetuses. The half-lives of between GD20 for fetuses and PND1 for the exposure
 9 group were excluded from the calculation because of the time lag due to birth.

10 As shown in Fig. 2, the bromine ion concentration in the brains of mothers was
 11 lower than that in the brains of virgin females. A reason for this might be that the
 12 bromine ion concentration was diluted because of increasing body weight. The average
 13 body weight of pups, w (g), was expressed using the following equation (Fig. 1):

$$15 \quad w = 0.00028T^{3.31} \quad (6)$$

16
 17 The average body weight of mothers, W (g), was calculated as the sum of that of virgin
 18 females ($\rho V = 271.1$ g) and of pups, w , (interpolated value for GDs):

$$20 \quad W = 271.1 + 27w \quad (7)$$

21
 22 where 27 is the constant, which was determined to give the best fit for the experimental
 23 data as shown in Fig. 1.

24 For virgin females, the uptake rate, R , of 2853 $\mu\text{g/h}$ was obtained to give the best fit

1 of equations (3) and (4) for the experimental data on GD21 in Fig. 2. Therefore, $R/\rho V =$
2 $R/W = 2853/271.1 = 10.5 \mu\text{g}/(\text{h}\cdot\text{g})$ for virgin females, and $R/\rho V = 2853/(271.1 + 27w)$
3 for mother rats was used in equation (3). For fetuses, R (bromine ion uptake rate from
4 mothers) was assumed to be proportional to body weight, and $R/\rho V = R/w = 22.0$
5 $\mu\text{g}/(\text{h}\cdot\text{g})$ was applied, which was obtained to give the best fit for the experimental data
6 on GD20 in Fig. 2. On PNDs, suckling (exposure to bromine ion from milk) was
7 assumed to occur at 2-h intervals. As shown in Fig. 3, the curve of bromine ion
8 concentration in the brains of the postnatal exposure group is convex. In addition, on
9 PND1, the concentration in pup stomachs with milk was high, and the level was higher
10 than that in the mother brain, as calculated using the one-compartment model (486.2
11 $\mu\text{g}/\text{g}$ -brain). Therefore, we assume that the uptake rate R of pups is high at first and then
12 decreases. In this work, R in the postnatal exposure group can be expressed by the
13 following equation:

$$14 \quad R = 388e^{-0.126(t-32)} \quad (8)$$

15
16
17 where 32 is the initial suckling (h) and 388 and 0.126 are the constants determined
18 experimentally. The bromine ion concentration in the exposure group was calculated as
19 the sum of the concentrations in the gestation exposure and postnatal exposure groups.
20 Conditions of the one-compartment model and the values of parameters obtained are
21 listed in Table 2. Solid, broken, and dotted lines in Fig. 2 indicate calculated lines for
22 fetuses, mothers, and virgin females, respectively. In Fig. 3, solid, broken, and dotted
23 lines indicate calculated lines of exposure, postnatal exposure, and gestation exposure
24 groups, respectively. The lines calculated using the proposed model could be estimated
25 from the experimental data with acceptable precision as shown in both figures.

1 The calculated bromine ion uptake rates per weight, $R/\rho V$, for adults and fetuses
2 were 10.5 and 22 $\mu\text{g}/(\text{h}\cdot\text{g})$, respectively. This result suggests that the bromine ion easily
3 transfers from mothers to fetuses, and the concentration in fetuses was higher than that
4 in mothers. R in postnatal exposure group was expressed as an exponential function, and
5 $R/\rho V$ of 55 $\mu\text{g}/(\text{h}\cdot\text{g})$ was obtained at initial suckling time. This value was large
6 compared to 22 $\mu\text{g}/(\text{h}\cdot\text{g})$, the calculated value at GD20, before birth. This suggests that
7 uptake rate of bromine ion via milk was higher than that via the placenta, and the
8 bromine ion concentration in the exposure group could be explained as the sum of that
9 in the gestation and postnatal exposure groups, which is shown in Fig. 3.

10 In summary, the results of this study suggest (1) the concentration of bromine ion in
11 mother rats was lower than that in virgin female rats, (2) bromine ion easily transferred
12 from mothers to fetuses and accumulated before birth, (3) bromine ion was concentrated
13 more in milk than in the brains of the mothers, and (4) bromine ion uptake rate in pups
14 was high immediately after birth.

15

16

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20 experiments.

21

1 **References**

- 2 1) Ichihara G. Neuro-reproductive toxicities of 1-bromopropane and 2-bromopropane.
3 Int Arch Occup Environ Health 2005; 78; 79-96.
- 4 2) The Japan Society for Occupational Health. Recommendation of occupational
5 exposure limits (2013-14). J Occup Health 2013; 55; 422-41.
- 6 3) Ohnishi A, Ishidao T, Kasai T, Arashidani K, Hori H. Neurotoxicity of
7 1-bromopropane in rats. J UOEH 1999; 21; 23-8.
- 8 4) Fueta Y, Ishidao T, Kasai T, Hori H, Arashidani K. Decreased paired-pulse inhibition
9 in the dentate gyrus of the brain in rats exposed to 1-bromopropane vapor. J Occup
10 Health 2000; 42; 149-51.
- 11 5) Fueta Y, Ishidao T, Arashidani K, Endo Y, Hori H. Hyperexcitability of the
12 hippocampal CA1 and the dentate gyrus in rats subchronically exposed to a
13 substitute for chlorofluorocarbons, 1-bromopropane vapor. J Occup Health 2002;
14 44; 156-65.
- 15 6) Fueta Y, Fukuda T, Ishidao T, Hori H. Electrophysiology and immunohistochemistry
16 in the hippocampal CA1 and the dentate gyrus of rats chronically exposed to
17 1-bromopropane, a substitute for specific chlorofluorocarbons. Neuroscience 2004;
18 124; 593-603.
- 19 7) Fueta Y, Ishidao T, Ueno S, Yoshida Y, Kunugita N, Hori H. New approach to risk
20 assessment of central neurotoxicity induced by 1-bromopropane using animal
21 models. Neurotoxicology 2007; 28; 270-3.
- 22 8) Ueno S, Yoshida Y, Fueta Y, Ishidao T, Liu JQ, Kunugita N, Yanagihara N, Hori H.
23 Changes in the function of the inhibitory neurotransmitter system in the rat brain
24 following subchronic inhalation exposure to 1-bromopropane. Neurotoxicology
25 2007; 28; 415-20.

- 1 9) Yoshida Y, Liu JQ, Nakano Y, Ueno S, Fueta Y, Ishidao T, Kunugita N, Yamashita U,
2 Hori H. 1-BP inhibits NF-kB activity and Bcl-xL expression in astrocytes in vitro
3 and reduces Bcl-xL expression in the brains of rats in vivo. *Neurotoxicology* 2007;
4 28; 381-6.
- 5 10) Yoshida Y, Nakano Y, Ueno S, Liu JQ, Fueta Y, Ishidao T, Kunugita N, Yanagihara
6 N, Sugiura T, Yamashita U, Hori H. Effects of 1-bromopropane, a substitute for
7 chlorofluorocarbons, on brain-derived neurotrophic factor (BDNF) expression. *Int*
8 *Immunopharmacol* 2009; 9; 433-8.
- 9 11) Ishidao T, Kunugita N, Fueta Y, Arashidani K, Hori H. Effects of inhaled 1-
10 bromopropane vapor on rat metabolism. *Toxicol Lett* 2002; 134; 237-43.
- 11 12) Ichihara G, Miller JK, Ziolkowska A, Itohara S, Takeuchi Y. Neurological disorders
12 in three workers exposed to 1-bromopropane. *J Occup Health* 2002; 44; 1-7.
- 13 13) Ichihara G, Li W, Ding X, Peng S, Yu X, Shibata E, Yamada T, Wang H, Itohara S,
14 Kanno S, Sakai K, Ito H, Kanefusa K, Takeuchi Y. A survey on exposure level,
15 health status, and biomarkers in workers exposed to 1-bromopropane. *Am J Ind Med*
16 2004; 45; 63-75.
- 17 14) Li W, Shibata E, Zhou Z, Ichihara S, Wang H, Wang Q, Li J, Zhang L, Wakai
18 K, Takeuchi Y, Ding X, Ichihara G. Dose-dependent neurologic abnormalities in
19 workers exposed to 1-bromopropane. *J Occup Environ Med* 2010; 52; 769-77.
- 20 15) Sekiguchi S, Suda M, Zhai YL, Honma T. Effects of 1-bromopropane,
21 2-bromopropane, and 1,2-dichloropropane on the estrous cycle and ovulation in
22 F344 rats. *Toxicol Lett* 2002; 126; 41-9.
- 23 16) Yamada T, Ichihara G, Wang H, Yu X, Maeda K, Tsukamura H, Kamijima M,
24 Nakajima T, Takeuchi Y. Exposure to 1-bromopropane causes ovarian dysfunction in
25 rats. *Toxicol Sci* 2003; 71; 96-103.

- 1 17) Furuhashi K, Kitoh J, Tsukamura H, Maeda K, Wang H, Li W, Ichihara S,
2 Nakajima T, Ichihara G. Effects of exposure of rat dams to 1-bromopropane during
3 pregnancy and lactation on growth and sexual maturation of their offspring.
4 *Toxicology* 2006; 224; 219-28.
- 5 18) Lau C, Thibodeaux JR, Hanson RG, Rogers JM, Grey BE, Stanton ME, Butenhoff
6 JL, Stevenson LA. Exposure to perfluorooctane sulfonate during pregnancy in rat
7 and mouse. II: postnatal evaluation. *Toxicological Sci* 2003; 74; 382-92.
- 8 19) Hori H, Hyakudo T, Oyabu T, Ishimatsu S, Yamato H, Tanaka I. Effects of inhaled
9 methyl bromide gas on the metabolic system and kinetics of bromine ion in rats. *J*
10 *UOEH* 2002; 24; 151-60.

Table 1. Experimental groups and ages of adult and fetal rats exposed to 700 ppm of 1-BP and of pups on sampling day

Groups (n)		Age (n) on sampling day
Virgin female	Exposure (5)	GD21 (2)
Mother	Exposure (11) Control (5)	GD20 (3)
Fetus	Exposure	GD20 (13)
Pup†	Exposure	PND1 (10), PND3 (10), PND5 (5), PND7 (5)
	Postnatal exposure	PND2 (5), PND4 (5), PND8 (5)
	Gestation exposure	PND4 (5), PND8 (5)
	Control	PND3 (5)

1-BP: 1-bromopropane, GD: gestation day, PND: postnatal day, †: Exposure = 1-BP exposed pups were raised by their birth mother exposed to 1-BP, Postnatal exposure = control pups were raised by 1-BP exposed mother, Gestation exposure = 1-BP exposed pups were raised by control mother, Control = control pups were raised by control mother

Table 2. Parameters of the one-compartment model

Groups	$T_{1/2}$ (days)	ρV (g)	R ($\mu\text{g/h}$)	Results	
GD	Virgin female	7.0	271.1	2853	Fig. 2
	Mother	7.0	$271.1+27w$	2853	Fig. 2
	Fetus	3.1	w	$22w$	Fig. 2
PND	Gestation exposure	3.1			Fig. 3
	Postnatal exposure	3.1	w	$388e^{-0.126(t-32)}$	Fig. 3
	Exposure	Gestation exposure + Postnatal exposure			Fig. 3
	Mother	7.0			Text†

†: the concentration in mother brain corresponding to PND1 ($486.2 \mu\text{g/g-brain}$),
 $w=0.00028T^{3.31}$ by equation (6)

Figure Captions

Fig. 1. The average body weight of mothers (W) exposed to 700 ppm of 1-BP up to GD20 and that of pups (w) after exposure. 1-BP: 1-bromopropane; GD: gestation day; PND: postnatal day

Fig. 2. Change in bromine ion concentration in rat brain exposed to 700 ppm of 1-BP on GDs. Symbols represent experimental data: ●, fetus; ▲, mother; △, virgin female. Solid, broken, and dotted lines indicate calculated lines for fetuses, mothers, and virgin females, respectively. 1-BP: 1-bromopropane; GD: gestation day

Fig. 3. Change in bromine ion concentration in pup brain during PNDs. Symbols represent experimental data: ●, exposure group (1-BP exposed pups were raised by their birth mother exposed to 1-BP); ◇, postnatal group (control pups were raised by 1-BP exposed mother); □, gestation exposure (1-BP exposed pups were raised by control mother). Solid, broken, and dotted lines indicate calculated lines for exposure, postnatal exposure, and gestation exposure groups, respectively. 1-BP: 1-bromopropane; PND: postnatal day

