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Role of soy isoflavones in the hypotriglyceridemic effect of soy protein in the rat

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Abstract

The present study was undertaken to determine whether isoflavones present in soy protein isolate contribute to the triglyceride-lowering effect of the protein relative to casein. Plasma triglyceride concentrations, their secretion rate into blood circulation, and post-heparin plasma lipoprotein lipase activity (a major determinant of intravascular catabolism of triglycerides) were measured in the fasted state in male Sprague-Dawley rats fed for 21 days one of three experimental diets varying in protein source (20% weight/weight): soy protein isolate, casein or casein to which 1.82 mg/g isoflavones (genistein and daidzein) were added to match the isoflavone content of soy protein isolate. Body weight gain was slightly lower in soy protein fed rats than in casein fed rats, but this effect was not statistically significant ($P = 0.22$). Casein plus isoflavones diet induced intermediary weight gain. A decrease in plasma total triglycerides was observed in rats fed soy protein and casein plus isoflavones compared with casein ($P < 0.05$), and there was a tendency to a positive correlation between weight gain and plasma triglyceride concentrations ($r = 0.35$, $P = 0.06$). However, no significant effect was observed on hepatic triglyceride concentrations, triglyceride secretion rate by the liver and post-heparin plasma lipoprotein lipase activity. These results show that soy protein isolate, in comparison with casein, has a hypotriglyceridemic effect in the rat and suggest that isoflavones may be responsible, at least in part, for this effect. The lowering effect of soy protein isolate and isoflavones on plasma triglyceride concentrations may be mediated by an alteration in energy balance, and possibly by the hepatic production of lipoproteins more susceptible to intravascular hydrolysis. Subtle but sustained changes in triglyceride secretion and post-heparin plasma lipoprotein lipase activity may also be implicated.

Keywords: Soy protein; Isoflavones; Triglycerides; Lipoprotein lipase; Rat

1. Introduction

Coronary heart disease (CHD) is the leading cause of mortality in Western countries. In the past 30 years, numerous studies have shown that soy consumption improves the lipid profile in plasma. Addition of soy in the diet or substitution of soy protein for animal protein has been shown to reduce plasma total and low-density lipoprotein (LDL) cholesterol and triglyceride concentrations in laboratory animals [1,2] and in humans [3,4]. The mechanisms by which soy protein lowers blood lipid concentrations include a reduction in intestinal absorption of cholesterol and bile acids [5] and in insulin-to-glucagon ratio [6], a rise in thyroid hormone concentrations [7] and in the activity of apolipoprotein B/E receptors [5,8]. However, the constituents of soy protein responsible for these favorable effects remain to be clearly identified.

Along with soy protein itself and its amino acid composition, phytoestrogenic isoflavones have been proposed to play a role in the hypolipidemic effect of soy protein observed in various animal models [9–12]. Balmir et al [11] reported that the incorporation of an ethanol isoflavone-rich extract from soy protein to a diet containing casein similarly lowered plasma cholesterol as did soy protein in hamsters. However, the exact composition of the ethanol extract was unknown and it could not be concluded that soy isoflavones were the only components responsible for the hypocholesterolemic effect. Another study in the gerbil showed a decrease in total plasma cholesterol concentrations and a dose-dependent increase in high-density lipoprotein (HDL) cholesterol concentrations after feeding soy protein provid-

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ing various amounts of isoflavones (0.05 to 6.2 mg/g protein) in comparison with casein [13]. The source of isoflavones used in that study was also an alcool extract from soy protein. Therefore, the effects of isoflavones were not isolated from the possible effects of other components present in the extract. Few data are currently available on the mechanisms by which soy isoflavones may affect plasma cholesterol concentrations. Results obtained in C57BL/6 (a model for low HDL-cholesterol) and low-density lipoprotein (LDL) receptor deficient mice [14] suggest that the cholesterol-lowering effect of soy isoflavones observed in humans [15,16] and some animal species [9,11,13,17] may be mediated by increased LDL receptor activity [14]. However, little data are available on the involvement of isoflavones in the regulation of triglyceridemia, which is also a major risk factor in the development of CHD. Nogowsky et al. [18] showed that the addition of purified genistein to the diet induced a decrease in serum triglyceride concentrations in rats although no effect was observed on cholesterolemia.

