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Short Communication

Hypolipidaemic and anti-atherosclerotic effects of lupin proteins in a rabbit model

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The biological activities of a protein isolate from lupin (*Lupinus albus*) were studied in a rabbit model of atherosclerosis. Focal plaque development was induced at both common carotid arteries by perivascular injury. After surgery, animals were fed three different diets for 90 d, all with 1 % cholesterol, 15 % SFA and 20 % protein; the protein source was casein (CAS), lupin proteins (LUP) or 50 % CAS + 50 % LUP (CAS + LUP). Lower cholesterolaemia was detected in the LUP *v.* the CAS group at 60 and 90 d of treatment (−40·3 and −33·5 %, respectively; $P<0·05$). Cryosection analyses of the carotids indicated a significant reduction in focal lesion progression in the LUP *v.* the CAS group (−37·4 %; $P<0·05$). In summary, in a rabbit model of atherosclerosis, a protein isolate from *L. albus* reduced cholesterolaemia and exerted a remarkable protective activity against atherosclerosis progression.

***Lupinus albus*: Protein isolates: Cholesterol: Atherosclerosis: Rabbits**

The growing use of legume proteins in human nutrition for their nutraceutical properties[1–4] has recently suggested detailed investigations on the possible clinical use of lupin proteins. Lupin beans are characterised by a lower content of antinutrients *v.* other legumes[5] and by an almost total absence of phyto-oestrogens[6,7]. This last feature, on the one hand, may avoid potential problems that have been recently indicated for these hormone-like components[8]; on the other, it allows a direct evaluation of the activity of 'proteins', independent of other components. Lupin protein isolates are nutritionally satisfactory[9,10] and have a neutral flavour[11], thus allowing the production of food items with optimal sensory characteristics[12].

A previous study from our group[7] investigated the potential hypolipidaemic effect of a total protein extract from *Lupinus albus*. When given to rats fed a classical cholesterol–cholic acid regimen, lupin proteins significantly reduced both plasma cholesterol and TAG levels *v.* control animals. The cholesterol reduction appeared to be associated with a mechanism shared with soya proteins, i.e. a direct up regulatory activity on LDL receptors[13–15].

Based on recent data by our group[16] and others[17,18] suggesting an anti-atherosclerotic effect of soya proteins, also characterised by a cholesterol-lowering activity[1,13,15], the impact on atherosclerosis progression of a diet containing *L. albus* proteins was tested in a rabbit model of focal lipid-rich soft plaques, generated at the common carotid arteries[19].

Materials and methods

Lupin protein preparation

A total protein isolate from *L. albus* seeds was manufactured by the Fraunhofer Gesellschaft, Fraunhofer-Institute (Freising, Germany), by an extraction and precipitation process followed by spray drying[11]. The protein percentage was 91·21 % DM. A detailed description of the composition and a proteomic investigation of this protein isolate have been previously reported[11,20].

Animals, diets and experimental protocols

Procedures involving animals and their care were conducted in compliance with national and European Union laws and policies.

Abbreviations: CAS, casein; LUP, lupin protein.

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Arterial plaque formation at the common carotid artery was induced in male New Zealand White rabbits by perivascular injury, as described previously[19,21]. Rabbits were divided into three groups of six rabbits, balanced for body weight. After surgery, rabbits were fed a 1 % cholesterol and 15 % SFA diet for 90 d, the protein source (20 % in each diet) being either casein (CAS), 50 % CAS + 50 % total protein isolate from *L. albus* (CAS + LUP) or total protein isolate from *L. albus* (LUP).

Fasting blood samples were taken before and after 30, 60 and 90 d of dietary treatments for plasma total cholesterol, TAG and HDL-cholesterol measurements, by a Roche Diagnostics Cobas autoanalyser (Nutley, NJ, USA). After 90 d diet, animals were anaesthetised with xylazine/ketamine and then killed. Injured arteries were excised, embedded in optimal cutting temperature compound for cryosections under liquid N_2 and stored at −80°C, until analyses. Plaque volume was evaluated by measuring cross-sectional areas of the intima every 0·25 mm within the area of plaque accumulation using an image analysis system (ImageJ 1.37v; National Institutes of Health, Bethesda, MD, USA) interfaced to a Zeiss Axioscope microscope (Carl Zeiss, Oberkochen, Germany). Selected sections were incubated as described[19] with mouse monoclonal antibodies directed against rabbit smooth muscle α-actin (HHF35; DAKO Corp., Glostrup, Denmark) and rabbit macrophages (RAM-11; DAKO Corp.). Sections were also stained with Oil red O to identify lipid accumulation within the plaque[19,21]. Quantification of the percentage of plaque area covered by lipids or macrophages (i.e. with positive staining for Oil red O or for the anti-RAM-11 antibody, respectively) was performed by using a Nikon Coolpix 950 digital camera interfaced with a Zeiss Axioscope microscope (Carl Zeiss), followed by computer-assisted planimetry.

