Processed Aconite Root Prevents Cold-Stress-Induced Hypothermia and Immuno-Suppression in Mice

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Processed aconite root (PA) is a crude drug used in traditional Chinese or Japanese medicine to generate heat in interior body and dispel cold. We evaluated the effects of PA on hypothermia and reduction in the activity of natural killer (NK) cells in mice exposed to chronic cold stress. Male mice were reared at 4 °C, and powdered PA was administered for 10 d as a food additive. Core body temperature of mice significantly decreased by approximately 1 °C after rearing in a cold environment, and PA administration significantly restored the reduction in core body temperature in a dose-dependent manner. After 10 d, splenic NK-cell activity of cold-stressed mice was significantly reduced, and the reduction was dose-dependently recovered by PA administration. An aconitine-type alkaloid fraction prepared from PA was ineffective when administered to cold-stressed mice, and the thermogenic effect on hypothermic mice was present in the fraction containing low-molecular-weight compounds without alkaloids. In cold-stressed mice, the weight of brown adipose tissue (BAT) and uncoupling protein (UCP)-1 level in BAT increased, whereas the weight of white adipose tissue decreased. The increase in UCP-1 level in BAT of cold-stressed mice was further augmented by PA treatment. These results indicate that PA exhibited a thermogenic effect on hypothermia induced by cold stress in mice by additional upregulation of UCP-1 level in BAT, which was already enhanced by hypothermia, and that the active ingredients present in PA are non-alkaloidal low-molecular-weight compounds.

Key words   processed aconite root; hypothermia; cold stress; alkaloid; uncoupling protein-1

Homeostasis is a mechanism by which internal environment of a living organism is maintained at a stable and constant condition in order to adapt to a broad range of external environments.[1] In endothermic animals, homeostatic regulation involves not only the maintenance of the core body temperature but also of the blood glucose level, blood pressure, and circadian rhythms. When an animal is temporarily exposed to a cold environment, several cold receptors sense this stimulus, and transmit the information to the hypothalamus, thereby resulting in contraction of periphery blood vessels, increase in metabolic rates and shivering thermogenesis to maintain constant core body temperature. However, chronic exposure to cold stress disturbs homeostasis, gradually resulting in an unstable internal environment and increased risk of illnesses. In animal experiments, chronic cold stress reduced the core body temperature,[2] induced hypertension,[3,4] anxiety-like behavior,[5] and suppressed immune functions, including the activity of natural killer (NK) cells[6] and antibody production.[7] Therefore, cold stress could lead to the development of dysfunctions in some organs.

The principal theory of traditional Chinese medicine (TCM) or traditional Japanese medicine (kampo) is the balance of "yin" (negative) and "yang" (positive). It is thought that the human body functions by balancing yin and yang, which are two opposing and competing aspects of one phenomenon in the body, and health is achieved by adopting the middle course of this balance. The function of the human body is to maintain a balance between yin and yang, and disturbance in this balance results in a diseased state. In medicinal treatment of TCM, the drugs to restore such imbalances were prescribed.[8] The concept of "yin-yang" is poorly understood from the viewpoint of modern sciences, but the idea that the body maintains several types of balance is very similar to the physiological concept of homeostasis. Cold or heat syndrome in TCM or kampo medicine is one of the principal

aspects of "yin-yang" balance. Cold syndrome involves not only hypothermia but also other symptoms such as paleness, peripheral chill, spasm and diarrhea. When patients are diagnosed to have interior cold syndrome behind their diseased state, drugs that generate heat within the body are prescribed. Such effects are not available in modern medicine except for the adverse effect of interferons, though there are wide varieties of antipyretics.

In TCM, the aconite root is a well-known crude drug that has strong effects to generate heat within the body and dispel the cold.[8] However, raw aconite root contains highly toxic alkaloids such as aconitine, mesaconitine, and hypaconitine; LD_{50} of the raw aconite root for oral administration in mice was reported to be 0.5—1.8 g/kg.[9] It is therefore very difficult for physicians or pharmacists to utilize raw aconite root, and various processing methods to reduce the toxicity have been developed since ancient times. Processed aconite root (PA), which is the dried material of heat-treated or autoclaved aconite root, is registered in the fifteenth edition of the Japanese Pharmacopoeia (JPXV).[10] Highly toxic aconitine-type alkaloids are degraded into less toxic alkaloids (*e.g.* benzoylmesaconine) by heating or autoclaving.[9] It was shown that PA exhibits analgesic activity, and that the active ingredients were aconitine-type alkaloids.[11—14] However, the thermogenic effects of PA and its active ingredients have yet to be evaluated, and thus may lead to discovery of novel drugs that can be used in hypothermia treatment.

In this study, we evaluated the effects of PA on hypothermia and reduction in the activity of NK cells in mice exposed to chronic cold stress. Moreover, we investigated the mechanisms of action and the active ingredients present in PA.

MATERIALS AND METHODS

Processed Aconite Root (PA) and Its Fractionation   PA

(JPXV; lot number 262114), autoclaved root of *Aconitum carmichaeli* DEBEAUX (Ranunculaceae), was purchased from Uchida Wakanyaku (Tokyo, Japan). According to the manufacturer, alkaloid contents of PA were: 0.5—1.4% for total alkaloids, 50 $\mu g/g$ for aconitine, and 150 $\mu g/g$ for mesaconitine. PA was supplied as small pieces by cutting the whole crude drug into 2—4 mm blocks. Powdered PA was prepared in our laboratory by a mill and maintained in a desiccated condition. HPLC fingerprint of PA is shown in Fig. 1A. Powdered PA (50 mg) was extracted with 70% EtOH (1.0 ml) by sonication for 30 min at room temperature. After centrifugation at 14000 g for 7 min, the supernatant (25 μl) was applied to HPLC analysis under the following conditions: systems, Shimadzu LC-10A$_{VP}$ (Kyoto, Japan); column, TSK-GEL ODS-80$_{TS}$ (4.6×250 mm; Tosoh, Tokyo); mobile phase, 0.05 M AcONH$_4$ (pH 3.6)/CH$_3$CN 90:10→0:100 (0→80 min), linear gradient; flow rate, 1.0 ml/min; column temperature, 40 °C; and detection, 200—400 nm by a photodiode array detector.