It was thus important to determine whether isoflavones can favorably affect the regulation of triglyceridemia. In the light of a previous study conducted in our laboratory showing a hypotriglyceridemic effect of soy protein in rats fed a high-cornstarch diet [19], the present study was undertaken to examine whether isoflavones could be responsible for the hypotriglyceridemic effect of soy protein. To isolate the respective effects of isoflavones and soy protein on triglyceridemia, three groups of rats were fed high-cornstarch diets containing either soy protein isolate with naturally occuring isoflavones (SPI), casein (Cas), or casein to which synthetic isoflavones (genistein and daidzein) were added in the same concentrations as those present in soy protein isolate (Cas+). To gain insight into the mechanisms by which soy protein and soy isoflavones may influence serum triglyceride concentrations, the secretion rate of triglycerides into the blood and postheparin plasma lipoprotein lipase activity, which are major determinants of intravascular metabolism of triglycerides, were assessed in the fasted state.

2. Methods and materials

2.1. Experimental animals

Sprague-Dawley rats (Charles River, St-Constant, Quebec, Canada) initially weighing ca. 200 g were housed individually in stainless-steel wire-bottom mesh cages. The temperature (20 ± 2°C) and humidity (45–55%) of the animal room were constant and the rats were kept under a daily inverted light-dark cycle (light : 21:00 to 09:00). Upon arrival, rats were fed a non purified commercial diet (Purina, St. Louis, MO) for 2–6 days to acclimate them to their new environment. They were then randomly assigned to one of three dietary groups (n = 12 per group). Purified diets and water were provided once daily on an ad libitum basis for a 21-day period. Food intake was measured daily and body weight was monitored three times a week. This experiment was approved by the Animal Care Committee of Laval University according to the guidelines of the Canadian Council on Animal Care.

Table 1
Composition of the purified diets

	Cas[a] (g/kg)	Cas+ (g/kg)	SPI (g/kg)
Ingredients			
Casein	225.4	225.4	—
Soy protein	—	—	234.6
Cornstarch	526.9	526.53	517.7
Cellulose	50	50	50
Beef tallow	100	100	100
Soybean oil	40	40	40
Cholesterol	10	10	10
Minerals	35	35	35
Vitamins	10	10	10
Choline bitartrate	2.5	2.5	2.5
BHT	0.2	0.2	0.2
Genistein	—	0.25	—
Daidzein	—	0.12	—

[a] Cas = casein; Cas+ = casein + 1.82 mg/g isoflavones; SPI = soy protein isolate.

2.2. Purified diets

Diets were similar except for the protein source with or without isoflavones (Table 1). They contained, by weight, 20% protein (soy protein isolate, casein or casein to which isoflavones were added at the same concentrations as those found in soy protein isolate), 14% fat (10% beef tallow plus 4% soybean oil) and 1% cholesterol. Soy protein isolate (SPI) of which the isoflavone content was known (aglycone daidzein: 0.59 mg/g protein, aglycone genistein: 1.23 mg/g protein) was provided by Protein Technologies International (St. Louis, MO). The casein plus isoflavones diet (Cas+) was prepared by adding to the casein diet (Cas) 1.82 mg isoflavones/g protein (synthetic genistein: 1.23 mg/g protein and synthetic daidzein: 0.59 mg/g protein) (Sigma-Aldrich Canada, Oakville, Ontario, Canada). Beef tallow was supplied by ICN Biomedicals Inc. (Aurora, Ohio) and soybean oil was purchased from a local supermarket. Alpha tocopherol, butylated hydroxyanisole and butylated hydroxytoluene (ICN Biomedicals Inc, Aurora, Ohio) were added to diets to minimize the oxidation of n-6 polyunsaturated fatty acids in soybean oil. Highly purified casein, cornstarch, cellulose (Alphacel), cholesterol, rat AIN-93 mineral mix and vitamin mix were purchased from ICN Biomedicals Inc. The protein content (N × 6.25) of soy protein and casein was assayed by the Kjeldahl method using a Kjeldahl-Foss autoanalyser (Model 16216; Foss Co, Hillerod, Denmark), and the level of protein in the diets was adjusted at the expense of cornstarch to obtain an isonitrogenous content. The residual lipid content of soy protein (0.17%) and casein (0.04%) was determined using a Goldfish Lipid Extractor

(Model 35001; Labconco Corporation, Kansas city, MO). The energy content of the diets was measured in an automatic adiabatic calorimeter (Model 1241; Parr Instruments, Moline, IL) and was similar in the SPI (20.1 kJ/g), Cas (20.4 kJ/g) and Cas+ (20.2 kJ/g) diets.