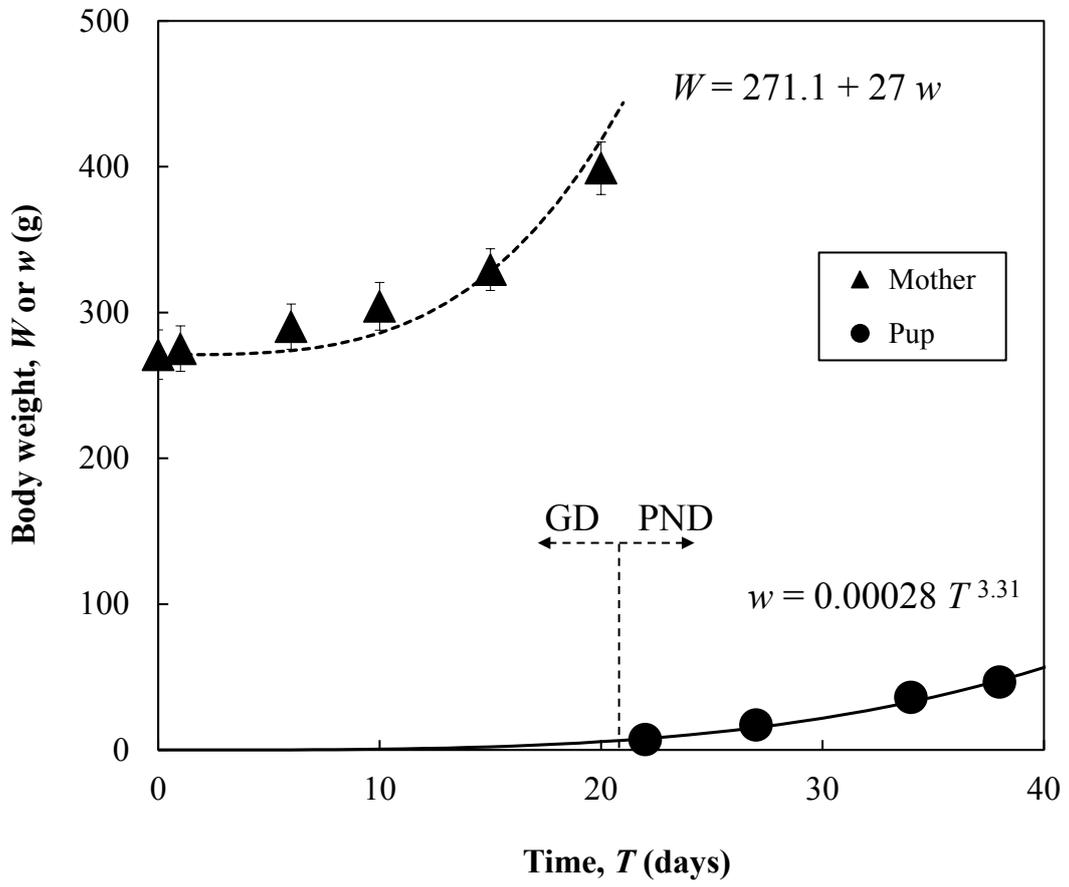


Fig. 1.

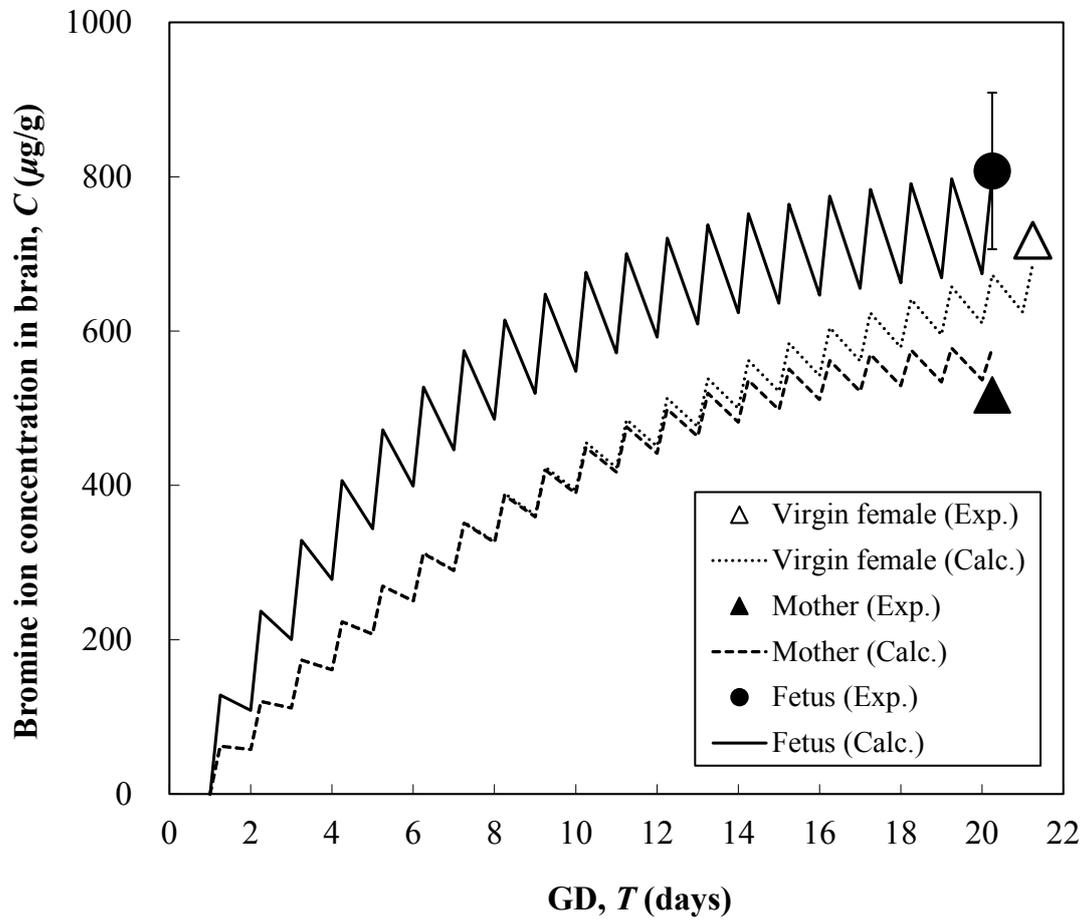


Fig. 2.

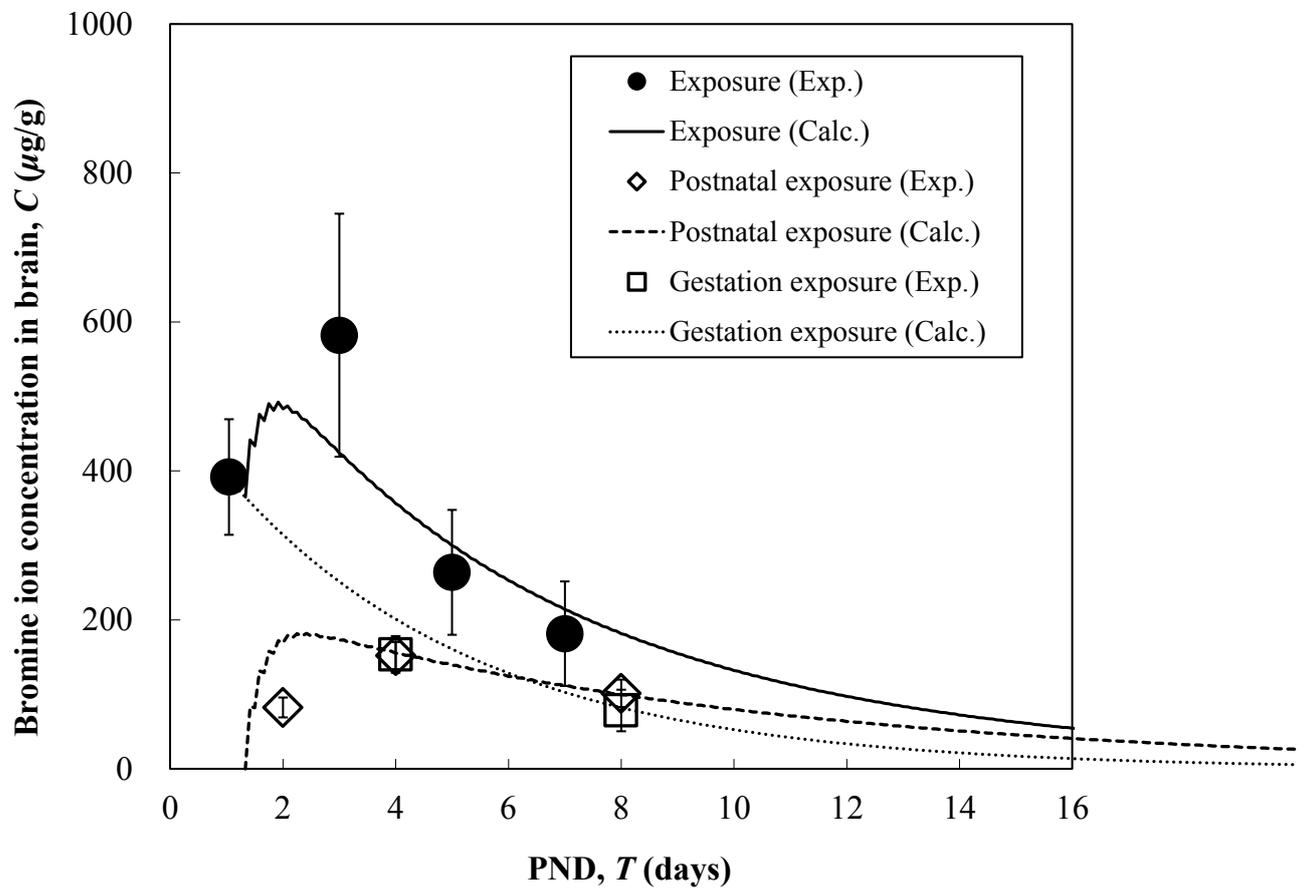


Fig. 3.

[Research Note]

Prenatal Exposure to 1-Bromopropane Suppresses Kainate-Induced Wet Dog Shakes in Immature Rats

Yukiko FUETA^{1*}, Masanari KANEMITSU¹, Sumie EGAWA¹, Toru ISHIDAO¹, Susumu UENO² and Hajime HORI¹

¹ Department of Environmental Management, School of Health Sciences, University of Occupational and Environmental Health, Japan. Yahatanishi-ku, Kitakyushu 807-8555, Japan

² Department of Occupational Toxicology, Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, Japan. Yahatanishi-ku, Kitakyushu 807-8555, Japan

Abstract : 1-Bromopropane (1-BP) is used in degreasing solvents and spray adhesives. The adverse effects of 1-BP have been reported in human cases and adult animal models, and its developmental toxicity has also been reported, but its effects on developmental neurotoxicity have not been investigated in detail. We evaluated the effects in rat pups of prenatal exposure to 1-BP on behaviors such as scratching and wet dog shakes (WDS), which were induced by injection of kainate (KA). Pregnant Wistar rats were exposed to vaporized 1-BP with 700 ppm from gestation day 1 to day 20 (6 h/day). KA at doses of 0.1, 0.5, and 2.0 mg/kg were intraperitoneally injected into a control group and a 1-BP-exposed group of pups on postnatal day 14. There was no significant difference in scratching between the control and the prenatally 1-BP-exposed groups, while suppression of the occurrence ratio of WDS was observed at the low dose of 0.1 mg/kg of KA in the prenatally 1-BP-exposed pups. Our results suggest that prenatal exposure to 1-BP affects neurobehavioral responses in the juvenile period.

Keywords : 1-bromopropane, prenatal exposure, developmental neurotoxicity, wet dog shake, rats.

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Introduction

The volatile organic compound 1-bromopropane ($\text{CH}_3\text{-CH}_2\text{-CH}_2\text{Br}$; 1-BP), a substitute for specific chlorofluorocarbons, is mainly used in degreasing solvents and spray adhesives. It has been reported that occupational exposure to 1-BP causes neurotoxicity, such as numbness, gait disturbance, prolongation of distal latency and memory dysfunction [1].

Animal models exposed to 1-BP have also shown central neurotoxicity, including ataxic gait, prolongation of distal latency, alteration of mRNA levels of neurotransmitter receptors [1], and hippocampal dis-

inhibition [2]. *In vitro* studies have revealed that the direct application of 1-BP enhanced the currents mediated by the activation of A type γ -aminobutyric acid (GABA_A) receptors, suppressed the currents mediated by neuronal nicotinic acetylcholine receptors, and potentiated feedback inhibition in the cornu ammonis 1 (CA1) subfield of hippocampal slices [3]. The gene expression of the B-cell lymphoma-extra large molecule (Bcl-xl), and the activity of nuclear factor-kappa B (NF- κ B), were suppressed in *in vitro* and *in vivo* studies [4]. The developmental effects of 1-BP have also been investigated [5], but little is known about the developmental neurotoxicity in offspring.

*Corresponding Author: Yukiko FUETA, Department of Environmental Management, School of Health Sciences, University of Occupational and Environmental Health, Japan. Yahatanishi-ku, Kitakyushu 807-8555, Japan, Tel: +81-93-603-1611, Fax: +81-93-691-2694, E-mail: yukiko@med.uoeh-u.ac.jp

In our previous study of the developmental neurotoxicity of 1-BP, prenatal exposure to 1-BP altered hippocampal excitability and the gene expression of the Na⁺ channel [6] and glutamate receptor subunits on postnatal day (PND) 14 [7]. These results raised the possibility that prenatal exposure to 1-BP affects brain development and its related behaviors. However, conventional behavioral tests for rodents are difficult to apply to pups. Thus, we focused on the particular behaviors of scratching and wet dog shakes (WDS), which can be observed in pups.