Quantification of benzoylmesaconine in aconite samples was conducted according to the method described in the JPVX.[10] In brief, 2 g of powdered PA was extracted with a mixture of 1.6 ml of 10% NH$_3$ and 20 ml of diethyl ether by sonication for 30 min at room temperature. After centrifugation (1500 g, 15 min), the ether layer was separated, and the extraction procedure repeated three times. All extracts were combined and evaporated under reduced pressure below 40 °C. The residue was dissolved in 1.0 ml of ethanol, and applied to HPLC. Benzoylmesaconine (Wako Pure Chemicals, Osaka, Japan) dissolved in ethanol was used as the standard. The HPLC conditions were as follows: column, Cosmosil 5C18-AR-300 (4.6×150 mm; Nacalai Tesque, Kyoto); mobile phase, 1.0 ml/min of 0.1% tetrafluroacetic acid/tetrahydrofran 10:1; column temperature, 40 °C; detection, 235 nm, retention time of benzoylmesaconine, 10.5 min; range of quantification, 0.149—1.19 μg of benzoylmesaconine hydrochloride/10 μl of the sample as benzoylmesaconine hydrochloride. The benzoylmesaconine hydrochloride content in PA used in the present study was 0.12 (w/w) %.

For preparation of the boiled water extract of PA, small pieces of PA (10 g) were boiled in 500 ml of water for 30 min, and the filtrated decoction was lyophilized. The extract yield was 2.7 g.

Alkaloids were extracted from PA according to the method described in the JPVX.[10] Powdered PA (10 g) was immersed in H$_2$O (60 ml), and 10% NH$_3$ (20 ml) and diethyl ether (400 ml) were added. After sonication for 30 min, the mixture was centrifuged at 1500 g for 15 min. The upper ether layer was separated, and 10% NH$_3$ (20 ml) and diethyl ether (400 ml) were added to the lower layer. The extraction procedure was repeated four times, and the combined ether layers were evaporated under reduced pressure to dryness. The yield of the alkaloids fraction (AL) was 0.15 g. The lower layer and the residue were suspended in H$_2$O, lyophilized to dryness, and regarded as the fraction without alkaloids (FWA). The yield of FWA was 9.8 g. When AL was analyzed by thin-layer silica gel chromatography (Silica gel 60 F$_{254}$; Merck, Darmstadt, Germany; mobile phase, AcOEt/EtOH/28% NH$_3$ solution 40:3:2; 15 cm) sprayed with Dragendorff reagent (Sigma-Aldrich, St. Louis, MO, U.S.A.) followed by 10% NaNO$_2$,[10] several positive spots appeared.

One of the spots was identified as benzoylmesaconine (*Rf*, 0.59) by comparison with the standard, whereas there were no Dragendorff-positive spots in FWA, indicating that FWA did not contain alkaloids. Based on HPLC analysis, it was observed that the AL fraction contained 5.0 (w/w) % of benzoylmesaconine, whereas benzoylmesaconine was undetectable in FWA.

FWA was boiled in 600 ml of water for 60 min. The decoction was filtered, and the residue was further boiled in 600 ml of water for 60 min. This process was repeated three times, and the filtrate mixed. The weight of insoluble residue (RE) was 5.5 g. The filtrate was lyophilized and dissolved in 200 ml of water to which 800 ml of EtOH was gently added with stirring. The suspension was centrifuged (1500 g, 10 min), and the supernatant was collected. The precipitate was dissolved in 200 ml of water, and this process was repeated again. The combined supernatant and the precipitate were both lyophilized, and the fractions of low-molecular-weight compounds (LMW, 1.0 g) and high-molecular-weight compounds (HMW, 2.1 g) were obtained. The HPLC fingerprint and fractionation scheme are shown in Figs. 1 and 2, respectively.

Animal Experiments Male ddY mice (4 week-old, Japan SLC, Hamamatsu, Japan) were housed in 3—4 mice per cage in a temperature-controlled room (24±1 °C) with lighting from 7:00 a.m. to 7:00 p.m. Mice were trained for the measurement of rectal core body temperature by inserting a cannula-type thermosensor (PTI-200 combined with PTC-301; Unique Medical, Tokyo) approximately 5 mm into the anus for 5 d. Mice were provided standard murine powdered chow (MF, Oriental yeast, Tokyo) and water *ad libitum* under conventional conditions in whole experimental days. For the
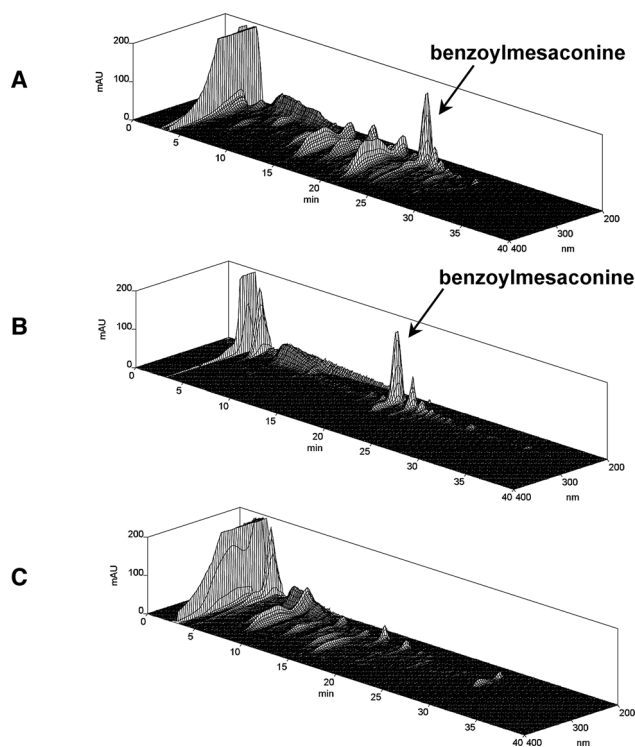

Fig. 1. HPLC-Fingerprints of Samples

(A) Processed aconite root (PA). (B) Alkaloid fraction of PA (AL). (C) Low-molecular-weight fraction without alkaloids (LMW). Each sample (50 mg) was extracted with 70% EtOH. See Materials and Methods for HPLC conditions.