2.3. Experimental procedures, measurement of post-heparin plasma lipoprotein lipase and hepatic lipase activities, and determination of triglyceride secretion rate

On day 16 of the experimental period, rats were cannulated into the jugular vein under isoflurane anesthesia. They were allowed to recover for three days before being used for the following procedures. On day 19, after a 12-hr fast, the rats were administered 200 IU/kg body weight of heparin through the jugular catheter. Ten minutes later, a 0.25 ml blood sample was withdrawn and centrifuged (3000 RPM, 4°C, 15 min), and plasma was stored at −80°C until later determination of lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL) activities. Postheparin plasma LPL and HTGL activities were assessed using a method described by Belahsen and Deshaies [20] in which enzyme activities are evaluated by measuring the amount of in vitro hydrolysis by postheparin plasma samples of a labeled triolein emulsion in the presence of 0.1 or 1 mol/L NaCl. The triglyceride secretion rate was measured on day 21 according to the method of Otway and Robinson [21]. After a 12-hr fast, rats were injected through the jugular catheter with Triton WR-1339 (300 mg/kg body weight), a detergent that prevents intravascular triglyceride catabolism. Blood samples (0.15 ml) were taken before (0 min) and 20, 40 and 60 min after Triton injection. The rate of very low-density lipoprotein (VLDL) triglyceride secretion was determined from regression analysis of triglyceride accumulation in plasma versus time, and adjusted for plasma volume. Rats were then killed by CO_2 after O_2/CO_2 anesthesia. The liver was removed and stored at −80°C until further analyses.

2.4. Serum, lipoprotein and hepatic lipid analyses

Blood samples were centrifuged (900 rpm, 4°C, 10 min) to isolate plasma. Total plasma triglyceride concentrations were determined in all blood samples by an enzymatic method using the Triglycerides/GB kit provided by Roche Diagnostics (Laval, Quebec, Canada). Total plasma cholesterol concentrations were also determined by an enzymatic method (Boehringer Manheim CHOD-PAP kit provided by Roche Diagnostics), but only in blood samples collected at 0 min (before injection of Triton WR-1339). Hepatic cholesterol and triglycerides were extracted by chloroform:methanol (2:1, vol/vol) according to Folch et al [22], and determined enzymatically as described above.

Table 2
Food intake and weight gain of rats fed the purified diets[a]

Dietary group	*n*	Food intake (*g/day*)	Weight gain (*g/21 days*)
Cas[b]	12	18.7 ± 0.7	94.4 ± 6.7
Cas+	12	18.4 ± 0.7	88.8 ± 8.6
SPI	12	18.6 ± 0.5	81.7 ± 5.8

[a] Values are means ± standard error of the mean.
[b] Cas = casein, Cas+ = casein + 1.82 mg/g isoflavones, SPI = soy protein isolate.

2.5. Statistical analyses

The results are expressed as mean ± standard error of the mean (SEM) and a P value of 0.05 was considered significant. Data were subjected to an analysis of variance (ANOVA) using the general linear model procedure of the Statistical Analysis System (SAS Institute, Cary, NC) to determine the main diet effects. When statistically significant diet effects were detected, Duncan's New-Multiple-Range test was performed to identify differences among diet groups.

3. Results

3.1. Food consumption and weight gain

Food intake and body weight gain of rats fed the various diets are presented in Table 2. Food consumption was similar among the three dietary groups. Weight gain was slightly (14%) lower in SPI fed rats than in Cas fed rats, but this effect was not statistically significant ($P = 0.22$). Cas+ feeding induced weight gain intermediate but not significantly different from those obtained with SPI and Cas feeding.

