

The mechanism of the cholesterol-lowering effect of water-insoluble fish protein in ovariectomised rats

Masaki Kato¹, Hiroshi Ogawa², Taro Kishida¹ and Kiyoshi Ebihara^{1*}

¹Department of Biological Resources, Faculty of Agriculture, Ehime University, 3-5-7 Tarumi, Matsuyama 790-8566, Japan

²Faculty of Human and Cultural Studies, Tezukayamagakuin University, 4-2-2 Harumidai, Minami-ku, Sakai-city, Osaka 590-0113, Japan

(Received 2 October 2008 – Revised 10 February 2009 – Accepted 11 February 2009 – First published online 1 April 2009)

The purpose of the present study was to investigate whether water-insoluble fish protein (IFP) from Alaska pollock (*Theragra chalcogramma*) prevents hypercholesterolaemia induced by ovarian hormone deficiency. Wistar female rats, aged 6 months, were subjected to sham-operation or ovariectomy, and fed a cholesterol-free diet containing casein or IFP as a protein source for 28 d. Body-weight gain and food intake increased in the ovariectomised rats as compared with the sham-operated rats. Plasma total cholesterol concentration was decreased and faecal bile acid excretion was increased by IFP in the ovariectomised rats, but not in the sham-operated rats. Plasma homocysteine concentration was decreased by IFP in the ovariectomised rats, but not in the sham-operated rats. Liver lipids and liver cholesterol concentrations were increased and cholesterol 7 α -hydroxylase (CYP7A1) activity was decreased by ovariectomy, but not by diet. Bile acid content and the ratio of cholic acid groups to chenodeoxycholic acid groups in bile were increased by ovariectomy, but decreased by IFP. Bile acid content in the small intestine was increased by IFP in the ovariectomised rats, but not in the sham-operated rats. 3-Hydroxy-3-methylglutaryl-CoA reductase and microsomal TAG transfer protein mRNA levels were decreased by ovariectomy and IFP, whereas LDL-receptor mRNA level was decreased by ovariectomy but unaffected by diet. Thus, the preventive effect of IFP on the ovarian hormone deficiency-associated increase in plasma cholesterol concentration seems to be mediated by accelerated faecal excretion of bile acids, coupled with an increase in the intestinal pool of bile acids.

Water-insoluble fish protein: Ovariectomised rats: Plasma lipids: Bile acids

It is well known that lipid metabolism is influenced by sex hormones in animals and humans^(1,2). Sex hormones such as oestrogen have a major impact on atherosclerotic processes, and studies in animal models have shown that oestrogen inhibits the development of atherosclerotic lesions^(3,4). Oestrogen deficiency is associated with changes in blood lipid levels. It is now clear that oestrogen deficiency plays a key pathogenetic role in the development of CHD in women, as supported by several epidemiological findings^(5,6). Oestrogen replacement therapy in postmenopausal women provides a protective effect against CHD. However, various side-effects, such as breast cancer, resumption of menses and weight gain, have consistently accompanied this treatment^(7,8).

Dietary fats and cholesterol have been among the most studied nutrients with regard to plasma cholesterol. Food also contains other nutrients, such as dietary proteins, which have important physiological and metabolic effects on cholesterol metabolism. It has been established that dietary proteins influence lipid metabolism in humans and animals⁽⁹⁾. Most studies have focused on the effects of soya protein compared with casein. It is often assumed that all animal proteins behave like casein and that all plant proteins behave like soya protein

with respect to lipid and lipoprotein metabolism⁽¹⁰⁾. However, it is well known that the nature of dietary protein influences blood lipid levels.

Fish consumption has been shown to be inversely associated with the risk of CHD⁽¹¹⁾. Many studies have reported that the lower incidence of CHD among populations consuming fish-rich diets has been attributed to a hypocholesterolaemic effect of fish oil⁽¹²⁾. Only a limited number of studies have investigated the hypocholesterolaemic effect of another macronutrient in fish, namely fish protein. Proteins from different fish species have been proved to reduce the plasma cholesterol concentration in animals when compared with casein^(13,14). Fish proteins play an important role in human nutrition worldwide. Surimi, the water-insoluble fish protein (IFP) prepared by washing minced fish meat with water, is a useful ingredient in processed sea foods such as fish cakes (kamaboko) and fish sausage in Japan.

Ovariectomy can minimise the interference of endogenous oestrogens and mimics postmenopausal conditions. Not much is known about the effects of dietary fish protein on lipid metabolism in ovariectomised rats. Therefore, in the present study, we investigated the effect of IFP from Alaska

Abbreviations: ACAT, acyl-CoA-cholesterol acyltransferase; CYP27A1, cholesterol 27-hydroxylase; CYP7A1, cholesterol 7 α -hydroxylase; IFP, water-insoluble fish protein; SREBP, sterol regulatory element-binding protein.

* **Corresponding author:** Dr Kiyoshi Ebihara, fax +81 89 946 9847, email ebihara@agr.ehime-u.ac.jp

pollock (*Theragra chalcogramma*) on lipid metabolism in ovariectomised rats. Casein was used as the reference.

Materials and methods

Test materials

Water-insoluble fish protein. IFP was donated by Nippon Suisan Kaisha, Ltd (Tokyo, Japan). IFP was prepared by washing minced Alaska pollock meat with water, a process that removes water-soluble proteins and fat. IFP was cut into small pieces, freeze-dried and powdered.

Casein. Acid-precipitated lactic casein (30 mesh) was purchased from New Zealand Daily Board (Wellington, New Zealand).

Protein and lipid content. The protein content of IFP and casein was determined by the Kjeldahl method, with an N:protein conversion factor of 6.25. The lipid content of IFP and casein was determined by the Soxhlet method. The protein content and lipid content of IFP and casein were 91.4 and 0.04 g/100 g, and 89.3 and 0.12 g/100 g, respectively.

Animals and diets

The present study was approved by the Laboratory Animal Care Committee of Ehime University. Rats were maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals of Ehime University.