Processed aconite root (PA) powder
(10 g)

Sonicated in diethylether and
NH$_3$ water （400 mL × 4）

Ether layer
Alkaroid fraction
(AL; 0.15 g)

Water layer and the residue
Fraction without alkaloids
(FWA; 9.8 g)

Dried up
Boiled in water (60 min x 3)
Centrifugation

Supernatant

Insoluble residue
(RE; 5.5 g)

Add 4-fold EtOH
Centrifugation

Supernatant
Low moleculer weight compounds
without AL fraction (LMW; 1.0 g)

Precipitate
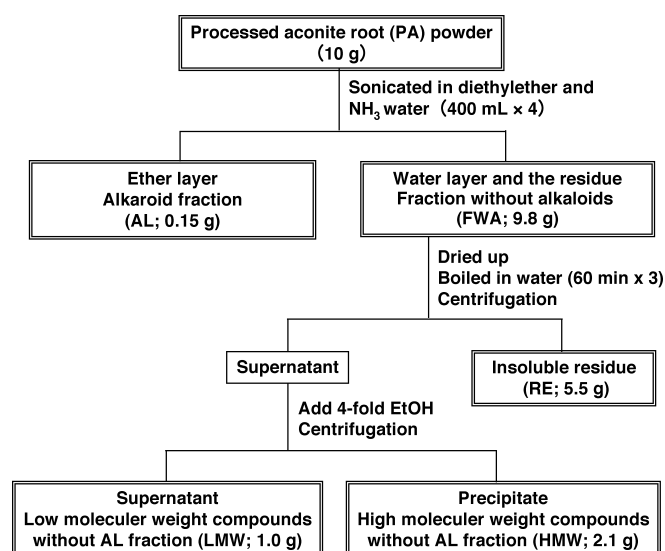High moleculer weight compounds
without AL fraction (HMW; 2.1 g)

Fig. 2. PA Fractionation

The contents of benzoylmesaconine (marker compound of alkaloids) are 0.12 (w/w) % in PA, 5.0 (w/w) % in AL, and undetectable in other fractions.

experiment, mice were housed in a room at normal temperature (24 °C; normal group, $n=7$), at cold temperature (4 °C) with chow containing no sample (control group, $n=7$) or the samples (sample-treated groups, $n=7$ each) for 10 d. Core body temperature and body weight of the mice were measured every 2 d starting from 1 d till 9 d after initiation of the cold-stress treatment, and also 3 d and 1 d before the treatment. The measurement was performed between 1:00 and 3:00 p.m. of each day. At the same time, the remaining chow was weighted and their food intakes for 2 d were calculated. On day 10, mice were sacrificed under excessive anesthesia with diethyl ether. Serum, spleen, interscapular brown adipose tissue (BAT), and epididymal white adipose tissue (WAT) were collected. These experimental procedures were approved by the Animal Care Committee at Graduate School of Pharmaceutical Sciences, Nagoya City University, in accordance with the guidelines of the Japanese Council on Animal Care.

PA powder, boiled water extract of PA, or the fractions of PA were separately mixed in powdered chow, and fed to the mice for 10 d. The dose of PA powder (1 g/kg/d) corresponded to ten-times the daily dosage for humans (6 g/d) converted by body weight. The concentration of samples in powdered chow was adjusted based on the changes of food intake and body weight of the mice. For normal and control mice, potato starch (Sigma-Aldrich) was mixed instead of the sample into the chow. In a separate experiment, it was confirmed that food intakes by mice were not significantly different between powdered and pelleted chows.

Measurement of NK-Cell Activity NK-cell activity was measured according to the method of Kraut and Greenberg.[15] Yac-1 cells (ATCC, Manassas, VA, U.S.A.) were suspended at a concentration of 1×10^6 cells/ml in RPMI 1640 medium (Invitrogen, Carlsbad, CA, U.S.A.) containing 5% fetal bovine serum (FBS; Japan Biosupply Center, Tokyo) labeled with 50 μCi of Na$_2$[^{51}Cr]O$_4$ (2.0 mCi/ml, MP Biomedicals, Irvine, CA, U.S.A.) for 12 h. Spleen cells collected from mice were suspended in RPMI 1640 medium, and lym-

phocytes were separated using Lympholyte-H (Cedarlane, Ontario, Canada) according to the manufacturer's instructions. Lymphocytes (1×10^5 cells in 100 μl), and ^{51}Cr-labeled Yac-1 cells (1×10^4 cells in 100 μl) were mixed and incubated for 12 h. The reaction mixture was then centrifuged and the radioactivity (RI) of the supernatant was measured using a gamma counter. RI of the culture supernatant of ^{51}Cr-labeled Yac-1 cells alone and 6% Triton X-114 (Sigma-Aldrich)-treated ^{51}Cr-labeled Yac-1 cells was measured for spontaneous release and maximum release, and NK-cell activity was calculated using the following formula: killing activity (%)=(RI of the sample−spontaneous RI release from Yac-1 cells)/(maximum RI release from Yac-1 cells−spontaneous RI release from Yac-1 cells)×100.

