Biochemical and Molecular Actions of Nutrients

Proteins of White Lupin Seed, a Naturally Isoflavone-Poor Legume, Reduce Cholesterolemia in Rats and Increase LDL Receptor Activity in HepG2 Cells[1]

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ABSTRACT White lupin (*Lupinus albus*, L.), a widely cultivated crop that has been consumed for many years in Western Europe, may provide a useful alternative for individuals wishing to substitute animal with plant proteins for cardiovascular disease prevention. Lupin seeds have a very low content of isoflavones, and lupin protein isolates are essentially isoflavone free. In rats fed a casein-based cholesterol + cholic acid diet, a relatively low daily intake (50 mg/d by gavage for 2 wk) of total lupin protein extract reduced plasma total and VLDL + LDL cholesterol concentrations by 21 and 30%, respectively (both $P < 0.001$). In an attempt to elucidate the lipid-lowering mechanism, LDL receptor activity was evaluated in a human hepatoma cell line (HepG2). In this model, the lupin total protein extract was essentially inactive, whereas one purified minor protein component, conglutin γ, had a remarkable upregulatory effect, with maximal increases of 53 and 21% (both $P < 0.05$) for LDL uptake and degradation, respectively. This initial study indicates that lupin, although isoflavone free, has hypocholesterolemic activity similar to that of other leguminous proteins in an established animal model. Further, the cholesterol reduction appears to be associated with stimulation of LDL receptors by a well-defined protein component of the lupin seeds as demonstrated by in vitro studies. J. Nutr. 134: 18–23, 2004.

KEY WORDS: • *lupin proteins* • *isoflavones* • *soybean globulins* • *hypercholesterolemia* • *LDL receptors*

Plant proteins in the diet have been shown to successfully reduce cholesterolemia in experimental animals and humans (1–3). The cholesterol-reducing effect, potentially leading to reduced cardiovascular risk, was the basis for the U.S. FDA approval of the health claim concerning the role of soybean proteins in reducing the risk of coronary disease (4).

Approval of the claim and the wide availability of numerous products derived from soybeans, has definitely led to a greater use of these food items. This has not been the case in Western Europe, however, where soy protein–based foods are still used to a modest extent (5), partly because these products are foreign to European food culture and partly because of public concern about the use of genetically modified foods (6).

In addition, doubts still exist concerning the mechanism(s) whereby soy protein–based foods may reduce cholesterolemia and cardiovascular risk. The claim that the isoflavones of soy, particularly daidzein and genistein, may affect cholesterolemia, was strongly supported by a major meta-analysis on the cholesterol-lowering effect of soybeans (3). This hypothesis is supported in part by experimental and clinical studies with hot ethanol-extracted isoflavone-free soybean products, apparently with reduced cholesterol-lowering potential (7,8). On the other hand, readdition of the ethanol-extracted material to the diet did not cause any relevant cholesterol reduction (9). A recent proteomic investigation by our group (10) showed that the extraction of isoflavones with ethanol changes the protein/peptide profile of the protein isolates with unpredictable effects on activity. In a study in which removal of the isoflavones from soy protein isolates was achieved by a chromatographic procedure (11), the milder purification did not alter their hypocholesterolemic properties. Further, both cholesterolemia and atherosclerosis appeared to be positively correlated with plasma isoflavone concentrations in primates following a soybean-containing diet (12); a very recent study also reported a direct correlation between plasma isoflavones and cholesterolemia in hamsters (13). Finally, the major clinical studies in type II hypercholesterolemic patients were conducted with isoflavone-poor soybean products (14), and in vitro studies have clearly indicated that specific protein fractions of soybean, in particular the α' subunit of the 7S globulin and peptides thereof, can directly activate LDL receptors in liver cells (15,16).

