Pulmonary, Gastrointestinal and Urogenital Pharmacology

Induction of aminolevulinic acid synthase gene expression and enhancement of metabolite, protoporphyrin IX, excretion by organic germanium

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ABSTRACT

Poly-trans-[(2-carboxyethyl) germasesquioxane], Ge-132 is a water-soluble organic germanium compound. Oral intake of dietary Ge-132 changes fecal color and we attempted to identify the fecal red pigment, which increased by the intake of dietary Ge-132. Sprague Dawley rats were given diets containing Ge-132 from 0 to 0.5% concentration. Fecal red pigment was extracted and purified for optical and structural studies. We examined the fecal red pigment content by high performance liquid chromatography (HPLC), and hepatic gene expressions relating to heme synthesis by reverse transcription polymerase chain reaction (RT-PCR). The purified red pigment had particular optical characteristics on the ultraviolet (UV)–visible spectrum (Soret band absorbance at 400 nm) and fluorescence emission at 600 nm by 400 nm excitation, and was identified as protoporphyrin IX by LC-MS analysis. Protoporphyrin IX significantly ($P<0.05$) increased 2.4-fold in the feces by the intake of a 0.5% Ge-132 diet. Gene expression analysis of the liver explained the increase of protoporphyrin IX by dietary Ge-132 as it enhanced ($P<0.05$) aminolevulinic acid synthase 1 (Alas1), a rate-limiting enzyme of heme synthesis, expression 1.8-fold, but decreased ferrochelatase (Fech) expression 0.6-fold ($P<0.05$). The results show that the intake of dietary Ge-132 is related to heme metabolism. Because protoporphyrin IX is used to treat chronic hepatitis, Ge-132 may be a beneficial substance to increase protoporphyrin IX in the liver.

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1. Introduction

Poly-trans-[(2-carboxyethyl) germasesquioxane], Ge-132, is the most common water-soluble organogermanium compound (Fig. 1); it has very low toxicity. Ge-132 was first synthesized by Tsutsui et al. (1976), and its immunomodulation activities have been revealed (Aso et al., 1989; Ebina et al., 2004; Aso et al., 1985). In particular, the activation of natural killer cells and macrophages (Aso et al., 1985) has been reported; therefore, interferon gamma is induced in the serum of humans (Miyao and Tanaka, 1988) and mice (Brutkiewicz and Suzuki, 1987) after the administration of Ge-132. Many physiological functions of Ge-132 have been reported: anti-rheumatoid (Arimori et al., 1990), anti-osteoporosis (Fujii et al., 1993), anti-inflammatory (Furusawa et al., 1987) and so on. Recently, Ge-132 has been used in functional foods and in cosmetics in Japan and the U.S.A.

On the other hand, an inorganic germanium dioxide, GeO_2, has fatal toxicity. The difference in the intake of GeO_2 and Ge-132 was reported by Sanai et al. (1991). GeO_2 is accumulated in the kidney and causes renal failure, whereas Ge-132 is not accumulated in any tissue. The structure of

the germanium compound is very important for safe use. In the past, fatal accidents occurred by the intake of health foods containing germanium compounds. In these cases, they were sold as organic germanium unless they contained inorganic germanium; therefore, it is necessary to avoid contamination with inorganic germanium.

The intake of Ge-132 changes the fecal color of mammals to yellow. Recently, we reported (Nakamura et al., 2010) that this color change was the increase of the fecal pigment stercobilin, a metabolite of bilirubin. Mammalian feces contain many porphyrin metabolic pigments, such as stercobilin, urobilinogen, bilirubin, protoporphyrin IX (PpIX) and so on. Urobilinoids are metabolites of bilirubin by clostridia (Vitek et al., 2006). We have demonstrated the anti-oxidation activity of urobilinogen previously (Nakamura et al., 2006). Dietary Ge-132 increased various antioxidants related to bilirubin and urobilinoids.

We also showed the increase of a red pigment in the feces by Ge-132 intake and considered the red pigment to be related to PpIX (Nakamura et al., 2010). PpIX is synthesized in many tissues, mainly bone marrow (Surinya et al., 1997), and is secreted into the feces in bile (Beukeveld et al., 1994). On the other hand, there are diseases such as porphyria; erythropoietic protoporphyria is a disease caused by excess PpIX as a result of ferrochelatase deficiency (Lecha et al., 2009). Meanwhile, protoporphyrin disodium is used to treat chronic hepatitis in Japan. This suggests that PpIX in the intestine affects the suppression of hepatic

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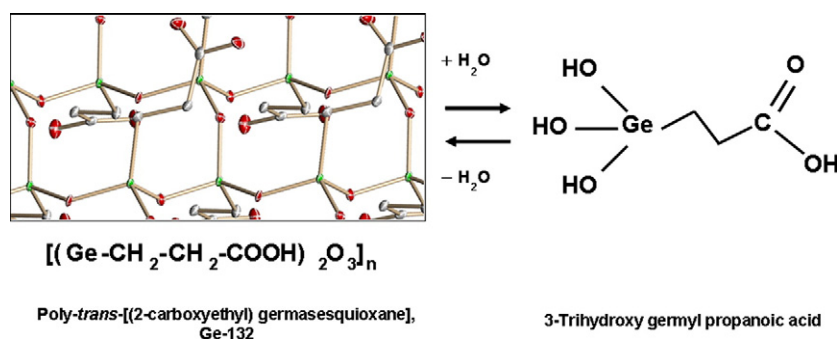E-mail address: nakamura@asai-ge.co.jp (T. Nakamura).