Scratching is defined as repetitive and quick flexion-extension movements of the hind limbs toward the neck or the head region. This behavior has been shown to be spontaneously induced in normal as well as pathological conditions and is used as an itch model in rodents [8], although the behavior in pups remains to be analyzed. WDS is characterized as brief and fierce shaking of the head, neck, and trunk, appearing when rodents are wet, as the name suggests [9]. Interestingly, it has been reported that both scratching and WDS can be induced by electrical stimulation of limbic structures [10], and by several pharmacological interventions, such as kainate (KA) [9, 11] and pentylenetetrazole. KA is the agonist of ionotropic glutamate receptors, which mediate excitatory neurotransmission and are predominantly distributed in the hippocampus, inner lamina of the neocortex, and ventral thalamus [12]. Thus, scratching and WDS induced by KA could be useful indices of changes in the excitatory neurotransmission of neuronal networks in pup brains. In this study, we examined the effect of prenatal exposure to 1-BP on behaviors in pups by evaluating the incidences of scratching and WDS induced by KA.

Materials and Methods

Animals and 1-BP inhalation

Thirty-two female and 16 male Wistar rats (designated the parental (P) generation) purchased from Kyudo Co. (Tosu, Japan) at 11 weeks of age were housed in plastic cages with paper-made chips (ALPHA-dri, Shepherd Specialty Papers, Milford, USA) on a 12 h light/dark cycle (light period: 07:00-19:00). The room temperature was kept at 23 ± 1°C. The relative humidity was about 70%. The animals had free

access to food and water. Proestrus stage was verified with an impedance checker (MK-10B, Muromachi Kikai Co., Ltd., Tokyo, Japan). When the impedance was over three kΩ, the F0 female rats were mated with male rats. In the morning of the following day, the existence of sperm in the vaginal smear or vaginal plug was verified as the gestation day (GD) 0. Fourteen dams from the colony were used in the experiment. The pregnant rats of the P generation were randomly divided into two groups (7 rats in each): one group as the control and the other for exposure to 1-BP.

1-BP was purchased from Kanto Chemical Co., Ltd. (Tokyo, Japan). Seven dams were exposed to 1-BP vapor at a concentration of 700 ppm (6 h/day) for 20 days from GDs 1 to 20 in an exposure chamber [13], whereas the other seven dams were provided fresh air in the same type of chamber. Both P generation groups were not allowed access to food and water during the inhalation period. Four weeks of 1-BP inhalation (700 ppm) resulted in apparent effects on the hippocampus in the adult rats [2]. Therefore, we first chose the concentration of 700 ppm to study the possible underlying mechanism of developmental neurotoxicity in prenatally 1-BP-exposed rats. The concentration of 1-BP was monitored with a gas chromatograph (GC353B FSL, GL Sciences Inc., Japan) equipped with a flame ionization detector.

All the pregnant rats gave birth to offspring (termed the first filial (F1) generation) on GD 21. The day of birth was defined as PND 0. We randomly gathered 26 F1 rats from the 7 control litters and 22 F1 rats from the 7 1-BP-exposed litters. All the F1 pups were bred with their mother rats during the lactation period. In this study, 24 female and 3 male F1 rats were obtained from the 7 dams in the control group, and 18 female and 4 male F1 rats were obtained from the 7 dams in the 1-BP-exposed group, respectively. We examined the F1 rats for the general toxicity of 1-BP inhalation exposure, such as litter size, sex ratio, testicular descent, vaginal opening, ear opening, and survival rate. The body weight of the F1 rats was measured on PND 14.

KA administration and behavioral observation

KA was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). KA (0.1, 0.5, and 2.0 mg/kg) was dissolved in phosphate buffered saline (PBS).

PBS or KA was intraperitoneally injected to the F1 rats at PND 14, after which the F1 rats were placed in a clear plastic cage, and the scratching and WDS were observed by video-recording for 180 min in a room for the behavioral observation. The room temperature was kept at about 25°C. The behavioral observation was conducted for 180 min between 09:30 and 15:30. The number of F1 rats that showed the scratching and the WDS behavior was counted and then the occurrence ratio was calculated. The duration and frequency of scratching and WDS were also measured. This experiment was approved by the Ethics Committee for Animal Care and Experimentation in accordance with the University of Occupational and Environmental Health, Japan.

All the chemicals used in this study were a reagent grade and purchased from commercial sources.

Statistical analysis

The difference in bodyweight between the F1 control and F1 1-BP-exposed groups was analyzed by Student *t*-test. The Mantel-Haenszel procedure was utilized to see the whole effect of the prenatal inhalation of 1-BP on the occurrence ratio of scratching and WDS. When appropriate, Fisher's exact test determined significant differences. A two-way analysis of variance (ANOVA) was performed to clarify the effects of prenatal exposure to 1-BP and/or a dose of KA on the frequency and the duration of scratching and WDS. When appropriate, *post hoc* analysis by Scheffe's test determined significant differences, respectively. The criteria of significant difference was $P < 0.05$ in all the statistical analyses. Data represent mean \pm standard error of the mean (SEM).

Results and Discussion

General toxicity of 1-BP inhalation exposure in F0 and F1 generations

There were no outward pathological signs related to 1-BP in the F0 rats. The body weights of the P generation dams treated with 1-BP were not significantly different from those in the control (fresh air) group (data not shown). None of the F1 rats died during the experimental period, indicating that the exposure seemed to cause little stress on the dams in this study. There

was no difference in the sex ratio, survival rate, or other clinical signs between the F1 control and F1 1-BP-exposed groups, with the exception of body weight. The body weight in the female F1 1-BP-exposed group (32.5 ± 0.5 g) was significantly lower ($P < 0.01$, Student *t*-test) than that in the female F1 control group (35.0 ± 0.4 g). The 1-BP-exposed male F1 rats also had a lower body weight (33.5 ± 0.3 g) compared to the male F1 control group (37.0 ± 0.8 g) ($P < 0.01$, Student *t*-test). Our results were consistent with previous studies showing that prenatal exposure to 1-BP has no effects on postnatal survival rate, excluding the body weight [5].

Effect of prenatal exposure to 1-BP on behavioral responses

KA administration elicits immobilization, followed by scratching, WDS, forelimb clonus, and status epilepticus (continuous chronic-tonic posturing of all 4 limbs) [11]. It is also known that a low dose less than 3 mg/kg of KA elicits scratching and WDS but hardly ever elicits epileptic convulsions. Our preliminary study also showed that doses of KA higher than 4 mg/kg induced convulsive behaviors as well as scratching and WDS, thus we chose doses of 0.1, 0.5, and 2.0 mg/kg of KA.

Behavioral data obtained from both genders is combined in Tables 1 and 2, because it has been reported that there are no sex differences in KA induced-behaviors in pups [14].

In the F1 control group, all of the tested pups showed scratching during the 180 min after injection of PBS or KA. The frequency and duration of the scratching was significantly higher only at the dose of 2.0 mg/kg (Table 1). WDS were observed in 80% of the PBS-injected control pups and in all of the KA-injected control pups. A significantly higher frequency of WDS was observed at the dose of 2.0 mg/kg (Table 2). The behavioral changes induced by the KA doses of 0.1 and 0.5 mg/kg were similar to those of PBS, thus it can be said that these two doses are subclinical.

Spontaneous scratching and WDS were also observed in the F1 1-BP-exposed group. The occurrence ratio of scratching was 100% at all doses of KA (Table 1), whereas that of WDS was 40 to 60% in 0 to 0.5 mg/kg and 100% in 2.0 mg/kg of KA (Tables 2). The effect of prenatal exposure to 1-BP was observed

in the occurrence ratio of WDS ($P < 0.01$; Mantel-Haenszel test). The occurrence ratio in the F1 1-BP-exposed group at 0.1 mg/kg KA was lower than that in the F1 control group ($P < 0.05$ by Fisher's exact test). The dose of 0.5 mg/kg KA tended to decrease the occurrence ratio in the F1 1-BP-exposed group, but did not reach a significant level. Taken together with the results of the 0.1 and 0.5 mg/kg KA (subclinical doses), the occurrence ratio (6 out of 12 rat pups) in the F1 1-BP-exposed group exhibited a lower value than that in the F1 control group (16 out of 16 rat pups, $P < 0.005$ by Fisher's exact test). This indicates that the effects of prenatal 1-BP exposure can be observed only at the subclinical doses of KA. The duration and the frequency of the scratching and the WDS increased at the dose of 2.0 mg/kg ($P < 0.01$), but we did not find any significant effect of prenatal 1-BP exposure on the duration and frequency of WDS at any of the doses

of KA. Our results suggest that prenatal exposure to 1-BP suppresses the occurrence of WDS only at a low dose of KA, possibly due to an effect on mechanisms underlying the generation of WDS.

WDS can be induced by electrical stimulation of limbic structures and by the administration of several chemicals, such as serotonergic compounds [15] and an opioid receptor agonist [16], as well as KA. KA-induced-WDS is depressed by μ -opioid receptor antagonists [16]. An antagonist of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/KA receptors suppresses WDS induced by serotonin receptor agonists [15]. The mechanisms of WDS induction by these chemicals are assumed to be related to each other. Besides those receptors, nitric oxide has also been demonstrated to play a regulatory role in KA- and getting-wet-induced-WDS [9]. These receptors and nitric oxide might be the target of prenatal exposure to 1-BP.

Table 1. The occurrence ratio, duration and frequency of scratching in F1 control and 1-BP-exposed groups

KA (mg/kg)	F1 control				F1 1-BP-exposed		
	S/N	duration (s)	frequency (counts)	S/N	duration (s)	frequency (counts)	
PBS	5/5	1.9 \pm 0.3	21 \pm 6	5/5	2.3 \pm 0.3	17 \pm 5	
0.1	11/11	2.0 \pm 0.1	14 \pm 2	7/7	2.0 \pm 0.1	26 \pm 10	
0.5	5/5	1.7 \pm 0.3	25 \pm 4	5/5	2.1 \pm 0.5	20 \pm 8	
2.0	5/5	2.9* \pm 0.4	557* \pm 164	5/5	3.6* \pm 0.2	517* \pm 41	

F1: first filial generation, 1-BP: 1-bromopropane, KA: kainate, PBS: phosphate buffered saline, S: the number of rats in which scratching was observed, N: the total number of rats used in the experiment, S/N: the occurrence ratio, *: significant effects of KA on the duration or the frequency by two-way ANOVA followed by Scheffé's test ($P < 0.01$), mean \pm SEM: mean \pm standard error of the mean

Table 2. The occurrence ratio, duration and frequency of WDS in F1 control and 1-BP-exposed groups

KA (mg/kg)	F1 control				F1 1-BP-exposed		
	S/N	duration (s)	frequency (counts)	S/N	duration (s)	frequency (counts)	
PBS	4/5	0.3 \pm 0.02	1.8 \pm 0.6	3/5	0.2 \pm 0.03	0.8 \pm 0.4	
0.1	11/11	0.3 \pm 0.03	3.4 \pm 0.6	4/7 ^a	0.3 \pm 0.09	1.0 \pm 0.4	
0.5	5/5	0.3 \pm 0.02	2.6 \pm 0.9	2/5	0.2, 0.3	0.8 \pm 0.6	
2.0	5/5	0.3 \pm 0.05	29.2* \pm 10.0	5/5	0.4 \pm 0.02	54.4* \pm 21.1	

WDS: wet dog shakes, F1: first filial generation, 1-BP: 1-bromopropane, KA: kainate, PBS: phosphate buffered saline, S: the number of rats in which WDS were observed, N: the total number of rats used in the experiment, S/N: the occurrence ratio, a: a significant difference between F1 control and F1 1-BP-exposed groups at the dose of 0.1 mg/kg in the Fisher's exact test ($P < 0.05$), *: significant effects of KA ($P < 0.01$) on the two-way ANOVA followed by Scheffé's test, mean \pm SEM: mean \pm standard error of the mean. The data of durations in the F1 1-BP-exposed group administered 0.5 mg/kg of KA are shown in the duration(s) column

There are studies suggesting that the hippocampus is the target of KA. KA receptors have been found in the hippocampus in rat pups [12], and epileptic discharges have been observed when KA-induced seizures occur [17]. Moreover, KA-induced WDS was accompanied by robust electrographic seizures recorded from the hippocampus [18]. On the other hand, Fueta *et al.* have reported that prenatal 1-BP exposure decreases the paired-pulse ratio of population spikes in the CA1 subfield of the dorsal hippocampus in PND14 rats [19]. A decrease in the paired-pulse ratio of the population spike is generally interpreted as an increase in an inhibition [2]. Thus, prenatal 1-BP exposure may disturb the propagation of hyperactivity in the hippocampus, such as electrographic discharges associated with KA-induced WDS. This may account for the suppression of WDS by prenatal exposure to 1-BP. However, it should also be considered that the dentate gyrus (DG) in the ventral hippocampus is thought to be necessary for chemical interventions such as KA-, μ -opioid-, and electrical stimulation-induced WDSs in adult rats [16, 20, 21]. Therefore, further studies are needed to investigate the excitability of the DG in the ventral hippocampus in prenatally 1-BP-exposed rats.

