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Identificaton of 2,3-Dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one Isolated from *Lactobacillus pentosus* Strain S-PT84 Culture Supernatants as a Compound That Stimulates Autonomic Nerve Activities in Rats

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ABSTRACT: Intestinal administration of various lactobacilli has been reported to affect autonomic neurotransmission, blood pressure, and body weight in rats. In this study, three molecules (peaks A, B, and C) were isolated from *Lactobacillus pentosus* strain S-PT84 (S-PT 84) culture supernatants. Intraduodenal (ID) injection of these molecules increased or inhibited renal sympathetic nerve activity (RSNA) in rats as follows: peak A, 134%; peak B, 40.1%; peak C, 408%. Furthermore, we identified peak C as 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP). ID injection of DDMP increased brown adipose tissue sympathetic nerve activity (BAT-SNA; 118 ± 15.3%), whereas intraoral injection of DDMP increased the body temperature above the interscapular brown adipose tissue (BAT-T; 0.72 ± 0.13 °C) in rats. These data suggest that S-PT84 produces molecules that modulate autonomic nerve activity. In addition, DDMP increased BAT-SNA and BAT-T, and these changes in BAT-T may be caused by changes in BAT-SNA.

KEYWORDS: *2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP), Lactobacillus, autonomic nerve, renal system, brown adipose tissue*

INTRODUCTION

Probiotic strains of *Lactobacillus* have been shown to affect autonomic nerve activities and alter physiological phenomena, such as blood pressure (BP), blood glucose, and body weight.[1−4] Specifically, intestinal administration of *Lactobacillus johnsonii* strain La1 was shown to reduce renal sympathetic nerve activity and BP[1] while suppressing adrenal sympathetic nerve activity (ASNA) and blood glucose levels in rats.[2] Additionally, ingestion of *Lactobacillus paracasei* ST11 (NCC2461) increased white adipose tissue sympathetic nerve activity (WAT-SNA) and brown adipose tissue sympathetic nerve activity (BAT-SNA) and reduced body weight in rats.[3] Moreover, intraduodenal (ID) injection of NCC2461 in rats suppressed gastric vagal nerve activity (GVNA), accelerated renal sympathetic nerve activity (RSNA), and raised BP.[4]

Although these responses have been characterized, the mechanisms underlying these responses have yet to be clarified. In a recent study conducted in our laboratory, we found that ID injection of the lower molecular weight (LMW) fraction (including molecules less than 10000 Da) from *Lactobacillus pentosus* strain S-PT84 (S-PT84) culture supernatants elevated BAT-SNA and reduced GVNA. Moreover, intraoral (IO) injection of the LMW fraction from S-PT84 culture supernatants increased the body temperature above the interscapular BAT temperature (BAT-T) in rats.[5] However, it remains unclear which molecules are responsible for the effects of the LMW fraction from S-PT84 on autonomic nerve activities.

In the current study, we isolated molecules from the LMW fraction of S-PT84 and investigated the effect of these molecules on RSNA. We also identified one molecule responsible for the effects on RSNA and investigated the effects of this molecule on BAT-SNA and BAT-T in rats. The details of the results are described herein.

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MATERIALS AND METHODS

Preparation of *Lactobacillus* Culture Supernatants. S-PT84 cells were cultured under stable conditions in MRS broth (Difco Laboratories, Detroit, MI, USA) at 37 °C for 48 h. Culture supernatants were then collected by centrifugation and subjected to ultrafiltration using an Amicon Ultra-15 (molecular weight cutoff value, 10000 Da; Millipore, Bedford, MA, USA). The LMW fraction, containing molecules of <10000 Da, was used in this study. The LMW fraction of uninoculated MRS broth was used as a control.

A sample of the S-PT84 strain was deposited at the National Institute of Advanced Industrial Plant Organism Depository (Tsukuba, Japan), Plant Microorganisms Depository, as FERM ABP-1002.

