Conversion of aconitine to lipoaconitine by human intestinal bacteria and their antinociceptive effects in mice

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Abstract

Aconitine was substantially converted to lipoaconitine (LA) after incubation with human intestinal bacteria. The Frit-FAB LC/MS and GC/MS indicated that the fatty acid composition of LA was similar to cellular fatty acids of the bacterial strain used; major fatty acids in LA produced by *Bacteroides fragilis* were anteiso-C15 : 0, n-C15:0 and n-C16 : 0, while LA produced by *Klebsiella pneumoniae* or by fecal flora contained n-C16 : 0 as the major fatty acid. LA from *Clostridium butyricum* contained C18 : 1, C18 : 0, C16 : 1, C16 : 0. LA was also obtained after incubation of aconitine with sterile bacterial cells or a precipitate of disrupted bacterial cells in phosphate buffer.

Aconitine at a dose of 0.1 mg/kg, significantly increased the nociceptive threshold in mice with nociceptive hypersensitivity, while 8-*O*-oleoylbenzoylaconine (OBA) was active but toxic at higher dose of 3.0 mg/kg, and 8-*O*-palmitoylbenzoylaconine (PBA) was only active at a dose of 30 mg/kg. These findings suggest that OBA and PBA do not play an important role in the antinociceptive action of aconite tuber and the alkaloid aconitine.

Key words Aconitine, antinociceptive effect, intestinal bacteria, LC/MS, lipoaconitine.

Abbreviations FAB, fast atom bombardment ; GC/MS, gas chromatography/mass spectrometry ; HPLC, high performance liquid chromatography ; IR, infrared ; LA, lipoaconitine ; LC/MS, liquid chromatography/ mass spectrometry ; MS, mass spectrum/spectra ; MW, molecular weight ; ^{13}C-NMR, carbon-13 nuclear magnetic resonance ; ^{1}H-NMR, proton nuclear magnetic resonance ; OBA, 8-*O*-oleoylbenzoylaconine ; PBA, 8-*O*-palmitoylbenzoylaconine ; TLC, thin layer chromatography.

Introduction

Aconite tuber is the most toxic and most important crude drug used in traditional Chinese medicine as an analgesic and anaesthetic agent for the treatment of rheumatism and neuralgia.[1] The majority of aconite alkaloids have an aconitane skeleton where aconitine is the most important representative alkaloid. It has been reported that the toxicity of this tuber apparently decreased after processing where the fatally toxic alkaloids, aconitine and mesaconitine, were converted to the less toxic alkaloids, aconine, benzoylaconine and lipoaconitine (LA).[2-5] On the other hand, lipomesaconitine, one of the lipo-alkaloids, was found to exhibit anti-inflammatory and analgesic activities.[4] Although the traditional use as well as illicit use of this tuber or its toxic alkaloids is increasing, the metabolic fate of aconite alkaloids has not been investigated so far. For better understanding of its biological disposition, we are presenting here the *in vitro* conversion of aconitine to LA by human intestinal bacteria and their antinociceptive effects in mice.

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Materials and Methods

Instruments : ^{1}H-NMR and ^{13}C-NMR spectra were measured by a JEOL GX400 spectrometer with tetramethylsilane (TMS) as an internal standard; fast atom bombardment-mass spectra (FAB-MS) were obtained with a JMS-DX 300 mass spectrometer with Xe as neutral gas; High performance liquid chromatography (HPLC) was carried out with a JASCO TRI ROTAR-V liquid chromatograph equipped with a JASCO UVIDEC100-V detector (JASCO, Tokyo, Japan) and a C-R6A chromatopac (Shimadzu, Kyoto, Japan). LC/MS was performed on a Frit-FAB LC/MS system (JEOL, Tokyo, Japan) equipped with a TRI ROTAR-V liquid chromatograph (JASCO, Tokyo, Japan). Infrared (IR) spectra were measured on a Hitachi 260-10 infrared spectrometer.

Chromatography : Merck Kieselgel 60 F_{254} plates (E. Merck, Darmstadt, Germany) were used for thin-layer chromatography (TLC) with solvent system A (benzene-1,4-dioxane-28% NH_4OH, 10 : 3 : 0.2) and spots were detected under UV light or after spraying with Dragendorff reagent.

Bacteria : *Bacteroides fragilis* ss *thetaotus, Klebsiella pneumoniae* ATCC 13883 and *Clostridium butyricum* were provided by Prof. T. Mitsuoka, the University of Tokyo.

Chemicals : Aconitine was obtained from Sigma Chem. Co. (St. Louis, MO, USA). General anaerobic medium (GAM) was purchased from Nissui Co. (Tokyo, Japan). Saturated fatty acids and oleic acid (C18 : 1) were purchased from Nacalai Tesque Co. (Kyoto, Japan), other unsaturated and methyl branched fatty acids were obtained from Larodan Fine Chemicals AB, (Malmo, Sweden). Tripalmitin was purchased from Wako Pure Chemical Co. (Osaka, Japan).