3.2. Plasma and hepatic lipids

Total plasma cholesterol and hepatic cholesterol concentrations are presented in Table 3. Plasma and hepatic triglyceride concentrations, and triglyceride secretion rates are

Table 3
Total plasma and hepatic cholesterol concentrations of rats fed the purified diets[a]

Dietary group	*n*	Plasma cholesterol (mmol/L)	*n*	Hepatic cholesterol (μmol/g)
Cas[b]	9	2.1 ± 0.2	12	93.0 ± 10.0
Cas+	10	2.1 ± 0.2	12	98.0 ± 5.7
SPI	9	2.1 ± 0.2	12	82.4 ± 5.4

[a] Values are means ± standard error of the mean.
[b] Cas = casein, Cas+ = casein + 1.82 mg/g isoflavones, SPI = soy protein isolate.

Table 4
Total plasma and hepatic triglyceride concentrations, and triglyceride secretion rates in rats fed the purified diets[a]

Dietary group	*n*	Plasma triglycerides (mmol/L)	Triglyceride secretion rates (μmol/min)	*n*	Hepatic triglycerides (μmol/g)
Cas[b]	9	0.23 ± 0.03[a,*]	3.6 ± 0.6[a]	12	48.9 ± 5.9[a]
Cas+	9	0.17 ± 0.02[b]	3.8 ± 0.6[a]	12	49.5 ± 4.9[a]
SPI	9	0.16 ± 0.02[b]	3.9 ± 0.3[a]	12	44.4 ± 4.6[a]

[a] Values are means ± standard error of the mean.
[b] Cas = casein, Cas+ = casein + 1.82 mg/g isoflavones, SPI = soy protein isolate.
* Values bearing different letters in the same column are significantly different at $P < 0.05$.

shown in Table 4. Total plasma triglyceride concentrations at 0 min (before injection of Triton WR-1339) were significantly lowered by 26% in SPI and Cas+ fed rats in comparison with Cas fed rats. A strong tendency to a positive correlation was observed between body weight gains and fasting plasma triglyceride concentrations ($P = 0.06$, r = 0.35) (Fig. 1). However, no significant effect of dietary protein or isoflavone supplementation was observed on secretion rates of triglycerides into the blood, as assessed from plasma triglyceride accumulation after Triton WR-1339 administration. Similar overall results were obtained when statistical analyses were performed on log transformed values of plasma triglyceride concentrations and triglyceride secretion rates.

3.3. Post-heparin plasma LPL and HTGL activities

Table 5 shows post-heparin plasma LPL and hepatic lipase activities. No significant difference in enzyme activities was observed between the three diets.

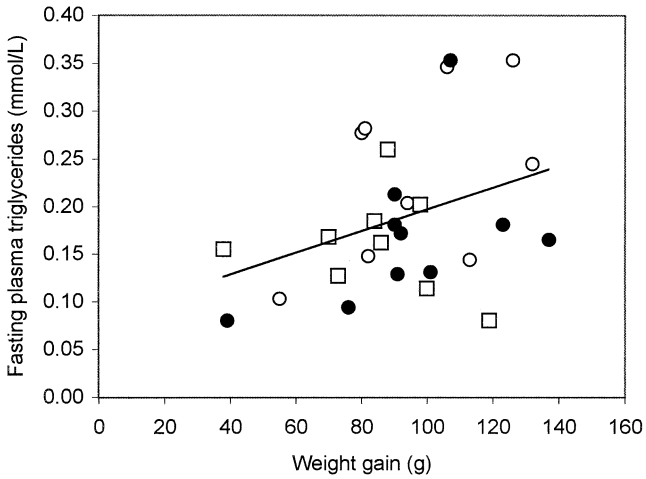

Fig. 1. Association between cumulative body weight gain and fasting plasma triglyceride levels in rats fed the purified diets (p = 0.06, r = 0.35, n = 28). ○ Casein, ● Casein + 1.82 mg/g isoflavones, and □ Soy protein isolate.

4. Discussion

The present study was designed to assess whether isoflavones partake in the effect of soy protein, relative to casein, on fasting triglyceridemia in the rat and to identify the determinants of triglyceride metabolism responsible for such an effect. Proteins and isoflavones were studied using a cornstarch-based diet to avoid carbohydrate-induced hypertriglyceridemia, and in the fasted state, such that treatment effects were assessed in relatively stringent conditions. To avoid the confounding effects of other components present in ethanol extracts of soy protein, the casein diet was supplemented with purified isoflavones. A decrease in fasting plasma total triglycerides was observed in rats fed SPI as well as in those fed casein plus isoflavones, indicating that isoflavones per se are responsible, at least in part, for the hypotriglyceridemic effect of soy protein.