Because potentially harmful effects of isoflavones have been reported in recent years (17), we decided to investigate alter-

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native legume protein products with a low isoflavone content; such products might be more acceptable to European consumers as an alternative to soybeans. The only other protein-rich grain legume is lupin (18), a common name that represents four domestic species, *Lupinus albus* (white lupin), *L. luteus* (yellow lupin), *L. mutabilis* (blue lupin) and *L. angustifolius* (narrow-leaf lupin). At present, the most important lupin-producing country worldwide is Australia, whereas in Europe, lupin is cultivated mostly in France, Poland, Russia, Hungary and Italy (19).

The alkaloids that confer a bitter taste to lupin kernels in modern varieties (sweet lupin) have been reduced to very low levels through conventional breeding techniques (20). In addition, an interesting feature of lupin is the very low (or nil) content of antinutritional factors, such as phytate, tannins, lectins, protease inhibitors and indigestible oligosaccharides (21–23).

Lupin seeds contain two classes of proteins which, according to Osborne's classification (24), belong to the albumin and globulin fractions, in the quantitative ratio of ~1:9. In turn, the globulins consist of the two classical major storage proteins, i.e., the 7S and 11S globulins, called conglutins β and α, respectively, in lupin and two minor distinct protein types, conglutins γ and δ (25). Conglutin γ is an oligomeric lupin seed glycoprotein comprising ~5% of the total lupin proteins (26). All of these proteins have been characterized at the molecular level, although only conglutin γ has a known amino acid sequence (27). Although lupin proteins have been thoroughly investigated for their potential food and feed applications for a long time, lupin's reputation as a minor crop and the lack, especially in Europe, of suitable industrial processing practices have prevented the wide diffusion of these proteins as important food components in the processed food chain.

Preliminary studies with seed meals and lupin fractions showed a substantial reduction of cholesterolemia in male rats (28); however, the chemical composition of the products used in that study and in a more recent one were not described fully (29). Because there is indication that lupin seeds may contain very low levels of isoflavones (30), the present study had the following objectives: *1*) to confirm the very low content of isoflavones in seeds of white lupin, the predominant species in Europe; *2*) to evaluate the cholesterol-lowering potential of lupin protein products in an established rodent model of hypercholesterolemia; and *3*) to investigate whether isolated lupin protein fractions exert an LDL receptor upregulatory activity in a human liver cell line.

MATERIALS AND METHODS

Lupin samples. White lupin seeds (*Lupinus albus* L.) were of the sweet Multitalia variety and were kindly provided by Dr. M. Fagnano, University of Naples, Italy.

Lupin protein preparation. The total protein extract (TPE)[3] from lupin seed was prepared as described below. Lupin seeds were manually dehusked and the kernels ground with a domestic coffee grinder. The flour was defatted in boiling hexane in a Soxhlet apparatus for 4 h. The defatted flour was then passed through a 60-mesh metal sieve. The resulting flour was then extracted in MilliQ water (Millipore, Billerica, MA), adjusted to pH 8.5–9.0 with diluted NaOH in a ratio of 1:20 (wt/v) with stirring at 5°C for 3 h. The pH was monitored regularly and readjusted to the original pH with diluted NaOH. Based on the solubility properties of lupin proteins, this step allowed their effective extraction. The slurry was then filtered on Miracloth (Calbiochem Corp., La Jolla, CA) tissue and centrifuged at 10,000 × *g* for 30 min at 20°C. The supernatant, consisting of the proteins, was treated with 90% ammonium sulfate at 4°C and the protein pellet was recovered by centrifugation as above. The pellet was then resuspended in MilliQ water and thoroughly dialyzed at 4°C for 24 h with frequent changes of water. The protein suspension was recovered and freeze-dried. The dried powder consisted of the lupin TPE with a protein content > 95 g/100 g (not shown).

Purification of lupin conglutin. Conglutin γ (Cγ) was purified essentially as described by Duranti et al. (31) by using a combination of anion and cation exchange chromatography. Purified Cγ was precipitated with ammonium sulfate (80% saturation) and then treated as the TPE. Homogeneity of the purified Cγ was verified by SDS-PAGE (not shown).

