Porcine Hemoglobin Promotes Lipid Excretion to Feces more Strongly than Globin Protein in Rats

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Abstract In an effort to clarify whether the lipid-lowering effect of hemoglobin is due to globin protein or heme, this study investigated the effects of dietary porcine hemoglobin (PH) and porcine globin (PG) on lipids contents of serum, liver, and feces in rats. Five-week-old male Wistar rats were divided into 3 dietary groups of 7 rats each, with one group receiving a control diet and the other groups receiving diets containing 1.25% (w/w) PH or 1.18% (w/w) PG for 4 weeks. The PH diet decreased triacylglycerol content in serum and cholesterol in serum and liver, whereas the PG diet reduced triacylglycerol content in serum and cholesterol in liver. Fecal lipid excretion in the PH group was significantly higher than in the PG group. Therefore, PH affected lipid excretion to feces due to globin protein as well as heme.

Keywords: porcine hemoglobin, globin, heme, lipid excretion, cholesterol

Introduction

In the slaughtering process, porcine blood is produced an abundant by-product. However, the blood of carnivores contains about 18–20% (w/w) protein, and small amounts of blood protein are used in food or feed. Porcine blood has been used as an ingredient of “blood sausage” in Germany and “blood tofu” in China, and is generally recognized to be safe (1). Blood protein includes two main components, plasma protein and hemoglobin, which together account for more than half of the total blood protein (2). Hemoglobin hydrolysates have been found to contain biologically active opioid peptides (3), a hypotriglyceridemic peptide (4), and a bacterial-growth stimulating peptide (5). Other reports suggested that hemoglobin hydrolysates decrease serum triacylglycerol (TAG) and cholesterol contents (6) and were capable of lipid peroxidation through the quenching of free radicals (7). In addition, heme-iron in hemoglobin is readily absorbed and used as a supplement for the prevention of iron deficiency in humans. Waste disposal and by-product management for environmental protection and sustainability continue to be a challenge for the food processing industry (8). Accordingly, the effective usage of porcine hemoglobin (PH) is expected to be generally beneficial.

Our previous report suggested that PH had lipid-lowering effects in the serum and liver, which was partly due to the enhanced excretion of fecal fatty acids, cholesterol, and bile acids (9). Hemoglobin is

composed of globin and heme, with each hemoglobin subunit consisting of a globular protein with an embedded heme. However, the individual contributions of globin protein or heme to the hypolipidemic effects of porcine hemoglobin remain unknown. In terms of the aim of developing lipid-lowering functional foods or supplements, we require the information in which component is a bioactive compound in PH. To examine the role of globin and heme in the lipid-lowering effect of hemoglobin, this study examined the effects of dietary PH and porcine globin (PG) on serum, liver, and fecal lipid contents, as well as the activity of enzymes related to fatty acid metabolism in rats.

Materials and Methods

Materials PH was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Acetyl-CoA, palmitoyl-CoA, malonyl-CoA, L-carnitine, flavin adenine dinucleotide, L-malic acid, glucose-6-phosphate, and nicotinamide adenine dinucleotide were purchased from Sigma-Aldrich Japan Co. Ltd. (Tokyo, Japan). Cornstarch, dextrinized cornstarch, sucrose, cellulose, casein, American Institute of Nutrition (AIN)-93G mineral mixture, and AIN-93 vitamin mixture were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Choline bitartrate, L-cystine, and *tert*-butylhydroquinone were purchased from Wako

Pure Chemical Industries Ltd. (Osaka, Japan). All other reagents were purchased from commercial sources and were reagent grade.

Globin preparation Globin was prepared by the method of Yip *et al.* (10) with some modifications. A PH solution at a concentration of 50 mL of 4% (w/v) was added dropwise at 4°C with vigorous stirring to an extraction mixture of 1 L of 0.025% (v/v) HCl in acetone. The globin precipitates were washed three times with cold acetone and centrifuged at 3,000 $\times$ g for 10 min. The globin precipitates were freeze dried and stored at –30°C, and were termed PG.

Chemical and amino acid analysis The iron content of casein, PH, and PG was measured using atomic absorption spectrophotometry (AA-6200; Shimadzu Co., Kyoto, Japan) after wet-ashing with nitric acid/perchloric acid (3:1). Protein content was determined according to the Kjeldahl method using an N-to-protein conversion factor of 6.25.