The findings clearly indicate that consumption of soy protein relative to casein lowered triglyceridemia, confirming earlier studies by us and others [1,19,23]. Regarding isoflavones, Nogowsky et al. [18] also showed in rats a decrease in serum triglyceride concentrations following the addition of purified genistein at two concentrations (0.01 or 0.1%). In humans, a weak negative correlation has been reported between triglyceridemia and urinary excretion of genistein, suggesting that consumption of isoflavones may affect triglyceridemia [24]. In contrast, studies comparing the effects of diets containing intact SPI or SPI with the isoflavones removed by ethanol extraction showed no effect

Table 5
Post-heparin LPL and hepatic lipase activities in rats fed the purified diets[a]

Dietary group	*n*	LPL activity (μU/mL/h)[b]	HTGL activity (μU/mL/h)	LPL-to-HTGL ratio
Cas[c]	10	37.4 ± 3.7	55.1 ± 4.1	0.70 ± 0.07
Cas+	11	40.1 ± 6.5	50.7 ± 6.3	0.85 ± 0.14
SPI	10	38.4 ± 5.3	46.8 ± 3.9	0.86 ± 0.13

[a] Values are means ± standard error of the mean.
[b] 1 μU = 1 μmol nonesterified fatty acids released per hour of incubation.
[c] Cas = casein, Cas+ casein + 1.82 mg/g isoflavones, SPI = soy protein isolate.

or a slight elevation of plasma triglyceride concentrations in male monkeys [9,17] and in hypercholesterolemic postmenopausal women [25,26]. When an ethanol extract from soy protein was added to a casein-based diet, no effect was observed on triglyceridemia in ovariectomized monkeys [12] or in hamsters [11]. Likewise, a supplement of isoflavones from soy [15] or red clover [27] had no effect on triglyceridemia in peri- and post-menopausal women. These divergent results in rats versus humans, monkeys or hamsters may be partly due to metabolic differences between species. The hormonal status of human subjects (menopausal or not) and animal models (ovariectomized or not), the bioavailability of isoflavones, the interindividual variability in absorption and metabolism of isoflavones, but also the different sources of isoflavones (purified forms versus ethanol extracts) may also be implicated, as it is possible that other compounds present in ethanol extracts (e.g. saponins, sugars and unknown compounds) may counteract the triglyceride-lowering effect of isoflavone consumption.

At the whole body level, the hypotriglyceridemic action of SPI and isoflavones may be related to their effects on energy balance, as suggested by the strong tendency toward a positive correlation between weight gain and triglyceridemia observed here. In support of this possibility is our study in the rat [28] which showed that SPI, compared with casein and in the presence of cornstarch, reduced metabolizable energy intake, total energy and fat gains, without significantly modifying total energy intake. This could be explained by the lower protein efficiency ratio of soy protein, which has a lower methionine content and lysine/arginine ratio than casein [28]. A lower body weight gain is associated with diminished plasma triglyceride concentrations in the rat [29], and a positive correlation has been observed in humans between body fat mass and plasma triglycerides [30,31]. Regarding soy isoflavones, the compounds are similar in structure to estrogens [32]. They have the capacity to bind to estrogen receptors [32,33] and may act as estrogen agonists and antagonists, a common characteristic of weak estrogens [33]. Ovarian hormones exert potent effects on energy balance, as ovariectomy of rats brings about a large increase in food efficiency (body weight gain per unit of food ingested) that is reversed by estrogen treatment [34, 35]. Hormonal replacement therapy and estrogen antagonists that possess pro-estrogenic actions also reduce fat accumulation and lipemia both in animal models [36] and in humans [37,38]. Therefore, the lowering effect of SPI and isoflavones on plasma triglycerides may be related, through various mechanisms, to alterations in energy balance, which may in turn impact determinants of triglyceride homeostasis. This possibility remains to be more firmly established, possibly with the use of diets favoring energy deposition that may reveal the full potential of soy and isoflavones on energy balance. Finally, it must be noted that the existence of direct metabolic actions of soy and isoflavones on triglyceride metabolism remains entirely possible.