In conclusion, we demonstrate here that prenatal exposure to 1-BP suppresses WDS induced by the administration of a low dose of KA. Our results indicate that prenatal 1-BP exposure may disturb the susceptibility to KA or the functions of neural networks related to the WDS. We also show that it may be advantageous to use pharmacological interventions with convulsants in investigations of the effects of environmental chemicals on behavioral responses in immature rats.

Conflict of Interest

No conflicts of interest to declare.

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References

1. Ichihara G, Kitoh J, Li W, Ding X, Ichihara S & Takeuchi Y (2012): Neurotoxicity of 1-bromopropane: Evidence from animal experiments and human studies. *J Adv Res* 3: 91–98
2. Fueta Y, Ishidao T, Ueno S, Yoshida Y, Kunugita N & Hori H (2007): New approach to risk assessment of central neurotoxicity induced by 1-bromopropane using animal models. *Neurotoxicology* 28: 270–273
3. Ueno S, Fueta Y, Ishidao T, Yoshida Y, Tsutsui M, Toyohira Y, Hori H & Yanagihara N (2006): The central neurotoxicity of 1-bromopropane (1-BP), a substitute for chlorofluorocarbons: Studies on the effects caused by direct and subchronic exposure to 1-BP on hippocampal function. *J Pharmacol Sci* 100 (Suppl 1): 140
4. Yoshida Y, Liu JQ, Nakano Y, Ueno S, Ohmori S, Fueta Y, Ishidao T, Kunugita N, Yamashita U & Hori H (2007): 1-BP inhibits NF- κ B activity and Bcl-xL expression in astrocytes in vitro and reduces Bcl-xL expression in the brains of rats *in vivo*. *Neurotoxicology* 28: 381–386
5. Huntingdon Life Sciences (2001): A developmental toxicity study in rat via whole body inhalation exposure. Study Number 98-4141. Document ID Title OAR-2002-0064: Document available in public dockets A-2001-07, OAR-2002-0064, and A-91-42. US EPA, Washington, DC
6. Ueno S, Fueta Y, Ishidao T, Yuhi T, Yoshida Y, Hori H & Yanagihara N (2008): Changes in the excitability and Na⁺ channel gene expression in the hippocampus of postnatal 14 days-aged rats prenatally exposed to 1-bromopropane. *Neurosci Res* 61(1 Suppl): S269
7. Fueta Y, Ueno S, Ishidao T, Yoshida Y & Hori H (2009): Effects of prenatally exposed to 1-bromopropane on the brain of the young offspring. *Neurosci Res* 65 (Suppl 1): S250
8. Nojima H & Carstens E (2003): Quantitative assessment of directed hind limb scratching behavior as a rodent itch model. *J Neurosci Methods* 126: 137–143
9. Koylu EO, Uz T, Manev H & Pogun S (2002): Nitric oxide synthase inhibition suppresses wet dog shakes and augments convulsions in rats. *Int J Neurosci* 112: 291–300

10. Frush DP & McNamara JO (1986): Evidence implicating dentate granule cells in wet dog shakes produced by kindling stimulations of entorhinal cortex. *Exp Neurol* 92: 102–113
 11. Albala BJ, Moshé SL & Okada R (1984): Kainic-acid-induced seizures: a developmental study. *Brain Res* 315: 139–148
 12. Miller LP, Johnson AE, Gelhard RE & Insel TR (1990): The ontogeny of excitatory amino acid receptors in the rat forebrain-II. Kainic acid receptors. *Neuroscience* 35: 45–51
 13. Ishidao T, Kunugita N, Fueta Y, Arashidani K & Hori H (2002): Effects of inhaled 1-bromopropane vapor on rat metabolism. *Toxicol Lett* 134 (1–3): 237–243
 14. Doucette TA, Strain SM, Allen GV, Ryan CL & Tasker RA (2000): Comparative behavioral toxicity of domoic acid and kainic acid in neonatal rats. *Neurotoxicol Teratol* 22: 863–869
 15. Gorzalka BB, Hill MN & Sun JC (2005): Functional role of the endocannabinoid system and AMPA/kainate receptors in 5-HT_{2A} receptor-mediated wet dog shakes. *Eur J Pharmacol* 516: 28–33
 16. Hong JS, Grimes L, Kanamatsu T & McGinty JF (1987): Kainic acid as a tool to study the regulation and function of opioid peptides in the hippocampus. *Toxicology* 46: 141–157
 17. Ben-Ari Y, Tremblay E, Berger M & Nitecka L (1984): Kainic acid seizure syndrome and binding sites in developing rats. *Brain Res* 316: 284–288
 18. Cherubini E, De Feo MR, Mecarelli O & Ricci GF (1983): Behavioral and electrographic patterns induced by systemic administration of kainic acid in developing rats. *Brain Res* 285: 69–77
 19. Fueta Y, Ueno S, Ishidao T & Hori H (2010): Long-lasting effects on hippocampal excitability of the offspring prenatally exposed to 1-bromopropane, a substitute for specific chlorofluorocarbons. *Neurosci Res* 68 (Suppl 1): e417
 20. Grimes LM, Earnhardt TS, Mitchell CL, Tilson HA & Hong JS (1990): Granule cells in the ventral, but not dorsal, dentate gyrus are essential for kainic acid-induced wet dog shakes. *Brain Res* 514: 167–170
 21. Barnes MI & Mitchell CL (1990): Differential effects of colchicine lesions of dentate granule cells on wet dog shakes and seizures elicited by direct hippocampal stimulation. *Physiol Behav* 48: 131–138
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1-プロモプロパンへの胎生期曝露は発達期ラットにおいてカイニン酸で誘導されるWet Dog Shakesを抑制する

笛田 由紀子¹, 金光 雅成¹, 江川 純恵¹, 石田尾 徹¹, 上野 晋², 保利 一¹

¹産業医科大学 産業保健学部 作業環境計測制御学

²産業医科大学 産業生態科学研究所 職業性中毒学

要 旨：1-プロモプロパン(1-BP)は洗浄やスプレー接着剤の溶剤として用いられている。1-BPの有害性はヒトの事例や成獣を用いた動物で報告されてきた。発達毒性も報告されているが、発達神経毒性についての詳細はわかっていない。我々は、1-BPの胎生期曝露が、発達期ラットへのカイニン酸投与により誘導される行動、すなわちscratching行動やwet dog shake様行動に及ぼす影響を調べた。ウイスター系妊娠ラットの妊娠1日目から20日目まで(6時間/日)、濃度700 ppmの1-BP蒸気を曝露した。生後14日目の対照群と1-BP曝露群にカイニン酸を0.1, 0.5, 2.0 mg/kgで腹腔内投与した。Scratching行動に関しては対照群と1-BP曝露群に違いは見られなかったが、wet dog shake様行動に関しては、低濃度である0.1 mg/kgにおいて発生率の低下が1-BP曝露群で見られた。1-BP胎生期曝露が発達期の神経行動に影響することが示唆された。

キーワード：1-プロモプロパン, 胎生期曝露, 発達神経毒性, wet dog shake, ラット。

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Ikariside A inhibits acetylcholine-induced catecholamine secretion and synthesis by suppressing nicotinic acetylcholine receptor-ion channels in cultured bovine adrenal medullary cells

Xiaojia Li¹ · Yumiko Toyohira¹ · Takafumi Horisita² · Noriaki Satoh³ · Keita Takahashi¹ · Han Zhang⁴ · Munekazu Inuma⁵ · Yukari Yoshinaga¹ · Susumu Ueno⁶ · Masato Tsutsui⁷ · Takeyoshi Sata² · Nobuyuki Yanagihara¹

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Abstract Ikariside A is a natural flavonol glycoside derived from plants of the genus *Epimedium*, which have been used in Traditional Chinese Medicine as tonics, antirheumatics, and aphrodisiacs. Here, we report the effects of ikariside A and three other flavonol glycosides on catecholamine secretion and synthesis in cultured bovine adrenal medullary cells. We found that ikariside A (1–100 μM), but not icariin, epimedin C, or epimedeside A, concentration-dependently inhibited the secretion of catecholamines induced by acetylcholine, a physiological secretagogue and agonist of nicotinic acetylcholine receptors. Ikariside A had little effect on catecholamine secretion induced by veratridine and 56 mM K⁺. Ikariside A (1–100 μM) also inhibited

influx and ⁴⁵Ca²⁺ influx induced by acetylcholine in a concentration-dependent manner similar to that of catecholamine secretion. In *Xenopus* oocytes expressing α3β4 nicotinic acetylcholine receptors, ikariside A (0.1–100 μM) directly inhibited the current evoked by acetylcholine. It also suppressed ¹⁴C-catecholamine synthesis and tyrosine hydroxylase activity induced by acetylcholine at 1–100 μM and 10–100 μM, respectively. The present findings suggest that ikariside A inhibits acetylcholine-induced catecholamine secretion and synthesis by suppression of nicotinic acetylcholine receptor-ion channels in bovine adrenal medullary cells.

Keywords Adrenal medulla · Catecholamine secretion · *Epimedium* · Flavonoids · Ikariside A · Nicotinic acetylcholine receptor

✉ Nobuyuki Yanagihara
yanagin@med.uoeh-u.ac.jp

¹ Department of Pharmacology, University of Occupational and Environmental Health, School of Medicine, 1-1, Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan

² Department of Anesthesiology, University of Occupational and Environmental Health, School of Medicine, Yahatanishi-ku, Kitakyushu, Japan

³ Shared-Use Research Center, University of Occupational and Environmental Health, Yahatanishi-ku, Kitakyushu, Japan

⁴ Research Center of Traditional Chinese Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin, China

⁵ Department of Pharmacognosy, Gifu Pharmaceutical University, Daigakunishi, Gifu, Japan

⁶ Department of Occupational Toxicology, Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, Yahatanishi-ku, Kitakyushu, Japan

⁷ Department of Pharmacology, Faculty of Medicine, University of The Ryukyus, Nishihara, Okinawa, Japan

Introduction

Flavonoids, a group of secondary metabolites with variable phenolic structure, which exist widely in plants (Nijveldt et al. 2001; Ren and Zuo 2012), may exert potential benefits associated with reduced risks of age- and life style-related diseases such as cardiovascular diseases, diabetes, and some cancers (Lu et al. 2013; Yanagihara et al. 2014). Ikariside A is one of the flavonol derivatives derived from plants of the genus *Epimedium*, which have been used in Traditional Chinese Medicine as tonics, antirheumatics, and aphrodisiacs (Dou et al. 2006). Previous studies reported that the total flavonoid fraction of *Epimedium* extract suppresses urinary calcium excretion and improves bone properties in ovariectomized mice (Chen et al. 2011), and that other extracts with structures similar to that of ikariside A, such as icariin, can stimulate osteogenic activities (Zhou et al. 2013) and have

anti-inflammatory effects (Lai et al. 2013). Furthermore, ikarisoside A also has pharmacological effects such as antioxidant and anti-inflammatory effects (Choi et al. 2008) as well as anti-osteoporosis effects (Choi et al. 2010).

In the human body, the most abundant catecholamines are adrenaline, noradrenaline, and dopamine, all of which are produced from phenylalanine and/or tyrosine. Catecholamines are produced mainly in the chromaffin cells of the adrenal medulla, the postganglionic fibers of the sympathetic nervous system, and the central nervous system. Catecholamines play very important roles in heart rate, blood pressure, blood glucose levels, and the general reactions of the sympathetic nervous system.

Adrenal medullary cells derived from embryonic neural crests are functionally homologous to sympathetic postganglionic neurons. In bovine adrenal medullary cells, catecholamine secretion is associated with the activation of three types of ionic channels: nicotinic acetylcholine receptor (nAChR)-ion channels, voltage-dependent Na^+ channels, and voltage-dependent Ca^{2+} channels (Wada et al. 1985b). ACh induces Na^+ influx via nAChR-ion channels, then, it induces Ca^{2+} influx and subsequent catecholamine secretion (Wada et al. 1985b). On the other hand, stimulation of catecholamine synthesis induced by ACh is associated with the activation of tyrosine hydroxylase in cultured bovine adrenal medullary cells (Yanagihara et al. 1987; Tsutsui et al. 1994). The conversion of tyrosine to L-3,4-dihydroxyphenylalanine (DOPA) is the rate-limiting step of catecholamine biosynthesis (Nagatsu et al. 1964). Adrenal medullary cells have provided a good model for the detailed analysis of a drug's actions on catecholamine secretion and synthesis (Kajiwara et al. 2002; Toyohira et al. 2005; Shinohara et al. 2007).

In our previous study, we isolated 20 flavonol glycosides from *Epimedium* species, including ikarisoside A, icariin, epimedeside A, and epimedin C (Mizuno et al. 1988). Ikariside A showed neurite outgrowth activity in cultured PC12h cells (Kuroda et al. 2000). There is, however, little evidence regarding ikariside A's effects on sympathetic nervous system activity. In the present study, we investigated the effects of four flavonol glycosides on bovine adrenal medullary cell functions and found that ikariside A, but not the other three flavonol glycosides, inhibited ACh-induced catecholamine secretion and synthesis by suppression of nAChR-ion channels in the cells.