Fig. 1. Structure of poly-*trans*-[(2-carboxyethyl) germasesquioxane], Ge-132, and its hydrolyzed compound, trihydroxy germyl propanic acid. The 3D structure model shows germanium in green, oxygen in red and carbon in gray, respectively. Hydrogen is omitted.

inflammation. Nagai et al. (1992) studied the anti-inflammation effect of zinc protoporphyrin and described the anti-inflammation effect of PpIX.

In this report we investigated the effect of oral Ge-132 intake on fecal pigmentation in rats. The increasing red pigment was purified from feces and identified as PpIX by TOF-MS analyses. The PpIX secretion in the feces affected by the dietary Ge-132 was then quantified by HPLC. Moreover, hepatic gene expressions were analyzed for heme synthesis and breakdown to bile pigment.

2. Materials and methods

2.1. Chemicals

Ge-132 (Lot No. 006316A) was synthesized at Asai Germanium Research Institute Co., Ltd (Hakodate, Japan). Its purity was over 99.99%, and it was used for all experiments. Protoporphyrin IX and protoporphyrin IX disodium salt were obtained from Sigma Chemical Co. (St. Louis, MO, USA). CD_3OD for nuclear magnetic resonance (NMR) analysis was obtained from Merck KGaA (Darmstadt, Germany). All organic solvents for extraction and HPLC were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Fecal color change by the intake of different amounts of Ge-132

Four male Sprague Dawley rats (four weeks old; Clea Japan Inc., Tokyo Japan) were housed in a divided cage. They were kept in an air-conditioned room (temperature 23–25 °C) on a 12 h light–dark cycle and maintained on a basal purified diet for a week. They were then given diets containing different amounts of Ge-132 for a week. The amount of Ge-132 in each diet was 0, 0.005, 0.5 and 0.5%, respectively (Table 1). We then observed the fecal color changes.

2.3. Characteristic studies and quantification of fecal red pigment

Sixteen male Sprague Dawley rats (four weeks old; Clea Japan Inc., Tokyo Japan) were housed in a divided cage and kept under the same conditions as described above. They were acclimated with the basal diet for a week and then randomly divided into two groups. One group was given the control diet and the other group was given the 0.5% Ge-132 diet for two weeks. Water was available ad libitum. The composition of each diet based on AIN 76 is shown in Table 1. On the final day, the animals were anesthetized with pentobarbituric acid and killed by bleeding from the abdominal aorta; total blood was collected into a heparin-treated tube, and the liver was removed and used for analysis. Each blood sample was centrifuged immediately to divide the serum. During the feeding period, the feces were collected for pigment analyses and kept at −80 °C.

2.4. Extraction of fecal pigments

2.4.1. HPLC analysis
Fecal pigments were extracted with organic solvent as follows: 4-fold ethyl acetate/acetic acid (4:1, vol/vol) was added to the feces and each sample mixture was homogenized with a polytron homogenizer. The homogenates were centrifuged and supernatants were collected. The pellets underwent extraction twice. The supernatant was mixed and filtrated, and then evaporated and resolved in aliquots with chloroform/methanol (2:1, vol/vol).

2.4.2. TOF MS analysis
Twenty grams of fecal sample was homogenized and then 100 ml chloroform/methanol (2:1) and 5 ml acetic acid were added. The sample was stirred for 2 h and filtered, and then 100 ml chloroform/methanol (2:1) was added to the filtered sample. The extraction was repeated 2 times. The total filtrate was mixed and evaporated, and then the pigments were purified by chromatography.

2.5. Separation of fecal red pigment

The total extract of fecal pigments was placed in a silicic acid column (Silicagel 60; Merck, Germany) for chromatography. Several solvents, chloroform, acetone and methanol, were used for elution. The eluted solutions were spotted on a silica gel thin layer chromatograph, TLC (Silicagel 60; Merck, Germany) and separated for confirmation by the developing solvent of chloroform/methanol/water (65:25:4, vol/vol). Fluorescence was detected by irradiating the TLC plate at 365 nm with a trans-illuminator (UVP; LLC, Upland CA, USA). The red pigment was eluted with the acetone fraction and then treated and attached to an OASIS sample extraction product, Oasis MAX Plus (Waters Co., Milford, MA, USA) ion exchange resin. The red pigment was eluted with 2% formic acid in acetone. The fraction containing red pigment obtained from ion exchange chromatography

Table 1
Composition of diets for Ge-132 administering in different concentration.