Isolation and Identification of the Molecule That Affects Autonomic Activities. *Large-Scale Reverse-Phase Column Chromatography.* Ten cubic centimeters of the LMW fraction of S-PT84 culture supernatants was lyophilized and diluted with 2 cm^3 of 0.1% formic acid/distilled water prior to application to a Develosil C30-UG-5 column (20 mm × 250 mm; Nomura Chemical Co., Ltd., Japan) pre-equilibrated with 0.1% formic acid/distilled water (buffer A). Fractions containing peaks A, B, and C were eluted using a gradient of buffer A and 0.1% formic acid/80% AcCN/distilled water (buffer B) with a flow rate of 6 cm^3/min, using an AKTA system (AKTA Explorer; GE Healthcare, UK) to monitor absorbance at 215 and 280 nm. The gradient was programmed as follows: 2% B, 0−20.0 min; 2−50% B, 20.0−100.0 min; 50% B, 100.0−110.0 min; 50−100% B, 110.0−111.0 min; 100% B, 111.0−120.0 min. Fractions containing peaks A, B, and C were collected and subjected to a second chromatography step as described below.

Small-Scale Reverse-Phase Column Chromatography for Analysis of Culture Supernatants. Two cubic centimeter aliquots of the LMW fraction of S-PT 84 culture supernatants and the fractions containing peaks A, B, and C were lyophilized and then resuspended with 2 cm^3 of buffer A prior to application to the Develosil C30-UG-5 column (4.6 mm × 150 mm; Nomura Chemical Co., Ltd., pre-equilibrated with buffer A). These solutions were eluted with a gradient of buffer A and buffer B with a flow rate of 0.5 cm^3/min, using the AKTA system (AKTA Explorer; GE Healthcare) to monitor the absorbance at 215 and 280 nm. The gradient was programmed as follows: 0% B, 0−18.0 min; 0−50% B, 18.0−51.0 min; 50−100% B, 51.0−52.0 min; 100% B, 52.0−60.0 min. Uninoculated MRS broth was used as a control. The three isolated peak fractions (peaks A, B, and C) were lyophilized and weighed prior to subjection to the following procedures and studies.

Mass Spectrometry. The mass spectrum of the peak C fraction was obtained using a Q-TOF mass spectrometer (Micromass) in electrospray ionization (ESI) mode. Peak C showed an [M + H] ion peak in the positive mode.

^{1}H and ^{13}C NMR Analyses. ^{1}H NMR (750 MHz) and ^{13}C NMR (750 MHz) data for the peak C fraction in methanol-d_4 were measured with an Advance DMX750 instrument (Bruker Biospin).

Preparation of DDMP. DDMP was synthesized according to previously described methods.[6] The structure and molecular weight of the synthesized DDMP were confirmed using mass spectrometry, ^{1}H NMR, and reverse-phase column chromatography (data not shown).

Electrophysiological Studies. All animal care and handling procedures were approved by the Institutional Animal Care and Use Committee of Osaka University. Male Wistar rats, age 11 weeks (300−350 g; Kiwa Laboratory Animals Co., Ltd., Wakayama, Japan), were used in this study. Animals were housed in a room maintained at 24 ± 1 °C under a 12/12 h light/dark cycle. Food and water were freely available. Rats were adapted to the environment for at least 1 week prior to the experiment. On the experimental day, food was removed 3−4 h prior to surgery. General preparation was performed as described previously.[1,7] Briefly, polyethylene catheters were inserted into the left femoral vein and the duodenal cavity for intravenous and ID injections, respectively, under anesthesia induced by an intraperitoneal (IP) injection of 1 g/kg urethane. Rats were then cannulated intratracheally and fixed in a stereotaxic apparatus, and their body temperatures were maintained at 37.0−37.5 °C. To record the efferent RSNA, the left renal sympathetic nerve was exposed retroperitoneally through a left flank incision under a dissecting microscope. To record the efferent BAT-SNA, the left sympathetic nerve innervating the interscapular BAT was exposed through a left dorsal incision. The distal end of each nerve was ligated and attached to a pair of silver wire electrodes for recording efferent nerve activity. The recording electrodes were immersed in a mixture of warm Vaseline and liquid paraffin oil for antidehydration of nerves and electrical insulation. Rats were allowed to stabilize for 30−60 min after placement of the recording electrodes.

