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PAPER

Bioavailability of orally administered water-dispersible hesperetin and its effect on peripheral vasodilatation in human subjects: implication of endothelial functions of plasma conjugated metabolites†

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Hesperetin is an aglycone of citrus flavonoids and is expected to exert a vasodilatation effect *in vivo*. We developed water-dispersible hesperetin by the process of micronization to enhance the bioavailability of hesperetin. This study aimed to assess the effect of this process on the bioavailability of hesperetin and to estimate its efficiency on vasodilatation-related functions using endothelial cells *in vitro* and a human volunteer study at a single dose *in vivo*. We found that water-dispersible hesperetin was absorbed rapidly, with its maximum plasma concentration (C_{max}) being 10.2 ± 1.2 μM, and that the time to reach C_{max}, which is within 1 h if 150 mg of this preparation was orally administered in humans. LC-MS analyses of the plasma at C_{max} demonstrated that hesperetin accumulated in the plasma as hesperetin 7-*O*-β-D-glucuronide (Hp7GA), hesperetin 3′-*O*-β-D-glucuronide (Hp3′GA) and hesperetin sulfate exclusively. Similar to hesperetin, Hp7GA enhanced nitric oxide (NO) release by inhibiting nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) activity in a human umbilical vein endothelial cell culture system, indicating that plasma hesperetin metabolites can improve vasodilatation in the vascular system. A volunteer study using women with cold sensitivity showed that a single dose of water-dispersible hesperetin was effective on peripheral vasodilatation.These results strongly suggest that rapid accumulation with higher plasma concentration enables hesperetin to exert a potential vasodilatation effect by the endothelial action of its plasma metabolites. Water-dispersible hesperetin may be useful to improve the health effect of dietary hesperetin.

Introduction

Hesperidin is a hesperetin (3′,5,7-trihydroxy-4′-methoxy-flavanone) glycoside with a linkage of rutinose at the 7-position of the A ring of hesperetin (Fig. 1). This glycoside is a predominant flavanone-type flavonoid present in citrus fruits, and is presumed to have a role in the beneficial effect of citrus fruits for human health because it exerts anti-oxidative, anti-inflammatory, anti-viral, and anti-carcinogenic activities.[1] Dietary supplementation of hesperidin has been used in subjects suffering from blood-vessel disorders (including fragile and

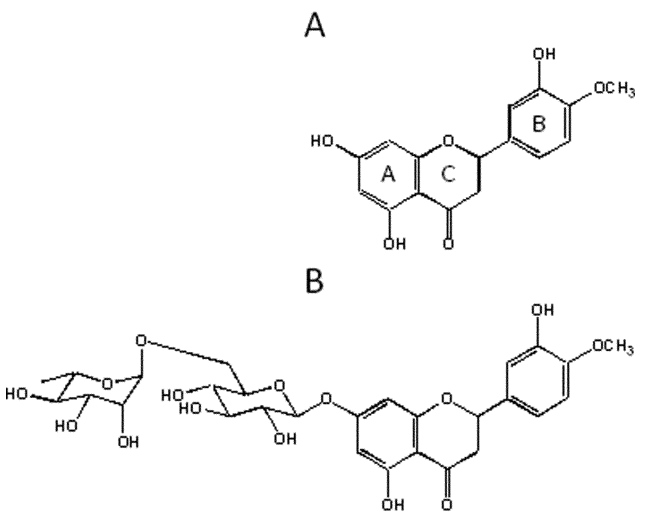

Fig. 1 Structures of hesperetin (A) and hesperidin (B).

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permeable blood vessels).[2] Morand *et al.*[3] reported, in a human volunteer study, that hesperidin contributes to the vascular protective effects of orange juice. Rizza *et al.*[4] revealed that hesperidin intake improved endothelial function and reduced inflammatory markers in patients with the metabolic syndrome.

In general, flavonoid glycosides are absorbed into the body after conversion to its aglycone through the hydrolytic activity of intestinal enzymes.[5] However, hesperidin has only limited bioavailability because rutinoside-form glycosides are poorly absorbed compared with their aglycone and monoglucoside forms.[6,7] In addition, hesperidin absorption is slower than its aglycone because rutinoside-form flavonoids are absorbed only in the distal part of the intestine after hydrolysis by the colonic microflora.[8–11] In contrast, aglycones can directly absorb in the upper part of the intestine without the assistance of colonic microflora. Therefore, the efficacy of hesperidin and its deglycosylation products on biological functions of humans should be intensively investigated.

Several attempts have been made to enhance the bioavailability of hesperidin.[9–11] Removal of rhamnose from the rutinose moiety of hesperidin was suggested to be effective in a similar way to quercetin monoglucoside.[12] We previously clarified that 4^G-α-glucopyranosylhesperidin (which is enzymatically synthesized from hesperidin) was ≈300-times more soluble than hesperidin in the aqueous phase,[13] and was absorbed more rapidly and more efficiently than hesperidin.[14] This implies that elevation of water solubility results in a higher bioavailability of hesperetin.

Here we developed micronized water-dispersible hesperetin to improve the bioavailability of hesperetin. This is because hesperetin would not be completely soluble in the aqueous phase due to its hydrophobic flavanone structure.[15] Thus, the aim of the present study was to assess the efficacy of this processing on the bioavailability and its influence on the biological activity of hesperetin in humans. In particular, the effect of this processed hesperetin on vasodilatation-related functions was estimated using endothelial cells *in vitro* and a human volunteer study *in vivo*. This approach is helpful to discover the physiological significance of hesperetin intake as well as to update the methodology for the development of highly bioavailable flavonoid products.

Materials and methods

Chemicals

Hesperetin (3′,5,7-trihydroxy-4′-methoxyflavanone) was purchased from Chengdu Wagott Pharmaceutical Co. (Chengdu, China). Hesperetin 7-*O*-β-D-glucuronide (Hp7GA) was obtained from Nagara Science Co. (Gifu, Japan). 4-Amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) was purchased from Sekisui Medical Co. (Tokyo, Japan). All other reagents were of analytical grade and used without purification.