Female Wistar rats, aged 6 months (twenty-four rats for experiment 1 and twenty-four rats for experiment 2), were housed individually in screen-bottomed, stainless-steel cages in a room maintained at $23 \pm 1^\circ\text{C}$ with a 12 h light–dark cycle (lights on 07.00–19.00 hours). The rats were acclimatised by being fed a commercial solid diet (MF; Oriental Yeast Co., Osaka, Japan) for 7 d. After acclimatisation, they were divided into two groups of twelve rats each. A bilateral ovariectomy was performed on one group, and a sham-operation on the other, under sodium pentobarbital anaesthesia (Nembutal, 30 mg/kg body weight, intraperitoneal injection; Abbott, North Chicago, IL, USA). The rats were then fed a commercial solid diet (MF; Oriental Yeast Co.) during the 8 d recovery period. The sham-operated rats and ovariectomised rats were each divided into two groups of six animals on the basis of body weight, and these groups were fed either the casein diet or the IFP diet for 28 d. The composition of the two test diets is shown in Table 1. The diets were based on AIN-93 guidelines for rodent diets⁽¹⁵⁾. For each rat, body weight and food intake were recorded daily in the morning before the food was replaced. The amino acid composition of the casein and fish diets was determined with an amino acid analyser (model JCL-555/V; Japan Electron Optics Laboratory Co., Ltd, Tokyo, Japan)⁽¹⁶⁾ (Table 2).

Experiment 1

Sampling and analytical procedures. Before the rats were killed, faeces were collected from each rat over the final 3 d of the experimental period. The faeces were freeze-dried, weighed and milled. On the last day of the experimental period, a blood sample was collected from the neck at midnight from fed rats into a blood collection tube (Vacutainer;

Table 1. Composition of experimental diets

Ingredients (g/kg)	Casein diet	IFP diet
Casein	200	–
IFP	–	196
Gelatinised maize starch	532	536
Sucrose	100	100
Cellulose*	50	50
Soyabean oil	70	70
Mineral mixture†	35	35
AIN-93 vitamin mixture†‡	10	10
L-Cystine	3	3

IFP, water-insoluble fish protein; AIN, American Institute of Nutrition.

* Cellulose powder, PC200 (Danisco Japan Ltd, Tokyo, Japan).

† Based on AIN-93G⁽¹⁵⁾.

‡ The vitamin mixture contained 20 g choline bitartrate per 100 g.

Becton Dickinson, Franklin Lakes, NJ, USA) that contained heparin as an anticoagulant. The plasma was separated by centrifugation at 1400 g at 4°C for 15 min and stored at -50°C until analysis. After blood collection, the liver was immediately perfused with cold saline (9 g NaCl/l), removed, washed with cold saline, blotted dry on filter paper, weighed, and stored at -50°C until analysis. After the liver had been removed, the small intestine was removed. The contents of the small intestine were transferred into a pre-weighed tube, freeze-dried and weighed.

Biochemical analysis. The concentration of total cholesterol and TAG in the plasma was determined enzymically by using commercial diagnostic kits (Cholesterol E-Test Wako and Triglyceride E-Test Wako; Wako Pure Chemical Industries, Osaka, Japan). Plasma lipoprotein fractions (VLDL, $d < 1.006$; LDL, $d = 1.006–1.063$; HDL, $d = 1.063–1.210$) were separated by stepwise density-gradient ultracentrifugation in a TL-100 ultracentrifuge (Beckmann Instruments Inc., Palo Alto, CA, USA). The cholesterol and TAG concentrations of the lipoprotein fractions were determined enzymically by using commercial kits (Cholesterol E Test Wako and Triglyceride E-Test Wako). The total lipid content in the liver was determined gravimetrically after extraction by the method of Folch *et al.*⁽¹⁷⁾. The liver

Table 2. Amino acid composition of experimental diets

Amino acid (g/kg diet)	Casein diet	IFP diet
Methionine	5.00	3.10
Cysteine	0.83	1.25
Glycine	4.14	8.20
Lysine	20.80	25.34
Arginine	7.72	12.66
Valine	15.69	12.28
Leucine	20.76	17.84
Isoleucine	10.06	9.11
Histidine	4.80	3.12
Tyrosine	12.63	4.26
Phenylalanine	11.21	7.69
Threonine	6.98	6.71
Serine	8.46	5.75
Alanine	6.80	13.03
Aspartic acid	15.17	21.72
Glutamic acid	53.91	41.06
Taurine	–	0.23

IFP, water-insoluble fish protein.

cholesterol and TAG concentrations were determined enzymically as previously described⁽¹⁸⁾. Cholesterol 7 α -hydroxylase (CYP7A1) activity in the liver was determined according to the method of Ogishima & Okuda⁽¹⁹⁾.

The serum total homocysteine concentration was measured by the HPLC method of Araki & Sako⁽²⁰⁾.

Steroids were extracted from faeces with a mixture of chloroform-methanol (1:1, v/v) at 70°C for 60 h⁽²¹⁾. The amount of bile acids in faeces was determined enzymically by the 3 α -dehydrogenase assay method of Sheltaway & Losowsky using taurocholic acid as a standard⁽²²⁾.

RNA extraction from the liver and reverse transcriptase polymerase chain reaction analysis of gene expression. Total RNA was extracted from frozen livers according to the method described by Chomczynski & Sacchi⁽²³⁾. RNA integrity was verified by agarose gel electrophoresis after purification of the mRNA with Oligotex-dT30 (Takara Bio, Shiga, Japan). A quantity of 1 μ g of mRNA was used for cDNA synthesis with 10 units of AMV RT (Takara Bio, Shiga, Japan) and 2 μ l of oligo(dT) primer (Novagen, Inc., Madison, WI, USA) according to the manufacturer's instructions. Expression of the mRNA for acyl-CoA-cholesterol acyltransferase (ACAT)-1, ACAT-2, cholesterol 27-hydroxylase (CYP27A1), cholesterol 12 α -hydroxylase (CYP8B1), farnesoid X receptor, 3-hydroxy-3-methylglutaryl-CoA reductase, LDL-receptor, liver X receptor, microsomal TAG transfer protein, sterol regulatory element-binding protein (SREBP)-1a, SREBP-1c, SREBP-2 and β -actin, as a housekeeping gene for normalisation, was determined by real-time monitoring of PCR using a Light Cycler instrument (Roche Diagnostics, Mannheim, Germany). cDNA (2 μ l) was amplified in a total volume of 20 μ l by using the 2 \times QuantiTect SYBR Green RT-PCR Master Mix (Qiagen, Hilden, Germany) and specific primers each at 0.5 M. After initial denaturation and activation of the polymerase at 95°C for 15 min, fifty cycles were performed with annealing at the temperatures shown in Table 3 for 25 s, synthesis at 72°C for 30 s, and denaturation at 94°C for 15 s. Fluorescence was measured at the end of the elongation step at 72°C. The sequences of the gene-specific primers (Carl Roth, Karlsruhe, Germany) that were used in the present study are listed in Table 3. Relative gene expression was calculated by using the crossing point of each target gene; the β -actin gene was used as the reference.