	Control	0.005%Ge-132	0.05%Ge-132	0.5%Ge-132
	(Weight %)			
Casein	23	23	23	23
Corn starch	61.5	61.495	61.45	61
Corn oil	5	5	5	5
DL-methionine	0.3	0.3	0.3	0.3
Vitamin mixture	1	1	1	1
Mineral mixture	4	4	4	4
Cellulose	5	5	5	5
Choline	0.2	0.2	0.2	0.2
Ge-132	-	0.005	0.05	0.5
Total	100	100	100	100

HPLC analysis was carried using control and 0.5%Ge-132 groups.
Gene expression was studied using control and 0.05%Ge-132 groups.

was placed on a TLC plate to purify. The TLC was developed with chloroform/methanol/water (65:25:4, vol/vol). The separated fraction of red pigment was scraped from the TLC plate for extraction. The red pigment attaching to silica gel was extracted twice with 1 ml methanol each time, and the extract was filtered by DISMIC (0.2 μm; ADVANTEC, Tokyo Japan). The obtained pigments were analyzed by an optical spectrometer and LC/MS.

2.6. Preparation of protoporphyrin IX methyl ester

Protoporphyrin IX (Sigma Chemical Co.) 10 mg was dissolved in 4 ml of 5% HCl in methanol. The mixture was then esterified at −20 °C for an hour, after which 8 ml chloroform and 3 ml water were added to the reaction mixture and mixed well. The lower phase was evaporated and dissolved in chloroform. The solution was added to a silicic acid column and eluted with chloroform/methanol (8:2, vol/vol) to isolate the two major red pigments. The obtained fractions containing red pigment were purified with silica gel TLC, as described above. Two red pigments possessing different Rf values on the TLC were obtained and named red pigment A and B. The obtained pigments were used for TOF/ MS and NMR analyses.

2.7. Structural analysis of synthesized protoporphyrin IX methyl ester

Two purified methyl esters of PpIX (PPME), named A and B, were identified by TOF/MS and NMR analyses. Both pigments were dissolved in 50% methanol for TOF/MS analysis. TOF mass spectra were recorded on an ESI: LCT (Waters Co.) mass spectrometer. Samples (approximately 10pmol) with 50% acetonitrile were injected and measured.

Purified PPME A and B were dissolved in CD_3OD and then analyzed by ^1H NMR using a Gemini 300 (Varian Technologies Japan Ltd., Tokyo Japan). Measurements were performed at 25 °C probe temperature. PPME A could be analyzed easily but PPME B did not show data with separate sharp peaks.

2.8. Analyses of separated synthesized or fecal red pigments

The spectra of purified red fecal pigment were recorded with a UV–visible (vis) spectrophotometer, UV2200 (Shimadzu, Kyoto Japan) and a fluorescence spectrophotometer, FP777 (Jasco, Tokyo Japan). The maximum absorption was confirmed by the UV–vis spectrophotometer. The pigment sample was then excited at the obtained maximum absorption wavelength and the fluorescence spectrum scanned using a fluorescence spectrophotometer.

2.9. Mass analysis of purified fecal red pigment

The LC/TOF mass spectra of purified fecal red pigment were recorded on an ESI: LCT (Waters Co.) mass spectrometer. Samples (approximately 10 pmol) with 50% acetonitrile were injected and separated by ODS-3 (1.5×150 mm; GL Science Inc., Tokyo Japan) using a semi-microcolumn. Elution was carried with 50% MeOH at a flow rate of 0.2 ml/ min. The separated peak at 10.8 min was analyzed for mass study.

2.10. HPLC analysis of rat fecal protoporphyrin

For HPLC analysis, the pigments were methylated by the same methods described above. They were then dissolved in 1 ml chloroform, and a 10 μl sample was injected onto an HPLC column. The pigments were quantified by HPLC equipped with a silicic acid column Chromosorb Silica 100 (Merck). The mobile phase was a mixture of n-hexane/ethyl acetate (1:1, vol/vol), and the flow rate was 2.5 ml/ min. Detection was carried out using a fluorescent spectrophotometric detector, FP821 (Jasco) at EX: 405 nm, Em: 630 nm.

2.11. Gene expression analysis of enzymes relating to heme metabolism

Fourteen male Sprague Dawley rats (four weeks old; Clea Japan Inc.) were housed in a divided cage and maintained under the same conditions as described above. They were acclimated with the basal diet for a week and then randomly divided into two groups. One group was given the control diet and the other group was given the 0.05% Ge-132 diet for two weeks. Water was available ad libitum. The composition of each diet based on AIN 76 is shown in Table 1. On the final day, the animals were anesthetized with diethyl ether and killed by bleeding from the abdominal aorta; their liver was removed and they were dipped in RNAlater (Applied Biosystems, Carlsbad, CA, USA) for gene expression analysis. They were kept at −30 °C.