Identification and quantitative analysis of lupin isoflavones. Because these isoflavones are not synthesized in dry lupin seeds but only during germination (30), we grew some lupin plants for 3 mo, and extracted and analyzed the isoflavones from the leaves by LC-MS/MS to have a correct identification of all of the peaks in lupin seeds (32). The extraction of isoflavones was performed as indicated for soybeans by Wang and Murphy (33). Each sample (1 g) was stirred for 2 h in a mixture of 5 mL acetonitrile, 2.5 mL distilled water and 1 mL 0.1 mol/L HCl. After filtration, the solvent was dried under a vacuum, and the residue was dissolved in 1 mL of 50% methanol and filtered on 0.45-μm filters. Analyses were performed on a microbore column, Alltima C18 (Alltech, Deerfield, IL), 3 μm, 150 × 2.1 mm under the following conditions: eluent A, 0.1% acetic acid in acetonitrile; eluent B, 0.1% acetic acid in water; gradient 15% A to 35% A in 50 min, then 35% A for 10 min; flow 0.2 mL/min; temperature 30°C. The isoflavone identification was completed by LC-electrospray ionization-MS/MS on an Agilent SL 100 Series LC/mass selective detector trap (Agilent, Palo Alto, CA) equipped with an Agilent 1100 binary pump and an Agilent 1100 Diode Array Detector (DAD) under the following conditions: source temperature 325°C; nebulizer 18 psi; drying gas 6 L/min; capillary voltage 4000 V; scan range 60–700 m/z. Analyses were performed in negative ion mode because the sensitivity is higher than in positive ion mode.

Quantitative analyses were carried out on flour from lupin seeds, TPE and Cγ. The extraction was performed as indicated for the leaves. The quantification was done with both diode array detection at 254 nm and MS with the external standard method. Isoflavone determinations had CV ranging between 2 and 10%. Isoflavone standards were kindly provided by Indena SpA Milano, Italy.

In vivo studies

Animals, diets and experimental protocol. Male Sprague-Dawley rats (Charles River Italia, Calco, Italy; body weight 200–225 g) were housed in a room with controlled lighting (12 h/d), constant temperature (18°C) and relative humidity (55–65%). During wk 1, they were fed a pelleted commercial nonpurified diet (Piccioni, Gessate, Italy) and then were divided into two groups of 10 rats on the basis of body weight, plasma lipids and glycemia so that the distribution among the groups was similar. The rats were then transferred to a Nath's hypercholesterolemic diet (1 g/100 g cholesterol and 0.5 g/100 g cholic acid) (34). Feed and water were freely available.

TPE from lupin was given daily by gavage at 0900 h at a dose of 50 mg/rat, dissolved in water with a small amount of carboxymethylcellulose for 2 wk; control rats were administered vehicle only. The Institutional guides for the care and use of laboratory animals were followed, and the experiments were supervised by the Laboratory Animal Welfare Service.

Plasma cholesterol, triglyceride and glucose levels were determined by enzymatic methods (Boehringer SA, Mannheim, Germany); plasma lipoproteins were separated by ultracentrifugation using pools from two rats (35) by appropriate density cuts.

In vitro studies

Cell culture. The established human hepatoma cell line (HepG2) was obtained from American Type Culture Collection

[3] Abbreviations used: Cγ, conglutin γ; FCS, fetal calf serum; HC, hypercholesterolemic; LDH, lactate dehydrogenase; LPDS, lipoprotein-deficient serum; MTT, methyltetrazolium salts; TPE, total protein extract.