Measurement of Free Triiodothyronin (FT$_3$) and Corticosterone in Serum The FT$_3$ concentration in murine serum was measured using the Active® Free T$_3$ EIA kit (Diagnostic Systems Laboratories Inc., TX, U.S.A.) according to the manufacturer's instructions. The concentration of corticosterone in serum was measured by HPLC/mass spectrometry system (Quattro II, Waters, Milford, MA, U.S.A.). Two hundred microliters of each sample and standard was spiked with 20 μl of dexamethazone (10 μg/ml). The sample was loaded onto the Oasis HLB μElution plate (Waters), which was pre-conditioned with MeOH followed by 0.1 M HCl solution. The plate was washed with 0.1 M HCl solution and the analytes were eluted with 50 μl of methanol. The analytical column was a Cadenza CD-C18, 50×2.1 mm, 3 μm (Intakt Inc., Kyoto) and the mobile phase was MeOH/0.2% AcOH 65 : 35 at a flow rate of 200 μl/min. Electrospray ionization in positive mode single-ion measurement mode was used for the detection of corticosterone ([M+H$^+$] 347.1, t_R 2.0 min) and dexamethazone ([M+H$^+$] 393.1, t_R 2.3 min).

Western Blot Analysis Samples of BAT and WAT were homogenized with ice-cold homogenate buffer (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 250 mM sucrose, 1% Triton X-114, 1 mM dithiothreitol, 1% protease inhibitor cocktail (Sigma-Aldrich)) by sonication for 10 s. After incubation on ice for 30 min, the samples were centrifuged (5000 g, 15 min) at 4 °C. Fat on the sample solution was aspirated, and the supernatant was collected. The protein concentration of the supernatant was measured using a BCA™ Protein Assay kit (Pierce, Rockford, IL, U.S.A.). Proteins (5 μg for BAT and 60 μg for WAT) were separated by SDS-PAGE using 12% polyacrylamide gel. Proteins were transferred onto the Immobilon™-P Transfer Membrane (Millipore, Billerica, MA, U.S.A.), which was incubated with 1 : 1500 diluted rabbit anti-uncoupling protein (UCP)-1 polyclonal antibody (AnaSpec, San Jose, CA, U.S.A.) or 1 : 200 diluted rabbit anti-β-actin (AnaSpec). Antigen-antibody complexes were detected by incubating the membrane with a 1 : 10000 dilution of goat anti-rabbit IgG-horse radish peroxidase (HRP) conjugate (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, U.S.A.). Signals were detected with Immobillon™ Western Chemiluminescent HRP Substrate (Millipore), and quantified by densitometry using an image analyzer, LAS 3000 (Fuji Film, Tokyo). UCP-1 levels were compensated by the levels of β-actin, and data expressed as fold changes compared with the level in control mice.

Real-Time PCR Total RNA was extracted from WAT and BAT samples in each group using TRIzol reagent (Invit-

Table 1. Primer List

	Sence primer	Antisense primer	Product size
UCP-1	CAGGATTGGCCTCTACGACTCA	TGAACACTGCCACACCTCCA	116
PGC-1α	GGAGCAATAAAGCGAAGAGCA	TGTGGGTTTGGTGTGAGGAG	113
PPAR-α	CGTCGAAGACAAAGAGGCAGA	CAAGGAGGACAGCATCGTGAA	187
PPAR-γ	GAGCCTAAGTTTGAGTTTGCTGTG	GCAGCAGGTTGTCTTGGATG	151
FAS	GTGTGGTGGGTTTGGTGAA	TGGGCTTGTCCTGCTCTAAC	219
HSL	AGCACTACAAACGCAACGAGACAG	CCAGAGACGACAGCACCTCAATC	183
LCAD	GGCTTGCTTGGCATCAACATC	TCATGGCTATGGCACCGATACA	229
Cyclophilin B	AATGGCTCACAGTTCTTCATAACCA	GGCTGTCTGTCTTGGTGCTCTC	127

UCP-1, uncoupling protein-1; PGC-1α, peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α; FAS, fatty acid synthase; HSL, hormone sensitive lipase; LCAD, long-chain acyl-CoA dehydrogenase.
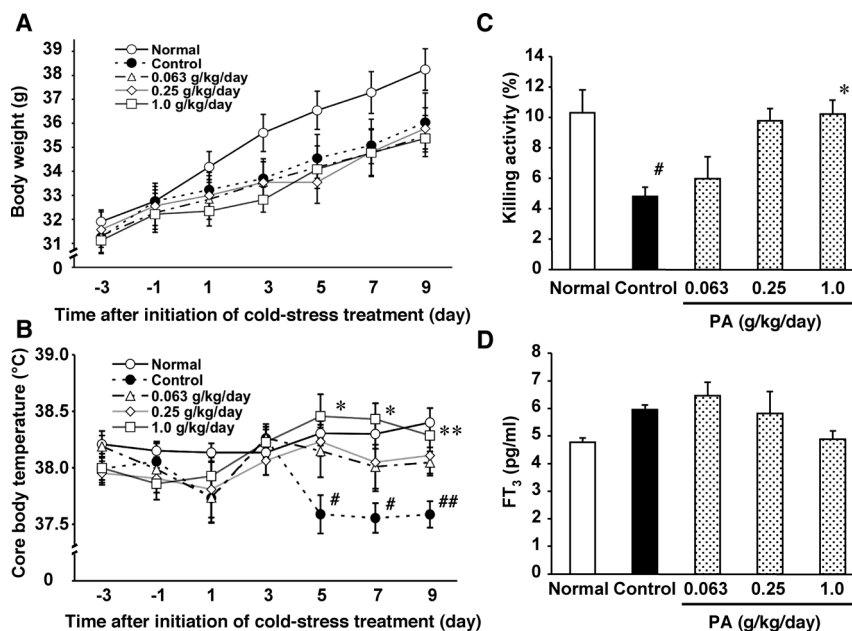

Fig. 3. Effects of PA on Physiological Changes in Mice Induced by Continuous Exposure to Cold

Mice were kept at 4 °C (control, closed circle); and PA (open triangle, 0.063 g/kg/d; open diamonds, 0.25 g/kg/d; open square, 1.0 g/kg/d) was administered for 10 d. Normal mice (open circle) were kept at 24 °C. The body weight and core body temperature were measured every 2 d starting from 1 d till 9 d after the initiation of the cold-stress treatment and also 3 d and 1 d before the treatment. The measurement was performed between 1 p.m. and 3 p.m. of each day. (A) Traces of alteration in body weight. (B) Effect of PA on core body temperature, which was measured in the rectum. (C) Effect of PA on activity of natural killer (NK) cells in the spleen. Lymphocytes were isolated from the spleen 10 d after initiation of the cold-stress treatment, and NK-cell activity determined by ^{51}Cr release assay. Killing activity implies the percentage of target cells (Yac-1) killed by effecter cells (splenic NK cells). (D) Free T$_3$ concentration in the serum collected on day 10. Significance is represented by #$p<0.05$, ##$p<0.01$ $vs.$ normal group, and *$p<0.05$, and **$p<0.01$ $vs.$ control group determined by the Bonferroni type multiple t-test.