Electrical activity of the renal sympathetic nerve and brown adipose tissue sympathetic nerve were amplified, filtered, and monitored using an oscilloscope. Raw data for nerve activity was converted to standard pulses by a window discriminator, and the signal was amplified with a bioelectric amplifier. These analogue signals were converted to digital signals though an A/D converter (Power-Lab model 4sp, AD Instruments, Colorado Springs, CO, USA), sampled, and stored on a hard disk for off-line analysis.

Baseline measurements of the RNSA were made for 5 min before ID injection of either 1.0×10^{-2} or 1.0×10^{-1} g/L of each of the three isolated peak fractions in distilled water (2 cm^3). Each sample was injected into one rat for the measurement of RSNA. Baseline BAT-SNA measurements were made for 5 min before ID injection of 1.0×10^{-2} g/L of DDMP in distilled water (2 cm^3). Each sample was injected into an equal number of rats (n = 3) for measurement of BAT-SNA. For both RNSA and BAT-SNA, injection of distilled water alone was used as the control.

The indicated parameters were recorded for 40 min (RSNA) or 60 min (BAT-SNA) after the injection. At the end of the experiment, hexamethonium chloride (10 mg/kg) was intravenously administered to block the action potentials arising from postganglionic neural activity to determine the noise level of the recording.

Telemetry Recording of Body Temperature. In some rats, a telemetry system (Star Medical Co., Tokyo, Japan) was used to measure the BAT-T as described previously.[7] A capsule containing a temperature sensor, battery, and transmitter (model 10T-T; Star Medical Co.) was implanted into the subcutaneous space above the interscapular BAT under pentobarbital anesthesia (35 mg/kg, IP injection) 3−4 days before IO injection of DDMP. Data obtained from the sensor were analyzed using the 16-channel Eight Star Program (Star Medical Co.). On the experimental day, food was removed 4−6 h prior to injection. Under unanesthetized conditions, baseline measurement of the BAT-T was performed for 5 min just prior to IO injection of 2 cm^3 of DDMP (1.0×10^{-2} g/L) or distilled water during the light period. Each sample was injected into an equal number of rats (n = 3), and the body temperature above the interscapular brown adipose tissue (BAT-T) was subsequently recorded for 120 min.

Statistical Analysis. BAT-SNA and BAT-T were measured during the 5 min periods before and after each injection, and these data were analyzed by digital signal processing analyses. BAT-SNA data are shown as the mean ± standard error of the mean (SEM) of activities expressed as a percentage of baseline (0 min) values. BAT-T data are expressed as the mean ± SEM of temperature differences from baseline (0 min) temperature values.

The Mann−Whitney U test was used to compare the baseline values in each group. Due to interindividual variability in the preinjection state, the percentage change and temperature change from baseline were calculated for the neural discharge data and temperature data, respectively.

To compare group responses for BAT-SNA and BAT-T, two-way analysis of variance (ANOVA) with repeated measures was first performed. When significant differences were confirmed by two-way ANOVA ($p < 0.05$: treatment), post hoc analysis was performed to provide more information on significant differences of means at different time points. A series of repeated two-way ANOVAs, using data from 5−10, 5−15, and 5−20 min, etc., up to 5 min as the last time point, was adopted for post hoc analysis. In this post hoc analysis, multiplicity was not taken into consideration. Analytical software (SPSS7.5.1J; IBM Japan Co., Ltd., Tokyo, Japan) was used to perform two-way ANOVA and post hoc analyses.[8]

RESULTS

Isolation and Identification of the Molecule Affecting Autonomic Nerve Activity from S-PT84 Culture Supernatants. Initially, we analyzed whole S-PT84 culture supernatants, the LMW fraction of the S-PT84 culture supernatant, and MRS broth as a control by reverse-phase column chromatography. Several peaks were detected from whole S-PT84 culture supernatants and the LMW fraction (Figure 1).