(Rockville, MD). Eagle's MEM, fetal calf serum (FCS), trypsin-EDTA (1X), penicillin (10^5 U/L), streptomycin (100 g/L), tricine buffer (1 mmol/L, pH 7.4) and nonessential amino acid solutions (100X) were from GIBCO (Madison, WI). Protease inhibitor cocktail for use with mammalian cell and tissue extracts was from Sigma-Aldrich (Milano-Italy). Petri dishes were from COSTAR (Cambridge, MA). Filters were from Millipore (Bedford, MA). The Protein Coomassie Plus Protein Assay kit was purchased from Pierce (Rockford, IL). ^{125}Iodine, carrier free, in 100 mmol/L NaOH, was from Perkin Elmer Life Sciences (Boston, MA). Sephadex G25 columns (PD10) were from Pharmacia Biotech (Uppsala, Sweden). All other chemicals were of analytical grade and were from Merck (Darmstadt, Germany).

Cells were grown in monolayers in 90-mm diameter Petri dishes, and maintained at 37°C in a humidified atmosphere of 95% air, 5% CO_2 in MEM supplemented with 10% FCS, non essential amino acid solution (1%, v/v), penicillin (10^5 U/L), streptomycin (0.1 g/L), tricine buffer (20 mmol/L, pH 7.4), NaHCO3 (24 mmol/L) and sodium pyruvate (0.11 g/L). For experiments designed to evaluate the LDL receptor modulation, cells were seeded in 35-mm plastic dishes ($3–5 \times 10^5$ cells) and used just before reaching confluence. In all cell culture experiments, the medium was changed every 2–3 d.

To assess cell viability, culture media from cells exposed to the different lupin preparations (all for TPE and the highest for Cγ) were tested by methyltetrazolium salts (MTT) assay, essentially as described by Lovati et al. (15). Cell enzyme leakage was determined by measuring lactate dehydrogenase (LDH) activity, using a kinetic (LDH/LD) diagnostic kit (Sigma Diagnostics).

Lipoproteins and lipoprotein-deficient serum. LDL ($1.019 \leq d \leq 1.063$ kg/L) were isolated by sequential preparative ultracentrifugation (35) from the plasma of clinically healthy normolipidemic volunteers. Lipoproteins were labeled according to the method of McFarlane as modified by Bilheimer et al. (36), as previously described (15). ^{125}I-LDL were sterilized by filtration (Millipore filters, 0.45-μm pore size) and stored at 4°C until use (<10 d after preparation). Human lipoprotein-deficient serum (LPDS) was prepared using the method of Goldstein et al. (37).

Uptake and degradation of ^{125}I-LDL. Monolayers of cells were preincubated for 24 h at 37°C in MEM supplemented with 5 g/100 g LPDS to upregulate the LDL receptors (37), in the presence or absence of TPE or Cγ at different concentrations. A fixed concentration (7.5 mg/L) of ^{125}I-LDL was then added to the medium and the incubation continued for a further 4 h at 37°C. Specific uptake (binding + internalization) and degradation of ^{125}I-LDL were evaluated as previously reported (15).

Statistical analyses. Statistical analyses of the individual differences in plasma lipids and lipoproteins were carried out with Student's *t* test. Differences in cell uptake and degradation of LDL after incubation with the different proteins were determined by ANOVA (SYSTAT 5.2, running on an Apple Macintosh LC 630) followed by Dunnett's test. Values are generally expressed as means ± SEM; *P*-values < 0.05 were considered to be significant.

RESULTS

Isoflavone identification and quantification. There are clear differences in the isoflavone pattern between white lupin (30) and soybeans (33). In lupin, genistein is present as genistin (7-*O*-glycoside) and malonylgenistin (6″-*O*-malonyl-7-*O*-glycoside) as in soybean, but also as the 4′-*O*-glycoside (**Fig. 1**). Daidzein and glycitein are not biosynthesized and are replaced by 2′-hydroxygenistein and 2′-hydroxygenistin (the 7-*O*-glycoside). Six isoflavones were identified in lupin leaves (**Table 1**); their relative retention times compared with genistein and the main ions of their mass spectra are reported.

At the end of this experiment, flour from dry seeds, TPE and purified Cγ underwent isoflavone quantification. In the seeds, only negligible amounts of genistin and genistein were present, whereas in TPE and Cγ, the isoflavones were all below the detection limits of our method (<0.1 nmol/g) (**Table 2**).

