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ORIGINAL CONTRIBUTION

Mechanisms underlying the beneficial effect of soy protein in improving the metabolic abnormalities in the liver and skeletal muscle of dyslipemic insulin resistant rats

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Abstract
Purpose The present study analyzes the effect of the replacement of dietary casein by soy protein on the mechanisms underlying dyslipidemia, liver steatosis and altered glucose and lipid metabolism in the skeletal muscle which developed in rats fed long-term a sucrose-rich diet (SRD).
Methods Wistar rats were fed a SRD for 4 months. From months 4 to 8, half the animals continued with the SRD, and the other half were fed a SRD in which the source of protein casein was replaced by soy. The control group received a diet with cornstarch as source of carbohydrate.
Results Compared to SRD-fed animals, the rats fed soy showed: A—in the liver: reduction of triglyceride and cholesterol storage and decreased steatosis; normalization of mature forms of the protein mass levels of SREBP-1 and the activities of lipogenic enzymes, while the protein mass level of PPAR-α and fatty acid oxidase activity increased. B—in the gastrocnemius muscle: normalization of the enhanced lipid storage and the altered glucose oxidation, improving glucose phosphorylation; decreasing protein mass level of nPKCθ in the membrane fraction; reversion of the impaired insulin-stimulated glucose transporter Glut-4, and glucose-6-phosphate and glycogen concentrations. Besides, dyslipidemia and glucose homeostasis returned to control values.
Conclusions This study provides new information concerning some key mechanisms related to the effect of dietary soy on hepatic lipid metabolism and insulin action in the skeletal muscle in the presence of pre-existing dyslipidemia and insulin resistance induced by a SRD.

Keywords Soy protein · Liver · Skeletal muscle · Dyslipidemia · Insulin resistance

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Introduction

Dysfunctional lipid metabolism is a key component in the development of the metabolic syndrome, a very frequent condition characterized by dyslipidemia, insulin resistance, abdominal adiposity and hypertension which are related to an elevated risk for type 2 diabetes mellitus [1]. This syndrome is physiologically related to both genetic factors and food intake habits, especially the consumption of a high-calorie, high-fat and high-carbohydrate diet.

Dietary interventions may induce changes in the prevention or improvement of many metabolic disorders included in this syndrome by modulating the expression of important genes involved in these chronic manifestations. It has been reported that the ingestion of vegetable protein instead of animal protein lowers the risk of coronary diseases. In this regard, several studies in human and experimental animals have demonstrated the cholesterol-lowering anti-lipogenic and anti-hypertensive effects of soy protein [2–4]. Moreover, additional health benefits have been suggested to include anti-diabetic effects: reduced weight gain and improved body composition, glucose homeostasis and insulin sensitivity [5–7]. In

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addition to its macronutrient content, soy protein contains bioactive components that directly or indirectly interact with hepatic genes to modulate serum lipids levels [6–8]. It has been reported that dietary soy protein in rodents had a hypotriglyceridemic effect in liver, mediated by sterol regulatory element-binding protein-1 (SREBP-1) dependent lipogenesis [9]. In addition, in hyperinsulinemic obese Zucker fa/fa rats, Tovar et al. [10] showed that soy protein compared to casein increased mRNA levels of peroxisome proliferator-activated receptor-α (PPAR-α). Thus, alterations of hepatic lipogenesis and fatty acid oxidation may be responsible for soy protein dependent decreases in lipoprotein production. Studies in a rodent model have also demonstrated the effect of soy protein isolates to reduce insulin resistance [9]. Besides, in healthy and diabetic animals, soybean peptides decreased blood glucose by increasing insulin sensitivity and improving glucose tolerance [11–13].

Skeletal muscle accounts for 75 % of whole body insulin-stimulated glucose uptake; therefore, this tissue plays an important role in maintaining whole body glucose homeostasis. Dietary proteins are important modulators of insulin signaling and action in rat skeletal muscle. Lu et al. [14] showed that the administration of the soy peptide aglycin during 4 weeks can attenuate or prevent hyperglycemia by increasing the insulin receptor signaling pathway in the skeletal muscle of streptozotocin/high-fat diet-induced diabetic BALB/C mice.

On the other hand, male Wistar rats have been used as an experimental model of insulin resistance, dyslipidemia, altered glucose homeostasis and visceral adiposity because of their susceptibility to these diseases in response to being chronically fed a high sucrose/fructose diet [15, 16]. At present, only few studies have investigated the capacity of soy protein to prevent, improve or reverse dyslipidemia and insulin resistance using this animal model. In this regard, Lavigne et al. [17] showed that Wistar rats fed during 4 weeks a sucrose-rich diet (SRD), in which the source of protein was isolate soy protein, improved fasting glucose levels, glucose tolerance test and whole body insulin action on glucose disposal compared with the isoenergetic amount of casein. We have recently demonstrated that substituting dietary casein by isolated soy protein in the SRD during the last 4 of the 8-month feeding period leads to reverse the pre-existing state of dyslipidemia by decreased hepatic VLDL-Tg secretion and increased plasma triglyceride removal. Besides, soy protein ameliorates the altered glucose homeostasis and the impaired whole body peripheral insulin sensitivity. In addition, soy protein was able to normalize the enhanced body weight and reverse the increased liver and skeletal muscle triglyceride storage [18].