Experiment 2

Sampling and analytical procedures. Before the rats were killed, faeces were collected from each rat over the final 3 d of the experimental period. At the end of the experiment, fed (non-fasting) rats were anaesthetised by intraperitoneal injection of sodium pentobarbital (40 mg/kg body weight). A mid-line laparotomy was performed, and the bile duct was exposed and ligated distally. The bile duct was then cannulated with a PE-10 polyethylene tube (Clay Adams, Parsippany, NJ, USA), and bile was collected into a pre-weighed tube that had been cooled on ice for 30 min. The bile volume was determined gravimetrically, assuming a bile density of 1.0. After bile collection, the small intestine and caecum were removed. The contents of the small intestine and caecum were transferred into a pre-weighed tube, freeze-dried and weighed. The amount of bile acids in the

Table 3. Primer sequence, product size and annealing temperature

Gene	Primer sequence		Product size (bp)	Annealing temperature (°C)
	Sense	Anti-sense		
Acyl-coenzyme A-cholesterol acyltransferase-1	ATGTGGGAAGTAAATGAAGC	AAATACTAGCCAGACCCGAAT	193	53
Acyl-coenzyme A-cholesterol acyltransferase-2	CGGTCATGCTGATCCCTCTTT	GTGTCACCAAGCTCCCAAAAT	204	55
ApoB	TTGACACACTGAAGTTCCCTA	ACATCAAACCCTGGTATTAG	124	60
CYP8B1	ATGAAGGCTGTGCGGAG	TCTTCCATCAGCGTGTG	127	59
CYP27A1	CTCAAGAGACTGTCCGCAC	CTTCCGATCTCTGGGT	114	57
Farnesoid X receptor	CGTTACGCGGAGATTTCAA	ATTTTCAGTCCCGACACT	140	57
3-Hydroxy-3-methylglutaryl-CoA reductase	GCTGGTGAGTTGTCCITTTGAT	CTTCTTGGTCATGTTCCCT	117	60
LDL-receptor	CTTGCCCTGAGGATGTGCTA	CTTGCGCTTCAGTGACACA	137	60
Liver X receptor	TGCTAAITGAAGCTGTGTA	AGAGGACACAGGAGAAACAGT	144	57
Microsomal TAG transfer protein	AGCGACATCACAGTGGACTC	GGCTCTCTTTCTCTCCGA	223	62
Retinoid X receptor	CAAACTGGGCTGAACC	AGCAGGATGACCTGGTCTG	148	57
Sterol regulatory element-binding protein-1a	CCGAGATGTGCGAACTGGAC	TGTCCTCGTTCACCCCCA	129	55
Sterol regulatory element-binding protein-1c	GGAGCCATGGATTGCACATT	AGGAAAGGCTTCCAGAGGA	191	53
Sterol regulatory element-binding protein-2	CACAATATCATTGAAAGCGGTACG	TTTTTCTGATTGGCCAGCTTCAGCA	200	55
β -Actin	CTATGAGCTGCCTGACGGTC	AGTTTCATGGATGCACAGG	115	53

CYP8B1, cholesterol 12 α -hydroxylase; CYP27A1, cholesterol 27-hydroxylase.

small intestine and caecum, and in faeces was determined by the same method used in experiment 1.

Analysis of bile. The amount of cholesterol in bile was analysed by a capillary GLC (model HP5890A; Hewlett Packard, Palo Alto, CA, USA) equipped with a flame-ionisation detector and a capillary column (30 m × 0.53 mm inner diameter) coated with DB-1 (J&W Scientific, Folsom, CA, USA)⁽²⁴⁾. The oven temperature was 260°C and the flow rate of the He carrier gas was 16.9 ml/min. 5 α -Cholestane (Nacalai Tesque Inc., Kyoto, Japan) was used as the initial standard for cholesterol analysis. The amount and composition of bile acid in bile were analysed by a capillary GLC (model HP5890A; Hewlett Packard, Palo Alto, CA, USA) equipped with a flame-ionisation detector and a capillary column (30 m × 0.25 mm inner diameter) coated with DB-210 (J&W Scientific, Folsom, CA, USA)⁽²⁵⁾. The oven temperature was programmed to increase from 60 to 235°C at the rate of 10°C/min, and the flow rate of the He carrier gas was 1.5 ml/min. Nordeoxycholic acid (Steraloid Inc., Wilton, NH, USA) was used as the initial standard for bile acid analysis. The standard bile acids, cholic acid, deoxycholic acid, 12-oxo-chenodeoxycholic acid, 12-oxo-lithocholic acid, chenodeoxycholic acid, α -muricholic acid, β -muricholic acid, ω -muricholic acid, lithocholic acid, hydoxycholic acid and ursodeoxycholic acid, were purchased from Steraloids Inc. (Steraloid Inc., Wilton, NH, USA).

Statistical analyses

Data are expressed as mean values with their standard errors ($n = 6$). Two-way ANOVA (StatView version 4.5; Abacus Concepts, Berkeley, CA, USA) was used to test the

significance of the effects of diet and ovariectomy, and their interaction. Individual comparisons were made by Tukey's multiple-range test using the Super ANOVA statistical software package (Abacus Concepts). Differences were considered to be significant at $P < 0.05$.

Results

Experiment 1

Body-weight gain and food intake were increased by ovariectomy, but were not affected by diet (Table 4). The concentration of plasma total cholesterol was not affected by diet in the sham-operated rats, but was decreased by the IFP diet in the ovariectomised rats. Plasma LDL-cholesterol concentrations was increased by ovariectomy, whereas plasma VLDL-cholesterol concentration was not affected. Plasma VLDL-cholesterol and LDL-cholesterol concentrations were decreased by the IFP diet. Plasma total, VLDL-, LDL- and HDL-TAG concentrations were not affected by diet or ovariectomy. Plasma apoA-1 concentration was increased by the IFP diet and ovariectomy. Plasma apoB concentration was increased in the casein diet-fed sham-operated rats only, and plasma apoE concentration was decreased in the IFP diet-fed sham-operated rats only. The plasma homocysteine concentration was not affected by diet in the sham-operated rats, but was decreased by the IFP diet in the ovariectomised rats.