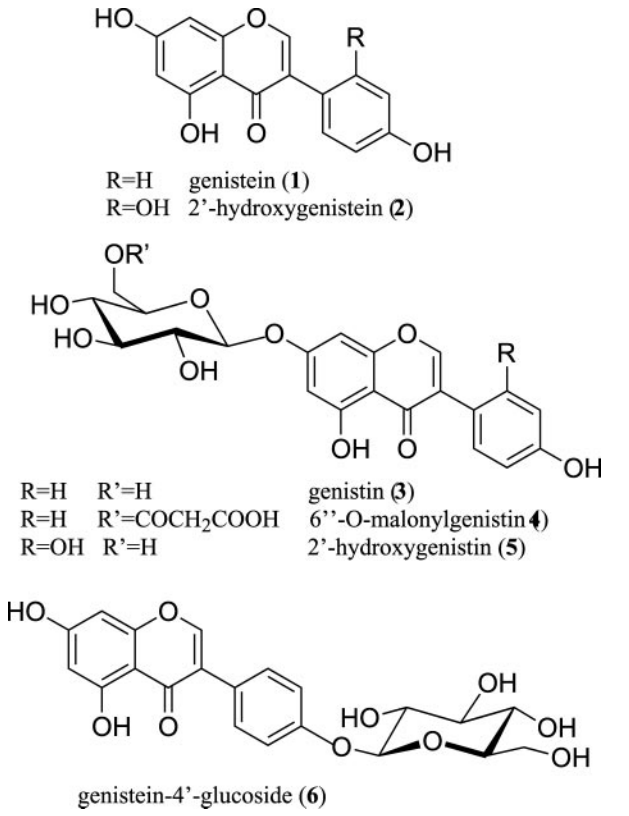

FIGURE 1 Chemical structures of the isoflavones found in the leaves of 3-mo-old white lupin plants.

The present study clearly shows the lack of any relevant presence of isoflavones in lupin seeds compared with soybeans, for which concentrations of 1500–2000 μg/g dry weight are generally reported (33). Moreover the procedures required for the purification of TPE and Cγ completely removed these antinutrients.

In vivo evaluation. HC (hypercholesterolemic) rats had lower plasma total (−21%) and VLDL-LDL cholesterol concentrations (−30%), and lower triglyceride levels (−17%) after administration of 50 mg/d lupin TPE (**Table 3**); HDL cholesterol levels tended to be 20% higher in lupin-treated rats at the end of the study ($P < 0.10$). Plasma glucose did not differ between the groups. The rats did not experience any side effects from treatment; body weight gain in rats fed lupin tended to be greater than that of controls ($P < 0.10$).

In vitro assays on HepG2 cells. For Hep G2 cells that were grown in the presence of LPDS to have maximal LDL receptor activation,we exposed them to both lupin TPE and isolated Cγ, at concentrations shown in previous studies with soy globulins from our group (12,13) to adequately upregulate LDL receptor activity. Although TPE appeared to be totally ineffective in this model system, Cγ, at the two maximal concentrations tested, stimulated LDL receptor–mediated uptake and degradation by ~53 and 21%, respectively (**Table 4**). At no concentration of either TPE or Cγ was there any evidence of cellular toxicity, as determined by the MTT and LDH assays.

DISCUSSION

The growing interest in diets rich in vegetable proteins has prompted a number of investigations on the potential value of

TABLE 1

Identification and quantification of isoflavonoids in leaves of white lupin plants[1]