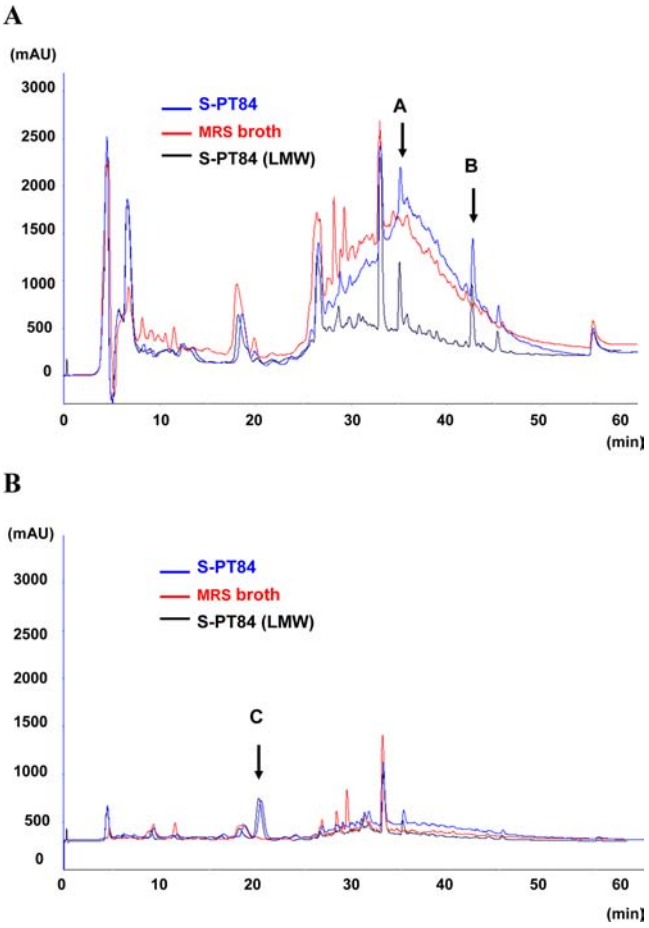

Figure 1. High-performance liquid chromatography (HPLC) chart of whole S-PT84 culture supernatants (S-PT84), the low molecular weight (LMW) fraction of S-PT84 culture supernatants [S-PT84 (LMW)], and MRS broth: (A) monitored at 215 nm; (B) monitored at 280 nm. Peaks A, B, and C appeared after cultivation of S-PT84. Each sample was analyzed by Develosil C30-UG-5 (4.6 mm × 150 mm).

Two major peaks (peaks A and B) were detected by measuring the absorbance at 215 nm (Figure 1A), and one major peak (peak C) was detected by measuring the absorbance at 280 nm (Figure 1B) in both whole S-PT84 culture supernatant and the LMW fraction of the S-PT84 culture supernatant. These three peaks were not detected in the uninoculated MRS broth (Figure 1), indicating that these three peaks were specific for the cultivation medium of S-PT84.

Therefore, we isolated three peak fractions from the LMW fraction of S-PT84 culture supernatants using reverse phase chromatography. First, the LMW fraction of the S-PT84 culture supernatant was applied to a Develosil C30-UG-5 column (20 mm × 250 mm). Fractions containing peaks A, B, and C were eluted using a gradient of buffers A and B at retention times of 57−59, 77−79, and 37−39 min, respectively. Subsequently, each fraction was applied to a new Develosil C30-UG-5 column (4.6 mm × 150 mm), and each peak was purified using a gradient of buffers A and B. This procedure allowed us to isolate three peak fractions (peaks A, B, and C) from the LMW fraction of the S-PT84 culture supernatant.