Preparation of water-dispersible hesperetin

Water-dispersible hesperetin was developed by the same method as that for micronized dispersible ferric pyrophosphate.[16,17] Briefly, water-dispersible hesperetin was prepared from hesperetin and emulsifiers composed of polyglyceryl fatty acid ester, glycerin fatty acid organic acid ester, and enzyme-degradable lecithin. Hesperetin and emulsifiers were mixed thoroughly at 80 °C and micronized using the Dyno-mil (Shinmaru Enterprises Corporation, Osaka, Japan). This resulted in hesperetin subnanoparticles with a mean diameter of 0.7 μm. This particle has the advantage of complete dispersion with the aqueous phase, and can be used to fortify liquid foods or drinks.[18,19]

Time course of plasma concentration of hesperetin conjugates after the intake of water-dispersible hesperetin

Healthy male volunteers ($n = 10$) were recruited into the study. Subjects were 22.0 ± 1.2 y old, weighed 68.6 ± 8.7 kg, and had a BMI of 22.6 ± 2.5 kg m^{-2}, systolic pressure 119.5 ± 7.3 mmHg, diastolic pressure 69.2 ± 8.5 mmHg (means ± SD). All subjects were assessed for eligibility on the basis of a health questionnaire and the medical interview. The following exclusion criteria applied: long term medical conditions, abnormality of hepatic, renal, or cardiorespiratory functions, diabetes, regular prescribed medication, pregnancy, perform vigorous exercise regularly. They were requested to avoid consumption of fruits, vegetables, seaweed, and spices, and to take only water as a beverage for 18 h before the ingestion. Each subject ingested a preparation of water-dispersible hesperetin containing 150 mg of hesperetin. Peripheral venous blood was collected into a heparinized tube just before and 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8 and 24 h after the ingestion. Subjects were provided meals without citrus fruits and which were free of hesperetin during the test period.

Comparison of plasma concentration of hesperetin after the intake of water-dispersible hesperetin and intact hesperetin

Healthy volunteers ($n = 9$; 4 men and 5 women; 41.3 ± 9.3 years old) were recruited into a cross-over study. Subjects were 41.3 ± 9.3 y old, weighed 54.7 ± 14.6 kg, and had a BMI of 20.0 ± 3.5 kg m^{-2}. They fulfilled the following criteria: no history of and not taking medication for any gastrointestinal disease, non-smokers, not pregnant, do not perform vigorous exercise regularly. Subjects avoided fruits, vegetables, seaweed, and spices, and drank only water as a beverage for 18 h before the ingestion. They only ate rice for breakfast before starting the test period. Each subject ingested a water-dispersible hesperetin preparation containing 150 mg hesperetin or intact hesperetin (150 mg) with a washout period of ≥7 days. Peripheral venous blood was collected into a heparinized tube just before and 1, 4, and 6 h after the ingestion. After blood sampling 4 h after the ingestion, subjects were served rice as the lunchtime meal.

Both human studies on the intake of hesperetin and measurement of plasma concentration were approved by the Ethical Committee of Ezaki Glico Co., Ltd (Osaka, Japan).

Determination of the plasma concentration of hesperetin conjugates

Plasma was obtained by centrifugation (9,000 × *g* for 10 min at 4 °C) of heparinized blood. The total content of hesperetin and

its conjugated metabolites was measured after deconjugation. Briefly, 50 μl of a plasma sample was diluted with 9 volumes of 50 mM sodium phosphate buffer (pH 5.0), and incubated at 37 °C for 2 h after mixing with 25 μl of 2 mg ml^{-1} of sulfatase H-1 from *Helix pomatia* (Sigma–Aldrich, St. Louis, MO, USA) which contains both glucuronidase and sulfatase activities for the deconjugation treatment of the hesperetin metabolites. The liberated hesperetin aglycone was extracted by solid-phase extraction using Oasis® HLB extraction cartridges (Waters Corp., Milford, MA, USA). After a plasma sample was put on the cartridge and rinsed with 1 ml of purified water, 1 ml of methanol was added to elute the liberated hesperetin. The methanol fraction was evaporated *in vacuo* and the residue dissolved in 50 μl of methanol. Hesperetin was determined by a high-performance liquid chromatography (HPLC) electrochemical detection system with a VP series HPLC instrument (Shimadzu Co., Kyoto, Japan). The column used was a TSK gel ODS-80Ts (i.d. 4.6 mm × 150 mm, Tosoh, Tokyo, Japan), and the mobile phase was composed of acetonitrile/100 mM sodium dihydrogen phosphate (33 : 67, v/v) with a flow rate of 0.5 ml min^{-1}. The eluent was monitored with a Coulochem III electrochemical detector (ESA, Chelmsford, MA, USA) with a working potential of 700 mV.

Identification of hesperetin metabolites

Plasma (50 μl) was diluted with 9 volumes of ascorbic acid solution (5 mM), and extracted with two volumes of methanol to analyze the hesperetin metabolites. Subsequently, all samples were centrifuged at 9,000 × *g* for 10 min at 4 °C. After centrifugation, each supernatant was evaporated *in vacuo* and the residue dissolved in 50 μl methanol. The eluent was monitored at 270 nm by a ultraviolet (UV) detector and subsequently a liquid chromatography–mass spectrometry (LC–MS) instrument (LC-MS QP-8000α, Shimadzu Co, Kyoto, Japan). A gradient elution was carried out with a C18 reverse-phase column (Phenomex Gemini, 5 μm, C18 110A). Solvent A was water containing 0.1% trifluoroacetic acid (TFA), and solvent B was 100% acetonitrile. The gradient program was set as follows: 0–2 min, 15% B; 10–15 min, liner gradient to 35%; 15–35 min, liner gradient to 50%, 35–45 min, 80% B hold; flow rate at 0.2 ml ml^{-1}. The mass spectrometer was operated in single ion monitoring (SIM) mode. The column effluent was directed without splitting into an ion trap mass spectrometer with an ESI source. The positive ion mode was employed for these experiments, using a cone voltage of 4.5 kV and desolvation temperature of 250 °C. N_2 gas was used for carrier gas.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Takara Bio. Inc. (Shiga, Japan). Cells were plated onto culture plates and cultured to confluence in EMB-2 medium (Takara Bio. Inc.) with heparin, hydrocortisone, fetal bovine serum (FBS), fibroblast growth factor, ascorbic acid, vascular endothelial growth factor, insulin-like growth factor-1, gentamicin, and amphotericin. Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