Statistical analyses

Data are expressed as mean values and standard deviations. Group differences were tested for statistical significance by multivariate ANOVA (repeated measures), followed by the Tukey *post hoc* test; a value of $P<0·05$ was considered statistically significant. The statistical analysis was performed using SYSTAT software (version 5.2; Systat Software, Inc., San Jose, CA, USA).

Results

Effect on plasma lipids of a total protein isolate from Lupinus albus *in rabbits*

The presence in each diet of 1 % cholesterol and 15 % coconut fat determined a marked increase in total cholesterol levels in all groups (Table 1). However, whereas in CAS and CAS + LUP animals total cholesterol concentrations increased progressively up to 60 d, reaching values close to 19 000 mg/l that were maintained until killing (90 d), in LUP-fed rabbits cholesterolaemia did not undergo marked variations after 30 d of treatment and reached a maximum of 12 500 mg/l at 90 d. As a consequence of the different responses to the dietary treatments, a significantly lower mean plasma cholesterolaemia was observed in LUP compared with CAS animals after both 60 and 90 d of experimental diet (Table 1). No significant variations were observed for HDL-cholesterol levels (Table 1).

A marked increase in TAG levels was observed after each dietary treatment, with no significant differences among groups (Table 1). However, whereas in the CAS group a progressive rise of TAG levels was observed, in LUP-fed rabbits triacylglycerolaemia significantly increased only after 90 d dietary treatment.

Effect on atherosclerosis development

The diet based on lupin proteins significantly affected atherosclerosis development (Fig. 1); at the end of the dietary treatments, plaque volume was significantly reduced in LUP

Table 1. Lipid levels in rabbits fed diets containing 20 % casein (CAS), 10 % CAS + 10 % isolate from *Lupinus albus* (CAS + LUP) or 20 % isolate from *L. albus* (LUP) as the protein source

(Mean values and standard deviations for six rabbits per group)*

	CAS		CAS + LUP		LUP	
	Mean	SD	Mean	SD	Mean	SD
Total cholesterol (mg/l)						
0 d	532·7[a]	91·3	530·9[a]	91·6	481·3[a]	77·6
30 d	9423·2[b]	3176·9	12 022·9[b]	2432·4	11 644·5[b]	2650·3
60 d	19 791·4[c]	5731·5	18 873·3[c]	3653·8	11 816·2[b]†	621·0
90 d	18 807·5[c]	2592·1	17 818·1[b,c]	4146·6	12 507·6[b]‡	732·8
HDL-cholesterol (mg/l)						
0 d	132·2[a]	24·3	135·1[a]	47·0	144·4[a]	12·7
30 d	490·7[a]	468·2	448·2[a]	309·5	619·4[a]	479·7
60 d	743·4[a]	407·8	622·5[a]	229·0	751·3[a]	465·8
90 d	598·6[a]	234·6	569·2[a]	426·8	599·2[a]	268·2
TAG (mg/l)						
0 d	750·0[a]	192·0	850·0[a]	385·6	675·0[a]	188·8
30 d	1945·0[a,b]	1121·3	3950·0[a]	5758·4	1370·0[a]	745·8
60 d	8547·5[b,c]	4731·4	6830·0[a]	5648·8	3682·5[a,b]	2470·3
90 d	12 055·0[c]	5340·2	7762·5[a]	1533·8	10 147·5[b]	6063·4

[a,b,c] Mean values within a column with unlike superscript letters were significantly different ($P<0·05$; ANOVA).

* For details of diets and procedures, see Materials and methods.

† Mean value was significantly different from that of the CAS-fed animals at 60 d ($P<0·05$).

‡ Mean value was significantly different from that of the CAS-fed animals at 90 d ($P<0·05$).

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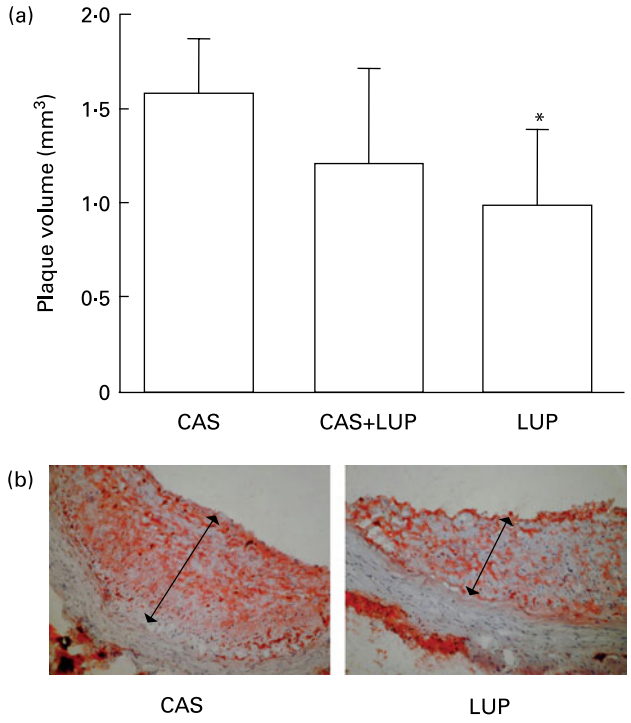