Materials and methods

Materials

Oxygenated Krebs-Ringer phosphate (KRP) buffer was used throughout unless stated otherwise. Its composition is as follows (in mM): 154 NaCl, 5.6 KCl, 1.1 MgSO_4 , 2.2 CaCl_2 ,

0.85 NaH_2PO_4 , 2.15 Na_2HPO_4 , and 10 glucose, adjusted to pH 7.4. Drugs and reagents were obtained from the following sources: Eagle's minimum essential medium (Eagle's MEM) (Nissui Pharmaceutical, Tokyo, Japan); collagenase (Nitta Zerachin, Osaka, Japan); calf serum (Cell Culture Technologies, Gravesano, Switzerland). ACh and veratridine were from Sigma (St. Louis, MO, USA). L-[U- ^{14}C]tyrosine was from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA); $^{45}\text{CaCl}_2$, $^{22}\text{NaCl}$, and L-[1- ^{14}C]tyrosine from Perkin-Elmer Life Sciences (Boston, MA, USA).

Isolation of flavonol glycosides from the leaves of *Epimedium* species

The leaves of *Epimedium diphyllum* were collected at Miyazaki Prefecture, Japan. Ikariside A and other flavonol glycosides were purified by high performance liquid chromatography, as reported previously (Mizuno et al. 1988). Ikariside A and other flavonol glycosides were dissolved in 100 % dimethyl sulfoxide (DMSO) and then diluted in a reaction medium before use at a final DMSO concentration not exceeding 0.5 %, unless otherwise specified. DMSO (0.5 %) did not influence the basal and ACh-induced catecholamine secretion in the present study (data not shown).

Primary culture of bovine adrenal medullary cells

Bovine adrenal medullary cells were isolated by collagenase digestion of adrenal medullary slices according to the method as reported previously (Yanagihara et al. 1979, 1996). Cells were suspended in Eagle's MEM containing 10 % calf serum, 3 μM cytosine arabinoside, and several antibiotics, and maintained in monolayer culture at a density of 4×10^6 cells/dish (35 mm dish; Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) or 10^6 cells/well (24-well plate; Corning Life Sciences, Lowell, MA, USA) at 37 °C under a humidified atmosphere of 5 % CO_2 and 95 % air. The cells were used for experiments between 2 and 5 days of culture.

Catecholamine secretion from cultured bovine adrenal medullary cells

The secretion of catecholamines was measured as described previously (Yanagihara et al. 1979). Cells (10^6 /well) were washed three times with oxygenated KRP buffer, then firstly preincubated with or without ikariside A (0.3–100 μM) or other flavonol glycosides (10 μM) at 37 °C for 10 min, and incubated with or without ikariside A (0.3–100 μM) or other flavonol glycosides (10 μM) in the presence or absence of various secretagogues (300 μM ACh, 100 μM veratridine or 56 mM K^+) at 37 °C for another 10 min. After the reaction, the incubation medium was transferred immediately to a test tube containing perchloric acid (final concentration, 0.4 M) for the

full stop of the reaction. Catecholamines (noradrenaline and adrenaline) secreted into the medium were adsorbed onto aluminum hydroxide and estimated by the ethylenediamine condensation method (Weil-Malherbe and Bone 1952) using a fluorescence spectrophotometer (F-2500; Hitachi, Tokyo, Japan) with excitation and emission wavelengths of 420 and 540 nm, respectively.

$^{22}\text{Na}^+$ and $^{45}\text{Ca}^{2+}$ influx

The influx of $^{22}\text{Na}^+$ and $^{45}\text{Ca}^{2+}$ was measured as reported previously (Wada et al. 1985a, b). After preincubation with or without ikarisoside A (0.3–100 μM) at 37 °C for 10 min, cells (4×10^6 /dish) were incubated with 1.5 μCi of $^{22}\text{NaCl}$ or 1.5 μCi of $^{45}\text{CaCl}_2$ at 37 °C for 5 min with or without 300 μM ACh and ikarisoside A (0.3–100 μM) in KRP buffer. After incubation, the cells were washed three times with ice-cold KRP buffer, solubilized in 10 % Triton X-100, and counted for radioactivity of $^{22}\text{Na}^+$ and $^{45}\text{Ca}^{2+}$ by a gamma counter (ARC-2005, Aloka, Tokyo, Japan) and a liquid scintillation counter (TRI-CARB 2900TR, PACKARD INSTRUMENT CO., Meriden, CT, USA), respectively.

^{14}C -Catecholamine synthesis from [^{14}C]tyrosine in the cells

After preincubation for 10 min, cells (4×10^6 /dish) were incubated with 20 μM L-[U- ^{14}C]tyrosine (1.0 μCi) KRP buffer in the presence or absence of various concentrations of ikarisoside A (0.3–100 μM) and 300 μM ACh at 37 °C for 20 min. After removing the incubation medium by aspiration, cells were harvested in 0.4 M perchloric acid and centrifuged at $1600 \times g$ for 10 min. ^{14}C -Catecholamines were separated further by ion exchange chromatography on Duolite C-25 columns (H^+ -type, 0.4×7.0 cm) (Yanagihara et al. 1987) and counted for the radioactivity by a liquid scintillation counter (TRI-CARB 2900TR, PACKARD INSTRUMENT CO., Meriden, CT, USA). ^{14}C -Catecholamine synthesis was expressed as the sum of the ^{14}C -catecholamines (adrenaline, noradrenaline, and dopamine).

Tyrosine hydroxylase activity in situ

After preincubation with or without ikarisoside A (0.3–100 μM) for 10 min, cells (10^6 /well) were exposed to 200 μl of KRP buffer with or without ikarisoside A (0.3–100 μM) and 300 μM ACh, supplemented with 18 μM L-[1- ^{14}C]tyrosine (0.2 μCi) for 10 min at 37 °C. Upon addition of the labeled tyrosine, each well was sealed immediately with an acrylic tube capped with a rubber stopper and fitted with a small plastic cup containing 200 μl of NCS-II tissue solubilizer (GE Healthcare UK Ltd, Little Chalfont, Buckinghamshire, UK) to absorb the $^{14}\text{CO}_2$ released by the

cells and counted for the radioactivity (Bobrovskaya et al. 1998).

Expression of $\alpha 3\beta 4$ nAChRs in *Xenopus* oocytes and electrophysiological recordings

The complementary DNAs (cDNAs) encoding the $\alpha 3$ and $\beta 4$ subunits of rat neuronal nAChR, subcloned into pcDNA1/Neo (Invitrogen, Carlsbad, CA) vector, were kindly provided from Dr. James W. Patrick (Division of Neuroscience, Baylor College of Medicine, TX, USA). After linearization of cDNA with *NotI*, complementary RNAs (cRNAs) were transcribed using T7 RNA polymerase from the mMACHINE kit (Ambion, Austin, TX, USA). Adult female *Xenopus laevis* frogs were obtained from Kyudo Co., Ltd. (Saga, Japan). *Xenopus* oocytes and cRNA microinjection were prepared as described previously (Ueno et al. 2004; Horishita and Harris 2008). cRNAs of $\alpha 3$ and $\beta 4$ subunits were co-injected at a same ratio (10–20 ng/50 nL) into *Xenopus* oocytes, and electrophysiological recordings were performed 2–6 days after injection. Oocytes were placed in a 100 μl recording chamber and perfused at 2 ml/min with extracellular Ringer solution (110 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 1.8 mM BaCl_2 , pH 7.5) containing 1.0 μM atropine sulfate. Ca^{2+} in the solution was replaced with Ba^{2+} to minimize the effects of secondarily activated Ca^{2+} -dependent Cl^- channels. Recording electrodes (1–3 M Ω) were filled with 3 M KCl, and the whole-cell voltage clamp was achieved through these two electrodes using a Warner Instruments model OC-725C (Warner, Hamden, CT, USA) at -70 mV. We measured the peak of the transient inward current in response to ACh that was applied for 30 s and examined the effects of ikarisoside A on a concentration of ACh that produced 50 % of the maximal effect (EC_{50}) of ACh. The EC_{50} was determined for each oocyte by 1 mM ACh that produces a maximal current. Ikarisoside A stocks were prepared in 100 % DMSO and diluted in bath solution to a final DMSO concentration not exceeding 0.1 %. Ikarisoside A was preapplied for 2 min to allow an equilibration with its site of interaction before ACh was added and its effect on the cation currents was determined. In all cases, between two currents, there was 10 min interval under washing with normal Ringer solution.

Statistical analysis

All experiments were performed in duplicate or triplicate, and each experiment was repeated at least three times. All values are given as means \pm SEM. The significance of differences between means was evaluated using one-way analysis of variance (ANOVA). When a significant *F* value was found by ANOVA, Dunnett's or Scheffe's test for multiple comparisons was used to identify differences among the groups. Values were considered statistically different when *P* was less than

0.05. Statistical analyses were performed using PRISM for Windows version 5.0J software (Abacus Concept, Berkeley, CA, USA).

Results

The structures of four flavonol glycosides isolated from *Epimedium*

The four flavonol glycosides ikarisoside A, icariin, epimedin C, and epimedoside A were isolated from the leaves of *E. diphyllum* as reported previously (Mizuno et al. 1988). The structures of these four flavonol glycosides are shown in Fig. 1.

Effects of the flavonol glycosides on catecholamine secretion induced by various secretagogues in adrenal medullary cells

None of the four flavonol glycosides (ikarisoside A, icariin, epimedin C, and epimedoside A) at 10 μM significantly affected the basal secretion of catecholamines (Fig. 2a). ACh (300 μM), an agonist of nAChRs, caused catecholamine secretion corresponding to $18.90 \pm 0.38\%$ of the total catecholamines in the cells. When the cells were treated with the same four flavonol glycosides at 10 μM for 10 min, ikarisoside A strongly reduced catecholamine secretion induced by ACh, to $6.83 \pm 0.51\%$ of the total, whereas the other three had little effect (Fig. 2a). Veratridine (100 μM), an activator of voltage-dependent Na^+ channels, and 56 mM K^+ , which depolarizes cell membranes and then activates voltage-dependent Ca^{2+}

channels, also caused catecholamine secretion corresponding to $26.52 \pm 0.88\%$ (Fig. 2b) and $20.51 \pm 0.70\%$ (Fig. 2c) of the total catecholamines, respectively. Treatment of cells with these flavonol glycosides at 10 μM did not affect catecholamine secretion induced by veratridine (Fig. 2b) and 56 mM K^+ (Fig. 2c).

Concentration-inhibition curves for the effects of ikarisoside A on catecholamine secretion, $^{45}\text{Ca}^{2+}$ influx, and $^{22}\text{Na}^+$ influx induced by ACh

We examined the effects of ikarisoside A on catecholamine secretion, $^{45}\text{Ca}^{2+}$ influx, and $^{22}\text{Na}^+$ influx induced by ACh. Treatment of cells with ikarisoside A at 1, 3, 10, 30, and 100 μM significantly inhibited ACh-induced secretion of catecholamines (18.22 \pm 0.16 % of the total catecholamines in the cells) to 15.36 \pm 0.38 %, 12.27 \pm 0.40 %, 7.68 \pm 0.30 %, 5.33 \pm 0.47 %, and 4.95 \pm 0.25 % of the total catecholamines in the cells, respectively (Fig. 3a). Ikariside A also inhibited ACh-induced $^{45}\text{Ca}^{2+}$ influx and $^{22}\text{Na}^+$ influx in a concentration-dependent manner (Fig. 3b, c). The half-maximal inhibitory concentration (IC_{50}) of ikarisoside A in catecholamine secretion, $^{45}\text{Ca}^{2+}$ influx, and $^{22}\text{Na}^+$ influx are 4.00, 9.90, and 2.96 μM , respectively.