Estimation of the vasodilatation-related properties of HUVECs

We examined the effectiveness of a plasma hesperetin metabolite on the release of nitric oxide (NO) and the activity of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) in endothelial cells. Hesperetin or Hp7GA were dissolved in methanol and diluted with the medium. The final methanol concentration of the cell culture was <0.1% and had no effect on the cell viability. Cells were placed in a dish (diameter, 15.5 mm) in an atmosphere of 5% CO_2 /95% air at 37 °C. Then the medium was replaced with fresh medium containing the test compounds. After treatment with test compounds (1, 5, 10, 25, and 50 μM) for 24 h, NO released from HUVECs was measured by the method of Balcerczyk *et al.*[20] using 4-amino-5-methylamino-2,7-difluorofluorescein (DAF-FM) as a fluorescence probe. For measurements of the effects of hesperetin and Hp7GA upon superoxide anion ($O_2^{\cdot-}$) release in HUVECs, confluent HUVECs were washed thrice with phosphate buffered saline (pH 7.4) and were subsequently exposed to the medium containing test compounds (1, 5, 10, 25, and 50 μM) for 6 h at 37 °C in the presence of 50 μmol l^{-1} cytochrome *c* in EMB-2 medium (phenol red and serum-free). Decrease of cytochrome *c* content was measured in the supernatant at 550 nm according to the method of Steffen *et al.*,[21] which was also applied for assessment of the NADPH oxidase activity of cultured cells. Confluent HUVECs were scraped off from T-175 cm^2 flasks and suspended in 5 ml serum-free culture medium. Cells were precipitated by centrifugation (9,000 × *g*, 4 °C, 5 min). The supernatant was discarded and the pellet resuspended in 800 μl of phosphate buffered saline (pH 7.4). Cells were then disintegrated by sonication using a probe sonifier for three cycles of 20 s at 0 °C. HUVEC lysates were added to 800 μl of 50 mM phosphate buffer (pH 7.4) containing 100 μM NADPH, 21 μM cytochrome *c*, and test compounds (1, 5, 10, 25, and 50 μM) dissolved in methanol with the solvent concentration not exceeding 1% by volume. The reaction was started by the addition of 90 μM SDS. The reaction was followed photometrically at 340 nm for 45 min at 25 °C.

Recovery of peripheral body temperature after cold water stress

We also studied the effect of the oral intake of processed hesperetin on the temperature of the human body. Six females (18–22 years old) with cold sensitivity were recruited into the study. They fulfilled the following criteria: no history of and not taking medication for any gastrointestinal disease, history of long-term medical conditions, non-smokers, not pregnant, do not perform vigorous exercise regularly. We determined if they were suffering from cold sensitivity using Terasawa's diagnostic criteria.[22] Subjects diagnosed as suffering from cold sensitivity participated in the experiment within 2 weeks after the menstrual period to avoid fluctuation of basal body temperature. We investigated the effectiveness of water-dispersible hesperetin for the treatment of blood-circulation disorders in women using a double-blind, placebo-controlled crossover protocol. Test samples were a beverage (100 ml) involving water-dispersible hesperetin (containing 34 mg hesperetin) kept at 37 °C. Flavoring had been added to both of the beverages, water-dispersible hesperetin beverage and a placebo beverage to mask its smell and

taste, and showed no influence on peripheral body temperature nor blood flow. A placebo not involving water-dispersible hesperetin was used for the control. Each subject took one of two beverages on the first day and the other beverage on the second day with a washout period about a week. Measurements were taken in a quiet, constant-temperature room (25 ± 0.5 °C) with a humidity of ≈50%. Each measurement was taken between 12:30 and 14:00, without lunch, to avoid the influence of a diurnal change in body temperature. Subjects abstained from food, drink, and exercise for 3 h before the experiment. The temperature of the surface of the middle finger of the left hand was measured with a compact Thermologger Type K instrument (Adachi Keiki Co., Tokyo, Japan). Each subject wore the same clothing for each measurement to eliminate the influence of clothing on the body-surface temperature. After each of the subjects entered the measurement room (25 ± 0.5 °C), a thermometer probe was placed on their body. The subjects wore a vinyl glove so as to avoid wetting the sensor. Then, left hands were soaked by placing wrists into water maintained at 33 °C to set an initial temperature for 30 min before cooling stress. Fifteen minutes after single administration of the test sample, left hands were also immersed into water at 15 °C for 5 min. We continued to measure finger temperature for the following 20 min (Fig. 2A).

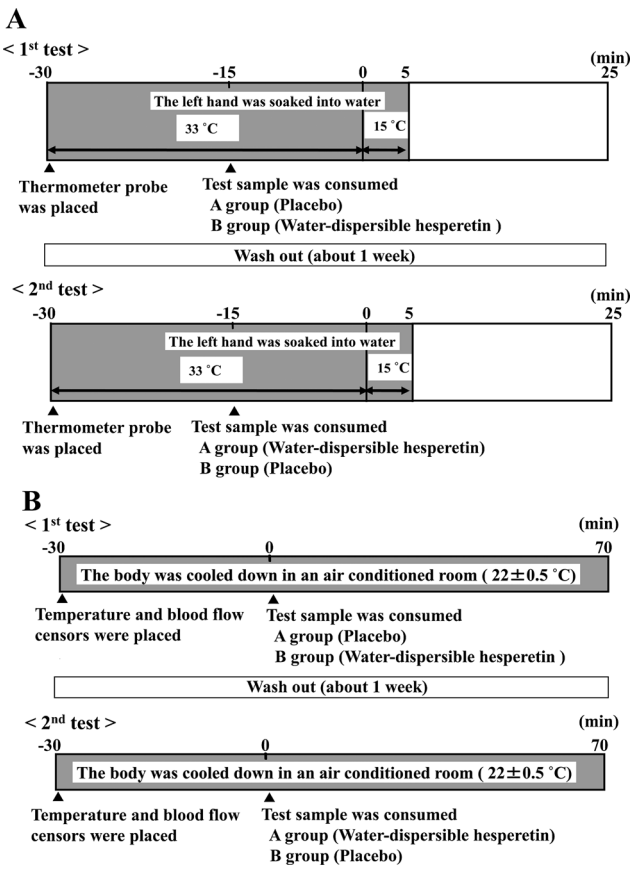

Fig. 2 Testing protocol for assessing the effect of the water-dispersible hesperetin on the peripheral body temperature and blood flow. A) Recovery of peripheral body temperature after cold water stress. B) Change in peripheral temperature and blood flow under staying in an air-conditioned room.