In animal experiments, aconitine was dissolved in dimethyl sulfoxide and diluted with 0.9 % saline. 8-*O*-Oleoylbenzoylaconine (OBA) and 8-*O*-palmitoylbenzoylaconine (PBA) were dissolved in dimethyl sulfoxide and suspended in olive oil. All drugs were administered subcutaneously (s.c.), in a volume of 0.1 ml/10 g of body weight.

Animals : Male ddY mice (6 weeks old, Nippon SLC, Shizuoka, Japan) were used. The animals were housed under controlled temp. (23-25°C) with a 12 hrs light/dark cycle (lights on from 08 : 00 to 20 : 00) for at least one week before the experiment. Food and water were freely available.

Metabolism of aconitine by Bacteroides fragilis : *B. fragilis* was anaerobically cultured in GAM broth (1 l) for 24 hrs at 37°C and the culture was centrifuged at 4500 × *g* for 5 min. The precipitates were suspended in 0.2 M phosphate buffer (pH 7.3 ; 100 ml). Aconitine (200 mg) was added to the suspension and the mixture was anaerobically incubated for 96 hrs at 37°C. The incubation mixture was extracted with EtOAc (100 ml × 4) and the EtOAc extract was evaporated *in vacuo* to give a residue. The residue was chromatographed on TLC plates developed with solvent system A to give a metabolite (LA) in a yield of 30 mg.

Metabolite (LA) produced by B. fragilis : Colorless oil, IR λ_{max} cm^{-1} : 3500 (OH), 2930, 1725 (ester C=O), 1280 and 1100. FAB-MS (positive ion mode) : *m/z* 842 $[M+H]^+$, 828 $[M+H]^+$, 814 $[M+H]^+$, 800 $[M+H]^+$, 586 [LA-fatty acid residue]$^+$, 105 (base peak, benzoyl), FAB-MS (negative) : *m/z* 255, 241, 227, 213, 121 (base peak, benzoate). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ : 1.10 (3H, t, *J*=7 Hz, NCH_2-C<u>H</u>$_3$), 3.34 (1H, d *J*=5 Hz, 16-H), 3.46 (1H, d, *J*=9 Hz, 18-H_b), 3.61 (1H, d, *J*=9 Hz, 18-H_a), 4.03 (1H, d, *J*=6.5 Hz, 6-H), 4.44 (1H, dd, *J*=5 and 2.5 Hz, 15-H), 4.86 (1H, d *J*=5 Hz, 14-H), 7.45 (2H, t, *J*=7.5 Hz, Ph-H), 7.57 (2H, t, *J*=7.5 Hz, Ph-H), 8.03 (1H, d, *J*=7.5 Hz, Ph-H), and 3.16, 3.27, 3.30 and 3.76 (4 × 3H, s, 4 × O-C<u>H</u>$_3$).

Synthesis of 8-O-oleoylbenzoylaconine (OBA) and 8-O-palmitoylbenzoylaconine (PBA) : Oleic or palmitic acid (6.3 g) was added to a solution of aconitine (500 mg) in anhydrous pyridine (1.8 ml). The mixture was stirred at 70°C for 4 hrs. After dilution with cold water, the mixture was extracted with EtOAc (50 ml × 3). The EtOAc layer was washed with water, dried over anhydrous $MgSO_4$ and concentrated *in vacuo* to give a residue. Column chromatography of the residue over neutral Al_2O_3 using benzene-$CHCl_3$ (1 : 1) afforded OBA (370 mg) or PBA (400 mg).

8-O-Oleoylbenzoylaconine (OBA) : Colorless oil, FAB-MS (positive ion mode): *m/z* 869 $[M+2H]^+$, 764 and 647. ^{1}H-NMR ($CDCl_3$, 500 MHz) δ : 0.88 (3H,

t, oleoyl-$C\underline{H}_3$), 1.09 (3H, t, $C\underline{H}_3$), 1.44 and 1.78 (each 1H, m, oleoyl-CH_2), 1.99 (4H, m, oleoyl-$C\underline{H}_2$-CH=CH-$C\underline{H}_2$-) and 5.35 (2H, m, oleoyl-CH_2-$C\underline{H}$=$C\underline{H}$-CH_2-). ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 128.6, 129.7, 130.0 and 133.3 (5×Ph-C), 129.6 (d, -$\underline{C}$H=$\underline{C}$H-), and 166.0 and 175.1 (2×ester $\underline{C}$=O).

8-O-Palmitoylbenzoylaconine (PBA) : Colorless oil, FAB-MS (positive ion mode) : *m/z* 842 $[M+H]^+$ and 646. ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 0.88 (3H, t, palmitoyl-$C\underline{H}_3$), and 1.09 (3H, t, $C\underline{H}_3$), 1.44 and 1.78 (each 1H, m, palmitoyl-$C\underline{H}_2$). ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 128.6, 129.6, 129.7 and 133.2 (5 × Ph-C), and 166.0 and 175.2 (2 × ester $\underline{C}$=O).