rogen). DNAse I (Promega, Madison, WI, U.S.A.)-treated RNA (500 ng) was reverse-transcribed using 100 units of reverse transcriptase (ReverTra Ace; Toyobo, Osaka) with 0.1 mM oligo(dT) primer in a total volume of 20 μl. Real-time PCR was carried out on an ABI PCR7300 System (Applied Biosystems, Carlsbad, CA, U.S.A.) using two-fold diluted Power SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences were obtained from an online library (Table 1). ΔCycle threshold (Ct) was determined by subtracting the mean Ct value of cyclophilin B from that of the targeted gene, and data were expressed as fold changes compared with the level in control mice.

Statistical Analyses Data were expressed as mean±S.E. (n=7). Statistical analyses were performed by repeated one-way analysis of variance (ANOVA), combined with the Bonferroni type multiple t-test. $p<0.05$ was considered statistically significant.

RESULTS

PA Restored Physiological Changes in Cold-Stressed Mice Alteration in the body weight of mice exposed to cold stress is shown in Fig. 3A. The growth rate of cold-stressed mice tended to be lower than that of normal mice. Food intake in control mice was higher than that in normal mice after their housing in cold temperature (normal, 4.4 g/d/mouse; control, 6.5 g/d/mouse). Core body temperature significantly reduced by approximately 1 °C after day 5 in mice exposed to cold stress. Addition of powdered PA, its extract or its fractions into their food did not affect the reduction of body weight and the increase of food intake in cold-stressed mice. When powdered PA was administered to cold-stressed mice, the reduction of the core body temperature was significantly restored ($p<0.01$) in a dose-dependent manner (Fig. 3B). NK-cell activity of spleen lymphocytes collected from cold-stressed mice on day 10 was significantly reduced ($p<0.05$) compared with that of normal mice, and

Table 2. Effect of Processed Aconite Root (PA) on Core Body Temperature and NK-Cell Activity of Mice Exposed to Cold Stress

	Core body temperature (°C)	NK-cell activity (%)
Normal group	37.8±0.1	14.1±3.0
Control group	36.8±0.2††	2.7±0.9††
PA powder-treated group	37.6±0.1**	11.4±2.0*
PA extract-treated group	37.4±0.1*	7.2±1.5

Mice were kept at 4 °C (control); and powdered PA, or PA extract was administered for 10 d. The doses were corresponded to powdered PA (1.0 g/kg/d). Normal mice were kept at room temperature (24 °C). Core body temperature was measured 9 d and NK-cell activity of spleen lymphocytes was measured 10 d after initiation of cold-stress treatment. ††$p<0.01$ *vs.* normal group. *$p<0.05$, **$p<0.01$ *vs.* control group by the Bonferroni type multiple *t*-test.

Table 3. Effect of PA on Core Body Temperature and NK-Cell Activity of Normal Mice

	Core body temperature (°C)	NK-cell activity (%)
Normal	37.5±0.1	14.2±1.9
PA-treated group	37.6±0.1	14.2±0.9

Mice were kept at 24 °C (normal); and powdered PA (1.0 g/kg/d) was administered for 10 d. Core body temperature was measured 9 d and NK-cell activity of spleen lymphocytes were measured 10 d after initiation of cold-stress treatment. There is no significant difference by the Bonferroni type multiple *t*-test.

was significantly recovered ($p<0.05$) by administration of PA powder in a dose-dependent manner (Fig. 3C). Serum FT_3 concentration of cold-stressed mice on day 10 was slightly increased ($p<0.10$) compared with normal mice; however, it tended to decrease by the treatment with PA powder in a dose-dependent manner (Fig. 3D). Serum concentration of corticosterone was not affected by cold stress. Moreover, it was not affected by administration of PA powder (normal, 23.3±2.1 pg/ml; control, 26.4±6.2 pg/ml, 1.0 g/kg of PA powder, 22.2±3.4 pg/ml, respectively).

The ameliorating effects of PA extract on reduced core body temperature and NK-cell activity of cold-stressed mice were compared with those of PA powder: the former was less active than the latter (Table 2). When PA powder was administered to normal mice reared at 24 °C, it did not affect their food intake, body weight, core body temperature, or the NK-cell activity of spleen lymphocytes (Table 3).

Fractionation of PA To find the active ingredients of PA with respect to thermogeneic effects, the ammonia-ether extract of PA was separated into AL and residual FWA (Fig. 2). According to the ratio of yields of the fractions, dosages were set at 0.015 g/kg/d for AL, and 0.98 g/kg/d for FWA, corresponding to the dosage of PA dosage (1.0 g/kg/d). Reduced core body temperature in the cold-stressed mice was significantly recovered by treatment with PA or FWA ($p<0.01$), whereas AL did not exhibit any effect (Fig. 4A). The reduced NK activity of spleen lymphocytes of cold-stressed mice was also significantly recovered by administration of PA or FWA ($p<0.01$ and $p<0.05$) (Table 4). FWA was further separated into insoluble RE, LMW, and HMW. Dosages were set at 0.55 g/kg/d for RE, 0.10 g/kg/d for LMW, and 0.21 g/kg/d for HMW based on the yield of each fraction. Reduced core body temperature in cold-stressed mice was significantly restored by treatment with LMW
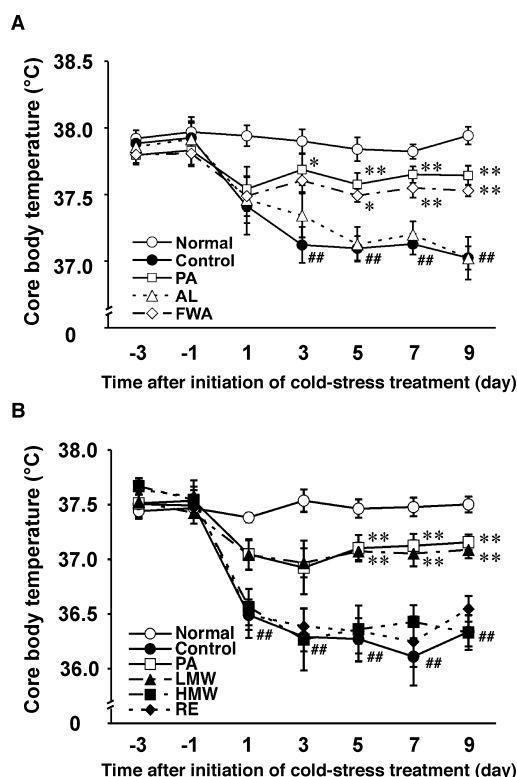