Change in peripheral temperature and blood flow under staying in an air-conditioned room

The subjects were 10 Japanese female volunteers (18–22 years old) with cold sensitivity determined using the diagnostic criteria set by Terasawa.[22] They fulfilled the following criteria: no history of and not taking medication for any gastrointestinal disease, history of long-term medical conditions, non-smokers, not pregnant, do not perform vigorous exercise regularly. We conducted a double-blind, placebo-controlled crossover study to more minutely elucidate the effects of water-dispersible hesperetin on peripheral body temperature. We measured the change in finger temperature and blood flow when subjects took water-dispersible hesperetin or placebo, and stayed in an air-conditioned room. The test samples were beverage (100 ml) containing water-dispersible hesperetin (17 mg or 170 mg) at 37 °C. Flavoring had been added to both of the beverages, water-dispersible hesperetin beverage and a placebo beverage to mask its smell and taste, and showed no influence on peripheral body temperature nor blood flow. A placebo not containing water-dispersible hesperetin was used for the control. The treatment order was randomized with a washout period of one week. The same lunch was given to each subject. Each measurement was taken between 15:00 and 17:00 to avoid the influence of a diurnal change in body temperature. The other situation of subjects was identical to that discussed in the experiment of the recovery of peripheral body temperature after cold water stress.

Body-surface temperature of the middle finger of the left hand was measured with a BAT-12 Thermistor (Physitemp Instruments, Clifton, NJ, USA). Temperature data were recorded automatically with a computer *via* an AM-7002 Data Collector (Adachi Keiki Co., Tokyo, Japan). The blood flow in the annular finger of the left hand was measured with an ALF21/21D laser blood flow meter (Advance Co.). Measurements were taken in a quiet, constant-temperature room (22 ± 0.5 °C) with a humidity of ≈50%. After subjects entered the measurement room, thermometer probes and blood-flow sensors were placed on their bodies. Subjects were instructed to first sit and rest quietly in the measurement room for 30 min. The test sample was then taken within 2 min. Measurements were taken for 70 min after administration while the subject remained seated (Fig. 2B).

Both of the human studies of the peripheral body temperature were approved by the Ethical Committee of the University of Shiga Prefecture (Shiga, Japan).

Statistical analyses

Results are means ± SEM. The area under the available plasma hesperetin curve *versus* time after treatment was calculated as previously reported.[10] Comparisons between mean values of water-dispersible hesperetin and placebo at certain timepoints were evaluated by a two-tailed paired *t*-test with SPSS (ver.14.0 for Windows; SPSS, Chicago, IL, USA). To evaluate the effect of hesperetin and Hp7GA on the vasodilatation-related properties of HUVECs, Dunnett's multi-comparison test was calculated with SPSS software. $p < 0.05$ was considered significant. To evaluate the effect of water-dispersible hesperetin on peripheral body temperature, a time *versus* treatment effect was evaluated by repeated analysis of variance.[23] Statistical values were

calculated with the StatView software package (Macintosh Version J5.0, Abacus Concepts, Berkeley, CA, USA).

Results

Plasma concentration of total hesperetin after consumption of a preparation of water-dispersible hesperetin

Fig. 3A shows the changes in plasma concentration of hesperetin during 24 h after the intake of a preparation of water-dispersible hesperetin. The area under the curve (AUC) for total plasma hesperetin after a β-glucuronidase/sulfatase treatment was 19.1 ± 7.8 μmol h l^{-1}. The kinetic curves showed that total plasma hesperetin returned to baseline 24 h after the intake of water-dispersible hesperetin. The maximum plasma concentration of total hesperetin (C_{max}) was 6.7 ± 3.2 μM and the corresponding time to reach maximum plasma concentration (T_{max}) was estimated to be within 1 h because the T_{max} of 6 subjects was 0.5 h and the T_{max} of 4 subjects was 1 h. No peak corresponding to hesperetin appeared in the chromatogram when β-glucuronidase/sulfatase treatment was omitted from the analytical procedures (data not shown). This indicated that not the hesperetin aglycone but that hesperetin conjugates exclusively accumulates in the plasma after the intake of water-dispersible hesperetin. Fig. 3B shows the difference in plasma hesperetin concentration between the intake of water-dispersible hesperetin preparation and that of intact hesperetin. The periods of blood collection were arranged at 1 h, 4 h and 6 h because maximum plasma concentration was attained within 1 h after taking water-dispersible hesperetin (Fig. 3A) and that the T_{max} of intact hesperetin was reported to be 4–6 h.[8,24] The maximum concentration of water-dispersible hesperetin (10.2 ± 1.2 μM) at 1 h was 5.5-fold higher than that of hesperetin (1.8 ± 0.8 μM) at 4 h. It was therefore clarified that water-dispersible hesperetin was incorporated into plasma significantly faster than intact hesperetin with a much higher concentration.