Inhibitory mode of ikarisoside A on ACh-induced catecholamine secretion in adrenal medullary cells

To investigate the mechanism by which ikarisoside A inhibits ACh-induced catecholamine secretion, we examined whether or not the inhibitory effect of ikarisoside A on catecholamine secretion is overcome when the ACh concentration is

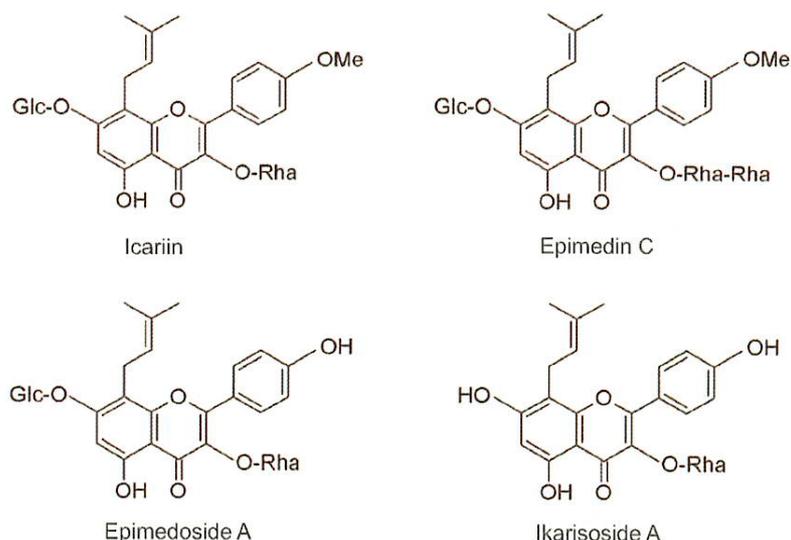


Fig. 1 Chemical structures of icariin, epimedin C, epimedoside A, ikarisoside A. The abbreviations Glc and Rha in the structures are glucose and rhamnose, respectively

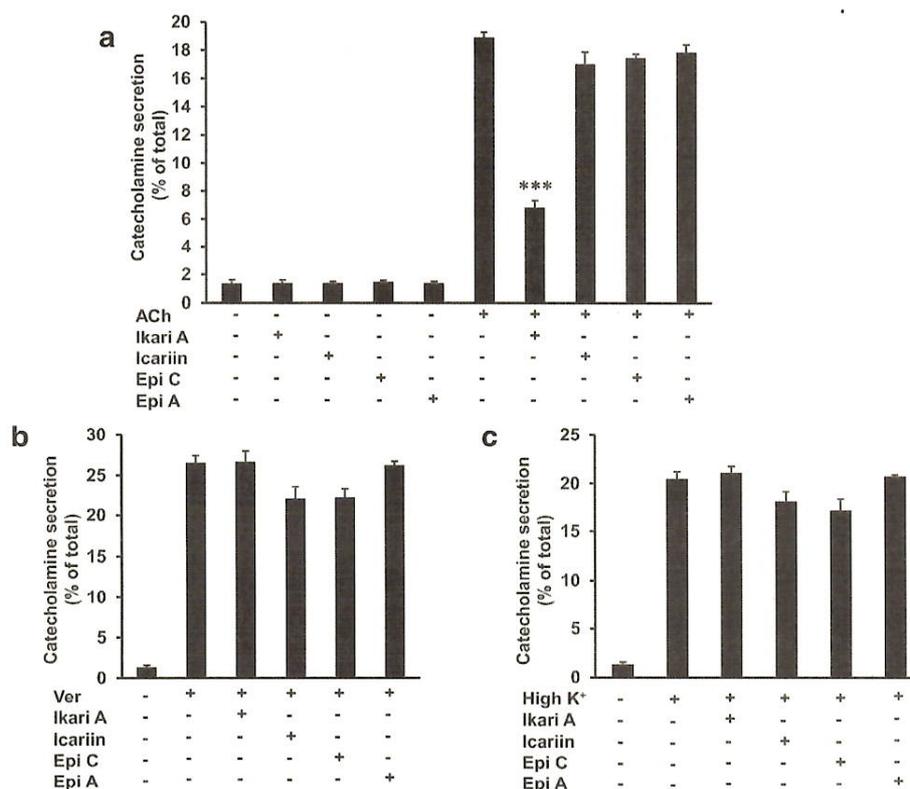


Fig. 2 Effects of ikarisoside A, icariin, epimedin C, or epimedeside A on catecholamine secretion induced by various secretagogues in cultured bovine adrenal medullary cells. After preincubation with cells with or without ikarisoside A (Ikari A) (10 μ M), icariin (10 μ M), epimedin C (Epi C) (10 μ M), and epimedeside A (Epi A) (10 μ M) for 10 min, the cells (10^6 /well) were incubated with or without these four flavonol

glycosides (10 μ M), ACh (300 μ M) (a), veratridine (100 μ M) (b), or 56 mM K⁺ (c) for another 10 min at 37 °C. Catecholamines secreted into the medium were expressed as a percentage of the total catecholamines in the cells. Data are means \pm SEM from three separate experiments carried out in triplicate. *** P < 0.001, compared with ACh alone (analyzed by one-way ANOVA with Dunnett's multiple comparison post hoc test)

increased. Even when the ACh concentrations in the incubation medium increased from 3 to 300 μ M, they did not overcome the inhibitory effect of ikarisoside A (Fig. 4a). Double-reciprocal plot analysis revealed that ikarisoside A exerts a noncompetitive type of inhibition on ACh-induced secretion of catecholamines (Fig. 4b).

Effects of ikarisoside A on ACh-induced inward current in *Xenopus* oocytes expressing $\alpha 3\beta 4$ nAChRs

We examined the direct effects of ikarisoside A on ACh responses in *Xenopus* oocytes expressing rat $\alpha 3\beta 4$ nAChRs. As shown in Fig. 5a, ikarisoside A reversibly inhibited ACh (0.2 mM)-induced currents. Ikarisoside A inhibited ACh-induced currents concentration dependently. It suppressed those currents to 80 ± 3 %, 69 ± 6 %, 43 ± 6 %, 32 ± 8 %, and 22 ± 5 % of the control at 0.1, 0.3, 1, 3, and 10 μ M, respectively, and the inhibitory effects were significant from 0.10 μ M onward; the IC₅₀ was 0.48 μ M (Fig. 5b).

Effect of aglycon of ikarisoside A on ACh-induced secretion of catecholamines

Ikarisoside A is a flavonol glycoside having one rhamnose at the 3 position in the chemical structure. 3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-8-(3-methylbut-2-enyl)-4H-chromen-4-one is the aglycon of ikarisoside A. We examined the effect of this aglycon on ACh-induced secretion of catecholamines. As shown in Fig. 6, the aglycon of ikarisoside A did not affect basal or ACh-induced secretion of catecholamines.

Effect of ikarisoside A on ¹⁴C-catecholamine synthesis from [¹⁴C]tyrosine and tyrosine hydroxylase activity

As shown in Fig. 7a, ACh (300 μ M) increased the synthesis of ¹⁴C-catecholamines from [¹⁴C]tyrosine about 3-fold in bovine adrenal medullary cells. The concurrent treatment of cells with ikarisoside A inhibited the stimulatory effect of 300 μ M ACh on ¹⁴C-catecholamine synthesis in a concentration (1–

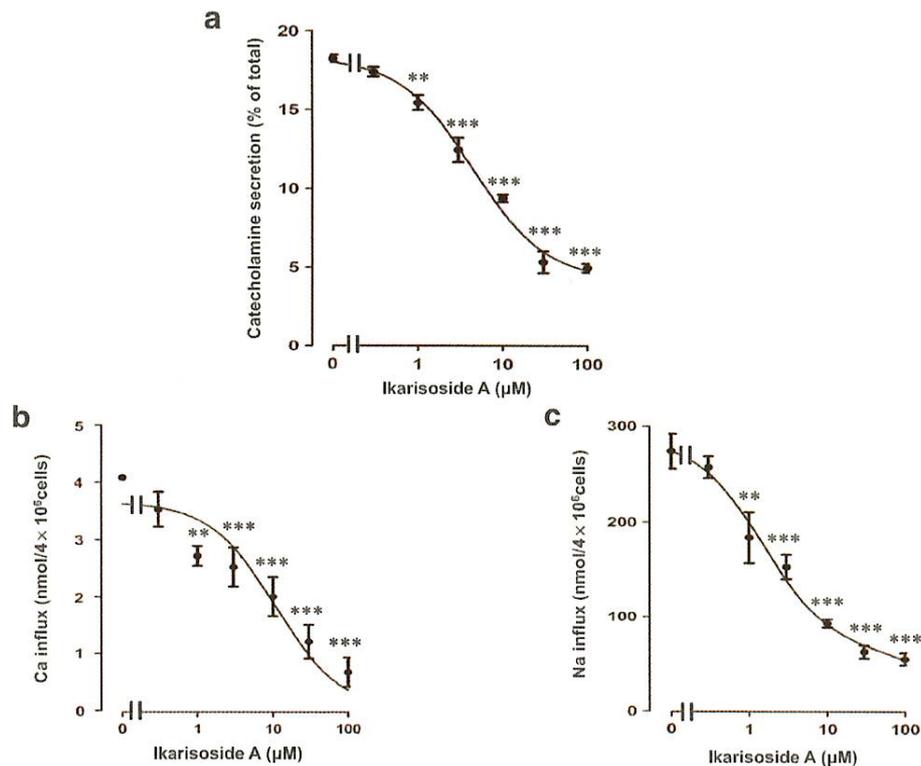


Fig. 3 Effects of ikarisoideside A on catecholamine secretion (a), $^{45}\text{Ca}^{2+}$ influx (b), and $^{22}\text{Na}^{+}$ influx (c) induced by ACh. (a) After preincubation for 10 min with or without ikarisoideside A (0.3–100 μM), cells were stimulated with ACh (300 μM) in the presence or absence of ikarisoideside A (0.3–100 μM) for another 10 min at 37 $^{\circ}\text{C}$. Catecholamines secreted into the medium were expressed as a percentage of the total catecholamines in the cells. b, c After preincubation for 10 min, cells were

stimulated with ACh (300 μM) and 1.5 μCi of $^{45}\text{CaCl}_2$ (b) or $^{22}\text{NaCl}$ (c) in the presence or absence of ikarisoideside A (0.3–100 μM) for another 5 min at 37 $^{\circ}\text{C}$. $^{45}\text{Ca}^{2+}$ influx and $^{22}\text{Na}^{+}$ influx were measured and were expressed as $\text{nmol}/4 \times 10^5$ cells. Data are means \pm SEM from three separate experiments carried out in triplicate. ** $P < 0.01$ and *** $P < 0.001$, compared with ACh alone (analyzed by one-way ANOVA with Dunnett's multiple comparison post hoc test)

100 μM)-dependent manner (Fig. 7a), yielding an IC_{50} value of 2.85 μM . Ikarisoideside A (1–100 μM) had little effect on the basal synthesis of ^{14}C -catecholamines.

We next examined the effect of ikarisoideside A on tyrosine hydroxylase activity in the cells. After preincubation with or without ikarisoideside A (0.1–100 μM) for 10 min, cells were incubated with 300 μM ACh in the absence or presence of ikarisoideside A (0.1–100 μM) for another 10 min at 37 $^{\circ}\text{C}$. Ikarisoideside A (10–100 μM) inhibited the tyrosine hydroxylase activity induced by ACh and tended to inhibit the basal enzyme activity (Fig. 7b). The IC_{50} value of ikarisoideside A for its inhibitory effect on the ACh-induced tyrosine hydroxylase activity was 9.13 μM (derived from the curve representing the difference between stimulated and basal tyrosine hydroxylase; not shown).

Discussion

In present study, we investigated the effects of four flavonol glycosides derived from the leaves of the genus *Epimedium*.

We demonstrated that ikarisoideside A, but not the other three, inhibited the secretion and synthesis of catecholamines induced by ACh in cultured bovine adrenal medullary cells. To our knowledge, this is the first direct evidence of an inhibitory effect of ikarisoideside A on catecholamine secretion and synthesis in cultured bovine adrenal medullary cells.

Inhibitory effect of ikarisoideside A on catecholamine secretion induced by ACh

The present study demonstrated that ikarisoideside A significantly inhibits catecholamine secretion induced by ACh, but not by veratridine or 56 mM K^{+} in adrenal medullary cells. We previously reported that ACh activates nAChR-ion channels, and induces Na^{+} influx, subsequent Ca^{2+} influx, and finally catecholamine secretion. On the other hand, veratridine activates voltage-dependent Na^{+} channels and 56 mM K^{+} depolarizes cell membranes to activate voltage-dependent Ca^{2+} channels (Wada et al. 1984, 1985b). In the present study, ikarisoideside A did not inhibit the stimulatory effects of veratridine and 56 mM K^{+} on catecholamine secretion. Therefore,

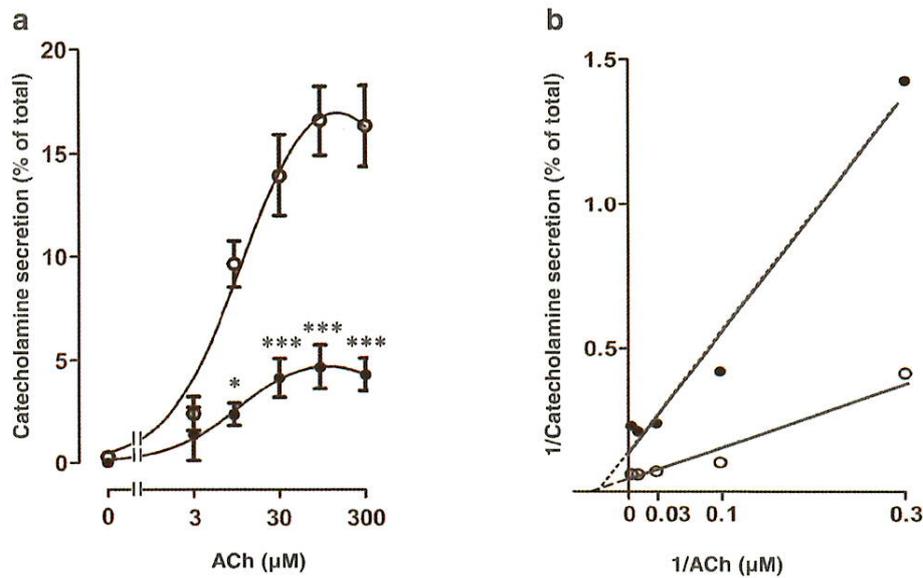


Fig. 4 Inhibitory mode of ikarisoside A on catecholamine secretion induced by ACh. **a** After preincubation for 10 min, cells were stimulated with (black circle) or without (white circle) ikarisoside A (10 μM) in the presence or absence of ACh (1–300 μM) for another 10 min at 37 °C. Catecholamines secreted into the medium were expressed as a percentage of the total catecholamines in the cells. Data

are means ± SEM from three separate experiments carried out in triplicate. The data of ACh plus ikarisoside A are shown by subtracting basal secretion obtained in the presence of ikarisoside A. **P* < 0.05 and ****P* < 0.001, compared with ACh alone (analyzed by one-way ANOVA with Dunnett's multiple comparison post hoc test). **b** Double-reciprocal plot analysis of the data in (a)

ikarisoside A seems to inhibit nAChR-ion channels but not voltage-dependent Na⁺ channels or voltage-dependent Ca²⁺

channels. Ikarisoside A inhibited Ca²⁺ influx and Na⁺ influx induced by ACh in a concentration-dependent manner similar