Preparation of GC/MS samples : The metabolite (5 mg) was dissolved in 2 ml of MeOH containing 0.5 % NaOMe and kept at room temperature for 8 hrs. The reaction mixture was evaporated to dryness *in vacuo*, and $CHCl_3$ and H_2O (2 ml each) were added. The $CHCl_3$ layer was separated and dried over $MgSO_4$. After $CHCl_3$ was evaporated *in vacuo*, the residue was dissolved in hexane and analyzed by GC/MS.

As for the GC/MS analysis of bacterial fatty acids, a suspension of *B. fragilis* (10 ml) was acidified with 0.1 N HCl and sonicated with a cell disruptor for 3 min. The suspension was extracted with ether (10 ml × 3) and the ether layer was dried over $MgSO_4$ and concentrated *in vacuo*. The ether extract (fatty acid fraction) was treated with CH_2N_2. Authentic samples of fatty acids were dissolved in ether and also methylated with CH_2N_2.

Time course of LA formation by B. fragilis : Tubes containing 2 mM aconitine in 0.2 M phosphate buffer (pH 7.3, 10 ml each) were anaerobically incubated at 37°C with viable and heat-killed cells of *B. fragilis*, and a supernatant and a precipitate obtained by sonication of the cells followed by centrifugation. A portion (1 ml) was taken out at 24 hours interval and applied to an extraction column, Chem Elut CE 1003 (Varian, USA). The EtOAc eluate (1 ml) was evaporated *in vacuo* to give a residue. The residue was dissolved in MeOH (500 μl) and an aliquot (2 μl) was analyzed by HPLC.

The heat-killed cells were prepared by autoclaving. The supernatant and precipitate of the preculturd cell suspension were obtained by sonication followed by centrifugation at 5700 × *g* for 5 min. The volumes of the supernatant and precipitate were adjusted to 10 ml each with 0.2 M phosphate buffer (pH 7.3).

Trasformation of aconitine to LA by Klebsiella pneumoniae or Clostridium butyricum : A 24 hour culture of *K. pneumoniae* or *C. butyricum* in GAM broth (200 ml) was centrifuged at 4500 × *g* for 5 min and the precipitate was suspended in 0.2 M phosphate buffer (pH 7.3). Tubes (10 ml) containing the bacterial cells and aconitine (32.3 mg/tube) were anaerobically incubated for 96 hrs at 37°C. The metabolites were separated by using the extraction column and analyzed by LC/MS.

Transformation of aconitine to LA by a bacterial mixture : A fresh fecal specimen (10 g) obtained from a healthy subject was suspended in 0.2 M phosphate buffer (pH 7.3, 100 ml). Tubes containig aconitine (32.3 mg, 0.05 mmol) and the fecal suspension (10 ml) were anaerobically incubated for 96 hrs at 37°C. Each incubation mixture was applied to an extraction column and worked up as described above. The metabolite was analyzed by LC/MS.

LC/MS measurement : A sample solution (2 μl) was injected into a column (25 cm × 4.6 mm i. d.) of Develosil ODS K-5 (Nomura Chem., Seto, Japan) attached to the HPLC system. The column was eluted with MeOH-H_2O-AcOH-glycerol (80 : 20 : 1 : 0.5) at a flow rate of 1.0 ml/min. The effluent was split in a ratio of 1:20 and introduced to an LC/MS interface (*ca.* 5-11/min). Mass detection was carried out in a positive ion mode by scanning repeatedly (10 s/scan) from *m/z* 100 to 1000 under the following conditions: fast atom bombardment (FAB) energy, 4 kV; emission current, 10 mA ; neutral gas, xenon.

GC/MS measurement : Fatty acid methyl esters were separated with a capillary column of Silicone OV-101 (30 m × 0.25 mm i. d., GL Science). The injector temperature was 250°C and column temperature was programmed from 150°C to 250°C at a rate of 4°C/min. Inlet to mass spectrometer was performed with a split mode and He was used as a carrier gas. Mass detection was carried out by scanning repeatedly (2 s/scan) from *m/z* 50 to 650 under the following conditions: ionization voltage, 70 eV ; ion source temperature, 200°C.

Repeated cold stress : The animals were exposed to repeated cold stress to induce nociceptive hypersensitivity using an automatic repeated cold stress apparatus (Hohdensha, Shizuoka, Japan). Repeated cold stress was performed as described previously.[6] Briefly, animals were exposed to cold environmental temp. (4°C) from 4 : 30 PM to 10 : 00 AM and for 30 min every hour between 10 : 00 AM and 4 : 30 PM for 3 days. They were kept at room temperature until the next day, when the antinociceptive effects ot drugs were examined.