Total RNA was extracted from approximately 50 mg liver tissue. The tissue was homogenized with 1 ml RNAiso Plus (Takara Bio, Shiga Japan). Total RNA was extracted according to the usual phenol–chloroform extraction method. The DNA of the extracted sample was digested with TURBO DNA-free (Applied Biosystems), and then the sample was checked for quality and quantified. Two micrograms of total RNA sample was used in reverse transcription to synthesize template cDNA with oligo deoxythymidine primer and Super Script III reverse transcriptional enzyme (Invitrogen). The reaction was performed at 50 °C for 60 min.

The synthesized cDNA was diluted 5-fold with PCR Easy Dilution (Takara Bio) and then used for quantitative real-time polymerase chain reaction (PCR). The expressions of genes relating to heme metabolism were measured as the expression ratio to beta actin as a housekeeping gene. The primers were as follows: Alas1 (Genbank accession No. NM_024484.2) forward 5′-TGT GGC CCA TGA GTT TGG AG-3′, Alas1 reverse 5′-GAA CGC TTT ACC GAG TGT TCC AG-3′, Alas2 (Genbank accession No. NM_013197.1) forward 5′-TGC GCC AGC TAC TAA TGG ACA G-3′, Alas2 reverse 5′-CGA GGC ACA GTT GGG TAG TTG A-3′, Fech (Genbank accession No. NM_ 001108434.1) forward 5′-ATT TCC AAG AAC AGT GCC ACC AG-3′, Fech reverse 5′-TTC AAG GCT ATC AAA CAC GGT CAA-3′, Hmox1 (Genbank accession No. NM_012580.2) forward 5′-AGG TGC ACA TCC GTG CAG AG-3′, Hmox1 reverse 5′-TCC AGG GCC GTA TAG ATA TGG TAC A-3′, Actb (Genbank accession No. NM_031144.2) forward 5′-GGA GAT TAC TFC CCT GGC TCC TA-3′ and Actb reverse 5′-GAC TCA TCG TAC TCC TGC TTG CTG-3′. The cDNA was amplified using SYBR Premix Ex Taq II (Takara Bio) on an Opticon 2 (Bio-Rad Laboratories, Hercules, CA, USA), which was programmed for 95 °C for 30 s, followed by 45 cycles of denaturation (95 °C for 5 s), annealing and extension (60 °C for 30 s).

2.12. Peripheral blood ferric ion

The serum sample was used for the quantitative study of ferric ion. The unsaturated iron binding capacity was measured using a UIBC test Wako kit (Wako Pure Chemical Industries, Ltd.). The measurement was performed according to the manufacturer's manual with a UV–vis spectrometer.

2.13. Ethics of animal experiments

All animal experiments were conducted according to the guidelines of the ethics committee of experimental care at Asai Germanium Research Institute Co., Ltd., which are based on public guidelines set by the Japanese Ministry of Education, Culture, Sports, Science and Technology.

2.14. Statistical analysis

The results of the experiments are presented as the mean ± standard error of the means (S.E.M.). Significant differences among two groups were analyzed using Student's t test. The statistical significance of all experiments was defined as $P < 0.05$.
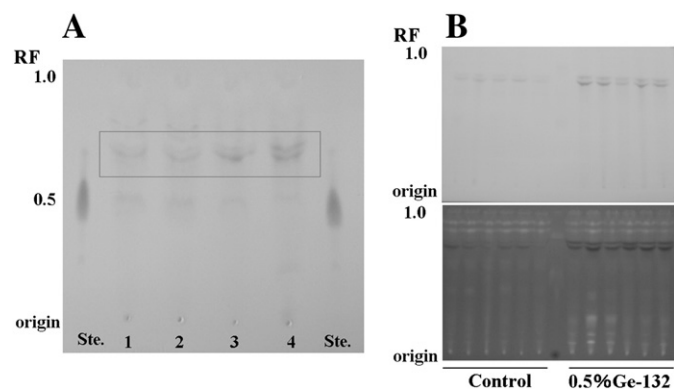
Fig. 2. Fecal color change by Ge-132 diets. Feces samples were collected from rats given 0, 0.005, 0.05 and 0.5% Ge-132 diets. The TLC pattern of extracted pigments is shown in (A). Extracts from the same amount of feces were spotted and developed by chloroform–methanol (95:12, v/v). Lanes are: 1: control (0% Ge-132), 2: 0.005% Ge-132, 3: 0.05% Ge-132, 4: 0.5% Ge-132, respectively. Lane ste. was spotted with commercial stercobilin as a standard. Each pigment extract from 5 g feces was dissolved to 1 ml. TLC separation of extracted pigment is shown in (B). Twenty microliters of extract was spotted in each lane. Commercial stercobilin was spotted between two groups as a reference. Developing solvent, C-M-W (65:25:4, v/v), was used. Lower image of the TLC plate was irradiated by ultraviolet light at 365 nm.