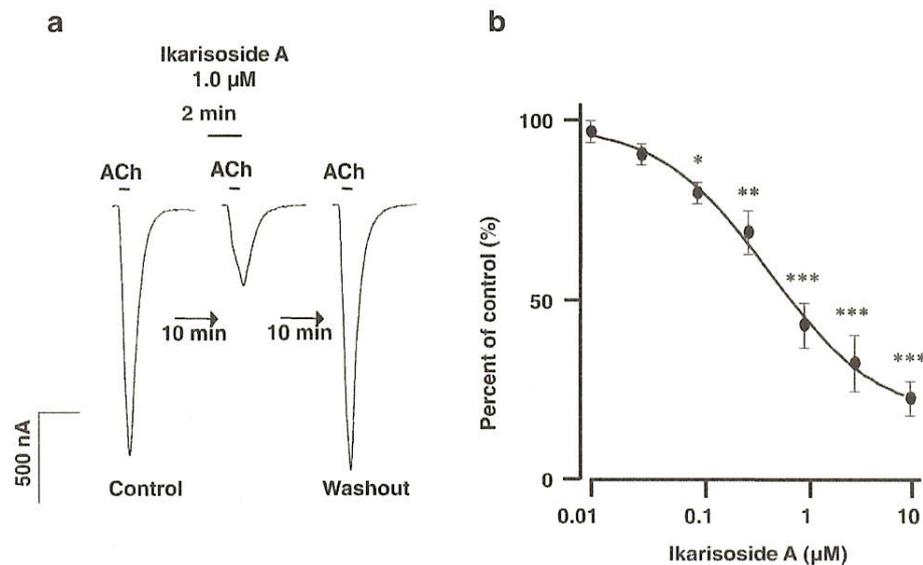


Fig. 5 Effects of ikarisoside A on peak ACh-induced inward currents in *Xenopus* oocytes expressing rat α3β4 nAChRs. **a** Representative traces from a single *Xenopus* oocyte are shown. The currents of ikarisoside A-treated oocytes were recorded 10 min after recording of the control currents, and the washout currents were obtained 10 min after ikarisoside A treatment. Ikarisoside A (1 μM) suppressed the currents induced by the EC₅₀ (0.2 mM) of ACh, and the inhibitory effects were reversible. **b** Concentration-response curve for the inhibitory effects of ikarisoside A

on ACh-induced currents. The peak current amplitude in the presence of ikarisoside A was normalized to that of the control and the effects are expressed as percentages of the control. Data are presented as means ± SEM from four separate experiments carried out in triplicate. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, compared to the control (based on one-way ANOVA with Dunnett's multiple comparison post hoc test). Nonlinear regression analysis was performed and the mean value of IC₅₀ for ikarisoside A is 0.48 μM

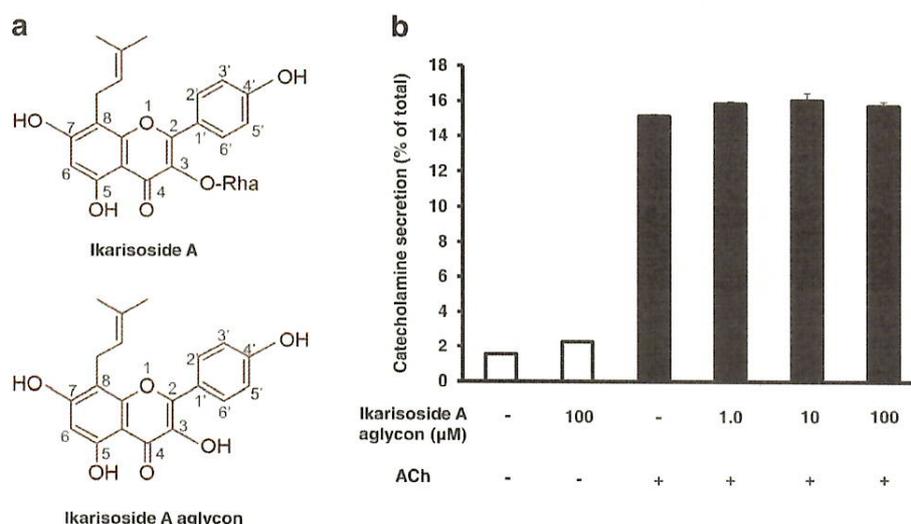


Fig. 6 Structure of ikarisoside A and its aglycon (**a**) and effect of aglycon of ikarisoside A on ACh-induced catecholamine secretion (**b**). **a** Structure of ikarisoside A and its aglycon (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-8-(3-methylbut-2-enyl)-4H-chromen-4-one). **b** After preincubation with cells with or without aglycon of ikarisoside A (1–100 μM) for 10 min, the

cells ($10^6/\text{well}$) were incubated with or without aglycon of ikarisoside A (1–100 μM) and ACh (300 μM) for another 10 min at 37 $^\circ\text{C}$. Catecholamines secreted into the medium were expressed as a percentage of the total catecholamines in the cells. Data are means \pm SEM from three separate experiments carried out in triplicate

to that of catecholamine secretion. In the exocytotic secretion of catecholamines, Ca^{2+} plays an indispensable role as the coupler in the stimulus-secretion coupling (Douglas and Rubin 1961, 1963). From these findings, it is likely that

ikarisoside A inhibits ACh-induced catecholamine secretion by suppressing nAChR-ion channels. We investigated the inhibitory mode of ikarisoside A on nAChR-ion channels. Even when the concentration of ACh was increased, the inhibitory

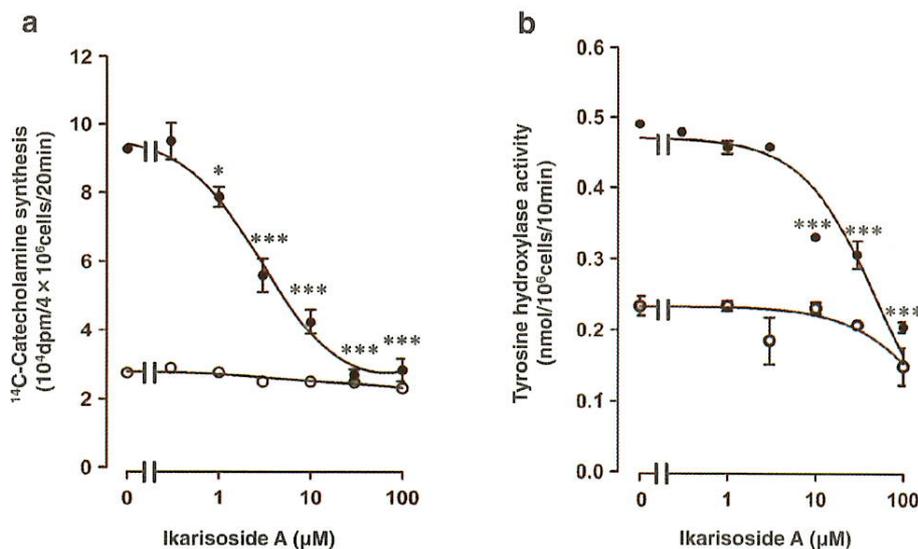


Fig. 7 Effects of ikarisoside A on ^{14}C -catecholamine synthesis from [^{14}C]tyrosine (**a**) and tyrosine hydroxylase activity (**b**) in the cells. **a** After preincubation for 10 min with or without ikarisoside A (0.1–100 μM), cells ($4 \times 10^6/\text{dish}$) were incubated with L-[U- ^{14}C] tyrosine (20 μM , 1 μCi) in the presence or absence of ikarisoside A (0.1–100 μM) and with (black circle) or without (white circle) 300 μM ACh at 37 $^\circ\text{C}$ for 20 min. The ^{14}C -catecholamines formed were measured. **b** After preincubation with or without ikarisoside A (0.1–100 μM) for 10 min,

cells ($10^6/\text{well}$) were incubated with L-[^{14}C] tyrosine (18 μM , 0.2 μCi) in the presence or absence of ikarisoside A (0.1–100 μM) and with (black circle) or without (white circle) 300 μM ACh at 37 $^\circ\text{C}$ for 10 min, and tyrosine hydroxylase activity was measured. Data are means \pm SEM from three separate experiments carried out in triplicate. * $P < 0.05$ and *** $P < 0.001$, compared with ACh alone (analyzed by one-way ANOVA with Dunnett's multiple comparison post hoc test)

effect of ikarisoside A on ACh-induced secretion of catecholamines was not overcome, suggesting a noncompetitive inhibition and that ikarisoside A acts at a site different from that for ACh binding. A previous review (Lena and Changeux 1993) reported that the site at which noncompetitive blockers act lies at the interface between the nicotinic receptor protein and the membrane lipids.

In the *Xenopus* oocytes expressed with $\alpha 3\beta 4$ nAChRs, ikarisoside A directly inhibited ACh-induced current. The IC_{50} values of ikarisoside A for $^{22}Na^+$ influx in adrenal medullary cells and for Na^+ current in the oocytes were 2.96 and 0.48 μM , respectively. The IC_{50} in the bovine adrenal medullary cells is 6.2-fold bigger than that of the drug in the oocyte system. Although the reason for the discrepancy of the IC_{50} between the two systems is not yet clear, the discrepancy may be explained in the following way. (1) A maximally effective concentration of ACh was used for the $^{22}Na^+$ influx experiments in bovine adrenal medullary cells but the half-maximal concentration was used for the Na^+ current in the oocyte system. (2) In the oocyte expression system, there may be some changes in the test compound potency compared to that of the method using mammalian cells, i.e., a decrease (Lambert et al. 2001; Akk et al. 2008) or an increase (Pintado et al. 2000) in the sensitivity of test compounds. (3) Bovine adrenal medullary cells express multiple nAChR subtypes such as $\alpha 3\beta 4$ (Criado et al. 1992; Garcia-Guzman et al. 1995), $\alpha 3\beta 4\alpha 5$ (Campos-Caro et al. 1997), and $\alpha 7$ (Lopez et al. 1998). We should study above possibilities and examine the effect of ikarisoside A on the function of nAChRs in other mammalian cells.

Structure-activity relationship of ikarisoside A for inhibition of nAChR-ion channels

In the present study, we used four flavonol glycosides derived from the *Epimedium* species. Ikariside A, but not the other three flavonols, inhibited the functioning of nAChR-ion channels. Judging from the differences in their structures, ikariside A has a hydroxyl group at the 7 position in the structure whereas other three have a glucose moiety at this position, suggesting that a glucose moiety at the 7 position may induce stereo-specific interference when flavonol glycosides interact with nAChRs. Furthermore, the inhibition of ACh-induced secretion by ikariside A disappeared by the removal of the rhamnose moiety at the 3 position from ikariside A. These findings suggest that the rhamnose moiety at the 3 position of ikariside A is essential to inhibit the function of nAChR-ion channels.

Inhibitory effect of ikarisoside A on catecholamine synthesis

Ikariside A inhibited not only catecholamine secretion but also reduced catecholamine synthesis in ACh-stimulated cells.

In the regulation of catecholamine synthesis, Ca^{2+} plays an important role as the coupler in the stimulus-synthesis coupling (Yanagihara et al. 1987) as well as in the stimulus-secretion coupling (Douglas and Rubin 1961, 1963). In the present study, we observed that ikariside A suppressed the $^{22}Na^+$ influx and the subsequent $^{45}Ca^{2+}$ influx by inhibiting nAChR-ion channels. Therefore, it is likely that ikariside A inhibits catecholamine synthesis and tyrosine hydroxylase activity induced by ACh via the suppression of Ca^{2+} influx in cultured bovine adrenal medulla cells. In harmony with this view, the IC_{50} values of ikariside A for inhibition of $^{22}Na^+$ and $^{45}Ca^{2+}$ influx and for inhibition of catecholamine synthesis and tyrosine hydroxylase are very similar.