Amino acid analysis was performed by hydrolyzing casein, PH, and PG with 6 M HCl at 110°C for 24 h. The amino acid composition of casein, PH, and PG was measured using high-performance liquid chromatography with UV detection following derivatization with phenylisothiocyanate, and using L- α -amino adipic acid as an internal standard (11). The identification of each amino acid was carried out using the retention times of commercially standard mixtures (Amino Acids Standard Solution Type H; Wako Pure Chemical Industries Ltd.).

SDS-PAGE analysis The molecular weight distribution of PH and PG was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which was performed in accordance with the method of Laemmli (12). Aliquots (5 μ g protein) of PH and PG were loaded on a 4% polyacrylamide stacking gel, and then separated on a 16% polyacrylamide running gel together with a marker of protein molecular weight (SDS-PAGE Molecular Weight Standard, Broad Range; Bio-Rad Laboratories Inc., Hercules, CA, USA). The resulting protein bands were stained with coomassie brilliant blue G and the staining gel was subsequently destained.

Animal diet and care The experimental protocol was reviewed and approved by the Animal Ethics Committee of Kansai Medical University and followed the “Guide for the Care and Use of Experimental Animals” issued by the Prime Minister’s Office of Japan. Five-week-old male Wistar rats obtained from Japan SLC, Inc. (Shizuoka, Japan) were housed in polycarbonate cages in a room maintained as follows: temperature, 21–22°C; humidity, 55–65%; lights on, 08:00–20:00. Twenty-one rats were divided into the following 3 dietary groups of 7 rats each: the CAS diet (AIN-93G), PH diet, and PG diet groups. Table 1 presents the composition of the experimental diets prepared according to the AIN93G formula (13). The iron content of the experimental diets was adjusted for 35 mg/kg by supplementing with iron as ferric citrate or PH. The globin of PG diet was adjusted for the same protein content of PH in the PH

Table 1. Composition of the experimental diets (g/kg)

Component ¹⁾	Groups		
	CAS	PH	PG
Casein	200	187.5	188.2
PH	-	12.5	-
PG	-	-	11.8
Dextrinized corn starch	132	132	132
Corn starch	397.486	397.486	397.486
Sucrose	99.89	100	99.80
Cellulose	50	50	50
Iron-free AIN-93G mineral mixture	35	35	35
Ferric citrate ²⁾	0.21	-	0.20
AIN-93 vitamin mixture	10	10	10
L-Cystine	3	3	3
Choline bitartrate	2.5	2.5	2.5
Soybean oil	70	70	70
<i>tert</i> -Butylhydroquinone	0.014	0.014	0.014

¹⁾PH, porcine hemoglobin; PG, porcine globin

²⁾The premixture consisted of 2.0 g of FeC₆H₅O₇·5H₂O mixed with 98.0 g of sucrose.

diet. The experimental diets and tap water were offered *ad libitum*. Food consumption and body weights were recorded daily. After feeding for 28 days, rats were weighed and sacrificed under pentobarbital sodium (Kyoritsu Seiyaku Co., Tokyo, Japan) anesthesia between 09:00 and 11:00. Rats were not deprived of food prior to being sacrificed because food deprivation is known to significantly down-regulate the expression of genes involved in fatty acid synthesis and cholesterol metabolism (14). Blood was collected from the abdominal aorta without the use of anti-coagulants. A portion of each blood sample was transferred to a heparinized tube, and serum was obtained by centrifugation at 2,000 $\times$ g for 15 min. The liver and abdominal white adipose tissue (WAT) from the epididymis, mesentery, perinephria, and retroperitoneum were removed rapidly, weighed, rinsed with cold saline, frozen in liquid nitrogen, and stored at –70°C until later analysis. Feces were collected from each group every 24 h for 7 days before being sacrificed, dried to a constant weight, and ground to a fine powder using a mill.

Analysis of biochemical components and lipids Blood and serum biochemical tests were performed by a commercial service (Japan Medical Laboratory, Osaka, Japan).