Plasma profiles of hesperetin metabolites of preparations of water-dispersible hesperetin

When HPLC was applied for the characterization of plasma hesperetin metabolites, the blood extracts from subjects collected at 1, 4, and 6 h after taking water-dispersible hesperetin or intact hesperetin were injected to the column without deconjugation. Fig. 4A shows the HPLC chromatogram of the extract collected 1 h after the intake of water-dispersible hesperetin, in which three prominent peaks (X, Y, and Z) appeared. Different from

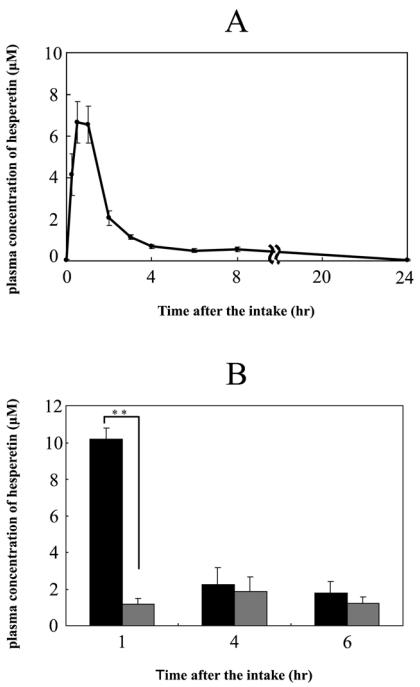

Fig. 3 Plasma concentration of hesperetin metabolites after intake of water-dispersible hesperetin or intact hesperetin. Concentration of hesperetin conjugates was determined by HPLC-ECD after the treatment with β-glucuronidase/sulfatase. A) Plasma concentration of hesperetin was measured after the ingestion of water-dispersible hesperetin preparation containing 150 mg hesperetin ($n = 10$). B) Plasma concentrations of hesperetin conjugates were determined at 1 h, 4 h and 6 h after the ingestion of water-dispersible hesperetin preparation or intact hesperetin at each 150 mg hesperetin equivalent ($n = 9$). Water-dispersible hesperetin (black) and intact hesperetin (gray). Values are means ± SE. *significantly different ($p < 0.01$ by paired t-test).

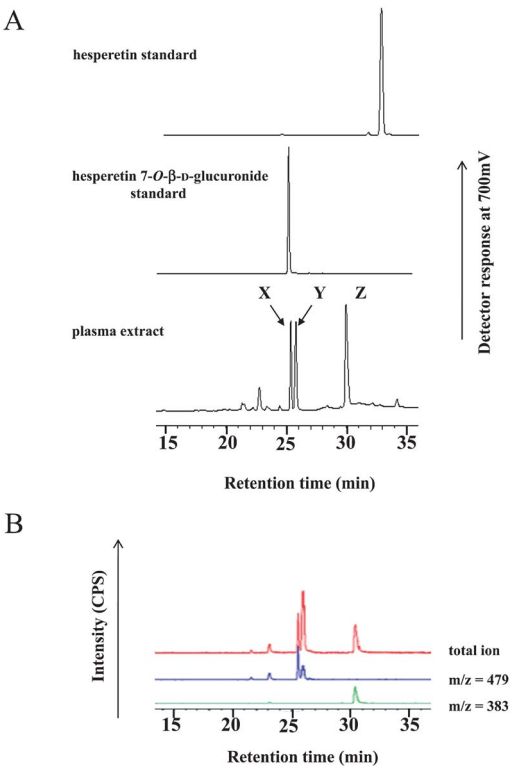

Fig. 4 HPLC profiles for hesperetin metabolites obtained from a representative plasma extract 1 h after the intake of water-dispersible hesperetin. A) HPLC-UV detection of standard hesperetin (retention time (RT): 32.6 min) and Hp7GA (RT: 25.6 min) and main peaks X, Y and Z as the plasma metabolites. B) LC-MS analyses in total ion collector mode and selective ion monitoring mode at m/z 479 for hesperetin monoglucuronide ($[M + H]^+ = 479$) and m/z 383 for hesperetin monosulfate ($[M + H]^+ = 383$). Peak X was identified as hesperetin 7-*O*-β-D-glucuronide (Hp7GA). Peak Y and Z were ascribed to hesperetin-monoglucuronide (a different structure from Hp7GA) and hesperetin-monosulfate, respectively.

Fig. 4A, many small peaks appeared as hesperetin metabolites in the chormatograms 4 h and 6 h after intake of water-dispersible hesperetin and at 1, 4, 6 h after the intake of intact hesperetin (data not shown). This means that only three major metabolites (corresponding to peaks X, Y and Z) were yielded in the plasma at a C_{max} of hesperetin in the case of intake of water-dispersible hesperetin. In other cases, a wide variety of metabolites accumulated in the plasma at much lower level than that 1 h after the intake of water-dispersible hesperetin. The retention time of peak X corresponded to the Hp7GA standard. In addition, the result of LC–MS (Fig. 4B) showed that peak X and peak Y were assigned to hesperetin-monoglucuronide because single ion monitoring (SIM) analysis demonstrated the presence of the ion ($[M + H]^+ = 479$) specific to hesperetin-monoglucuronide. Peak Z was also assigned to hesperetin-monosulfate because the SIM analysis showed the presence of ($[M + H]^+ = 383$) due to hesperetin-monosulfate. Therefore, peak X,Y, and Z were identified as Hp7GA, hesperetin-monoglucuronide (a different structure from Hp7GA) and hesperetin-monosulfate, respectively (Fig. 5).

Effects of hesperetin and Hp7GA on the vasodilatation-related properties of HUVECs

We examined the stimulation of NO production from HUVECs by the exposure of hesperetin or Hp7GA for 24 h. Both compounds significantly stimulated NO production (Fig. 6A). In either case, enhancement of NO production was concentration-dependent with significant differences at ≥25 μM. Next, the effect of these compounds on $O_2^{\cdot-}$ production from HUVECs was measured (Fig. 6B). Both compounds suppressed $O_2^{\cdot-}$ production with concentration-dependence by the treatment with HUVECs for 6 h. Fig. 6C shows the effect of these compounds on the NADPH oxidase activity of disintegrated HUVECs by treatment with hesperetin or HpGA. The inhibition was concentration-dependent, with significance at ≥5 μM in