Fig. 4. Effects of the Fractions Prepared from PA on Low Core Body Temperature in Mice Induced by Continuous Exposure to Cold

Mice were kept at 4 °C (control, closed circle); and PA (open square), fraction with alkaloids (AL, open triangle), and without alkaloids (FWA, open diamonds) (A), or low-molecular-weight compounds of PA without alkaloids (LMW, closed triangle), high-molecular-weight compounds of PA without alkaloids (HMW, closed square), or the residue of PA (RE, closed diamonds) (B) were administered for 10 d. Normal mice (open circle) were kept at room temperature (24 °C). Core body temperature was measured every 2 d starting from 1 d till 9 d after the initiation of the cold-stress treatment and also 3 d and 1 d before the treatment. The measurement was performed between 1 and 3 p.m. of each day. Significance is represented by ##$p<0.01$ *vs.* normal group, and *$p<0.05$, and **$p<0.01$ *vs.* control group determined by the Bonferroni type multiple *t*-test.

Table 4. Effect of PA Fractions on NK-Cell Activity of Mice Exposed to Cold Stress

	NK-cell activity (%)
Normal group	15.6±1.5
Control group	6.1±0.2††
PA-treated group	12.0±1.4**
AL-treated group	6.0±0.5
FWA-treated group	13.8±2.0*

Mice were kept at 4 °C (control); and PA, its alkaloid fraction (AL), or its alkaloid-free fraction (FWA) was administered for 10 d. The doses were corresponded to powdered PA (1.0 g/kg/d). Normal mice were kept at room temperature (24 °C). NK-cell activity of spleen lymphocytes was measured 10 d after initiation of cold-stress treatment. ††$p<0.01$ *vs.* normal group. *$p<0.05$, **$p<0.01$ *vs.* control group by the Bonferroni type multiple *t*-test.

($p<0.01$); the extent was almost similar to that of PA. In addition, neither HMW nor RE had any effect on reduced core body temperature (Fig. 4B).

PA and Its LMW Fraction Upregulated UCP-1 Expression in BAT The weight of WAT in cold-stressed mice on day 10 was significantly reduced ($p<0.01$), whereas that of BAT significantly increased ($p<0.01$) compared with that in normal mice (Table 5). The weight of WAT tended to further reduce by treatment with either PA or LMW, but increase in

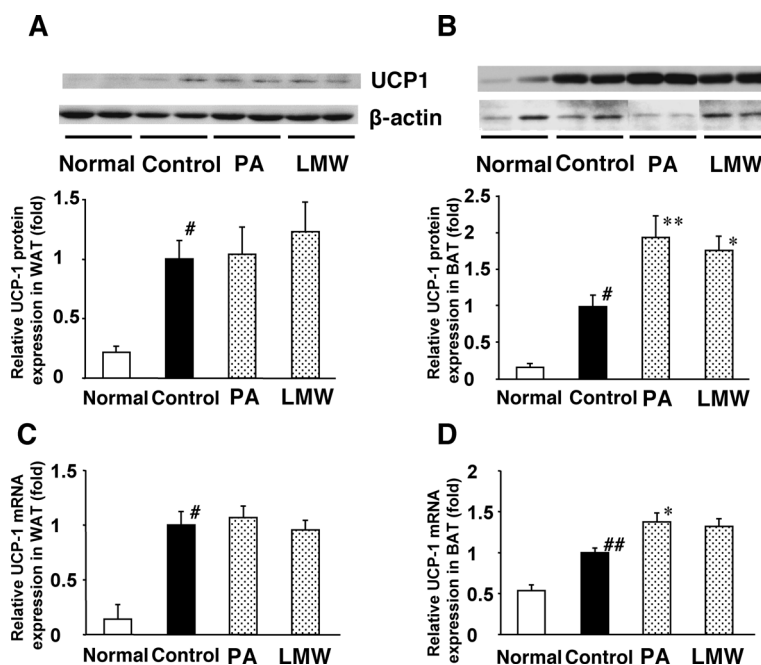**A**   **B**

Fig. 5.   UCP-1 Protein and mRNA Levels in White Adipose Tissue (WAT) and Brown Adipose Tissue (BAT) of Mice Exposed to Cold Stress

Mice were kept at 4 °C (control); and PA, the fraction containing low-molecular-weight compounds of PA without alkaloids (LMW) were administered. Normal mice were kept at room temperature (24 °C). Ten days after cold exposure, WAT and BAT of mice were collected and UCP-1 protein and mRNA levels were evaluated. The top panel shows UCP-1 protein levels assessed by Western blotting in BAT (5 μg protein, A) and WAT (60 μg protein, B). The UCP-1 protein levels were compensated by the expression of β-actin. The bottom panel shows UCP-1 mRNA levels assessed by real-time PCR in BAT (C) and WAT (D). The UCP-1 mRNA levels were compensated by the expression of cyclophilin, and values are shown as fold changes compared with levels in control mice. Significance is represented by ## $p < 0.01$ vs. normal group, and * $p < 0.05$ and ** $p < 0.01$ vs. control group determined by the Bonferroni type multiple t-test.