Total liver lipids were extracted by the method of chloroform/methanol/water. Each total lipid sample was dissolved in an equal volume of 2-propanol, and the TAG content was determined using an enzymatic assay kit (Wako Triglyceride-E-Test; Wako Pure Chemical Industries Ltd.). Phospholipid (PL) content was determined by phosphorus analysis (15). The cholesterol content was analyzed using a gas-liquid chromatography system (GC-14B; Shimadzu Co.) with a SE-30 column (Shinwa Chemical Industries, Kyoto, Japan), in which 5 α -cholestane was used as an internal standard.

Fecal fatty acid content was determined by the method of van de Kamer *et al.* (16). The fecal neutral steroid content was determined

by a gas-liquid chromatography system as described above, and the fecal acidic steroid content was measured using an enzymatic assay kit (Total Bile Acid Test Kit; Wako Pure Chemical Industries Ltd.). Fecal nitrogen content was assessed using the Kjeldahl method, as described above.

Analysis of enzyme activities The hepatic crude, mitochondrial, and cytosolic fractions were prepared using an ultracentrifuge, as described previous report (17). Acyl-CoA oxidase (ACOX) activity of the crude fraction was measured as described previously (18). Carnitine palmitoyltransferase-2 (CPT-2) activity in the mitochondrial fraction was measured using the method of Markwell *et al.* (19). Acetyl-CoA carboxylase (ACC) (20), fatty acid synthase (FAS) (21), malic enzyme (ME) (22), and glucose-6-phosphate dehydrogenase (G6PDH) (23) activities in the cytosolic fraction were assayed spectrophotometrically. The protein content of each fraction was determined according to the method of Lowry *et al.* using bovine serum albumin as a standard (24).

Statistical analysis Data represent the means and standard errors of the mean (SEM). The significance of differences was evaluated using the one-way analysis of variance and Tukey's multiple comparisons test. Correlations were tested using Pearson's correlation coefficients. Means were considered significantly different at $p < 0.05$. Analyses were performed using GraphPad Prism6 software (GraphPad Software Inc., La Jolla, CA, USA).

Results and Discussion

Amino acid composition and molecular weight of PH and PG The protein and iron content in casein, PH, and PG are shown in Table 2. Preparation of PG using HCl/acetone resulted in the removal of about 95% of the heme content, calculated as iron content. The amino acid compositions of casein, PH, and PG are shown in Table 3. PH and PG, compared with casein, were rich in alanine, arginine, phenylalanine, serine and glycine, whereas the levels of glutamic acid, lysine, proline, and threonine were low. PG showed higher levels of leucine and lysine compared with PH. The difference in amino acid composition between PH and PG is likely due to the heme removal process.

The molecular weights of PH and PG are presented in Fig. 1. Two prominent bands of around 45 and 21.5 kDa likely correspond to the dimeric and monomeric globin forms (37 and 16 kDa, respectively), as described previously (7). PH and PG were analyzed by SDS-PAGE under reducing conditions (add the 2-mercaptoethanol), with the dimers and monomers likely formed during the heat treatment step in PAGE sample preparation.

Hypolipidemic effects of the PH and PG in rats Growth parameters, relative organ weights, biochemical and lipid parameters in serum,

Table 2. Crude protein and iron content in casein, PH, and PG.

	Casein	PH ¹⁾	PG
Crude protein (g/100 g)	86.2	88.7	88.9
Iron (mg/100 g)	0.07	2.8	0.12

¹⁾PG, porcine globin; PH, porcine hemoglobin

Table 3. Amino acid composition of casein, PH, and PG (mol%)

	Casein	PH	PG
Alanine	4.0	13.0	12.7
Arginine	3.3	6.7	7.2
Aspartic acid ¹⁾	5.1	5.5	5.0
Glutamic acid ²⁾	15.4	8.3	7.3
Glycine	3.1	6.2	5.6
Histidine	2.2	3.3	2.8
Isoleucine	4.8	3.7	3.2
Leucine	9.2	11.7	15.7
Lysine	10.6	4.4	6.7
Methionine	1.9	2.1	1.7
Phenylalanine	4.1	9.5	8.6
Proline	14.0	0.3	0.4
Serine	6.9	12.7	11.7
Threonine	4.5	0.6	0.5
Tyrosine	4.4	5.1	4.6
Valine	6.6	7.0	6.3

¹⁾Aspartic acid: Aspartic acid+Asparagine.