Nociceptive test : The nociceptive threshold for mechanical stimulation of the mouse was determined using an analgesimeter (Ugo Basile, Milan, Italy). Pressure stimulation was applied to the tail with a wedge-shaped piston at a loading rate of 16 g/sec, and the pressure eliciting struggle responce was determined as nociceptive threshold adopting the method reported by Kuraishi *et al.*[7]

Data processing : Unless otherwise mentioned, results given are the means±S.E.M. Statistical significance was analyzed with one-way analysis of variance (ANOVA), followed by *post hoc* Dunnett's test ; $p<0.05$ was considered significant.

Results

Metabolites produced by Bacteroides fragilis

After incubation of aconitine with a bacterial suspension of *B. fragilis,* four spots (R_f=0.18, 0.46, 0.56 and 0.72) were detected by TLC. The two of them (R_f =0.18 and 0.46) were identified as benzoylaconine and aconitine, respectively by comparison of the R_f values and mass spectral data of authentic samples. The ^{1}H-NMR spectrum of a colorless oily substance (R_f=0.56) showed signals characteristic for the aconitine skeleton with four methoxy groups and five aromatic protons. The signals at δ 3.34 (H-16), 3.46 (H_b-18), 3.61 (H_a-18), 4.03 (H-6), 4.44 (H-15) and 4.86 (H-14), and other signals remained essentially unaffected (see experimental). When compared with that of aconitine, methyl protons of the acetyl group were replaced by methylene protons which appeared as multiples around at δ 1.33. In the ^{13}C-NMR spectrum, most of the carbon signals were quite similar to those of aconitine,[8] except for the downfield shift of the ester carbonyl carbon signal at C-8 (+2.5 ppm) and the presence of signals for the methylene carbons at δ 22-37. The FAB mass spectrum (positive ion mode) showed four protonated molecular ion peaks $[M+H]^+$ at m/z 842, 828, 814 and 800, a weak peak at m/z 586 and a base peak at m/z 105. Comparing with the ^{1}H-NMR, ^{13}C-NMR, and the FAB mass spectral data of LA (OBA and PBA) synthesized by reacting aconitine with oleic acid and palmitic acid, respectively, the metabolite was identified as LA (see experimental). However, the fatty acid composition in LA was different from that of OBA and PBA, and also different from that of LA isolated from processed aconite tuber.[3,5] By the use of Frit-FAB LC/MS, the fatty acid composition at C-8 of LA was estimated, where the molecular weight for LA was calculated to be "586+MW of fatty acid." The mass chromatograms showed seven protonated molecular ion peaks in the mass range of m/z 800 to 868 (Fig. 1). These peaks corresponded to LA acylated with a mixture of fatty

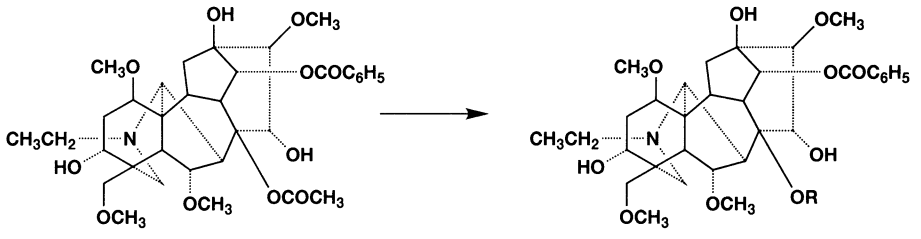

Chart 1. Conversion of aconitine to lipoaconitine by *B. fragilis*.

Table I Fatty acid compositions of LA formed by *B. fragilis* and of the bacterial cells.

A Mass number		B % of Fatty acids		C Fatty acid identified
$[M+H]^+$ of LA	$[M]^+$ of fatty acid	LA	Bacterial cells	
800	214	4.3	5.4	anteiso-C13 : 0
814	228	1.6	2.1	n-C14 : 0
828	242	18.7	30.4	n-C15 : 0
		28.2	26.1	anteiso-C15 : 0
842	256	12.7	15.6	n-C16 : 0
868	282	8.5	13.0	n-C18 : 1

A, from Frit-FAB LC/MS of LA ; **B** and **C**, from GC/MS

acids having chain lengths of 13-18 carbon atoms. The observation that two intense peaks were seen at the same molecular ion peak (*m/z* 828) and that their individual mass spectra were almost identical suggested that these two peaks were of lipoaconitine containing two different fatty acid (branched and non-branched) residues with the same chain length (C15 : 0) (Fig. 1 and Table I). The peaks at *m/z* 800 and 842 were relatively abundant and correspond to LA containing C13:0 and C16:0 fatty acid residues, respectively.

After methanolysis of LA, GC/MS analysis was carried out in order to determine the fatty acid composition in LA. When the retention times and mass spectra were compared with those of standard fatty acid methyl esters under the same analytical conditions, the fatty acid composition in LA was confirmed to be a mixture of saturated fatty acids (n-C14 : 0, n-C15 : 0, n-C16 : 0, n-C18 : 0), branched fatty acids (anteiso-C13 : 0, anteiso-C15 : 0), and unsaturated fatty acid (C18 : 1) (Fig. 2 and Table I).