Table 5.   Effect of PA and Its Fraction on the Weight of White Adipose Tissue (WAT) and Brown Adipose Tissue (BAT) of Mice Exposed to Cold Stress

	WAT (% of body weight)	BAT (% of body weight)
Normal group	2.23±0.26	0.48±0.03
Control group	1.18±0.08††	0.67±0.03††
PA-treated group	0.92±0.05	0.65±0.03
LMW-treated group	0.90±0.08	0.62±0.03

Mice were kept at 4 °C (control); PA or its fraction containing low-molecular-weight compounds without alkaloids (LMW) was administered for 10 d. The doses were corresponded to powdered PA (1.0 g/kg/d). Normal mice were kept at room temperature (24 °C). WAT and BAT were collected 10 d after initiation of cold-stress treatment. †† $p < 0.01$ vs. normal group by the Bonferroni type multiple t-test.

Table 6.   Quantification of mRNA Levels in BAT and WAT of Mice Exposed to Cold Stress

BAT	PGC-1α	PPAR-α	PPAR-γ
Normal	0.57±0.12	1.17±0.22	1.16±0.13
Control	1.00±0.07	1.00±0.13	1.00±0.12
PA	1.82±0.28*	0.97±0.17	1.18±0.10
LMW	1.76±0.23	0.81±0.18	1.20±0.09

WAT	FAS	HSL	LCAD
Normal	2.66±0.13	0.51±0.15	0.29±0.13
Control	1.00±0.24	1.00±0.20	1.00±0.09
PA	1.16±0.20	1.25±0.15	1.06±0.17
LMW	1.49±0.11	0.91±0.14	0.93±0.05

Mice were kept at 4 °C (control); PA or its fraction containing low-molecular-weight compound without alkaloids (LMW) was administered for 10 d. The doses were corresponded to powdered PA (1.0 g/kg/d). Normal mice were kept at room temperature (24 °C). WAT and BAT were collected 10 d after initiation of cold-stress treatment, and mRNA levels of peroxisome proliferator-activated receptor (PPAR)-γ coactivator 1α (PGC-1α), PPAR-α, PPAR-γ in BAT, and fatty acid synthase (FAS), hormone sensitive lipase (HSL), and long-chain acyl-CoA dehydrogenase (LCAD) in WAT were evaluated. mRNA levels were compensated by cyclophilin, and values are shown as fold changes compared with the levels in control mice. * $p < 0.05$ vs. control group by the Bonferroni type multiple t-test.

the weight of BAT was not affected by PA or LMW (Table 5). mRNA and protein levels of UCP-1 in WAT and BAT of cold-stressed mice were significantly higher than those of normal mice ($p < 0.05$ or $p < 0.01$; Fig. 5). Although increased UCP-1 expression in WAT was not affected by treatment with PA or LMW, increased UCP-1 protein expression in BAT appeared to be significantly further upregulated in the PA- or LMW-treated mice ($p < 0.01$ and $p < 0.05$, respectively), and its mRNA expression in BAT of PA-treated mice was significantly higher than that of control mice ($p < 0.05$, Fig. 5). mRNA expressions of upstream genes for UCP-1 were also evaluated. In BAT, although mRNA expressions of peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ between the cold-stressed mice and the control were not different, PPAR-γ coactivator (PGC)-1α mRNA expression in cold-stressed mice was higher than that of normal mice, and was further upregulated by treatment with PA

($p < 0.05$, Table 6). In WAT, mRNA expression of fatty acid synthase (FAS) tended to be lower and that of hormone-sensitive lipase (HSL), and long-chain acyl-CoA dehydrogenase (LCAD) tended to be higher in cold-stressed mice than in normal mice. Treatment with PA or LMW did not exhibit a significant effect on mRNA expressions of these genes.

DISCUSSION

The present investigation clearly revealed that oral administration of PA restored the reduction in core body temperature and splenic activity of NK cells caused by chronic cold stress in mice in a dose-dependent manner. The degrees of recovery in hypothermia and reduced activity of NK cells correlated with each other in murine groups at different doses of PA, suggesting that restoration of NK-cell activity was due to stable core body temperature maintained by PA administration. The fact that PA did not increase core body temperature and NK-cell activity in mice under normal conditions suggested that PA did not directly stimulate thermogenesis but rather facilitated for the thermoregulation intercepted by cold stress. This is consistent with the characteristics of TCM drugs that maintain the balance of "yin and yang".

It is well known that application of various types of stress leads to the suppression of immune functions in rodents. Stress stimulates the secretion of adrenocorticotropic hormone from the pituitary gland, following which "stress hormones" such as corticosterone or cortisol are then secreted from the adrenal cortex.[1] Elevated levels of serum glucocorticoids were reported to play an important role in the stress-induced modulation of NK-cell activity.[16,17] However, it was reported that while serum corticosterone level increased in mice exposed to acute cold stress for 2 h, the level was normalized after they were exposed to cold stress for 2 weeks.[18] In accord with this study, serum concentration of corticosterone did not significantly vary in mice exposed to 10 d of cold stress. Furthermore, oral administration of PA did not affect serum concentration of corticosterone, but prevented reduction of NK-cell activity in cold-stressed mice. Therefore, reduction of NK-cell activity in chronic cold-stressed mice may not be related to serum concentrations of glucocorticoids. Instead, activation of the autonomic nervous system may be involved in chronic cold stress-induced reduction of NK-cell activity in mice because intraperitoneal injection of the β-blocker propranolol or intracerebroventricular injection of hydroxydopamine to break down the autonomic nervous system has recently been shown to alleviate the reduction of NK-cell activity by long-term cold stress in rats.[19] Dopamine may also be involved in this relationship because cold stress augmented dopamine levels in rat brains.[20] Moreover, rats with a hyper-reactive dopaminergic system exhibited reduced splenic activity of NK cells.[21] PA may restore reduced activity of NK cells in cold-stressed mice through the regulation of the autonomic nervous system.