Liver weight and the concentration of total lipids and TAG in the liver were increased by ovariectomy (Table 5). In the sham-operated rats, the concentration of total cholesterol in the liver was increased by the casein diet. The concentration of non-esterified cholesterol in the liver was not affected by

Table 4. Effects of water-insoluble fish protein (IFP) on body weight, body-weight gain, food intake, plasma lipids, apolipoproteins and homocysteine in ovariectomised (OVX) rats*

(Mean values with their standard errors for six rats per group)

	Sham-operated				OVX				Two-way ANOVA		
	Casein diet		IFP diet		Casein diet		IFP diet		Diet	OVX	Interaction
	Mean	SE	Mean	SE	Mean	SE	Mean	SE			
Body-weight gain (g/28 d)	10	3	12	3	28	3	34	6	NS	$P < 0.01$	NS
Food intake (g/28 d)	323	13	324	4	351	6	355	14	NS	$P < 0.01$	NS
Plasma lipids (mmol/l)											
Cholesterol											
Total	2.87 ^a	0.07	2.68 ^a	0.08	3.36 ^b	0.11	2.75 ^a	0.10	$P < 0.01$	$P < 0.01$	$P < 0.05$
VLDL	0.04	0.01	0.07	0.02	0.02	0.01	0.05	0.01	$P < 0.05$	NS	NS
LDL	1.32	0.14	0.96	0.02	1.63	0.12	1.26	0.07	$P < 0.01$	$P < 0.01$	NS
HDL	1.38	0.05	1.29	0.03	1.48	0.06	1.38	0.05	NS	$P < 0.01$	NS
TAG											
Total	3.41	0.52	3.74	0.51	2.93	0.25	3.47	0.14	NS	NS	NS
VLDL	2.28	0.39	2.80	0.37	1.92	0.18	2.47	0.16	NS	NS	NS
LDL	0.77	0.18	0.93	0.21	0.75	0.11	0.76	0.03	NS	NS	NS
HDL	0.16	0.03	0.25	0.05	0.18	0.03	0.18	0.01	NS	NS	NS
Apolipoproteins (mg/l)											
ApoA-1	848	16	938	16	942	20	971	17	$P < 0.01$	$P < 0.01$	NS
ApoB	87.7 ^b	0.8	71.7 ^a	2.8	63.4 ^a	3.4	73.4 ^a	3.6	NS	$P < 0.01$	$P < 0.01$
ApoE	1020 ^b	40	871 ^a	8	1050 ^b	30	1090 ^b	20	NS	$P < 0.01$	$P < 0.01$
Homocysteine (μ mol/l)	8.72 ^a	0.46	9.47 ^{a,b}	0.23	9.85 ^b	0.34	8.88 ^a	0.38	NS	NS	$P < 0.05$

^{a,b} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

* Rats were fed one of the test diets for 28 d.

Table 5. Effects of water-insoluble fish protein (IFP) on liver weight, liver lipids, cholesterol 7 α -hydroxylase (CYP7A1) activity and faecal bile acid excretion in ovariectomised (OVX) rats*

(Mean values with their standard errors for six rats per group)

	Sham-operated				OVX				Two-way ANOVA		
	Casein diet		IFP diet		Casein diet		IFP diet		Diet	OVX	Interaction
	Mean	SE	Mean	SE	Mean	SE	Mean	SE			
Liver weight (g)	6.03	0.21	6.01	0.21	6.31	0.21	6.61	0.21	NS	<i>P</i> <0.05	NS
Liver lipids											
Total lipids (mg/g liver)	61.1	3.1	62.6	1.8	82.0	8.4	70.8	3.9	NS	<i>P</i> <0.01	NS
Cholesterol (μ mol/g liver)											
Total	10.2	0.3	9.5	0.4	9.3	0.2	9.5	0.2	NS	NS	NS
Non-esterified	7.82	0.1	8.07	0.1	8.25	0.2	8.38	0.2	NS	<i>P</i> <0.05	NS
Esterified	2.42 ^b	0.15	1.41 ^a	0.37	1.05 ^a	0.11	1.08 ^a	0.08	<i>P</i> <0.05	<i>P</i> <0.01	<i>P</i> <0.05
TAG (μ mol/g liver)	20.4	2.4	17.7	0.5	31.6	4.2	22.2	1.7	<i>P</i> <0.05	<i>P</i> <0.01	NS
CYP7A1 activity (pmol/min per mg protein)	32.3	4.1	22.9	6.6	16.6	3.6	14.8	4.9	NS	<i>P</i> <0.01	NS
Faecal excretion of bile acids (μ mol/d)	8.8 ^a	0.4	9.7 ^{a,b}	0.3	11.6 ^b	0.5	14.6 ^c	0.4	<i>P</i> <0.05	<i>P</i> <0.01	<i>P</i> <0.05

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different (*P*<0.05).

* Rats were fed one of the test diets for 28 d.

diet, but was increased by ovariectomy. The concentration of hepatic esterified cholesterol was decreased in the casein diet-fed sham-operated rats only. Hepatic CYP7A1 activity was decreased by ovariectomy, and that in the sham-operated rats was decreased by the IFP diet. The faecal excretion of bile acids was increased by ovariectomy, and was increased further by the IFP diet.

The levels of ACAT-1, farnesoid X receptor, RXR, SREBP-1a and SREBP-2 mRNA were not affected by diet or ovariectomy (data not shown). The levels of 3-hydroxy-3-methylglutaryl-CoA reductase and microsomal TAG transfer protein mRNA were decreased by the IFP diet and ovariectomy, whereas the level of SREBP-1c mRNA was increased by the IFP diet and ovariectomy (Table 6). The levels of ACAT-2, CYP27A1, CYP8A1 and liver X receptor- α mRNA tended to be decreased by the IFP diet (*P*=0.0632, 0.0869, 0.0680 and 0.0712), but were not affected by ovariectomy (data not shown). The level of LDL-receptor mRNA was decreased by ovariectomy, and tended to be decreased by the IFP diet (*P*=0.1078).