Isoflavone	Relative retention time	Negative ions in mass spectra (base peak in bold)	Dry leaves, *μg/g*
2′-Hydroxygenistin (5)	0.478	895 [dimer-H]; 507 [M-H + AcOH]; **447** [M-H]; 285 [M-H-Gluc]	577 ± 62
2′-Hydroxygenistein (2)	0.870	571 [dimer-H]; **285** [M-H];	244 ± 14
6″-*O*-Malonylgenistin (4)	0.751	1035 [dimer-H]; 517 [M-H]; 473 [M-H-CO_2]; **269** [M-H-MalGluc]	540 ± 78
Genistin (3)	0.619	863 [dimer-H]; **491** [M-H + AcOH]; 431 [M-H]; 269 [M-H-Gluc]	870 ± 108
Genistein-4′-glucoside (6)	0.698	863 [dimer-H], 491 [M-H + AcOH]; **431** [M-H]	1373 ± 159
Genistein (1)	1.000	539 [dimer-H]; **269** [M-H]	207 ± 35

[1] Concentrations are means ± SEM, $n = 3$.

such dietary alternatives for a variety of conditions, ranging from hypercholesterolemia and atherosclerosis (4), to prostate and breast cancer prevention (38,39), to cognitive function (40), to osteoporosis prevention (41) and to menopausal symptom treatment (42). These studies have often provided inconsistent results, mainly because of the difficulty in singling out the relative benefits of the proteins in soybean from other components responsible for the therapeutic effect. In particular, the value of the isoflavone/phytoestrogen content of soybeans, considered by a large number of investigators as the key factor in the potential benefits of soybeans in menopause, for example (42), has been disputed by generally equivocal results (43,44). A number of very recent reports from a working group of the UK Committee on Toxicity of Chemicals in Food (COT) expressed general skepticism concerning the health benefits of soy isoflavones, in particular for cholesterol reduction (45).

The role of isoflavones in the mechanism of cholesterol reduction exerted by soy products was scrutinized in particular because most studies on hypercholesterolemic individuals, displaying the most marked cholesterol reductions, were conducted with isoflavone-free products (3,14). In addition, very recent clinical studies in subjects with moderate cholesterol elevations (probably not very responsive to soy intake), indicated that isoflavone-depleted soy products reduced cholesterolemia just as well as intact products (46). More interestingly, administration of soy foods with highly variable isoflavone contents to hypercholesterolemic patients resulted in essentially identical cholesterol reductions (47). In the present study, the presence of isoflavones in lupin proteins was clearly ruled out; data on the lupin protein effects can be analyzed per se because other components that might influence lipid metabolism were not present.

Evaluation of the cholesterol-lowering effect of lupin proteins was carried out with a total protein extract (TPE) due to the lack of availability of adequate amounts of purified fractions for animal studies. In this established animal model with consumption of a casein diet with cholesterol and cholic acid (34), a daily dose of 50 mg TPE/rat, equivalent to 250 mg/kg, a frequently used dosage for lipid-lowering medications, e.g., of the fibrate class (48), reduced total plasma and VLDL-LDL cholesterol, with a somewhat lesser effect on triglycerides over 2 wk, and tended to increase HDL cholesterol. The rats had a normal body growth over the treatment period.

In an effort to investigate the mechanism of the reduction of cholesterolemia, we evaluated the effect of TPE and isolated components on LDL receptor activity in a human hepatoma cell line (Hep G2), using an approach similar to that used in previous studies by our group on soy proteins (15,16). Interestingly, there was no direct effect of the TPE on LDL receptor activity, whereas there was clear evidence of upregulatory activity by the Cγ component (31). Although we showed by 2D-electrophoretic mapping (not shown) that the TPE used in the cell assay did contain Cγ, the amount contained in the TPE maximal dosage was below the effective dose of purified Cγ. These findings will thus require further confirmation, but indicate an area in which to investigate protein components that more directly affect cholesterolemia, i.e., similar to what we reported previously in the case of the α′ globulin from soy (16).