Physiological thermogenenesis can be classified into "shivering" and "non-shivering" thermogenesis. Contraction of skeletal muscle is the principal mechanism for increasing heat output in the form of shivering and/or voluntary exercise. Non-shivering thermogenesis usually occurs in BAT, where heat is produced through the metabolism of free fatty acids in the mitochondria. Non-shivering thermogenesis in BAT plays an important role in thermoregulation in rodents exposed to cold environments.[22] In human, BAT was considered to be present only in infant age up to six months, however, recent research revealed that thermoregulation is mediated by BAT in most mammals including human adults.[23] Latest article described that the activity of BAT was observed in 23 of the 24 healthy men during cold exposure.[24] UCP-1 is a mitchondrial protein carrier, which causes H^+ influx into the matrix of mitochondria and bypasses the ATP synthase channel, thereby dissipating heat; it is essential for non-shivering thermogenesis in mammalian.[25,26] UCP-1 is specifically expressed in BAT in normal conditions, and in mice cold stress highly upregulates UCP-1 expression not only in BAT but also in WAT.[27] In this study, the weight of WAT in cold-stressed mice decreased, whereas that of BAT increased. These results suggested that cold stress stimulated thermogenesis by burning fat in WAT and non-shivering thermogenesis in BAT to regulate core body temperature. mRNA expression of FAS was slightly reduced, whereas expressions of HSL and LCAD were slightly increased in WAT of cold-stressed mice. Although neither mRNA expression of PPAR-α nor PPAR-γ was changed, mRNA and protein levels of UCP-1 in WAT and BAT were augmented in mice exposed to cold stress compared with the normal group. Increased expressions of UCP-1 and PGC-1α in BAT were further upregulated by administration of PA and its fraction LMW, whereas that in WAT was unchanged. PGC-1α is considered to be a master regulator of adaptive thermogenesis and UCP-1 expression in BAT,[28] and PPAR-γ is a central transcriptional regulator of the differentiation of brown and white adipocytes.[29] A recent report revealed that the PPAR-γ agonist rosiglitazone upregulated mRNA expressions of PPAR-α, PGC-1α, and UCP-1 in cultured adipocytes and that norepinephrine could stimulate the expressions of PGC-1α and UCP-1 without upregulating PPAR-α mRNA expression.[30] These results suggested that the preventive effect of PA on hypothermia in mice exposed to cold stress might not arise through a PPAR-γ agonistic mechanism but by sympathetic-mediated recruitment of PGC-1α and UCP-1 in BAT to augment the thermogenesis in BAT. The hypothesis that PA prevented hypothermia by some sympathetic-mediated mechanisms was also supported by the result that PA did not increase core body temperature and NK-cell activity in mice under normal conditions.

It was reported that chronic cold stress resulted in increased blood pressure and heart rate with elevated plasma adrenaline and noradrenaline concentrations in rats.[3] When rats were kept at 4 °C, heart rate was transiently augmented from day 2 to day 4, and then recovered to the normal level 6 d after the begining of chronic cold exposure, while blood pressure was gradually increased for 4 d and thereafter the elevated value became stable 6 d after the begining of cold exposure.[4] These results suggest that the rodents are acclimatized to the chronic cold stress at least 6 d after the stress-treatment started. This is consist with the present results that the core body temperature of the cold-stressed mice was unstable on day 1 and 3, and then became steady 5 d after the cold exposure. In the present study, however, we did not measure the blood pressure and plasma adrenaline and noradrenaline concentrations of the mice. Further studies about the thermogenous mechanisms of PA *via* these sympathetic-mediated events are required.

Thyroid hormones are also known to have long-term control of the metabolic rate, and thus, regulate heat production. In fact, serum concentration of FT_3 tended to increase in cold-stressed mice. However, this increase was suppressed by PA treatment in a dose-dependent manner, suggesting that

the thermogenic effect of PA is independent of the thyroid function.

PA is usually used as an analgesic to treat severe pain by rheumatoid arthritis, spondyloarthritis, or fibromyalgia syndrome.[31] It has been considered that the analgesic properties of aconite root are attributed to the presence of aconitine-type alkaloids, which act *via* the opioid κ receptor.[12,14] However, the analgesic effect of aconite root was greatly reduced by heat processing that hydrolyzed the ester linkage in aconitine-type alkaloids.[13] Rats with chronic constriction injury exhibited a reduced threshold to mechanical and thermal pain stimulus and augmentations of pain-related behaviors when reared in a decreased temperature condition by 7°C,[32] suggesting that hypothermia would be involved in the symptoms, including allodynia, hyperalgesia, and spontaneous pain. The theory of TCM or kampo medicine suggests that PA can be effective in patients with interior cold symptoms. The clinical analgesic effects of PA may be provided not only by the opioid-mediated action of aconitine-type alkaloids but also by the improvement of hypothermia and hyperalgesia due to non-alkaloidal thermogeneous compounds.

Although aconitine-type alkaloids and their degradation compounds obtained by processing are the characteristic constituents of PA, the present results unambiguously indicated that the active principles for the thermogeneous effect of PA are non-alkaloidal low-molecular-weight compounds. PA powder exhibited notably higher activity than the boiled water extract of PA, suggesting that the active ingredients are not quite soluble in water. In TCM or kampo medicine, the general dosage form of crude drugs is a decoction, whereas PA is usually prescribed as a powdered form.[31] This might reflect the long-term clinical experiences that the powdered form is more effective to treat hypothermia than the decoction. In addition, isolation and identification of the active compounds of the non-alkaloidal compounds in PA has not been reported before, only the sugars and amino acids have been studied.[33,34]

In conclusion, oral administration of PA prevented the reduction in core body temperature and activity of NK cells in the spleen. The thermogenic effect of PA was mediated by the upregulation of UCP-1 expression in BAT. The active ingredients were non-alkaloidal low-molecular-weight compounds. Further study to isolate and identify the active ingredients of PA is underway, and thus, may lead to the discovery of a novel drug that can be used in treating cold symptoms or excessive sensitivity to cold.

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