Experiment 2

Bile flow in the rats fed the casein diet was decreased by ovariectomy; however, that in the rats fed the IFP diet was not affected by ovariectomy. The concentration of biliary bile acids was increased by ovariectomy, but was decreased by the IFP diet (Table 7). The concentration of biliary cholesterol tended to be decreased by ovariectomy (*P*=0.0954). The cholic acid group:chenodeoxycholic acid group ratio of biliary bile acids was decreased by ovariectomy, but was not affected by diet.

The amount of bile acids in the small intestine and caecum was increased by the IFP diet in the ovariectomised rats, but was not affected by diet in the sham-operated rats. The dry weight of the small intestine and caecum contents was not affected by diet. The dry weight of faeces extracted per d was not affected by diet. The amount of bile acids extracted per d in faeces was not affected by diet in the sham-operated rats, but was increased by the IFP diet in the ovariectomised rats.

Table 6. Effects of water-insoluble fish protein (IFP) on mRNA levels (arbitrary units) of cholesterol 7 α -hydroxylase (CYP7A1), 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, LDL-receptor, microsomal TAG transfer protein (MTP) and sterol regulatory element-binding protein-1c (SREBP-1c) in ovariectomised (OVX) rats†

(Mean values with their standard errors for six rats per group)

	Sham-operated				OVX				Two-way ANOVA		
	Casein diet		IFP diet		Casein diet		IFP diet		Diet	OVX	Interaction
	Mean	SE	Mean	SE	Mean	SE	Mean	SE			
CYP7A1	5.50	2.72	1.95	0.48	1.99	0.32	2.73	0.74	NS	NS	NS
HMG-CoA reductase	1.92	0.44	1.13	0.15	1.09	0.12	0.77	0.14	<i>P</i> <0.05	<i>P</i> <0.05	NS
LDL-receptor	1.03	0.08	1.00	0.07	0.97	0.06	0.74	0.09	NS	<i>P</i> <0.05	NS
MTP	1.56	0.26	1.05	0.08	1.07	0.10	0.92	0.06	<i>P</i> <0.05	<i>P</i> <0.05	NS
SREBP-1c	0.68	0.09	1.54 [*]	0.23	1.35	0.17	1.73	0.22	<i>P</i> <0.01	<i>P</i> <0.05	NS

* Mean value was significantly different from that of the sham-operated rats fed the casein diet (*P*<0.05).

† Rats were fed one of the test diets for 28 d.

Table 7. Effects of water-insoluble fish protein (IFP) on bile flow, bile acid and cholesterol concentrations in bile, bile acid composition of bile, bile acids in small intestine and caecum, and faecal bile acid excretion in ovariectomised (OVX) rats*

(Mean values with their standard errors for six rats per group)

	Sham-operated				OVX				Two-way ANOVA		
	Casein diet		IFP diet		Casein diet		IFP diet		Diet	OVX	Interaction
	Mean	SE	Mean	SE	Mean	SE	Mean	SE			
Bile											
Bile flow (g/30 min)	0.21 ^b	0.01	0.20 ^b	0.01	0.18 ^a	0.00	0.20 ^{a,b}	0.01	NS	<i>P</i> < 0.01	<i>P</i> < 0.05
Bile acid (μmol)	2.79	0.26	2.04	0.15	3.07	0.15	2.74	0.21	<i>P</i> < 0.01	<i>P</i> < 0.05	NS
Cholesterol (μmol)	0.040	0.004	0.035	0.003	0.032	0.003	0.032	0.003	NS	NS	NS
Composition of bile acid (%)											
Cholic acid group											
Cholic acid	30.1	2.4	27.0	1.3	23.0	1.7	25.3	1.2	NS	<i>P</i> < 0.05	NS
Deoxycholic acid	3.64	0.49	3.22	0.47	3.51	0.35	3.22	0.35	NS	NS	NS
12-Oxo-chenodeoxycholic acid	1.12	0.25	1.20	0.11	1.59	0.37	0.72	0.04	NS	NS	<i>P</i> < 0.05
12-Oxo-lithocholic acid	0.31	0.04	0.51	0.11	0.22	0.01	0.32	0.04	<i>P</i> < 0.05	<i>P</i> < 0.05	NS
Chenodeoxycholic acid group											
Chenodeoxycholic acid	1.26	0.07	1.62	0.16	1.56	0.16	2.46	0.16	<i>P</i> < 0.01	<i>P</i> < 0.01	NS
Lithocholic acid	2.07	0.36	2.08	0.20	4.55	0.88	3.49	0.19	NS	<i>P</i> < 0.01	NS
Hyodeoxycholic acid + ursodeoxycholic acid	36.2 ^a	1.5	41.5 ^{a,b}	1.6	49.9 ^c	1.7	46.4 ^{b,c}	1.4	NS	<i>P</i> < 0.01	<i>P</i> < 0.01
α-Muricholic acid	8.95 ^c	0.64	4.06 ^b	0.30	2.16 ^a	0.13	4.13 ^b	0.22	<i>P</i> < 0.01	<i>P</i> < 0.01	<i>P</i> < 0.01
β-Muricholic acid	0.31	0.04	0.62	0.06	0.23	0.03	0.47	0.03	<i>P</i> < 0.01	<i>P</i> < 0.05	NS
ω-Muricholic acid	15.4	0.6	16.9	0.6	13.1	0.5	13.0	0.6	NS	<i>P</i> < 0.01	NS
Cholic acid group:chenodeoxycholic acid group ratio	0.56	0.06	0.51	0.04	0.40	0.03	0.42	0.02	NS	<i>P</i> < 0.01	NS
Small-intestinal contents											
Dry weight (g)	0.33	0.01	0.33	0.01	0.35	0.02	0.36	0.01	NS	NS	NS
Bile acids (μmol/contents)	33.1 ^{a,b}	2.0	30.3 ^a	2.7	29.0 ^a	2.7	36.3 ^b	1.8	NS	NS	<i>P</i> < 0.05
Caecal contents											
Dry weight (g)	0.47	0.02	0.47	0.01	0.47	0.02	0.52	0.02	NS	NS	NS
Bile acids (μmol/contents)	8.7 ^{a,b}	0.6	8.1 ^a	0.8	6.9 ^a	0.4	11.2 ^b	0.8	<i>P</i> < 0.05	NS	<i>P</i> < 0.01
Faecal excretion											
Dry weight (g/d)	0.55	0.03	0.61	0.02	0.67	0.02	0.69	0.03	NS	<i>P</i> < 0.01	NS
Bile acids (μmol/d)	10.4 ^a	0.6	11.6 ^{a,b}	0.3	12.4 ^b	0.5	15.6 ^c	0.5	<i>P</i> < 0.01	<i>P</i> < 0.01	<i>P</i> < 0.05

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different (*P*< 0.05).