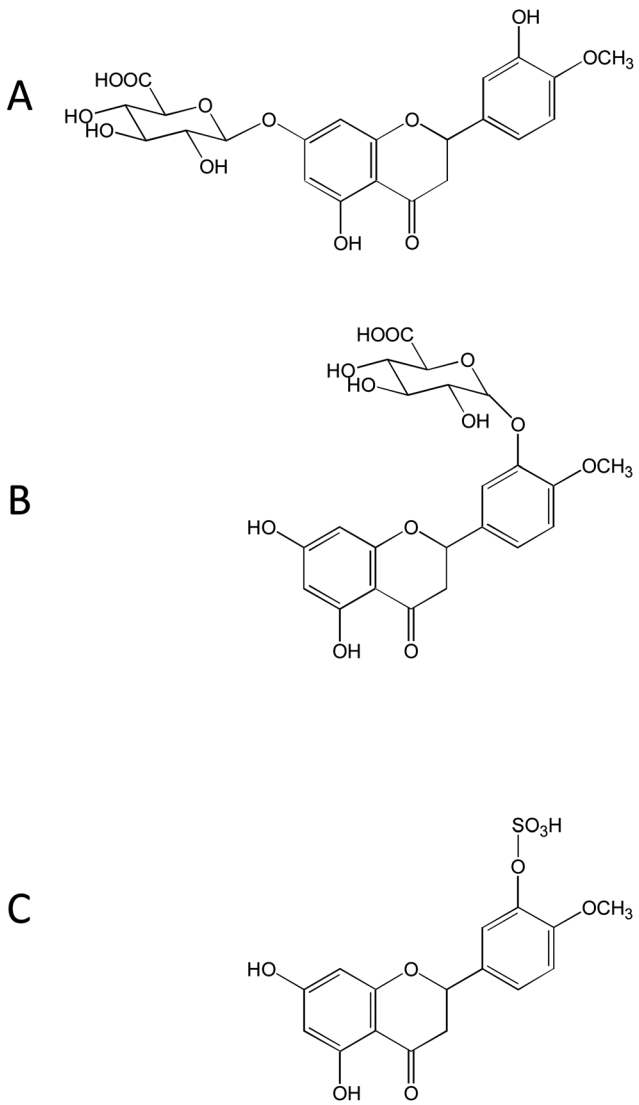

Fig. 5 Structures of hesperetin metabolites identified in plasma after the intake of water-dispersible hesperetin; Hp7GA (A), Hp3′GA (B), and hesperetin-3′-*O*-sulfate (C).

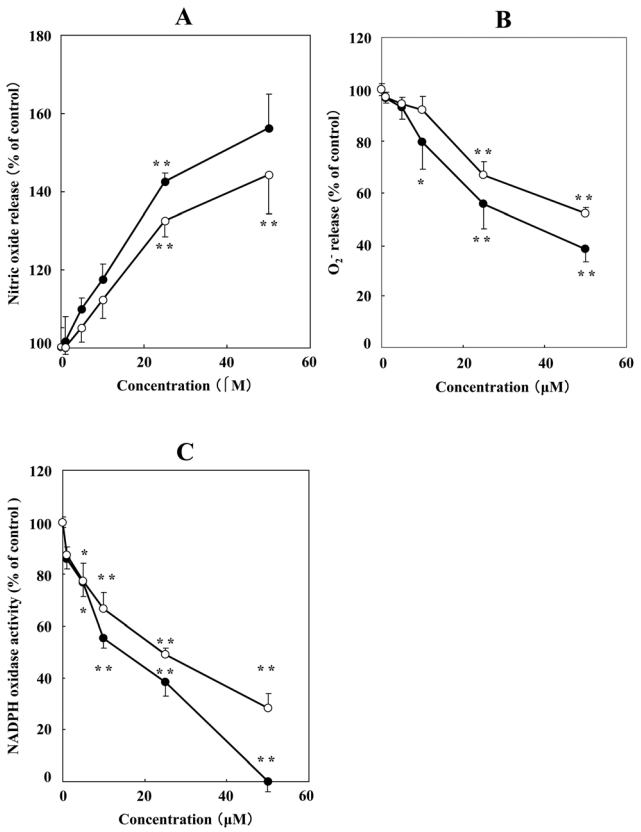

Fig. 6 Effectiveness of hesperetin and a hesperetin metabolite, Hp7GA, on the vasodilatation-related properties of cultured endothelial cells. hesperetin (●) and Hp7GA (○) at 1, 5, 10, 25, 50 μM. Values are means ± SE. $^{**}p < 0.01$, $^{*}p < 0.05$ (Dunnett's multi-comparison test). A) Release of NO from HUVECs was measured by a fluorescence assay using DAF-FM ($n = 5$).[20] B) $O_2^{\cdot-}$ released from HUVECs was measured using a cytochrome *c* assay ($n = 3$)[21] and the percentage of $O_2^{\cdot-}$ was calculated from the ratio of [(control − sample)/control] in the reduction of cytochrome *c* measured in the supernatant at 550 nm. C) NADPH oxidase activity of disintegrated HUVECs was measured ($n = 3$)[21] and the percentage of NADPH oxidase activity calculated by the ratio of [(control − sample)/control] in the consumption of NADPH measured in the supernatant at 340 nm after the reaction for 45 min at 25 °C.

either hesperetin or Hp7GA. The efficacy of Hp7GA was lower than that of hesperetin in all experiments, including stimulation of NO production, inhibition of $O_2^{\cdot-}$ production and inhibition of NADPH oxidase activity.

Effect of water-dispersible hesperetin on the peripheral body temperature and blood flow

In experiment 1, the temperature of fingers soaked in water was kept at 33 °C for 30 min. That is, the initial temperature was set intendedly. When the left hand was placed in water at 15 °C for 5 min, the temperature of the finger decreased to 16 °C in both test samples. After cooling stress, the temperature of the finger recovered. The recovery of finger temperature for 20 min was significantly higher in the water-dispersible hesperetin than in the placebo control ($p < 0.01$) (Fig. 7A). Changes in the surface temperatures of the finger after intake of water-dispersible hesperetin or placebo control in experiment 2 are shown in Fig. 7B. Differences between the respective basal values before the intake of water-dispersible hesperetin or placebo control were not statistically significant. For example, finger temperatures before the intake of water-dispersible hesperetin or placebo control were 32.0 °C to 33.5 °C. The surface temperature of the finger decreased after taking the test samples, regardless of being the water-dispersible hesperetin or the placebo control, in the air-conditioned room set at 22 °C. Intake of water-dispersible hesperetin significantly maintained the peripheral surface temperature of the finger as compared with placebo control ($p < 0.0001$). Furthermore, intake of 170 mg of the water-dispersible hesperetin was more effective than that of its 17-mg intake. In this experiment, intake of water-dispersible hesperetin at 17 mg or 170 mg significantly suppressed the drop of blood flow in the air-conditioned room at 22 °C ($p < 0.0001$) (Fig. 7C).