3. Results

3.1. Characteristic studies of fecal red pigment enhanced by Ge-132 administration

Ge-132 is a water-soluble organogermanium compound with a polymer structure that is hydrolyzed to 3-trihydroxy germyl propanoic acid in water. Its structure is shown in Fig. 1. Dietary Ge-132 changes mammalian fecal color and we administrated different amounts of Ge-132 orally to rats; their feces changed color clearly. The control feces were dark, and feces after administration of 0.005% Ge-132 diet were not so different; however, administration of the 0.05% Ge-132 diet made the fecal color lighter than the control, whereas the 0.5% Ge-132 diet made the feces slightly redder than 0.05% Ge-132. The extracted fecal pigments were separated on a silica gel TLC plate, as shown in Fig. 2A. The red pigment increased in a dose-dependent manner.

The rat fecal pigments after ingesting both diet compositions, shown in Table 1, were extracted by organic solvent. The TLC pattern of extracted fecal pigments is shown in Fig. 2B. Several spots of fecal pigments were found and the content of dietary Ge-132 affected the amount of red pigment, with an Rf value at 0.8. The concentration of red pigment in the feces after Ge-132 intake was higher than the
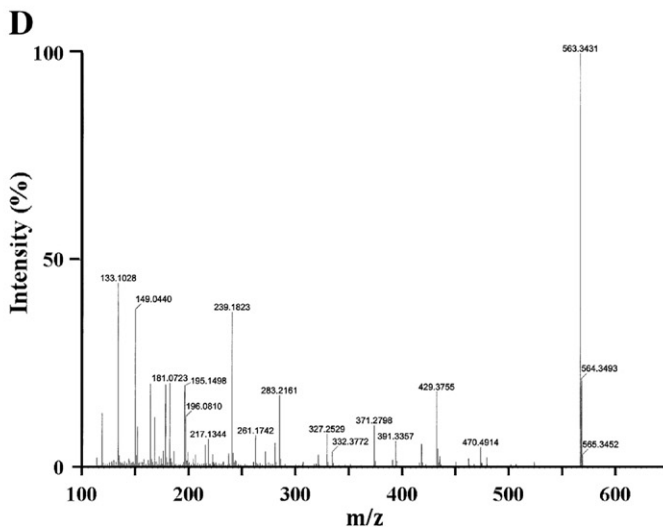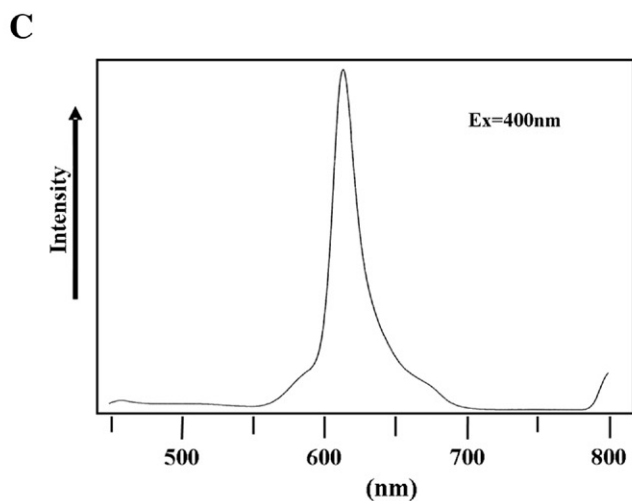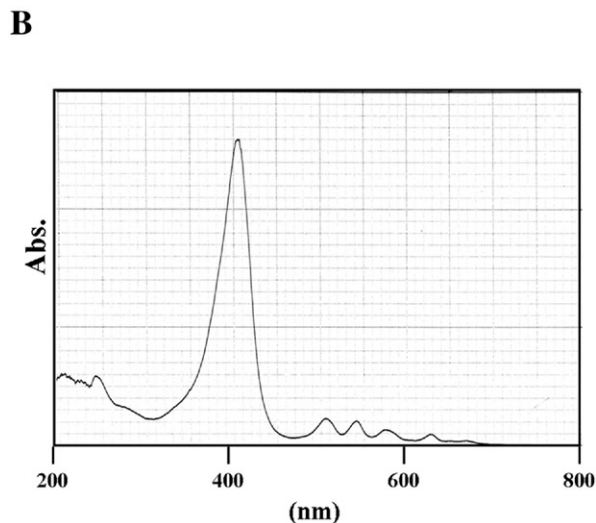

Fig. 3. Final identification of protoporphyrin IX. Assignment chemical shifts of synthesized protoporphyrin IX dimethyl ester by NMR are shown in (A). Purified red pigment from rat feces was dissolved in methanol. UV–vis spectrum was measured from 190 to 800 nm and is shown in (B). Fluorescent spectrum was measured using 400 nm for excitation and scanned emission from 420 to 820 nm, as shown in (C). TOF mass spectrum was used to measure the purified fecal red pigment, which increased with dietary Ge-132, as shown in (D). The red pigment was identified as protoporphyrin IX. The sample was dissolved in methanol, injected onto LC-MS, and separated by 50% methanol eluent. The peak detected at 10.8 min was analyzed by TOF/MS.

control. On the other hand, UV irradiation showed strong red fluorescence of the red pigment, which was increased by dietary Ge-132. We purified this red pigment using silicic column separation. The pigment was purified by ion exchange chromatography and then used for optical characteristic and structural studies.