Analysis of the cellular fatty acids of *B. fragilis* by GC/MS (Table I) showed the high percentages of n-C15 : 0, anteiso-C15 : 0 and n-C16 : 0 together with appreciable amounts of anteiso-C : 13 : 0, n-C14 : 0 and C18:1, and a quite low amount of n-C18 : 0 (see Fig. 2). From this finding, it was evident that the fatty acid composition in LA produced after incubation with *B. fragilis* was similar to that of bacterial cells. This was further suggested by the observation that LA produced by different bacterial strains and fecal flora observed in the mass chromatogram traced at different *m/z*. LA produced by *Klebsiella pneumoniae*

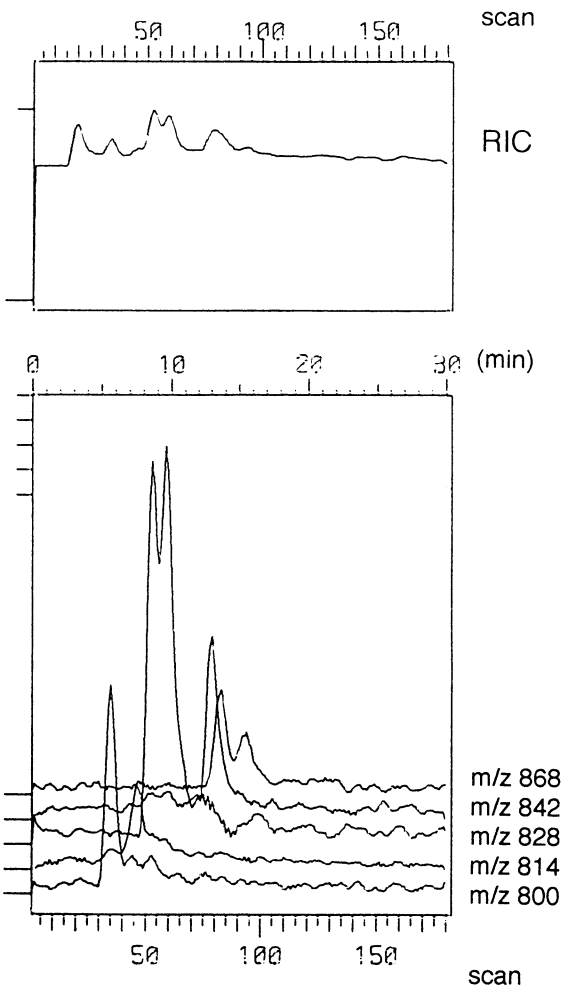

Fig. 1 Frit-FAB LC/MS chromatogram of LA formed after incubation of aconitine with *B. fragilis*.
Column, Nuclosil 5CN (25 cm × 4.6 mm i.d.) ; mobile phase, CH_3CN-H_2O-AcOH-glycerol (50 : 50 : 1: 0.5) ; flow rate, 0.8 ml/min ; FAB energy, 4 kV; detection, positive ion mode.

Table II Fatty acid compositions of LA formed by *K. pneumoniae, C. butyricum* and a human fecal suspension.

$[M+H]^+$ (m/z)	Expected fatty acid	*K. pneumoniae*	*C. butyricum*	Fecal suspension
800	C13 : 0	ND	ND	+
814	C14 : 0	+	ND	ND
840	C16 : 1	ND	+	+
842	C16 : 0	+ *	+	+ *
854	C17 : 1	+	ND	ND
868	C18 : 1	+	+ *	+
870	C18 : 0	ND	+	+
882	C19 : 1	+	ND	ND

* Major fatty acid. Symbol : +, detected ; ND, not detected.

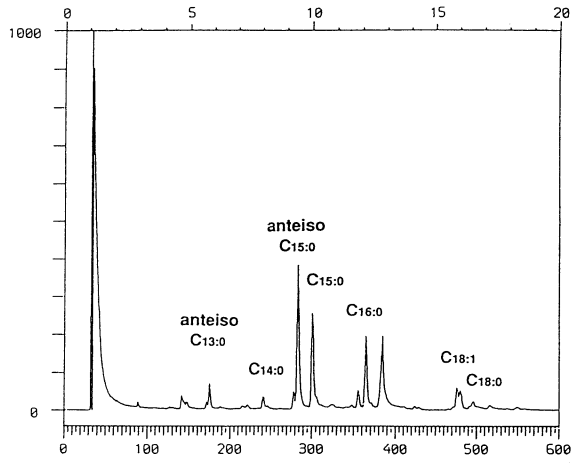

Fig. 2 GC/MS ion chromatogram of the fatty acid methyl esters of LA formed after incubation of aconitine with *B. fragilis*.
Column, silicone OV-101 (30 m × 0.25 mm i.d.) ; column temperature, 150-250°C, 4°C/min ; carrier gas, He (50 ml/min) ; inlet mode, split ; ionization voltage, 70 eV.