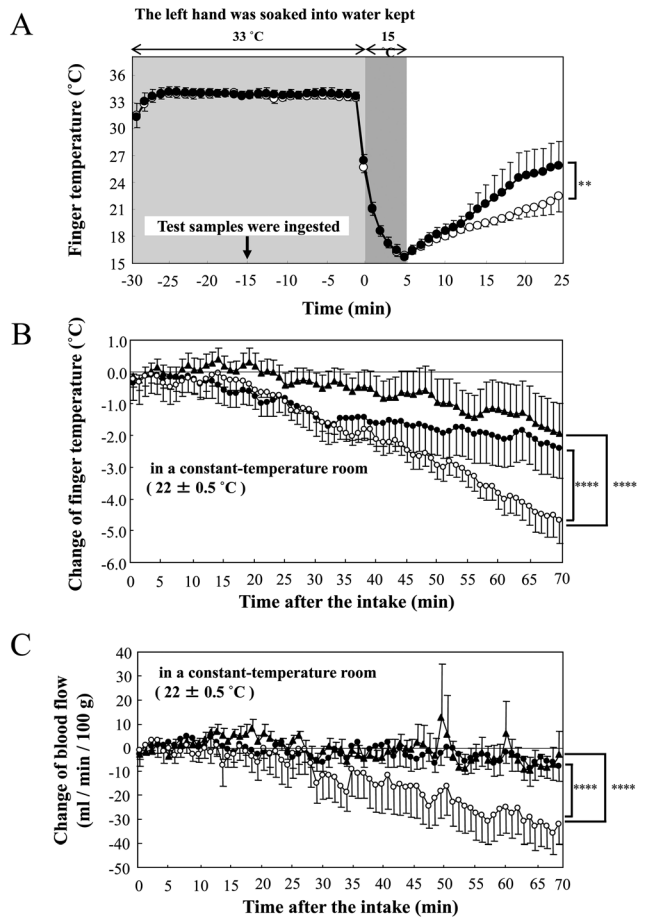

Fig. 7 Effect of the intake of water-dispersible hesperetin preparation on peripheral body temperature and blood flow in healthy volunteers. A) Volunteers ingested a water-dispersible hesperetin preparation with 34 mg of hesperetin equivalent (●) or placebo control (○) and then the recovery of peripheral body temperature after cold water stress (15 °C) was measured ($n = 6$). **Significantly different (**$p < 0.01$). B and C) Volunteers entered the constant-temperature room (22 ± 0.5 °C) just after the intake of 170 mg water-dispersible hesperetin (▲), 17 mg of the water-dispersible hesperetin (●), or placebo control (○). Each value is expressed as the difference of the finger temperature (B) and the blood flow (C) between after the intake and before the intake of test samples ($n = 10$). Values are means ± SE. **** $p < 0.0001$ (repeated analysis of variance).

Discussion

Hesperidin has attracted much attention in relation to vascular function[1] since Szent-Györgyi first demonstrated that hesperidin prevents capillary permeability by strengthening blood vessel walls, and he proposed the name "vitamin P" to bioflavonoids including hesperidin.[25] In our preceding article, we clarified that conjugated hesperetin metabolites accumulated in the aortic tissue of rodents after dietary supplementation for 4 weeks.[26] This phenomenon suggests that the vascular function of hesperidin is exerted by its successive supplementation. Nevertheless, the bioavailability of this compound seems to be low, and its physiological activity in the target sites inadequate because it is thought that dietary flavonoids are primarily excreted into the feces without absorption into the body.[27,28] Therefore, flavonoids barely function in the vascular system in the case of single doses. In our previous study,[14] a highly water-soluble hesperidin derivative was found to be absorbed more efficiently than hesperidin. Thus, the increase in water-solubility is likely to be essential to improve hesperidin bioavailability. Hesperetin, an aglycone of hesperidin, may be a suitable alternative for rapid absorption into the body because the hydrophobic property of flavonoid aglycone is suggested to accelerate passive transport mechanisms at the small intestine.[12]

Here we developed water-dispersible hesperetin to improve the bioavailability of hesperetin because hesperetin possesses rather lipophilic properties because of its diphenylpropane skeleton. Studies reported that the T_{max} of plasma hesperetin concentration was 4–6 h after the intake of hesperetin.[8,24] It was confirmed that water-dispersible hesperetin was absorbed more rapidly with a T_{max} of <1 h (Fig. 3A). Then we compared the plasma concentration of hesperetin between the intake of water-dispersible hesperetin and intact hesperetin. Interestingly, the maximum concentration of the water-dispersible hesperetin (10.2 ± 1.2 μM) at 1 h was 5.5-fold higher than that of intact hesperetin (1.8 ± 0.8 μM) (Fig. 3B), indicating that the processing for water-dispersible hesperetin dramatically increased the rate of absorption as well as the absorption efficiency of hesperetin into the body. Therefore, we succeeded in the preparation of hesperetin which reached the blood circulation rapidly

and efficiently to exert its physiological functions toward blood vessels.