In the preliminary experiment, we examined the effects of ID injection of each of the three peak fractions on the RSNAs in rats. ID injection of peak A slightly increased the RSNA, ID injection of peak B inhibited the RSNA, and ID injection of peak C increased the RSNA (Figure 2). Because ID injection of

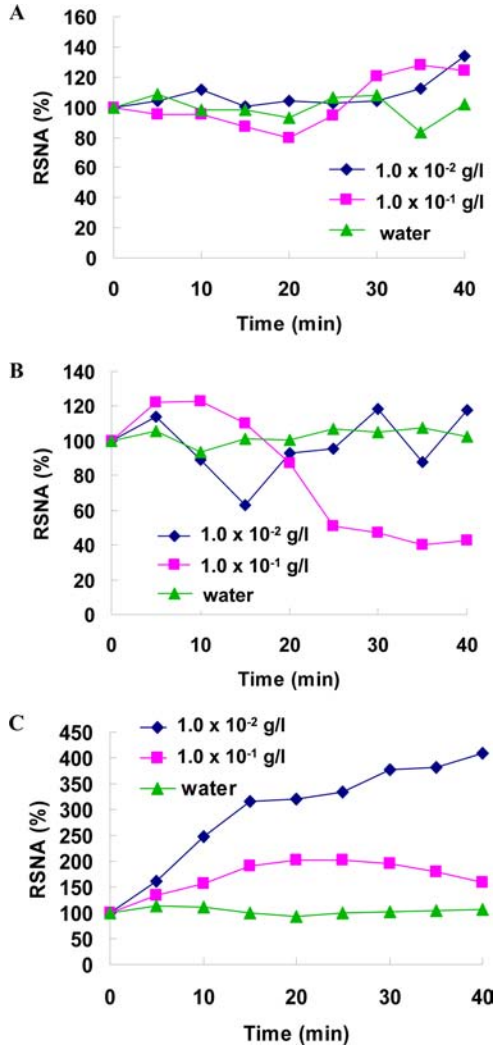

Figure 2. Effects of intraduodenal injection of isolated peaks from the LMW fraction of S-PT84 culture supernatants on renal sympathetic nerve activity (RSNA). Time courses of RSNA data are expressed as percentages of the values observed at 0 min. ID injections of 1.0×10^{-2} or 1.0×10^{-1} g/L of peak A (A), peak B (B), and peak C (C) were administered.

1.0×10^{-2} g/L of peak C markedly increased the RSNA (Figure 2C), we speculated that peak C may have the most potent effects on autonomic nerve activity among the three peak fractions.

Therefore, to determine the chemical structures of constituents of the peak C fraction, we analyzed peak C using mass spectrometry and ^{1}H and ^{13}C NMR analyses. Peak C showed an $[M + H]^+$ ion peak m/z of 145 in the positive

mode. The ^{13}C and ^{1}H NMR assignments are shown in Table 1.

Table 1. NMR Spectroscopic Data of 2,3-Dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) in Methanol-d_4

position	$^{13}C^a$ (M^b)	$^{1}H^a$ (J in Hz)
1	72.8 (t)	4.08 (dd, 9.6, 11.3), 4.33 (dd, 4.8, 11.3)
2	69.2 (d)	4.17 (dd, 4.8, 9.6)
3	189 (s)	
4	161 (s)	
5	133 (s)	
6	15.6 (q)	2.04 (s)

$^a\delta$ in ppm (750 MHz). ^{b}Multiplicity.

From these data, we identified one of the constituents of the peak C fraction as 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) ($C_6H_8O_4$; exact mass, 144.01). The planar structure of DDMP is shown in Figure 3.