The present findings should, of course, be viewed with caution because the lack of major lupin globulin amino acid sequences prevented direct comparison with the soybean 7S globulin, although sequence homologies between the 7S globulins of lupin and soybean seeds can be expected on the basis

TABLE 2

Isoflavonoid concentrations in seeds of Lupinus albus[1,2]

Isoflavonoid	Dry seeds, *nmol/g*	Dry seeds, *μg/g*
Genistin	8.70 ± 1.36	3.76 ± 0.59
Genistein	6.83 ± 0.39	1.84 ± 0.1
Total	15.53	5.60

[1] Values are means ± SEM, $n = 3$.
[2] Genistin and genistein were undetectable (<0.1 nmol/g) in TPE and conglutin γ.

TABLE 3

Body weights and plasma lipid and glucose concentrations in rats fed diets containing cholesterol and cholic acid and orally administered vehicle or 50 mg total lupin protein extract (TPE) daily for 2 wk[1,2]

	Vehicle	TPE
	mmol/L	
Total cholesterol	5.48 ± 0.17	4.34 ± 0.20*
VLDL + LDL cholesterol	4.90 ± 0.24	3.42 ± 0.17*
HDL cholesterol	0.70 ± 0.04	0.91 ± 0.03
Triglycerides	0.83 ± 0.06	0.70 ± 0.03**
Glucose	5.33 ± 0.24	5.33 ± 0.37
Body weight, *g*		
Initial	232.5 ± 12.6	216.6 ± 8.4
Final	305.7 ± 7.1	298.2 ± 10.0

[1] Values are means ± SEM, $n = 10$ pools of 2 rats each. * $P < 0.001$; ** $P < 0.01$ vs. vehicle.

TABLE 4

LDL receptor modulation in HepG2 cells exposed to total lupin protein extract (TPE) and conglutin γ[1,2]

	Uptake	Degradation
	ng ^{125}I-LDL/mg cell protein	
LPDS	107.8 ± 7.3	106.2 ± 9.0
TPE, *g/L*		
0.125	113.4 ± 8.7	95.8 ± 8.1
0.250	104.2 ± 12.4	88.3 ± 5.0
0.500	95.4 ± 12.6	76.8 ± 4.4
Conglutin γ, *g/L*		
0.125	104.1 ± 10.3	111.9 ± 2.0
0.250	154.5 ± 12.6**†	119.0 ± 6.0*†
0.500	165.1 ± 7.5**†	128.0 ± 1.6*†

[1] Values are means ± SEM of 3 separate experiments. * $P < 0.05$, ** $P < 0.001$ vs. LPDS; † $P < 0.001$ vs. TPE.

[2] Confluent monolayers of Hep G2 cells were preincubated for 24 h at 37°C in MEM with 5% lipoprotein-deficient serum (LPDS), in the presence of TPE or conglutin γ, at the listed concentrations. After the addition of ^{125}I-LDL (7.5 mg/lipoprotein/L of medium), cells were incubated at 37°C for a further 4 h and processed as described in Materials and Methods.

of the general homology in this class of legume proteins (49). Nevertheless, important differences, such as the endogenous extensive proteolytic cleavage of lupin 7S globulin, which gives rise to a number of noncovalently linked polypeptide fragments in the native protein conformation, have been described (50). The cloning and sequencing of 7S and 11S lupin globulin cDNAs are currently in progress in our laboratory. No homology between the Cγ amino acid sequence and the soybean 7S globulin has been found. Thus, the biological activity described in this paper might have a different molecular basis than that of soybean globulin. However, more detailed investigations on the various molecular properties of the lupin proteins in relation to cholesterol metabolism are required. Finally, the lack of adequate amounts of lupin proteins did not allow the evaluation of the effect of a total substitution in the rats' diet, in term of both lipids and other nutritional aspects.

This report clearly indicates that protein from a naturally isoflavone-poor legume can effectively reduce cholesterolemia and, most likely, upregulate LDL receptor activity, a widely accepted mechanism of cholesterol reduction associated with the intake of vegetable proteins (51). The growing interest in this product and its widespread availability clearly suggest its potential therapeutic use in hypercholesterolemia, particularly in individuals in whom soy treatment has significantly reduced cholesterol concentrations, probably associated with reduced cardiovascular risk.

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