²⁾Glutamic acid: Glutamic acid+Glutamine.

Fig. 1. SDS-PAGE patterns of PH and PG. SDS-PAGE was performed using 16% polyacrylamide separation gel; Lane (M), molecular weight marker; Lane (PH), porcine hemoglobin; Lane (PG), porcine globin. SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

blood, liver, and feces are presented in Table 4. Final body weights, body weight gain, and food intake in the PH and PG groups were significantly higher than in the CAS group. However, there were no significant differences in food efficiency among the experimental groups. The increased final body weight and body weight gain observed in the PH and PG groups were reflected in the corresponding increases in food intake. A previous study reported that protein

Table 4. Growth parameters, relative organ weights, biochemical and lipid parameters in serum, blood, liver, and feces

	CAS	PH	PG
Growth parameters			
Initial BW (g)	126.4±2.5 ¹⁾	126.9±2.6	126.5±2.4
Final BW (g)	292.1±7.3 ²⁾	325.4±9.1 ^b	321.5±7.3 ^b
BW gain (g/day)	5.92±0.22 ^a	7.09±0.28 ^b	6.96±0.22 ^b
Food intake (g/day)	16.8±0.9 ^a	19.6±1.9 ^b	18.4±1.9 ^b
Food efficiency ³⁾ (g/g)	0.352±0.013	0.362±0.014	0.378±0.012
Relative organ weights (g/100 g BW ⁴⁾)			
Liver weight	3.96±0.22	3.79±0.29	3.68±0.14
Small intestine weight	2.45±0.16	2.18±0.14	2.06±0.13
Epididymal WAT weight	1.51±0.07	1.52±0.16	1.44±0.05
Mesentery WAT weight	1.26±0.10	1.31±0.15	1.18±0.07
Perirenal and retroperitoneal WAT weight	1.23±0.17	1.45±0.14	1.29±0.14
Total WAT weight ⁵⁾	4.00±0.32	4.29±0.43	3.91±0.23
Serum parameters			
TAG (mg/dL)	51.1±3.6 ^b	37.7±2.8 ^a	38.9±3.1 ^a
Cholesterol (mg/dL)	69.6±2.9 ^b	60.1±2.2 ^a	60.4±3.0 ^{ab}
PL (mg/dL)	126±5	119±3	130±4
Iron (fg/dL)	221±14	238±25	266±19
TIBC ⁶⁾ (fg/dL)	501±34	563±31	531±26
Blood parameter			
Hemoglobin (g/dL)	15.5±0.5	14.7±0.4	14.8±0.3
Liver parameters			
TAG (mg/g)	38.7±5.5	26.1±6.9	29.8±4.2
Cholesterol (mg/g)	3.17±0.19 ^b	2.00±0.06 ^a	2.43±0.20 ^a
PL (mg/g)	38.8±6.6	35.3±4.2	41.1±2.4
Iron (μg/g)	90.3±5.4 ^c	45.5±5.3 ^a	69.3±5.0 ^b
Feces parameters			
Dry weight (g/day)	2.13±0.12 ^a	2.70±0.16 ^b	2.64±0.14 ^b
Fatty acid (mg/day)	36.6±4.1 ^a	114.1±9.6 ^c	81.2±6.0 ^b
Neutral steroid (mg/day)	3.77±0.19 ^a	5.28±0.47 ^b	4.43±0.40 ^{ab}
Acidic steroid (mg/day)	3.02±0.15 ^a	5.89±0.49 ^b	4.09±0.28 ^a
Total steroid ⁷⁾ (mg/day)	6.79±0.25 ^a	11.17±0.88 ^b	8.52±0.67 ^a
Nitrogen (mg/day)	27.9±1.7 ^a	38.3±1.4 ^b	30.4±1.8 ^a

¹⁾Data represent means±SEM (n=7).²⁾Values in the same row not sharing a common superscript are significantly different at $p<0.05$ using a Tukey's multiple comparisons test.³⁾Food efficiency (g/g)=BW gain (g/day)/Food intake (g/day).⁴⁾BW, body weight; PL, phospholipids; TAG, triacylglycerol; WAT, white adipose tissue.⁵⁾Total WAT shows the sum of WAT weights from the epididymis, mesentery, perinephria, and retroperitoneum.⁶⁾Total iron binding capacity (TIBC): serum iron+unsaturated iron binding capacity (UIBC).⁷⁾Total steroid: neural steroid+acidic steroid.

sources of whey and casein differ in their effect on food intake (25). Further research is necessary to clarify the effects of PH and PG on food intake. There were no significant differences in relative liver, small intestine, epididymal, mesenteric, perirenal, retroperitoneal, and total WAT weights among the groups.