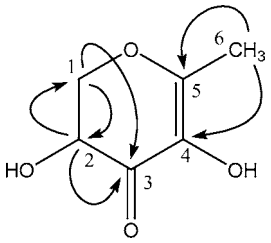

Figure 3. Planar structure of 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP). The arrows indicate HMBC correlations.

Taken together, these results suggest that strain S-PT84 may produce molecules that both suppress and stimulate RSNA in rats. Furthermore, DDMP is probably one of the molecules that stimulate RSNA.

Effects of ID Injection of DDMP on BAT-SNA. To examine the functions of DDMP, the effects of ID injection of 1.0×10^{-2} g/L of synthetic DDMP on BAT-SNA were examined in urethane-anesthetized rats. Changes in the BAT-SNA after ID injection of either DDMP or distilled water are shown in Figure 4. Compared to BAT-SNA values after the ID injection of water, BAT-SNA was elevated (118 ± 15.3%) 35 min after ID injection of DDMP, and this elevated value was maintained at over 105% until 60 min after injection. However, ID injection of distilled water slightly reduced the BAT-SNA, with the minimum BAT-SNA value (77.8 ± 13.5%) observed at 60 min after ID injection of DDMP (Figure 3B).

Because of significant differences in BAT-SNA values between water- and DDMP-treated rats in two-way ANOVA ($p < 0.05$: treatment), post hoc analysis was performed to confirm significant differences in means between time points. From this analysis, we found that significant differences in BAT-SNA values between water- and DDMP-treated rats became evident at 20 min and continued until 60 min after ID injection ($p < 0.05$). Therefore, these data suggested that BAT-SNA values in DDMP-treated rats remained higher than those of water-treated rats from 20 to 60 min after ID injection. Additionally, the Mann−Whitney U test revealed no significant differences in baseline BAT-SNA values at 0 min between water- and DDMP-treated groups (water-treated group, 222 ± 53 spikes/5 s; DDMP-treated group, 246 ± 11 spikes/5 s).

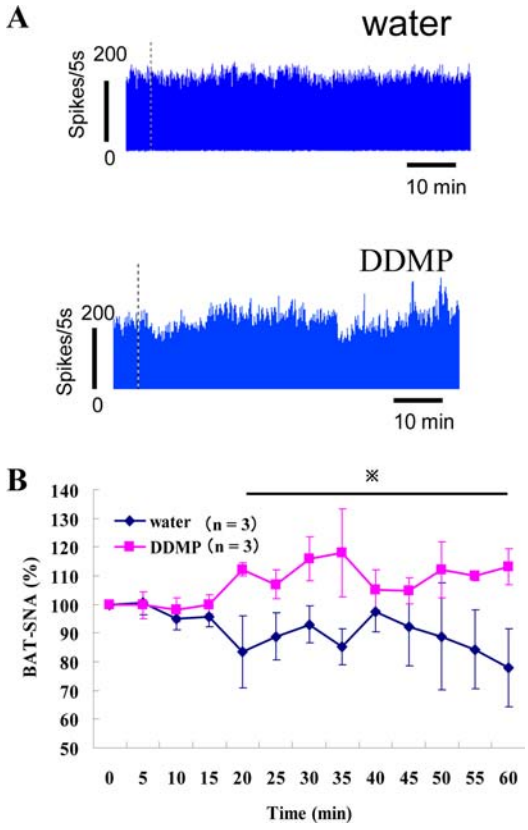

Figure 4. Effects of intraduodenal injection of DDMP on brown adipose tissue sympathetic nerve activity (BAT-SNA): (A) typical BAT-SNA recordings from rats injected with 2 cm^3 of distilled water or 1.0×10^{-2} g/L of DDMP (horizontal bars indicate a duration of 10 min, and vertical scale bars to the right of the recording represent neural discharge rates of 200 spikes/5 s); (B) time course of BAT-SNA data expressed as percentage of the value observed at 0 min (mean ± SEM) (number of animals used shown in parentheses). BAT-SNA values in DDMP-treated rats were significantly ($p < 0.05$) higher than those of water-treated rats from 20−60 min after ID injection, as determined using two-way ANOVA and post hoc analyses.