* Rats were fed one of the test diets for 28 d.

Hypolipidaemic effect of pollock protein

Discussion

The concentration of plasma total cholesterol was significantly decreased by the IFP diet in the ovariectomised rats, but not in the sham-operated rats. Because the experimental diets did not contain cholesterol, it is clear that this hypocholesterolaemic effect of the IFP diet must be due to changes in the endogenous cholesterol metabolism of the rats.

Oestrogen is known to influence lipoprotein and hepatic cholesterol metabolism⁽²⁶⁾. Oestrogen up-regulates LDL-receptor gene expression in rat liver^(27,28). In the present study, the level of LDL-receptor mRNA was decreased by ovariectomy.

Shukla *et al.*⁽²⁹⁾ have reported that the LDL-receptor mRNA level in rats fed fish protein is significantly higher than that in rats fed casein. Therefore, the higher mRNA level of LDL-receptor in the liver of rats fed fish protein might possibly contribute to increased removal of LDL from the circulation, resulting in a decrease in plasma cholesterol concentration. In the present study, plasma LDL-cholesterol concentration was decreased by the IFP diet.

Ovariectomy in the rat has been shown to result in an increase in the plasma concentrations of cholesterol, apoB and apoE⁽³⁰⁾. ApoB is the primary apolipoprotein of LDL and is absolutely required for LDL formation. However, there was not a significant correlation between the concentration of apoB and that of LDL-cholesterol.

Although the mechanism by which dietary proteins exert differential actions on plasma cholesterol has not been fully elucidated, one plausible explanation for the hypocholesterolaemic effect of plant proteins would be decreased intestinal absorption, and increased faecal excretion, of cholesterol and bile acids. The possibility that indigestible protein and peptides remaining after digestion can specifically bind bile acids and inhibit the micellar solubility of cholesterol, thereby leading to an increase in its faecal excretion, has attracted much attention and remains a major area of investigation with regard to the mechanism. Both hydrophobic peptides and the undigested high-molecular fraction of soya protein can bind bile acids^(31,32). Soya protein isolate is known to decrease the intestinal absorption of cholesterol and bile acids, and to increase their faecal excretion⁽³³⁾. The protease-resistant fraction of smoked, dried bonito can bind bile acids (N Mori and K Ebihara, unpublished results). In the present study, the faecal excretion of bile acids was increased by the IFP diet in the ovariectomised rats only. Thus, IFP may be able to bind bile acids.

The acidic pathway, which is regulated by CYP27A1 and produces mainly chenodeoxycholic acid, may contribute >50% of the total bile acid synthesis in the rat⁽³⁴⁾. In the intestine, cholic acid is absorbed much more efficiently than chenodeoxycholic acid⁽³⁵⁾. The amount of bile acids in the small intestine and caecum, as well as the faecal excretion of bile acids, was greater in the ovariectomised rats fed the IFP diet than in those fed the casein diet. Therefore, greater faecal bile acids excretion as a consequence of a reduction in intestinal absorption would be largely attributable to the hypocholesterolaemic effect of IFP in the ovariectomised rats. In the present study, however, the level of CYP27A1 mRNA was not affected by diet and the amount of cholic acid in bile was greater than that of chenodeoxycholic acid

in the ovariectomised rats. Another plausible explanation is an increase in the biliary production of bile acids. The activity and mRNA level of CYP7A1, the rate-limiting enzyme in the bile acid biosynthetic pathway, as well as bile acid synthesis, are reduced in oestrogen-treated rats⁽³⁶⁾. In addition, the level of CYP7A1 mRNA is decreased by ethinyl oestradiol in rats⁽³⁷⁾. In the present study, the level of CYP7A1 mRNA was not affected by ovariectomy and diet. In the ovariectomised rats, however, the excretion of bile acids via the faeces was significantly increased by the IFP diet as compared with the casein diet. Therefore, a decrease in plasma cholesterol concentration in the ovariectomised rats fed the IFP diet as compared with the casein diet would depend on an increase in the faecal excretion of bile acids. In the ovariectomised rats, the amount of bile acids present in the small intestine and caecum was significantly increased by the IFP diet as compared with the casein diet, which suggests that the reabsorption of bile acids from these organs was prevented by IFP. In our previous study, we found that the mRNA level of ileal bile acid transporter (IBAT) was significantly lower in rats fed the IFP diet compared with rats fed the casein diet (M Kato and K Ebihara, unpublished results). Thus, the increased amount of bile acids present in the small intestine and caecum might be coupled with decreased reabsorption of bile acids from the ileum through a decrease in IBAT.

Although the mechanism of the hypocholesterolaemic effect of plant proteins is not yet fully understood, proteins with different amino acid composition show differences in their hypocholesterolaemic effects. Dietary proteins with a higher Arg:Lys ratio lower the serum cholesterol of rats⁽³⁸⁾ and can result in an increase in hepatic CYP7A1 activity⁽³⁹⁾. The Arg:Lys ratio (w/w) was higher in the IFP diet (0.50) than in the casein diet (0.37). Plasma total cholesterol was decreased by the IFP diet. Conversion of cholesterol to bile acids is the major regulated pathway by which cholesterol is removed from the body. An increased faecal excretion of bile acids is also considered as a factor of the hypocholesterolaemic effect of IFP. Hepatic CYP7A1 is an enzyme catalysing the rate-limiting step of the bile acid synthetic pathway. However, hepatic CYP7A1 activity was decreased by the IFP diet. It should be mentioned that data arguing against the above-mentioned hypothesis have also been reported⁽⁴⁰⁾; therefore, this idea remains to be proved.