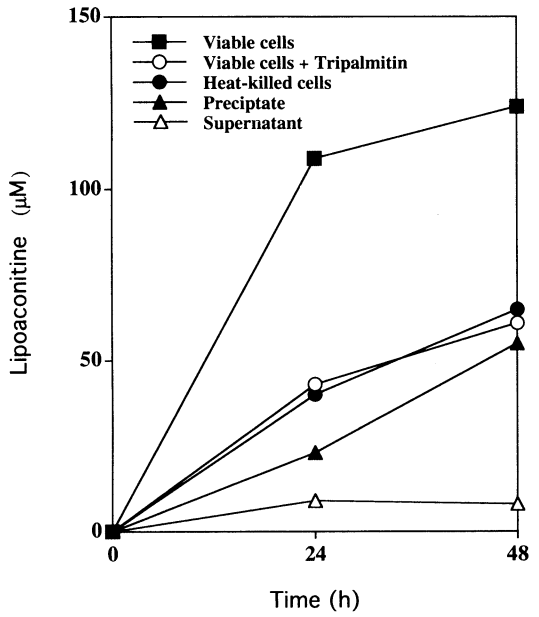

Fig. 3 Time course for the formation of LA after incubation of aconitine with viable and heat-killed cells of *B. fragilis*, and the supernatant and precipitate of the sonicated cells in phosphate buffer.
Viable cells of *B. fragilis* (■), the heat-killed cells (●), the viable cells in the presence of 2 mM tripalmitin (○), the supernatant (△) and precipitate (▲) of the sonicated cells.

contained saturated fatty acids (C14 : 0, C16 : 0) and unsaturated fatty acids (C17 : 1, C18 : 1 and C19 : 1), and hexadecanoic acid (C16 : 0) was found to be the major one. However, LA produced by *Clostridium butyricum* contained C16 : 0. C18 : 0, C : 16 : 1 and C18 : 1, and C18 : 1 was considered as the major fatty acid residue, while LA obtained after incubation with a human fecal suspension contained C16 : 0 as the major one (Table II).

Formation of LA from aconitine was further inverstigated with suspensions of viable, heat-killed and sonicated cells of *B. fragilis*. LA was formed up to a concentration of 109 μM by incubation with the viable cells for 24 hrs, then the concentration was slowly increased to reach 124 μM at 48 hrs (Fig. 3). On the other hand, the incubation with the supernatant obtained from the sonicated bacterial suspension resulted in the formation of a negligible amount of LA even after 48 hrs, while the precipitate led to the

formation of a relatively small amount of LA. Formation of LA was also observed by incubation of aconitine with palmitic acid but not with the triglyceride, tripalmitin, in the absence of cells (data not shown).

Antinociceptive effects of lipoaconitine

In naive, healthy mice, aconitine at doses of 0.1 and 0.15 mg/kg significantly elevated the nociceptive threshold (Fig. 4A). The effect of a dose of 0.10 mg/kg peaked 45 min after administration and the nociceptive threshold was increased by 74.2±13.8 g (n=6) from the pre-administration level (99.8±1.9 g). Although gross behaviors of mice given 0.10 mg/kg were not apparently different from untreated ones, a higher dose of 0.15 mg/kg produced abnormal behaviors such as salivation, tremor and decrease in locomotor activity. 8-*O*-Oleoylbenzoylaconine (OBA) was almost inactive at doses of 0.3 and 1.0 mg/kg. OBA at the highest dose tested (3.0 mg/kg) produced a short-lasting increase in nociceptive threshold (Fig. 4B) but showed abnormal behaviors similar to those of aconitine. However, 8-*O*-Palmitoylbenzoylaconine (PBA) at doses of 1, 10 and 30 mg/kg did not alter the nociceptive threshold (Fig. 4C) and gross behaviors of naive mice.

Three-day exposure of repeated cold stress produced 35.5 % (±8.4, S.D., n=6) decrease in nociceptive threshold (Fig. 5). When administered into such