3.2. Structural studies of synthesized protoporphyrin methyl ester

We synthesized the methyl ester of protoporphyrin (PPME) for standard HPLC analysis. Two different pigments were obtained by chromatography separation and were named A and B. Synthesized PPME A and B were purified using a silicic column and Silicagel TLC, and analyzed by TOF/MS and NMR analyses. PPME A was identified as a protoporphyrin dimethyl ester, PPDM, by TOF/MS analysis. Distinct peaks accorded with the fecal red pigment and were mainly observed at m/z 591 [M^+], 517, 503, 459, 445 and 431. The fragment ion peaks suggested that the sample was protoporphyrin IX dimethyl ester, PPDM. In addition, proton NMR analysis data showed that all the peaks originating from the twelve groups bonding to the skeleton of porphyrin were PPDM. The assigned chemical shifts of each radical of synthetic PPDM are shown in Fig. 3A. On the other hand, PPME B was identified as a protoporphyrin monomethyl ester, PPMM, by TOF/MS analysis. The parental peak was detected at m/z 577. PPDM was used as the standard in optical analyses, TLC and HPLC analysis.

3.3. Optical and structural characteristics of purified fecal red pigment

The observations of purified fecal red pigment with a UV–vis spectrometer and a fluorescent spectrometer are shown in Fig. 3B and C. The red pigment extracted from feces had several absorption maxima at 405, 505, 540 and 575 nm. Optic absorption was highest at 405 nm, which is specific to porphyrin products. Strong absorption at about 400 nm, so-called Soret band absorption, and excitation at this wavelength highlights strong red fluorescence of about 600 nm (Fig. 3C.). The characteristics of these optical analyses were very similar to fecal PpIX, synthesized PPDM and PPMM.

The red pigment separated from feces was analyzed by a TOF mass spectrometer equipped with LC. The red pigment was separated with LC with 50% MeOH as the elution solvent. The peak of the red pigment was identified at 10.8 min. The TOF mass spectrum of the peak is shown in Fig. 3D. The parental peak in the mass spectrum was observed at m/z 563 [$M + H^+$]; therefore, the red pigment was identified as PpIX.

3.4. HPLC analysis of rat fecal protoporphyrin IX

Fecal PpIX in the extracted sample was methylated and then identified by HPLC equipped with a silicic column. The content of PpIX in the fecal extract from rat feces is shown in Fig. 4. The amount of PpIX in the control group was 9.3 and in the Ge-132 group was 21.9 (μg/g wet feces), which was about two-fold significance (P<0.05). On the other hand, the cecal content was not different between groups. Feces were strongly concentrated from the cecal content, and the absorption of PpIX might have differed in the rectum. One reason for the increase of PpIX excretion in feces is considered to be the progression of synthesis in the bone marrow or the liver.

3.5. Gene expression of enzymes relating to heme metabolism in liver

The gene expressions of aminolevulinic acid synthase (Alas) 1, Alas 2, ferrochelatase (Fech) and heme oxigenase 1 (Hmox1) in rat liver were evaluated. 5-Aminolevulinic acid (ALA) is made from glycine and succinyl-CoA catalyzed by Alas. This reaction is the first step in porphyrin synthesis as it limits the rate of the whole synthesis of heme. The expressions of Alas 1, Alas 2, Fech and Hmox 1 in the liver are shown in Fig. 5. No change in Alas2 was found in either group. On the other hand, the expression of Alas1 was increased about 1.8-fold (0.65
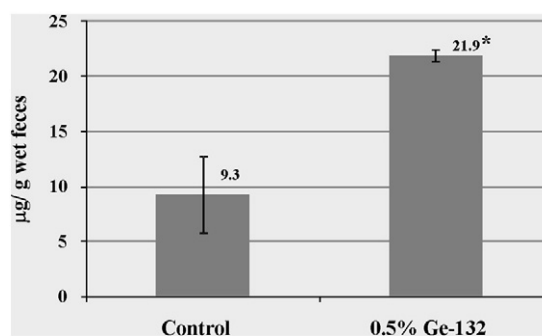

Fig. 4. Effect of Ge-132 administration on protoporphyrin amount in rat feces. Fecal protoporphyrin of rats was measured by HPLC. Extracted protoporphyrin was methylated and identified as protoporphyrin dimethylester. Pigments were quantified by HPLC equipped with a silicic acid column, Chromosorb Silica 100 (Merck, Germany). The mobile phase was a mixture of n-hexane/ethyl acetate (1:1, vol/vol), and the flow rate was 2.5 ml/min. Detection was carried out using a fluorescent spectrophotometric detector, FP821 (JASCO, Japan) at EX: 405 nm, Em: 630 nm. The intake of 0.5% Ge-132 diet increased fecal protoporphyrin. Data are presented as the mean. Error bars are presented as S.E.M. Value with asterisk is significantly different from the control group (P<0.05).