Plasma triglyceride concentrations in the fasted state are determined by their secretion into the blood from the liver and their intravascular hydrolysis by LPL. Therefore, in an attempt to identify the mechanisms of the hypotriglyceridemic action of soy protein and isoflavones, liver lipid content (a crude index of production), liver triglyceride secretion rates and the global intravascular availability of LPL were assessed. Whereas both SPI and Cas+ induced lower triglyceridemia than casein, no significant effect of SPI or isoflavone supplementation of casein was detected on hepatic cholesterol and triglyceride concentrations or on triglyceride secretion rates. These findings are at variance with those of Nogowsky et al. [18], who observed a decrease in triglyceride output from perfused rat livers after the addition of genistein in the perfusion medium. A reduction in hepatic and adipose lipogenesis from glucose was also noted. Importantly however, Pfeuffer and Barth [39] showed that soy protein lowered triglyceride secretion rates compared with casein in rats fed a high-sucrose diet but not in rats fed a high-cornstarch diet. In contrast with cornstarch, sucrose promotes *de novo* fatty acid synthesis in the liver, fatty acid esterification and VLDL secretion [39]. It must be noted that, in the present study, the hypotriglyceridemic effect of soy protein and isoflavone supplementation of casein was clearly significant, but somewhat limited in absolute terms, most likely because of the already low triglyceridemia that prevails in fasted rats chronically fed cornstarch. It is therefore possible that the cornstarch-based diet may have dampened the differences in the response to diet of VLDL secretion rate to below-detectable levels, its quantitation being inherently more prone to inter-individual variation than triglyceridemia. The fact that rats were studied in the fasted state also likely contributed to dampen the modulation of determinants of triglyceride metabolism. In this regard, our previous study showed that the hypotriglyceridemic effect of soy protein relative to casein is more pronounced in the postprandial than in the fasted state [23].

The intravascular hydrolysis of triglycerides by LPL is another major determinant of triglyceridemia [40]. Two previous animal studies conducted in our laboratory suggested that dietary proteins may modify LPL activity [19, 41]. Soy protein decreased post-heparin plasma LPL activity in rabbits when compared with cod protein [41], and lowered LPL activity in muscle, but not in other tissues, relative to casein feeding [19]. In the present study, SPI feeding resulted in post-heparin plasma LPL activity slightly higher (16%) than Cas, with intermediate values in Cas+ fed rats, such effects not being statistically significant. Modifications in HTGL activity neither seemed to be implicated in the lowering effect of SPI and Cas+ diets on triglyceridemia. A previous study carried out in humans showed that a decrease in LPL-to-HTGL ratio was associated with an increase in triglyceridemia [42]. In the present study, SPI and Cas+ feeding resulted in a 22% higher LPL-to-HTGL ratio than casein feeding, a difference that did not reach significance due to the large interindividual variation of lipase activities. The possibility remains that

subtle, but sustained, changes leading to a higher LPL-to-HTGL ratio may have contributed to the lowering effect of SPI and Cas+ diets on triglyceridemia.

Although plasma triglyceride concentrations declined with SPI and isoflavone feeding in this study, no parallel decrease in plasma cholesterol was seen. Here again, the use of cornstarch as opposed to sucrose may explain the lack of diet-related differences in cholesterolemia. Indeed, as observed previously by Pfeuffer et Barth [39] and Hurley et al [43], protein effects on cholesterolemia are less pronounced in the presence of cornstarch relative to sucrose. In addition, supplementation of casein with an ethanol extract of soy protein in a high-sucrose diet decreased LDL-cholesterol concentrations in the rat [11], but no effect of genistein on serum cholesterol was observed when starch from crude cereals was used as the carbohydrate source [18].

In conclusion, the present study demonstrated that the addition of isoflavones (genistein and daidzein) to a casein-based diet reproduced the hypotriglyceridemic effect of SPI, indicating that isoflavones are responsible, at least in part, for the hypotriglyceridemic effect of soy protein. The lowering effect of SPI and isoflavones on plasma triglyceride concentrations may be related to changes in energy balance, but neither changes in triglyceride secretion rate nor in post-heparin plasma LPL and HTGL activities were of sufficient magnitude to explain effects on triglyceridemia. The contribution of subtle but sustained alterations in triglyceride secretion and post-heparin plasma lipase activity cannot however be excluded. Possible additional levels of action of SPI and isoflavones such as liver apo B expression, and VLDL composition, which could affect their susceptibility to intravascular hydrolysis, await further assessment.

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