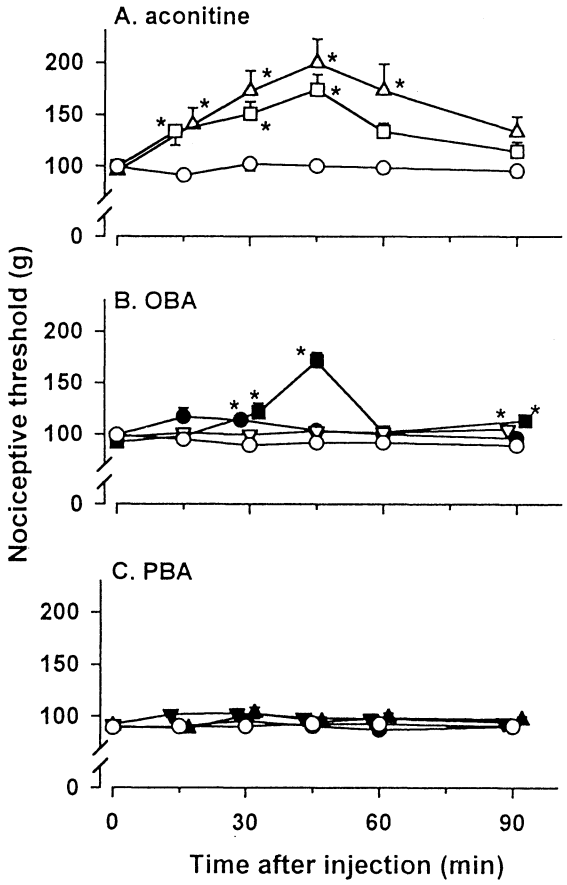

Fig. 4 Effects of aconitine, OBA and PBA on the nociceptive threshold of naive mice.
The mouse was given a subcutaneous injection of aconitine (A), OBA (B), PBA (C), at doses of 0.1 (□), 0.15 (△), 0.3 (▽), 1.0 (●), 3.0 (■), 10 (▲) and 30 mg/kg (▼), or vehicle (○). Values are the means and S.E.M. of 5-6 animals. *p<0.05 as compared with vehicle.

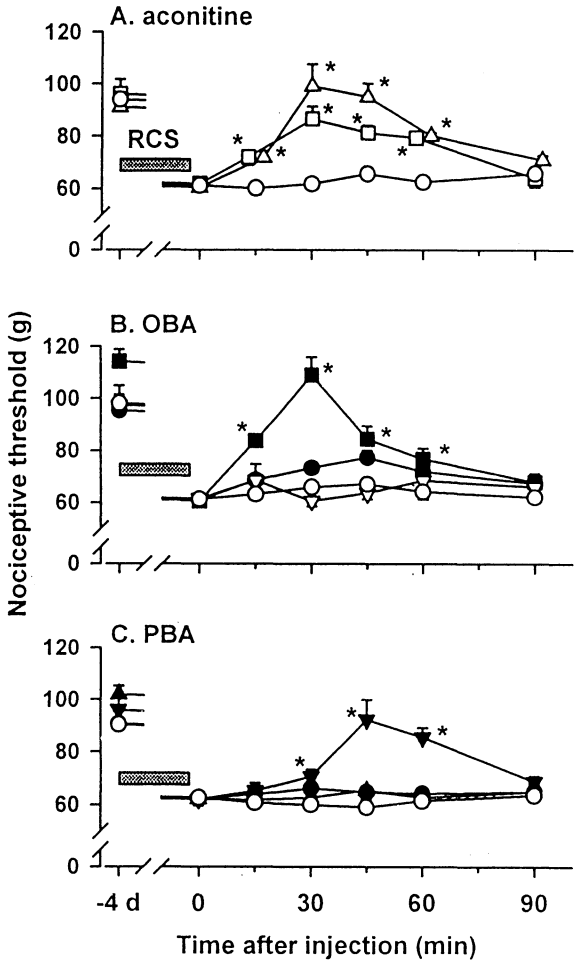

Fig. 5 Effects of aconitine, OBA and PBA on the nociceptive threshold of mice with nociceptive hypersensitivity induced by repeated cold stress (RCS).
The mouse was exposed to RCS (indicated by hatched bars; see text for details) and then given a subcutaneous injection of aconitine (A), OBA (B), PBA (C), at doses of 0.1 (□), 0.15 (△), 0.3 (▽), 1.0 (●), 3.0 (■), 10 (▲) and 30 mg/kg (▼), or vehicle (○). Values are the means and S.E.M. of 5-6 animals. *p<0.05 as compared with vehicle.

mice with nociceptive hypersensitivity, aconitine (0.10 and 0.15 mg/kg) produced a significant increase in nociceptive threshold (Fig. 5A). The effect of a dose of 0.10 mg/kg peaked at 30 min and the nociceptive threshold was increased by 24.8±5.2 g (n=6). OBA increased the nociceptive threshold at a toxic dose of 3.0 mg/kg, but lower doses of 0.3 and 1.0 mg/kg were without effects (Fig. 5B). PBA at a dose of 30 mg/kg produced a significant increase in the nociceptive threshold without other behavioral effects (Fig. 5C). At lower doses of 1.0 and 10 mg/kg, PBA was without effects on nociceptive threshold and gross behaviors.