Postmenopausal women have higher homocysteine levels than premenopausal women^(41,42). Recent studies suggest that increased blood homocysteine levels are as important as high blood cholesterol levels. A high plasma homocysteine concentration induces pathological changes in the arterial wall and thus is strongly associated with an increased risk of atherosclerosis⁽⁴³⁾. Plasma homocysteine levels have been shown to correlate significantly with LDL-cholesterol^(44,45). However, some studies did not find an association between serum cholesterol and homocysteine⁽⁴⁶⁾, and found that elevated homocysteine concentration was not associated with an increased risk of atherosclerosis⁽⁴⁷⁾.

In the present study, plasma homocysteine concentration was not affected by ovariectomy; in ovariectomised rats, however, it was significantly decreased by the IFP diet.

Because hepatic cholesterol homeostasis is achieved by a balance of biosynthesis, storage, catabolism and export, processes that influence cholesterol excretion may have

contributed to the observed accumulation of cholesterol in the livers of sham-operated rats fed the casein diet. Normally, excess cholesterol is eliminated from the liver mainly via bile acids, and CYP7A1 is an enzyme involved in the synthesis of bile acids from cholesterol⁽⁴⁸⁾. In the sham-operated rats, the relative mRNA concentration of CYP7A1 in the liver was increased by the casein diet; however, the amount of bile acids excreted via the faeces was not affected by diet. Therefore, the accumulations of total and esterified cholesterol in the livers of the sham-operated rats fed the casein diet might not be due to its reduced excretion via bile acids.

It is known that the expression of fatty acid synthase is promoted by the activation of SREBP-1c^(49,50). Fatty acids are the major components of TAG. Liver weight and TAG concentration in the liver were increased by ovariectomy. The level of SREBP-1c mRNA was increased by ovariectomy. The level of SREBP-1c mRNA was higher in the rats fed the IFP diet than in those fed the casein diet; however, the IFP diet decreased the concentration of TAG in the liver. Faecal excretion of bile acids was increased by the IFP diet feeding. The reduction of TAG concentrations in the liver by the IFP diet could be due, at least in part, to a reduction of intestinal fat absorption.

In conclusion, IFP decreased plasma total cholesterol concentration and increased faecal bile acid excretion in ovariectomised rats, but not in sham-operated rats. The preventive effect of IFP on the ovarian hormone deficiency-associated increase in plasma cholesterol seems to be mediated by accelerated faecal excretion of bile acids, coupled with an increase in the intestinal pool of bile acids.

Acknowledgements

This research was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan and a grant from the All-Japan Kamaboko Makers Association.

M. K. carried out the experimental plan, summarised the experimental results and discussed the experimental results with the other researchers. H. O. advised on all aspects of the experiment and discussed the experimental results. T. K. helped with all aspects of the experiment and discussed the experimental results. K. E. designed the experiment and prepared the manuscript.

There are no conflicts of interest to declare.

References

1. Gevers Leuven JA (1994) Sex steroids and lipoprotein metabolism. *Pharmacol Ther* **64**, 99–126.
2. Shono N, Kumagai S & Sasaki H (1996) Obesity, glucose and lipid metabolism, and steroid hormones (article in Japanese). *J Health Sci* **18**, 21–44.
3. Sullivan TR Jr, Karas RH, Aronovitz M, *et al.* (1995) Estrogen inhibits the response-to-injury in a mouse carotid artery model. *J Clin Invest* **96**, 2482–2488.
4. Chen SJ, Li H, Durand J, *et al.* (1996) Estrogen reduces myointimal proliferation after balloon injury of rat carotid artery. *Circulation* **93**, 577–584.
5. Colditz GA, Willett WC, Stampfer MJ, *et al.* (1987) Reproducibility and validity of self-reported menopausal status in a prospective cohort study. *Am J Epidemiol* **126**, 319–325.
6. Rosano GM & Fini M (2002) Postmenopausal women and cardiovascular risk: impact of hormone replacement therapy. *Cardiol Rev* **10**, 51–60.
7. Judd HL, Meldrum DR, Deftos LJ, *et al.* (1983) Estrogen replacement therapy: indications and complications. *Ann Intern Med* **98**, 195–205.
8. Henderson BE, Ross RK & Pike MC (1993) Hormonal chemoprevention of cancer in women. *Science* **259**, 633–638.
9. Kritchevsky D (1995) Dietary protein, cholesterol and atherosclerosis: a review of the early history. *J Nutr* **125**, 589S–593S.
10. Kritchevsky D, Tepper SA, Czarnecki SK, *et al.* (1982) Atherogenicity of animal and vegetable protein. Influence of the lysine to arginine ratio. *Atherosclerosis* **41**, 429–431.
11. Kromhout D, Bosschieter EB & de Lezenne Coulander C (1985) The inverse relation between fish consumption and 20-year mortality from coronary heart disease. *N Engl J Med* **312**, 1205–1209.
12. Harris WS (1989) Fish oil and plasma lipid and lipoprotein metabolism in humans: a critical review. *J Lipid Res* **30**, 785–807.
13. Bergeron N & Jacques H (1989) Influence of fish protein as compared to casein and soy protein on serum and liver lipids, and serum lipoprotein cholesterol levels in the rabbit. *Atherosclerosis* **78**, 113–121.
14. Zhang X & Beynen AC (1993) Influence of dietary fish proteins on plasma and liver cholesterol concentrations in rats. *Br J Nutr* **69**, 767–777.
15. Reeves PG, Nielsen FH & Fahey GC (1993) AIN-93 purified diets for laboratory rodents: Final Report of the American Institute of Nutrition Ad Hoc Writing Committee on the reformulation of AIN-76A rodent diet. *J Nutr* **123**, 1939–1951.
16. Hasegawa T & Kuroda M (1990) Automated amino acids determination. *Med Technol* **18**, 121–127.
17. Folch J, Lees M & Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissue. *J Biol Chem* **226**, 497–509.
18. Ebihara K, Shiraishi R & Okuma K (1998) Hydroxypropyl-modified potato starch increases fecal bile acid excretion in rats. *J Nutr* **128**, 848–854.
19. Ogishima T & Okuda K (1986) An improved method for assay of cholesterol 7 α -hydroxylase activity. *Anal Biochem* **158**, 228–232.
20. Araki A & Sako Y (1987) Determination of free and total homocysteine in human plasma by high performance liquid chromatography with fluorescence detection. *J Chromatogr* **42**, 43–52.
21. Eneroth P, Hellstrom K & Sjoval J (1968) A method for quantitative determination of bile acids in human feces. *Acta Chem Scand* **22**, 1729–1744.
22. Sheltaway MJ & Losowsky MS (1975) Determination of fecal bile acids by an enzymic method. *Clin Chem Acta* **64**, 127–132.
23. Chomczynski P & Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**, 156–159.
24. Kishida T, Ishikawa H, Tsukaoka M, *et al.* (2003) Increase of bile acids synthesis and excretion caused by taurine administration prevents the ovariectomy-induced increase in cholesterol concentrations in the serum low-density lipoprotein fraction of Wistar rats. *J Nutr Biochem* **14**, 7–16.
25. Liu X, Sawauchi H, Ogawa H, *et al.* (2006) Retrograded tapioca starch prevents ovarian hormone deficiency-induced hypercholesterolemia. *J Nutr Sci Vitaminol* **52**, 134–141.
26. Bayrak A, Aldemir DA, Bayrak T, *et al.* (2006) The effect of hormone replacement therapy on the levels of serum lipids, apolipoprotein AI, apolipoprotein B and lipoprotein (a) in Turkish postmenopausal women. *Arch Gynecol Obstet* **274**, 289–296.
27. Di Croce L, Bruscalupi G & Trentalancia A (1996) Independent behavior of rat liver LDL receptor and HMGCoA reductase under estrogen treatment. *Biochem Biophys Res Commun* **224**, 345–350.