The serum iron, total iron binding capacity (TIBC), and blood hemoglobin levels were not different among the experimental groups. In addition, liver iron contents in the PH and PG groups were lower than in the CAS group. Heme-iron consists of an iron held in a porphyrin and is absorbed by the body more efficiently than non-heme iron (26). However, other reports suggest that the bioavailability of heme-iron is lower than ferrous sulfate in experimental animals (27). In addition, our previous research suggested that iron nutritional

status, which consists of serum iron, TIBC, blood hemoglobin, and organ iron contents, in rats fed a diet containing 20 μg/g PH was not different from rats fed a diet containing ferric citrate (28). In the present study, rats fed the AIN93G-based diets containing iron at a level of 35 μg/g as ferric citrate (CAS group) or porcine hemoglobin (PH group) were not significantly different in their serum iron, TIBC, and blood hemoglobin content; however, liver iron content in the PH group was lower than in the CAS group. Further studies are necessary to clarify the effect of dietary PH as an iron source on iron nutritional states in rats.

There were no significant differences in the PL contents in serum and liver among the groups. Compared to the CAS diet, the diet containing 12.5 g/kg PH decreased serum TAG, serum cholesterol

Table 5 Liver enzyme activities related to fatty acid metabolism (nmol/min/mg protein)

	CAS	PH	PG
CPT-2 ¹⁾	0.51±0.07 ²⁾	0.33±0.05	0.53±0.07
ACOX	0.72±0.09 ^{a3)}	1.62±0.11 ^b	0.77±0.13 ^a
FAS	3.88±1.67	4.16±1.56	3.67±1.64
ACC	67.5±6.5	77.3±11.6	83.4±7.9
G6PDH	5.94±0.82	6.34±1.07	8.40±1.28
ME	1.39±0.19	1.88±0.13	1.71±0.20

¹⁾ACC, acetyl-CoA carboxylase; ACOX, acyl-coenzyme A oxidase; CPT-2, carnitine palmitoyltransferase-2; FAS, fatty acid synthase; G6PDH, glucose-6-phosphate dehydrogenase; ME, malic enzyme.

²⁾Data represent means±SEM (*n*=7).

³⁾Values in the same row not sharing a common superscript are significantly different at *p*<0.05 using a Tukey's multiple comparisons test.

and liver cholesterol contents, whereas the diet containing 11.8 g/kg PG decreased serum TAG and liver cholesterol contents. Previous studies reported that diets containing 200 g/kg soybean β -conglycinin (29), 50 g/kg salmon protamine (30), and 40 g/kg sericin (31) reduced the lipid content in the serum and liver compared with a 200 g/kg casein diet. In the present study, decreased serum and liver lipid contents were produced with 12.5 g/kg PH or 11.8 g/kg PG diets, which is lower than in other studies. Therefore, PH and PG have the potential to become functional food materials or supplements with hypolipidemic effects.