Discussion

It was not surprising that aconine and benzoylaconine were intestinal bacterial metabolites of aconitine as a result of bacterial hydrolysis, as shown in the present experiment. However, the finding that conversion of aconitine to LA was effectively proceeded by intestinal bacteria provided conclusive evidence that anaerobic bacteria of the human intestine play an important role in reducing the toxicity of aconitine which owes much of its toxicity to the acetyl group at C-8. This conclusion relied on the finding that fatty acid composition in LA produced by *B. fragilis* was similar to that of the cellular fatty acids of the strain used and different from that contained in LA isolated from processed aconite tubers.[3,5] The observation that the anteiso-C15:0 fatty acid was found as a major residue in LA produced by *B. fragilis* was in accordance with the fact that anteiso-C15 : 0 is the major cellular fatty acid in genus *Bacteroides*.[9] Similar correlations were also obtained when *K. pneumoniae* (major acid residue in LA was C16 : 0) and *C. butyricum* (major acid in LA was C18 : 1) were used (data not shown). This valuable information was obtained by the use of Frit-FAB LC/MS, although separation of LA with the same carbon number of fatty acids (normal or branched) was not satisfactory. Within the first 24 hrs, the time for maximal growth of most bacterial strains, viable bacteria showed high ability to convert aconitine to LA, and then the rate of LA formation was almost equivalent in the presence or absence of bacteria. This observation led us to investigate the effect of pH on the yield of LA. In the absence of bacteria, LA formation significantly proceeded at neutral pH, when aconitine was incubated with palmitic acid in 0.2 M phosphate buffer (data not shown). In the presence of bacteria, the pH of the medium turned acidic (pH 6.8) after prolonged incubation (48 hrs) probably due to the release of volatile fatty acids (like acetic, propionic and lactic acids), and this acidity may negatively affect the conversion rate of aconitine to LA. On the other hand, under the slightly alkaline condition of the becterial cytoplasm (pH 7.5),[10] aconitine taken up by the cell membrane was possibly transesterified by the cellular fatty acids. When tripalmitin was added to a suspension of viable bacteria, the amount of LA formed was significantly lowered to reach that formed after incubation of aconitine with a suspension of heat-killed bacteria. This was in accordance with the fact that lipophilic substances tend to destroy the energized state of the bacterial membrane and thus prevent the uptake and release of compounds.[10] However, when lipase was added to the incubation mixture containing tripalmitin, the amount of LA produced was significantly increased after 24 hrs (data not shown). This finding emphasized the role of intestinal bacteria and its enzymes (lipase and esterase) in the liberation of fatty acids and the conversion of aconitine to LA.

Aconitine at a dose as small as 0.10 mg/kg inhibited nociceptive response of healthy mice, without effect on gross behaviors. However, abnormal behaviors were also elicited by a little higher dose of 0.15 mg/kg. Thus this alkaloid has strong antinociceptive and toxic potencies. OBA was less potent in antinociceptive action and toxicity than aconitine, and both antinociceptive and toxic effects became apparent at the same dose (3.0 mg/kg). PBA was much less potent in antinociceptive action and toxicity than aconitine; it did not alter the nociceptive threshold and gross behaviors of healthy animals at all doses tested (1-30 mg/kg). These results suggest that even if aconitine is substantially converted to OBA and PBA by human intestinal bacteria, OBA and PBA do not play an important role in the antinociceptive action of aconite root and the alkaloid aconitine.

Kei-kyo-so-so-oh-shin-bu-to (桂姜棗草黃辛附湯), a Kampo prescription containing aconite tubers, at per-oral dose of 30-300 mg/kg produced dose-depen-

dent antinociceptive effects in mice and rats with nociceptive hypersensitivity induced by repeated cold stress, while it was without effect in healthy animals.[6,7] In the present experiments, PBA produced antinociceptive effect in mice with nociceptive hypersensitivity, without effects in healthy animals. However, as the antinociceptive dose (30 mg/kg) was relatively high, PBA may not be primarily involved in the antinociceptive action of this Kampo prescription. Aconitine and OBA inhibited nociceptive responses in healthy mice and mice with nociceptive hypersensitivity at relatively low doses. However, their effects were less in nociceptive hypersensitive mice than in healthy ones. Although lipoaconitine (the intestinal bacterial metabolites of aconitine) may not play an important role in the antinociception of aconite tuber, it seems of particular interest to investigate other pharmacological effects of the less toxic lipoaconitine.

和文抄録

Aconitineはヒト腸内細菌とインキュベーションすることにより，lipoaconitine (LA) に変換された。Frit-FAB LC/MSやGC/MSによる分析結果から，このLAの脂肪酸組成は用いた菌種の脂肪酸組成と類似していることが明らかとなった。すなわち，*Bacteroides fragilis* から生成するLAの主な脂肪酸はanteiso-C15 : 0, n-C15 : 0, n-C16 : 0であり，*Klebsiella pneumoniae* や糞便の細菌叢によって生成するものはn-C16 : 0であった。また，*Clostridium butyricum* からのLAはC18 : 1, C18 : 0, C16 : 1, C16 : 0であった。LAはまた，リン酸緩衝液中でaconitineと加熱処理した細菌や細胞破砕後の沈殿物とのインキュベーションによっても生成した。

Aconitineは0.1 mg/kgの用量で侵害受容高感受性マウスにおける侵害受容閾値を増大させたが，8-*O*-oleoylbenzoylaconine (OBA) は3.0 mg/kgの高用量で活性を示し，毒性も見られた。8-*O*-Palmitoylbenzoylaconine (PBA) は30 mg/kgで活性を示した。これらの知見からORA, PBAは附子やそのアルカロイド (aconitine) の抗侵害受容作用に重要な役割を演じてはいないことが示唆された。

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