28. Parini P, Angelin B, Stavr us-Evers A, *et al.* (2000) Biphasic effects of the natural estrogen 17 β -estradiol on hepatic cholesterol metabolism in intact female rats. *Arterioscler Thromb Vasc Biol* **20**, 1817–1823.
29. Shukla A, Bettzieche A, Hirche F, *et al.* (2006) Dietary fish protein alters blood lipid concentrations and hepatic genes involved in cholesterol homeostasis in the rat model. *Br J Nutr* **96**, 674–682.
30. van Lenten BJ, Melchior GW & Roheim PS (1983) Lipoprotein metabolism in the ovariectomized rat. *J Lipid Res* **24**, 1475–1484.
31. Kahlon TS & Woodruff CL (2002) *In vitro* binding of bile acids by soy protein, pinto beans, black beans and wheat gluten. *Food Chem* **79**, 425–429.
32. Sugano M, Goto S, Yamada Y, *et al.* (1990) Cholesterol-lowering activity of various undigested fractions of soybean protein in rats. *J Nutr* **120**, 977–985.
33. Nagata Y, Ishiwaki N & Sugano M (1982) Studies on the mechanism of antihypercholesterolemic action of soy protein and soy protein-type amino acid mixtures in relation to the casein counterparts in rats. *J Nutr* **112**, 1614–1625.
34. Fuchs M (2003) Bile acid regulation of hepatic physiology: III. Regulation of bile acid synthesis: past progress and future challenges. *Am J Physiol Gastrointest Liver Physiol* **284**, G551–G557.
35. Uchida K, Nomura Y & Takeuchi N (1980) Effects of cholic acid, chenodeoxycholic acid, and their related bile acids on cholesterol, phospholipid, and bile acid levels in serum, liver, bile, and feces of rats. *J Biochem* **87**, 187–194.
36. Davis RA & Kern F Jr (1976) Effects of ethinyl estradiol and phenobarbital on bile acid synthesis and biliary bile acid and cholesterol excretion. *Gastroenterology* **70**, 1130–1135.
37. Bravo E, Cantafora A, Cicchini C, *et al.* (1999) The influence of estrogen on hepatic cholesterol metabolism and biliary lipid secretion in rats fed fish oil. *Biochim Biophys Acta* **1437**, 367–377.
38. Sugano M, Ishiwaki N & Nakashima K (1984) Dietary protein-dependent modification of serum cholesterol level in rats. *Ann Nutr Metab* **28**, 192–199.
39. Vahouny GV, Adamson I, Chalcarz W, *et al.* (1985) Effects of casein and soy protein on hepatic and serum lipids and lipoprotein lipid distributions in the rat. *Atherosclerosis* **56**, 127–137.
40. Gibney MJ (1983) The effect of dietary lysine to arginine ratio on cholesterol kinetics in rabbits. *Atherosclerosis* **47**, 263–270.
41. Morris MS, Jacques PF, Selhub J, *et al.* (2000) Total homocysteine and estrogen status indicators in the Third National Health and Nutrition Examination Survey. *Am J Epidemiol* **152**, 140–148.
42. Hak AE, Polderman KH, Westendorp IC, *et al.* (2000) Increased plasma homocysteine after menopause. *Atherosclerosis* **149**, 163–168.
43. Refsum H, Ueland PM, Nygard O, *et al.* (1998) Homocysteine and cardiovascular disease. *Annu Rev Med* **49**, 31–62.
44. Tribouilloy CM, Peltier M, Iannetta Peltier MC, *et al.* (2000) Plasma homocysteine and severity of thoracic aortic atherosclerosis. *Chest* **118**, 1685–1689.
45. Wu LL, Wu J, Hunt SC, *et al.* (1994) Plasma homocyst(e)ine as a risk factor for early familial coronary artery disease. *Clin Chem* **40**, 552–561.
46. Malinow MR, Kang SS, Taylor LM, *et al.* (1989) Prevalence of hyperhomocyst(e)inemia in patients with peripheral arterial occlusive disease. *Circulation* **79**, 1180–1188.
47. Siri PW, Verhoef P & Kok FJ (1998) Vitamins B₆, B₁₂, and folate: association with plasma total homocysteine and risk of coronary atherosclerosis. *J Am Coll Nutr* **17**, 435–441.
48. Vlahcevic ZR, Pandak WM & Stravitz RT (1999) Regulation of bile acid biosynthesis. *Gastroenterol Clin North Am* **28**, 1–25.
49. Swinnen JV, Ulrix W, Henys W, *et al.* (1997) Coordinate regulation of lipogenic gene expression by androgens: evidence for a cascade mechanism involving sterol regulatory element binding proteins. *Proc Natl Acad Sci U S A* **94**, 12975–12980.
50. Bennett MK, Lopez JM, Sanchez HB, *et al.* (1995) Sterol regulation of fatty acid synthase promoter. Coordinate feedback regulation of two major lipid pathways. *J Biol Chem* **270**, 25578–25583.