Pharmacological significance of the inhibitory effects of ikarisoside A on adrenal medullary functions

The human serum concentration of ikariside A has not been reported yet. Several previous in vitro studies reported that ikariside A at 5.0–20 μM inhibits osteoclastogenic differentiation and nitric oxide synthase in murine monocyte/macrophage cell line RAW264.7 cells (Choi et al. 2008, 2010) and induces neurite outgrowth activity in PC12h cells at 10 μM (Kuroda et al. 2000). In the present study, we observed a significant inhibition of ikariside A at 0.1 and 1.0 μM in ACh-induced current in *Xenopus* oocytes and ACh-induced synthesis and secretion of catecholamines, respectively.

It is well known that adrenaline and noradrenaline have an important role in the regulation of normal function in the central and peripheral sympathetic nervous systems. Under strong and prolonged stress, an increased catecholamine release may occur, which possibly induces cardiovascular diseases such as hypertension, atherosclerosis, coronary heart disease, and heart failure (Yanagihara et al. 2014). Chronic heart failure is reported to be associated with the activation of the sympathetic nervous system as manifested by increased circulating catecholamines (Westfall and Westfall 2011). Furthermore, Hara et al. (2011) reported that the stress hormone adrenaline stimulates β_2 -adrenoceptors, which activates the Gs protein/cyclic AMP-dependent protein kinase and the β -arrestin-mediated signaling pathway, reduces the p53 level, and induces DNA damage.

Our previous studies reported that daidzein, a soy isoflavone, (Liu et al. 2007) and nobiletin, a citrus polymethoxy flavone, (Zhang et al. 2010) suppress the secretion and synthesis of catecholamines induced by ACh in cultured bovine adrenal medullary cells. In addition to these flavonoids, ikariside A also may protect the hyperactive catecholamine system induced by strong stress or emotional excitation which evokes the secretion of ACh from the splanchnic nerves. Further in vivo experiments will provide more conclusive information on ikariside A and promote the development of a

therapeutic drug for stress-induced disorders associated with mental or cardiovascular diseases.

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Conflict of interest The authors declare that they have no competing interests.

References

- Akk G, Li P, Bracamontes J, Reichert DE, Covey DF, Steinbach JH (2008) Mutations of the GABA_A receptor $\alpha 1$ subunit M1 domain reveal unexpected complexity for modulation by neuroactive steroids. *Mol Pharmacol* 74:614–627
- Bobrovskaya L, Cheah TB, Bunn SJ, Dunkley PR (1998) Tyrosine hydroxylase in bovine adrenal chromaffin cells: angiotensin II-stimulated activity and phosphorylation of Ser¹⁹, Ser³¹, and Ser⁴⁰. *J Neurochem* 70:2565–2573
- Campos-Caro A, Smillie FI, Dominguez del Toro E, Rovira JC, Vicente-Agullo F, Chapuli J, Juiz JM, Sala S, Sala F, Ballesta JJ, Criado M (1997) Neuronal nicotinic acetylcholine receptors on bovine chromaffin cells: cloning, expression, and genomic organization of receptor subunits. *J Neurochem* 68:488–497
- Chen WF, Mok SK, Wang XL, Lai KH, Lai WP, Luk HK, Leung PC, Yao XS, Wong MS (2011) Total flavonoid fraction of the herba epimedii extract suppresses urinary calcium excretion and improves bone properties in ovariectomized mice. *Br J Nutr* 105:180–189
- Choi HJ, Eun JS, Park YR, Kim DK, Li R, Moon WS, Park JM, Kim HS, Cho NP, Cho SD, Soh Y (2008) Ikariside A inhibits inducible nitric oxide synthase in lipopolysaccharide-stimulated RAW 264.7 cells via p38 kinase and nuclear factor- κ B signaling pathways. *Eur J Pharmacol* 601:171–178
- Choi HJ, Park YR, Nepal M, Choi BY, Cho NP, Choi SH, Heo SR, Kim HS, Yang MS, Soh Y (2010) Inhibition of osteoclastogenic differentiation by Ikariside A in RAW 264.7 cells via JNK and NF- κ B signaling pathways. *Eur J Pharmacol* 636:28–35
- Criado M, Alamo L, Navarro A (1992) Primary structure of an agonist binding subunit of the nicotinic acetylcholine receptor from bovine adrenal chromaffin cells. *Neurochem Res* 17:281–287
- Dou J, Liu Z, Liu S (2006) Structure identification of a prenylflavonol glycoside from epimedium koreanum by electrospray ionization tandem mass spectrometry. *Anal Sci* 22:449–452
- Douglas WW, Rubin RP (1961) Mechanism of nicotinic action at the adrenal medulla: calcium as a link in stimulus-secretion coupling. *Nature* 192:1087–1089
- Douglas WW, Rubin RP (1963) The mechanism of catecholamine release from the adrenal medulla and the role of calcium in stimulus-secretion coupling. *J Physiol* 167:288–310
- Garcia-Guzman M, Sala F, Sala S, Campos-Caro A, Stulmer W, Gutierrez LM, Criado M (1995) α -Bungarotoxin-sensitive nicotinic receptors on bovine chromaffin cells: molecular cloning, functional expression and alternative splicing of the $\alpha 7$ subunit. *Eur J Neurosci* 7:647–655
- Hara MR, Kovacs JJ, Whalen EJ, Rajagopal S, Strachan RT, Grant W, Towers AJ, Williams B, Lam CM, Xiao K, Shenoy SK, Gregory SG, Ahn S, Duckett DR, Lefkowitz RJ (2011) A stress response pathway regulates DNA damage through β_2 -adrenoreceptors and β -arrestin-1. *Nature* 477:349–353
- Horishita T, Harris RA (2008) n-Alcohols inhibit voltage-gated Na⁺ channels expressed in Xenopus oocytes. *J Pharmacol Exp Ther* 326:270–277
- Kajiwara K, Yanagita T, Nakashima Y, Wada A, Izumi F, Yanagihara N (2002) Differential effects of short and prolonged exposure to carvedilol on voltage-dependent Na⁺ channels in cultured bovine adrenal medullary cells. *J Pharmacol Exp Ther* 302:212–218
- Kuroda M, Mimaki Y, Sashida Y, Umegaki E, Yamazaki M, Chiba K, Mohri T, Kitahara M, Yasuda A, Naoi N, Xu ZW, Li MR (2000) Flavonol glycosides from *Epimedium sagittatum* and their neurite outgrowth activity on PC12h cells. *Planta Med* 66:575–577
- Lai X, Ye Y, Sun C, Huang X, Tang X, Zeng X, Yin P, Zeng Y (2013) Icaritin exhibits anti-inflammatory effects in the mouse peritoneal macrophages and peritonitis model. *Int Immunopharmacol* 16:41–49
- Lambert JJ, Belelli D, Harney SC, Peters JA, Frenguelli BG (2001) Modulation of native and recombinant GABA_A receptors by endogenous and synthetic neuroactive steroids. *Brain Res Brain Res Rev* 37:68–80
- Lena C, Changeux JP (1993) Allosteric modulations of the nicotinic acetylcholine receptor. *Trends Neurosci* 16:181–186
- Liu M, Yanagihara N, Toyohira Y, Tsutsui M, Ueno S, Shinohara Y (2007) Dual effects of daidzein, a soy isoflavone, on catecholamine synthesis and secretion in cultured bovine adrenal medullary cells. *Endocrinology* 148:5348–5354
- Lopez MG, Montiel C, Herrero CJ, Garcia-Palmero E, Mayorgas I, Hernandez-Guijo JM, Villarroja M, Olivares R, Gandia L, McIntosh JM, Olivera BM, Garcia AG (1998) Unmasking the functions of the chromaffin cell $\alpha 7$ nicotinic receptor by using short pulses of acetylcholine and selective blockers. *Proc Natl Acad Sci U S A* 95:14184–14189
- Lu MF, Xiao ZT, Zhang HY (2013) Where do health benefits of flavonoids come from? Insights from flavonoid targets and their evolutionary history. *Biochem Biophys Res Commun* 434:701–704
- Mizuno M, Iinuma M, Tanaka T, Sakakibara N, Hanioka S (1988) Flavonol glycosides in *Epimedium* species. *Chem Pharm Bull* 36(9):3487–3490
- Nagatsu T, Levitt M, Udenfriend S (1964) Tyrosine hydroxylase. The initial step in norepinephrine biosynthesis. *J Biol Chem* 239:2910–2917
- Nijveldt RJ, van Nood E, van Hooft DE, Boelens PG, van Norren K, van Leeuwen PA (2001) Flavonoids: a review of probable mechanisms of action and potential applications. *Am J Clin Nutr* 74:418–425
- Pintado AJ, Herrero CJ, Garcia AG, Montiel C (2000) The novel Na⁺/Ca²⁺ exchange inhibitor KB-R7943 also blocks native and expressed neuronal nicotinic receptors. *Br J Pharmacol* 130:1893–1902
- Ren ZL, Zuo PP (2012) Neural regeneration: role of traditional Chinese medicine in neurological diseases treatment. *J Pharmacol Sci* 120:139–145
- Shinohara Y, Toyohira Y, Ueno S, Liu M, Tsutsui M, Yanagihara N (2007) Effects of resveratrol, a grape polyphenol, on catecholamine secretion and synthesis in cultured bovine adrenal medullary cells. *Biochem Pharmacol* 74:1608–1618
- Toyohira Y, Kubo T, Watanabe M, Uezono Y, Ueno S, Shinkai K, Tsutsui M, Izumi F, Yanagihara N (2005) Selective blockade of nicotinic acetylcholine receptors by pimobendan, a drug for the treatment of heart failure: reduction of catecholamine secretion and synthesis in adrenal medullary cells. *Naunyn Schmiedeberg's Arch Pharmacol* 371:107–113
- Tsutsui M, Yanagihara N, Miyamoto E, Kuroiwa A, Izumi F (1994) Correlation of activation of Ca²⁺/calmodulin-dependent protein kinase II with catecholamine secretion and tyrosine hydroxylase activation in cultured bovine adrenal medullary cells. *Mol Pharmacol* 46:1041–1047

- Ueno S, Tsutsui M, Toyohira Y, Minami K, Yanagihara N (2004) Sites of positive allosteric modulation by neurosteroids on ionotropic γ -aminobutyric acid receptor subunits. *FEBS Lett* 566:213–217
- Wada A, Izumi F, Yanagihara N, Kobayashi H (1985a) Modulation by ouabain and diphenylhydantoin of veratridine-induced ^{22}Na influx and its relation to ^{45}Ca influx and the secretion of catecholamines in cultured bovine adrenal medullary cells. *Naunyn Schmiedeberg's Arch Pharmacol* 328:273–278
- Wada A, Takara H, Izumi F, Kobayashi H, Yanagihara N (1985b) Influx of ^{22}Na through acetylcholine receptor-associated Na channels: relationship between ^{22}Na influx, ^{45}Ca influx and secretion of catecholamines in cultured bovine adrenal medulla cells. *Neuroscience* 15:283–292
- Wada A, Yashima N, Izumi F, Kobayashi H, Yanagihara N (1984) Involvement of Na influx in acetylcholine receptor mediated secretion of catecholamines from cultured bovine adrenal medulla cells. *Neurosci Lett* 47:75–80
- Weil-Malherbe H, Bone AD (1952) The chemical estimation of adrenaline-like substances in blood. *Biochem J* 51:311–318
- Westfall TC and Westfall DP (2011) Adrenergic agonists and antagonists, in Goodman & Gilman's the pharmacological basis of therapeutics, 12th edn (Brunton LL, Chabner BC, and Knollmann BC, eds), pp. 277–330. McGraw-Hill, NY
- Yanagihara N, Isosaki M, Ohuchi T, Oka M (1979) Muscarinic receptor-mediated increase in cyclic GMP level in isolated bovine adrenal medullary cells. *FEBS Lett* 105:296–298
- Yanagihara N, Oishi Y, Yamamoto H, Tsutsui M, Kondoh J, Sugiura T, Miyamoto E, Izumi F (1996) Phosphorylation of chromogranin A and catecholamine secretion stimulated by elevation of intracellular Ca^{2+} in cultured bovine adrenal medullary cells. *J Biol Chem* 271:17463–17468
- Yanagihara N, Wada A, Izumi F (1987) Effects of α_2 -adrenergic agonists on carbachol-stimulated catecholamine synthesis in cultured bovine adrenal medullary cells. *Biochem Pharmacol* 36:3823–3828
- Yanagihara N, Zhang H, Toyohira Y, Takahashi K, Ueno S, Tsutsui M (2014) New insights into the pharmacological potential of plant flavonoids in the catecholamine system. *J Pharmacol Sci* 124:123–128
- Zhang H, Toyohira Y, Ueno S, Shinohara Y, Itoh H, Furuno Y, Yamakuni T, Tsutsui M, Takahashi K, Yanagihara N (2010) Dual effects of nobiletin, a citrus polymethoxy flavone, on catecholamine secretion in cultured bovine adrenal medullary cells. *J Neurochem* 114:1030–1038
- Zhou J, Chen KM, Ge BF, Ma XN, Guo XY, Cheng K, Gao YH, Yan LJ, Shi WG (2013) Comparison between icaritin and genistein in osteogenic activity of marrow stromal cells. *Zhongguo Zhong Yao Za Zhi* 38:1783–1788