Fig. 1. Histological analysis of rabbit carotid plaques. (a) Carotid plaque volume evaluated in rabbits after perivascular injury followed by 90 d of high-fat, high-cholesterol diet containing 20 % casein (CAS), 10 % CAS + 10 % isolate from *Lupinus albus* (CAS + LUP) or 20 % isolate from *L. albus* (LUP) as the protein source. Data are mean values (six rabbits per group), with standard deviations represented by vertical bars. * Mean value was significantly different from that of the CAS-fed group ($P<0{\cdot}05$). (b) Oil red O staining of carotid sections from rabbits fed high-fat, high-cholesterol diets containing 20 % casein (CAS) or 20 % isolate from *L. albus* (LUP) as protein source (magnification × 50). ↔, Plaque formation.

compared with CAS rabbits ($P<0{\cdot}05$) (Fig. 1 (a)). Rabbits fed CAS + LUP displayed an intermediate plaque size between CAS and LUP animals, but statistical significance *v.* either comparator was not reached.

As previously described in this animal model fed the same diet[16], CAS rabbits developed plaques characterised by a neointimal formation devoid of smooth muscle cells and mostly constituted by macrophages and extracellular lipids. Compared with CAS animals, atherosclerotic plaques of LUP rabbits displayed a reduced macrophage (−23·4 %) and lipid accumulation (−36·2 %), this latter reaching statistical significance ($P<0{\cdot}05$) (Fig. 1 (b)).

Discussion

In the present study, lupin proteins inhibited the cholesterol rise induced by a lipid-rich diet, confirming results previously obtained in rats[7]. No significant differences were instead observed for triacylglycerolaemia among groups. It should be noted, however, that, differently from the CAS group, the increase of TAG levels in the LUP-fed rabbits occurred only at the end of the dietary treatment (90 d). It may be speculated that the long duration of the high-fat, high-cholesterol dietary challenge required for the development of atherosclerotic plaques may have overcome a possible hypotriacylglycerolaemic effect of lupin proteins in this model. Similarly, the aggressive dietary treatment required to induce atherosclerotic plaque formation may have hidden a possible hypolipidaemic effect played by lupin proteins in the CAS + LUP group. The main objective of the present study was, however, the investigation of the impact of lupin proteins on atherosclerosis progression. To investigate this issue, an appropriate animal model was selected, i.e. the rabbit that, differently from the rat, is highly susceptible to atherosclerosis development. In this animal model, perivascular manipulation at the common carotid arteries, followed by a hyperlipidaemic diet, induces the formation of focal plaques, mostly constituted by extracellular lipids and macrophages[19], thus reflecting the main features of the human arterial plaques, defined as unstable, frequently associated with acute ischaemic events[22]. This same rabbit model has proven to be sensitive to local interventions with recombinant apolipoproteins[21], as well as to dietary treatments[16].

The diet exclusively based on *L. albus* proteins clearly reduced atherosclerosis progression, possibly as a consequence of the observed hypocholesterolaemic activity. The reduction of circulating atherogenic lipoproteins by lupin proteins could easily explain the lower lipid accumulation within carotid plaques of the LUP compared with the CAS group. In the CAS + LUP group, where no hypocholesterolaemic effect was observed, plaque volume was not statistically different from that observed in the CAS rabbits. However, the CAS + LUP group displayed a trend toward a lower extent of atheromas compared with the CAS group, suggesting that the anti-atherogenic activity exerted by lupin proteins may be partially explained by other mechanisms, in addition to the observed reduction of cholesterolaemia. This issue will be the object of future investigations.

While there is evidence for a vascular protective effect of soya proteins[16–18], no data are available on lupin proteins, except for those related to the hypolipidaemic activity[7,23]. Differently from soya, a relevant characteristic of lupin is the total absence of isoflavones[6,7], which permits us to conclude that the observed vascular protective properties should certainly be ascribed to the protein component.

When the nutraceutical properties of a food are due to the proteins, it becomes very important to assess their quality and integrity. A proteomic analysis of the protein isolate from *L. albus* used in the present study was recently reported[20]. The results show that the protein isolate had undergone limited damage, demonstrating that the manufacturing process had been very mild, different from the case of commercial soya protein isolates[24].

Interestingly, in a recent study, *L. albus* proteins were given as a beverage to subjects with moderate hypercholesterolaemia in an amount corresponding to a daily intake of approximately 35 g[25]. This dietary supplementation significantly reduced both cholesterolaemia and blood pressure, suggesting that the beneficial effects of lupin proteins observed in animal studies[7,26], including the present study, may occur also in humans. While these preliminary clinical data will require confirmation in appropriate controlled investigations, the results of the present study suggest the potential positive impact of lupin proteins on atherosclerosis prevention and treatment.

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