to 1.15) by dietary Ge-132, suggesting that porphyrin synthesis in the liver is enhanced by Ge-132 administration, while Fech, which is also a rate-limiting enzyme, was significantly decreased about 0.6-fold (1.29 to 0.79). This enzyme regulated the insertion of ferric ion to PpIX. Hmox 1 catalyzed the breakdown of hemin to biliverdin and was increased 1.7-fold (0.96 to1.63), with no significance.

3.6. Ferric ion

We evaluated the concentration of peripheral blood ferric ion by the intake of Ge-132. The unsaturated iron binding capacity of the control and 0.05% Ge-132 was 4.93 and 5.02 μg/ml, respectively. No change was found by the intake of Ge-132.

4. Discussion

The effects of dietary Ge-132 on fecal color change were evaluated. We previously reported the fecal color change by Ge-132 intake (Nakamura et al., 2010) and mainly described tetra pyrols, metabolites of heme; however, we found unknown red spots in silicagel TLC analysis of fecal pigments. In this study, we purified the red pigment, which was increased by dietary Ge-132, showed its optical characteristics and
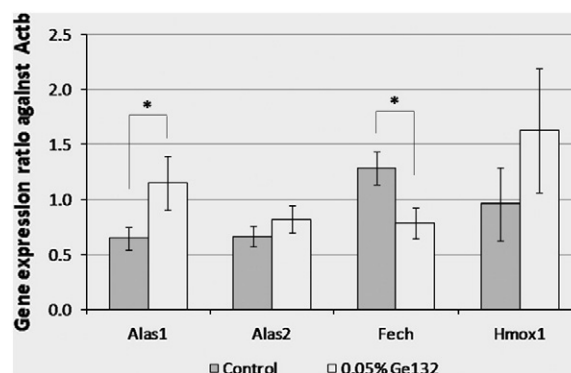

Fig. 5. Effect on gene expression of Alas1 in liver by Ge-132 administration. Hepatic gene expressions of Alas1, Alas2, Fech and Hmox1 against Actb were measured by qPCR. The diets, Ge-132 free (Control) and 0.05% Ge-132 (containing 0.05% Ge-132), were given to the rats for two weeks. Samples of total RNA were extracted from the liver of rats. Value with asterisk is significantly different from the control group (P<0.05).

identified the structure. Moreover, an increased excretion amount was identified by HPLC.

The administration of 0.5% Ge-132 diet to rats changed the fecal color clearly as a result of the red pigment (Fig. 2), which had specific optical characteristics (Fig. 3). Absorption maxima were found at 405, 505, 540 and 575 nm, and were Soret band absorption (Rimington, 1960), and alpha, beta and gamma band absorption, respectively, suggesting that it was a porphyrin product. In addition, with excitation at 400 nm, the pigment had strong fluorescence emission at 600 nm. These data also reflect the characteristics of porphyrin (shown in Fig. 3).

Analysis of the data peak of TOF-MS confirmed that the red compound was PpIX (Fig. 3). Rimington studied the spectral absorption coefficients of some porphyrins in the Soret band region (Rimington, 1960), and found that the Soret bands of protoporphyrin dimethyl ester and protoporphyrin were at 407.5 nm and 408 nm, respectively. These are almost identical, and we found the same pattern with synthesized PPDM and PpIX purified from fecal extract in this study. It is known that PpIX is a minor fecal component. The porphyrins are synthesized mainly in bone marrow (Surinya et al., 1997) and PpIX is the last precursor of hemin before insertion of a ferric ion (Beukeveld et al., 1994); therefore, PpIX is one of the most important compounds for mammalian respiration. Heme, which is synthesized in different organs, is used in different ways in the bone marrow and liver. The former is used as a component of hemoglobin in red blood cells (Surinya et al., 1997), and the latter in cytochrome enzymes (Sato et al., 1986). The increase of PpIX in the feces by dietary Ge-132 was confirmed by HPLC analysis (Fig. 4), and a two-fold increase was detected. We described the secretion of bilirubin in a recent study (Nakamura et al., 2010). Bilirubin is a metabolite of heme, therefore shortening heme, although the hematocrit value was not changed by Ge-132 intake; hence, supplying the heme system must keep the hematocrit value constant, suggesting that heme synthesis must occur at the same time to maintain homeostasis. Excess synthesized porphyrin, increased by dietary Ge-132, is excreted in feces as fecal protoporphyrin. We evaluated the PpIX increase with a 0.5% Ge-132 diet, which is too high for daily intake. The content was defined to identify the significant statistical difference. We confirmed the PpIX increase with 0.05% Ge-132, as shown in Fig. 2; therefore, the gene expression was studied using the 0.05% Ge-132 diet.