In general, a large part of flavonoids are converted into inactive metabolites by phase-II enzymes in the intestine, and the liver even absorbed into the body because flavonoids are recognized as xenobiotics by the body.[29] Dietary hesperetin and hesperidin are known to circulate in the blood-stream as mainly hesperetin glucuronide and sulfate conjugates.[9,27,30] In the present study, we clarified that conjugated hesperetin metabolites with glucuronidation or sulfation (but not aglycone) accumulated in human plasma after oral intake of water-dispersible hesperetin. Interestingly, water-dispersible hesperetin gave a simple profile of its conjugated metabolites at the highest plasma concentration, in which three major metabolites. From the comparison of the authentic standard, Hp7GAwas found to be involved in the metabolites. Earlier studies identified Hp7GA, Hp3′GA and hesperetin 3′-sulfate, hesperetin-*O*-diglucuronide, and hesperetin-*O*-glucuronidesulfate as hesperetin metabolites in human plasma after its oral administration.[27,30,31] Brett *et al.*[30] found hesperetin-3′-*O*-sulfate (not hesperetin-7-*O*-sulfate) in human plasma after oral administration of hesperidin. Brand *et al.*[31] presented the reason for the absence of hesperetin 7-*O*-sulfate in humans by sulfonation kinetics. Therefore, peak Y and peak Z in the Fig. 4A can be ascribed to Hp3′GA and hesperetin 3′-sulfate, respectively (Fig. 5).

In contrast, various metabolites, including glucuronide-sulfate and diglucuronide, seem to be yielded after taking a blood sample at a longer period. Rapid absorption at intestinal epithelial cells and after transport to the portal vein may limit the action of phase-II enzymes in the liver as well as the intestine.

NO released from endothelial cells *via* endothelial nitric oxide synthase (eNOS) is well known as a potential vasodilatation agent by which guanyl cyclase is activated to lead the enhancement of cyclic GMP level in the smooth muscle cells. NO/cGMP signaling cascade is of importance in the cardiovascular and nervous systems, where it controls smooth muscle relaxation. NO is also suggested to be a pivotal vasoprotective molecule.[32,33] In the vascular wall, $O_2^{\cdot-}$ can be generated by several enzyme systems, including endothelial NADPH oxidase.[34,35] This reactive oxygen species (ROS) can diminish the vascular level of NO by a spontaneous reaction with NO to produce the highly reactive peroxynitrite.[32] We investigated the effect of Hp7GA on NO release from HUVECs. Similar to hesperetin, this hesperetin metabolite led to a significant increase in NO release in a concentration-dependent manner (Fig. 6A). We also demonstrated that $O_2^{\cdot-}$ release from HUVECs and the NADPH activity of HUVECs were inhibited by Hp7GA (Fig. 6B and C), although the efficacy of inhibition by the conjugated metabolite was lower than that of the parent compound. These results strongly suggest that Hp7GA can inhibit the activity of endothelial NADPH oxidase, resulting in the suppression of $O_2^{\cdot-}$ release and following peroxynitrite formation. In addition, it was found that glucuronidation did not affect the inhibitory activity of hesperetin. This idea seems to be rational because Steffen *et al.*[21] demonstrated that apocynin-like inhibition of endothelial NADPH oxidase happens in the flavonoid subgroup containing the *O*-methyl group at the neighbor position of the phenol group in the B-ring. Thus, Hp7GA, as well as hesperetin aglycone, possesses this partial structure in the skeleton, leading it to act as an inhibitor of NADPH oxidase. Our preliminary experiment using paraquat-exposed HUVECs implied the direct $O_2^{\cdot-}$ scavenging by Hp7GA contributed little to the effect on vascular NO level (data not shown). It was already demonstrated that several flavonoids are able to enhance NO release from endothelial cells. For example, Lui *et al.*[36] reported that hesperetin up-regulated endothelium nitric oxide synthase (eNOS) expression and enhanced NO release from endothelial cells. In addition, Yamamoto *et al.*[37] found that glucosyl hesperidin decreased mRNA expression of NADPH oxidase subunits in the aorta of spontaneously hypertensive rats. Nevertheless, hesperetin is also known to be mostly present as conjugated metabolites after oral administration and the effect of conjugated hesperetin on endothelial function has been obscure. Here, we presented, for the first time, that plasma hesperetin metabolites can increase NO release from endothelial cells by inhibiting NADPH oxidase activity.

Finally, we assessed the effect of water-dispersible hesperetin on recovery of the peripheral body temperature from cooling stress. Intensively, water-dispersible hesperetin was effective in recovering finger temperature (Fig. 7A). Furthermore, we found that water-dispersible hesperetin could keep the blood flow in accordance with inhibition of the decrease of finger temperature (Fig. 7 B and C). These results demonstrated that water-dispersible hesperetin could promote blood flow and keep the peripheral temperature higher. Although the cold tolerance and blood flow tests were not gold standard for testing endothelial function,[38,39] this study was attempted to estimate the peripheral vasodilation in human volunteers. It should be noted that the amount of water-dispersible hesperetin used for the measurement of the effect on peripheral vasodilitation is similar to the amount used for the measurement of bioavailability studies and is small enough to be added to a portion of food, drink, or supplement. Therefore it is likely that the result obtained here can be applied in the practice. However, due to the small sample size in this study, future research is required to specify the effects of water-dispersible hesperetin on peripheral temperature and blood flow.

In conclusion, we showed that water-dispersible hesperetin is unexpectedly absorbed into the blood circulation rapidly at high concentrations in human subjects as compared with intact hesperetin. Because there is rapid absorption at high concentrations in human plasma, it is advantageous to exert the direct vascular function with a single dose of hesperetin. One of the main metabolites in human plasma, Hp7GA, could exert enhancement of NO release from cultured endothelial cells by inhibiting NADPH oxidase activity. Our results confirmed the findings of other human volunteer studies showing the effectiveness of a single dose of water-dispersible hesperetin to recover blood flow and body temperature under cold temperature stress. The mechanism involving the inhibition of NADPH oxidase is likely to participate in the validation action of water-dispersible hesperetin. Hesperetin is definitely a promising flavonoid as a component of functional foods and is expected to help in maintaining vascular health.

Abbreviations

Hp7GA	hesperetin 7- *O*-β-D-glucuronide

Hp3′GA	hesperetin 3′-*O*-β-D-glucuronide
NO	Nitric Oxide
$O_2^{\cdot-}$	superoxide anion
NADPH oxidase	nicotinamide adenine dinucleotide phosphate-oxidase
C_{max}	maximum plasma concentration
T_{max}	time to reach maximum concentration

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