Effects of Oral Injection of DDMP on the BAT-T. We also examined whether the 1.0×10^{-2} g/L injection of synthetic DDMP affected the BAT-T in conscious rats with the same potency as that of DDMP on BAT-SNA. Oral injection of DDMP initially produced a decrease in BAT-T, followed by a gradual and marked increase in BAT-T, with the greatest elevation occurring at 110 min (0.72 ± 0.13 °C; Figure 5A). As a control, oral injection of distilled water initially produced an increase in BAT-T, followed by a gradual and slight decrease in BAT-T (Figure 5B). Because of the significant differences in BAT-T values between water- and DDMP-treated rats, as revealed by two-way ANOVA ($p < 0.05$: treatment), post hoc analysis was performed to confirm significant differences in means between different time points. These analyses demonstrated that significant differences in BAT-T values between water- and DDMP-treated rats became evident at 40 min and continued until 120 min after ID injection ($p < 0.05$). Therefore, we observed that BAT-SNA values in DDMP-treated rats remained higher than those of water-treated rats from 40 to 120 min after ID injection. Moreover, the Mann−Whitney U test revealed no significant differences between the

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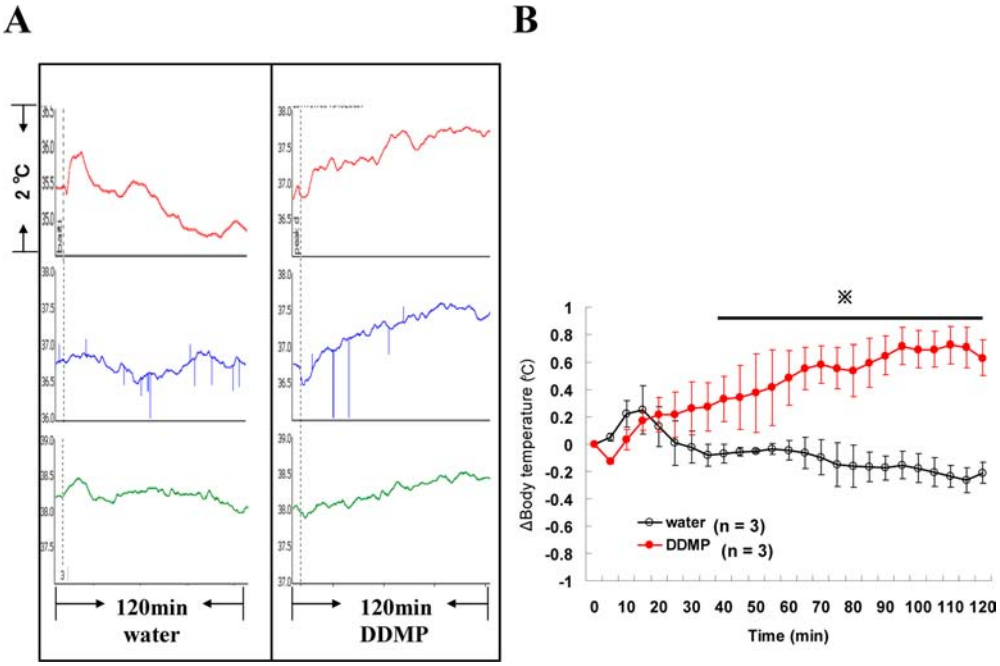

Figure 5. Effects of intraoral injection of DDMP on temperature in the subcutaneous space above the interscapular brown adipose tissue (BAT-T): (A) recordings of BAT-T in rats injected with 2 cm^3 distilled water or 1.0×10^{-2} g/L of DDMP; (B) BAT-T time course data are expressed as the mean ± SEM (number of animals used shown in parentheses). BAT-T values in DDMP-treated rats were significantly ($p < 0.05$) higher than those of water-treated rats from 40 to 120 min after ID injection, as determined using two-way ANOVA and post hoc analyses.

baseline values of the two groups at 0 min (DDMP-treated group, 37.2 ± 0.41 °C; water-treated group, 36.8 ± 0.80 °C).