We confirmed that PpIX synthesis in the liver was increased. In Fig. 5, the gene expression of Alas1 was increased, while Fech was decreased in the liver by Ge-132 intake. In heme synthesis by two enzymes, Alas1 and Alas2, which catalyze the first step, and Fech, which catalyzes the last step, both are rate-limiting enzymes; therefore, the increase of Alas1 expression and decrease of Fech expression can upregulate the PpIX pool level in the liver. Hemin is produced by Fech, an enzyme inserting ferric ion into PpIX. The PpIX level is regulated according to the amount of hemoglobin and unsaturated iron. Surplus PpIX may be excreted into the intestine when dietary Ge-132 is ingested, and excessed PpIX in blood can cause protoporphyria (Lecha et al., 2009); however, we could not find any indication of protoporphyria symptoms in animals or humans administrated dietary Ge-132 (data not shown). Increased fecal PpIX suggests its increased secretion via bile. Rapid secretion of PpIX by dietary Ge-132 may protect against disease from excessive PpIX. Tephly et al. revealed that Alas was induced by various drugs in the rat liver (Tephly et al., 1971) and they discussed the results relating to the induction of cytochrome P-450. Cytochrome P-450 enzyme catalyzes many drugs and endogenous compounds by oxidation or reduction, and reduces the toxicity (phase I reaction) to the liver from harmful compounds (Boron and Boulpaep, 2009). Ge-132 may also induce cytochrome P-450 by Alas in detoxification. These results of a gene expression study suggest an increasing supply of PpIX and excretion of excessive porphyrin as PpIX and bile pigments. Fech may be controlled by the need for cytochrome P-450. The peripheral free ferric ion was not changed as a result of only increasing PpIX.

We confirmed the increasing tendency of Hmox1 gene expression in the liver by the intake of Ge-132. This change is in agreement with our previous report on bile pigment secretion (Nakamura et al., 2010). Hmox1 is inducible by stimulation with hemin, many non-heme substances (for example, heavy metals) and oxidative stress, and this enzyme is a stress protein. Ishikawa et al. (1983) reported the induction of Hmox1 by exogenous factors, showing the high induction of Hmox1 from 4.8- to 25.5-fold by hemin and cadmium. In this study we also found increased Hmox1 but it was milder than other inducing factors. Hmox1 prevented inflammation by carbon monoxide, a reaction product (Sass et al., 2003); therefore, the intake of dietary Ge-132 may act as a mild inducer of anti-inflammation by carbon monoxide from heme degradation.

PpIX is used to treat chronic hepatitis. The absorption and excretion of PpIX were revealed by Yokoyama et al. (1973) The pharmacological functions of PpIX are the regulation of hepatic function (Sato et al., 1986), increase of oxygen used by tissues (Yamamoto et al., 1966), acceleration of synthesize hepatic proteins (Takahashi et al., 1984) and anti-inflammation (Nagai et al., 1992). Additionally, hepatic protection effects have been reported (Takeuchi et al., 1980; Ueno et al., 1978; Kawakami et al., 1978). These properties of PpIX are similar to the physiological actions of Ge-132 previously reported (Sasaki et al., 1984); for example, on the one hand, PpIX is used to treat hepatic inflammatory disease as a pharmaceutical, whereas Ge-132 was reported to have a preventive effect against the elevation GOT and GTP activity in the serum of carbon tetrachloride-induced acute hepatitis (Sasaki et al., 1984). Many protective effects of Ge-132 on hepatic function have been shown (unpublished data).

Dietary Ge-132 increases PpIX in the intestine, which may protect hepatic function. It is suggested that some physiological functions of dietary Ge-132 are expressed through the role of induced PpIX. Dietary Ge-132 increases heme metabolites in the intestine as bile secretion. Pigments bilirubin, urobilinogen and PpIX are beneficial for health; namely, bilirubin and urobilinogen act as antioxidants (Nakamura et al., 2010) and PpIX acts as a protective compound for hepatic function. Meanwhile, Ge-132 has an immune regulatory function and its safety is well known, so daily intake of this compound as a functional food will maintain health. In this study, we evaluated the genes relating to heme metabolism in the liver. It would be interesting to study the effect of Ge-132 on erythroid in the bone marrow because heme is synthesized mainly in progenitor cells of erythroid cells, not in adult RBCs. Moreover, it should be studied how to regulate the upstream of the gene expression of heme metabolism by Ge-132. Further studies are expected.

5. Conclusion

We evaluated the function of a water-soluble organic germanium compound, Ge-132, in the induction of a red pigment, which was identified as PpIX. Ge-132 affected the regulation of the gene expression of rate-limiting enzymes of heme metabolism. PpIX is using to treat chronic hepatitis; therefore, the beneficial effect of Ge-132 for chronic hepatitis is expected.

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