The observed lipid-lowering data support the serum and liver lipid-lowering action of PH reported in our previous research (9). In the previous paper, the reduced lipid content was due, in part, to fecal lipid excretion in rats fed the PH diet compared with the casein diet. Therefore, the effect of dietary PH and PG on fat absorption by determining the total amount of fatty acids, neutral steroid, acidic steroid, and nitrogen in feces were analyzed. The PH and PG diets increased the fecal dry weights compared with the CAS diet. A previous study showed that peptides isolated from a bovine hemoglobin hydrolysate exhibited a growth-stimulating activity (5). Dietary PH and PG increased fecal dry weights could be due to the promotion of intestinal fermentation. The fecal fatty acids in the PH and PG groups were higher than in the CAS group, and the PH diet strongly increased fecal fatty acid excretion compared with the PG diet. Previous studies reported that PH and PG hydrolysates produce a potent hypotriglyceridemic effect through the inhibition of pancreatic lipase (4,9). It is predicted that the enhancement of fecal fatty acid excretion in the PH and PG groups occurs through the inhibition of pancreatic lipase by the protein hydrolysates generated by PH and PG. The PH diet enhanced fecal nitrogen and steroids in neutral, acidic, and total excretions, compared with the CAS and PG diets, whereas the PG group did not affect fecal steroid and nitrogen excretions compared to the CAS diet. Compared with casein, several dietary proteins have been reported to have cholesterol-lowering activities in serum, which is dependent on their amino acid composition (32). However, the amino acid composition of the PH

and PG diets was found to be similar to that of the CAS diet, because relatively small proportions of casein were replaced with PH or PG (6.25 and 5.90% w/w, respectively; Table 1); therefore, the observed effects cannot be explained in terms of differences in dietary amino acid composition. Nagata *et al.* found that the degree of serum cholesterol-lowering depends on the extent of fecal excretion of steroids (33). Higaki *et al.* demonstrated that the increase in fecal bile acid by dietary soybean protein was accompanied by an increase in fecal nitrogen excretion (34). In the present study, total fecal steroids were positively correlated with the fecal nitrogen content (*p*<0.001 and *R*²=0.782). Previous research suggested that the insoluble peptide fraction prepared from buckwheat could bind to bile acid by hydrophobic interactions and inhibit cholesterol and bile acid reabsorption (35). The observed increase in fecal nitrogen excretion indicates that the enhanced fecal steroid excretion by PH is at least partly due to the indigestible protein and peptides remaining after digestion bind the cholesterol and bile acid by hydrophobic interactions. However, an *in vitro* study reported that the degree of PH hydrolysis was not different than casein (9). Previous studies have reported that rodents receiving a diet supplemented with heme leads to oxidative stress responses in the mucosa and hyperproliferation of cells in the crypts of the colon (36,37). Therefore, the enhancement of nitrogen excretion by dietary PH might be related to epithelial cell damage in the intestine and colon. Further research is needed in order to clarify the effects of heme on the state of intestinal epithelial cells. The PG diet did not influence fecal steroid excretion, however liver cholesterol content was reduced. Dietary PG might affect the activity of cholesterol or bile acid synthesis in liver, which is also a topic requiring further experimentation.

Hepatic enzyme activity Liver enzyme activities related to fatty acid metabolism are presented in Table 5. There were no significant differences in CPT-2, a key enzyme of fatty acid β -oxidation in mitochondria, FAS and ACC, which are key enzymes for fatty acid and malonyl-CoA synthesis, G6PDH and ME, which are key enzymes in the production of cellular NADPH, activities among the experimental groups. Surprisingly, the PH diet enhanced the liver ACOX activity, a key enzyme of fatty acid β -oxidation in peroxisomes, compared with CAS and PG diet. It was demonstrated here that dietary PG did not influence the enzymes related to fatty acid metabolism, indicating that dietary PG does not alter fatty acid synthesis and oxidation. It is possible that the active component that stimulates ACOX activity is heme, which is removed from PH during HCl/acetone treatment. Transcriptome analysis revealed that dietary heme up-regulates lipid metabolism-related peroxisome proliferator-activated receptor- α target genes, such as ACOX, in mucosa of mice (36). However, the TAG-lowering effect is equivalent between PH and PG, despite the enhanced hepatic ACOX activity associated with the PH diet. At present, it is unclear whether dietary PH-induced liver ACOX activity is responsible for reducing TAG content in the serum and liver.

In conclusion, this study showed that PH decreased serum and

liver lipid content, in part as a result of enhanced fecal fatty acid, neutral steroid, and acidic steroid excretion. Fecal lipid excretion with the PG diet was significantly weaker than with the PH diet. Dietary PH elevated lipid excretion in feces owing to the presence of both the globin protein and heme in PH. Therefore, the observed hypolipidemic effect of PH suggests that PH can possibly be used as a supplement or food material to prevent hyperlipidemia.

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