DISCUSSION

In the present study, we obtained the following results: (i) S-PT84 produces molecules with both RSNA-suppressing and -stimulating effects (Figure 2); (ii) one of the molecule affecting autonomic nerve activity was identified as DDMP (Figure 3); (iii) ID injection of DDMP significantly elevated BAT-SNA (Figure 4); and (iv) oral injection of DDMP significantly increased BAT-T (Figure 5).

Because *L. pentosus* is classified as a heterofermentative *Lactobacillus*,[8] S-PT84 may produce a wide variety of molecules, such as acetic acid, propionic acid, and ethanol, in addition to lactic acid. In the present study, we found that ID injection of peak A and C fractions increased the RSNA, whereas ID injection of the peak B fraction inhibited the RSNA (Figure 1). These results suggest that S-PT84 produces both RSNA-suppressing and -stimulating molecules.

In a recent study published by our laboratory, we found that ID injection of the LMW fraction (molecules <10000 Da) of the S-PT84 culture supernatant elevated BAT-SNA and reduced GVNA.[5] However, the specific molecules that affected autonomic nerve activities remained unknown. In the present study, we isolated and identified DDMP as one of the molecules affecting autonomic nerve activity.

DDMP has been reported to be present in a wide variety of foodstuffs, such as orange juice,[9] rambutan fruit,[10] popcorn,[11] and onions,[12] and has been shown to have biological activities, including radical scavenger activity[13] and antiproliferation and pro-apoptotic effects.[12] However, the effects of DDMP on autonomic nerve activities have yet to be reported. Therefore, the present study demonstrates, for the first time, that DDMP affects autonomic nerve activity.

In this study, we found that ID injection of DDMP (peak C) may accelerate RSNA but have no affect on BP (data not shown). This finding suggested that intake of DDMP may carry a low risk of cardiovascular effects.

Excitation of the sympathetic nerve innervating BAT is known to produce heat via activation of uncoupling protein 1 from ATP production in the mitochondria.[14,15] In another study, researchers suggested that ID injections of either the oligomeric proanthocyanidin Flavangenol or *Eucommia* leaf extracts caused an acceleration of thermogenesis due to changes in BAT-SNA.[16,17] Moreover, it was shown that administration of *L. paracasei* ST11 (NCC2461) elicited elevations in both BAT-SNA and body temperature.[4] Therefore, the present results suggest that the administration of DDMP may cause an increase in body temperature through the facilitation of BAT-SNA.

In a recent study, intralateral cerebral ventricular (LCV) injection of L-carnosine was shown to affect BAT-SNA and body temperature in rats, and bilateral lesions of the hypothalamic suprachiasmatic nucleus (SCN) abolished the effects L-carnosine on BAT-SNA and body temperature.[7] These findings suggest that L-carnosine affects BAT-SNA and body temperature in rats and that the SCN may be involved in these effects. Moreover, these finding also suggest that the hypothalamus is responsible for regulating sympathetic nerve activity and metabolism. The mechanisms through which DDMP affects sympathetic nerve activity and metabolism must be determined in future studies.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

DDMP, 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one; ESI, electrospray ionization; BP, blood pressure; RSNA, renal sympathetic nerve activity; ASNA, adrenal sympathetic nerve activity; WAT-SNA, white adipose tissue sympathetic nerve activity; BAT-SNA, brown adipose tissue sympathetic nerve activity; CASNA, cutaneous sympathetic nerve activity; GVNA, gastric vagal nerve activity; BAT-T, brown adipose tissue temperature; ID, intraduodenal; IP, intraperitoneal; LMW, low molecular weight.

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