In order to shed new light on this topic by further exploring the mechanism/s underlying the beneficial effect of dietary soy protein on liver lipid metabolism and impaired insulin action in the skeletal muscle of dyslipemic insulin resistant rats fed a SRD, the aim of this study was twofold: (i)—to analyze in the liver the protein mass level of transcription factor SREBP-1 and nuclear receptor PPAR-α and their target enzymes activities involved in "de novo lipogenesis" and "fatty acid oxidation". These collectively could affect the fate of fatty acid metabolism and contribute to improved/reversed liver steatosis and dyslipidemia; (ii)—to study in the skeletal muscle several biochemical and metabolic parameters involved in the glucose metabolism at the basal state and under insulin stimulation, and the protein mass levels of nPKCθ. This study was conducted in rats fed a SRD for 8 months, in which the metabolic abnormalities mentioned above were pre-existing before the source of dietary protein (casein) was replaced by an isocaloric amount of isolated soy for the last 4 months of the experimental period in half the animals.

Materials and methods

Animals and diets

Male Wistar rats initially weighing 180–190 g were maintained with unrestricted access to water and food under controlled temperature (22 ± 1 °C), humidity and air flow conditions, with a fixed 12 h light–dark cycle (light 07.00–19.00 h). They were initially fed a standard nonpurified diet (Ralston, Purina, St Louis, MO). After one week of acclimatization period, the rats were randomly divided into two groups (control and experimental) and were housed individually. The experimental group received a purified sucrose-rich diet (SRD) containing by weight 62.5 (g/100 g) sucrose, and the control group received the same purified diet but with sucrose replaced by cornstarch [control diet (CD)]. Details on the composition of the diets are given in Table 1. Both groups received each diet for 4 months, after which the SRD group of rats was randomly subdivided into two subgroups. The rats in the first subgroup continued on the SRD up to 8 months of feeding. The second subgroup (SRD-S) received the SRD in which the source of protein casein was replaced by soy protein isolate (MP Biomedicals, Solon, OH, USA) for the next 4 months. The composition of the soy protein isolate is given in Table 2. The control group received the control diet throughout the experimental period (Table 1). All diets provided approximately 16.30 kJ/g of food and were available ad libitum. Diets were prepared every week [18]. The weight of each animal and the energy intake were recorded twice per week during the experimental period in all groups and subgroups of rats. At the end of the experimental period, food was removed at 07.00 h, and unless

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Table 1 Composition of the experimental diets (based on the AIN-93 diet)

Diet ingredients	CD		SRD		SRD-S[a]	
	g/100 g	% Energy	g/100 g	% Energy	g/100 g	% Energy
Cornstarch	62.5	65	–	–	–	–
Sucrose	–	–	62.5	65	62.5	65
Casein-free vitamin	18	19	18	19	–	–
Soy protein	–	–	–	–	18	19
Corn oil	7	16	7	16	7	16
Vitamin mix[b]	1		1		1	
Cellulose	7.5		7.5		7.5	
Salt mix[c]	3.5		3.5		3.5	
Choline bitartrate	0.2		0.2		0.2	
DL- Methionine	0.3		0.3		0.3	

CD control diet, *SRD* sucrose-rich diet, *SRD-S* SRD with soy protein

[a] Soy protein isolated (MP Biomedicals, Solon, OH, USA)

[b] AIN-93 VX

[c] AIN-93M-MX

Table 2 Composition of the soy protein isolate

Composition	Soy protein isolate (g/100 g)
Protein	92
Water	6.0
Ash	4.1
Fat	0.8
Fiber	0.25
Carbohydrate	2.85
Isoflavones	0.0175
Calcium	0.15
Phosphor	0.8
Potassium	0.05
Sodium	1.3
Trypsin inhibitor (mg/g)	4.0–7.3

Soy protein isolate was purchased from MP Biomedicals, Solon, OH, USA

otherwise indicated experiments were performed between 07.00 and 09.00 h.

Rats from the three dietary groups were anesthetized with intraperitoneal sodium pentobarbital (60 mg/kg body weight). Blood samples were obtained from the jugular vein and rapidly centrifuged. Plasma was either immediately assayed or stored at −20 °C. The liver and gastrocnemius muscle were totally removed and immediately frozen and stored at the temperature of N_2 liquid. The experimental protocol was approved by the Human and Animal Research Committee of the School of Biochemistry, University of Litoral, Santa Fe, Argentina.

Analytical methods

Plasma triglyceride, free fatty acids, total cholesterol and glucose levels were determined by spectrophotometric methods and insulin by immunoreactive assays as previously described [18–20]. The insulin assay was calibrated against rat insulin standard (Novo Nordisk, Copenhagen. Denmark).

Enzymatic activity assays in liver tissue

Acetyl CoA carboxylase was assayed according to Zimmermann et al. [21]. Briefly, the cytosolic fraction was obtained after centrifugation of the supernatant at 30,000 rpm for 1 h at 4 °C, and Acetyl CoA carboxylase was measured by an NADH-linked assay. Fatty acid synthase activity was assayed on the cytosolic fraction of liver by measuring malonyl-CoA-dependent NADPH oxidation at 37 °C as described recently [22]. Malic enzyme was measured according to Hsu et al. [23], Liver tissue glucose-6 phosphate dehydrogenase activity was measured according to Cohen et al. [24] as described recently [22]. Peroxisomal fatty acid oxidase was measured in liver by the procedure reported by Vamecq [25] with the addition of 0.1 g/L Brij 58 to the reaction mixture as described by Hein et al. [26]. Carnitine palmitoyltransferase-1 was measured spectrophotometrically using the method described by Karlic et al. [27].

Western blot analysis of liver protein mass levels of PPAR-α and sterol regulatory element-binding protein-1 (SREBP-1)

Liver homogenates were prepared for PPAR-α protein mass level analysis, and Western blots were run under reduced–denatured protein (Laemmli buffer) as described by Hein et al. [26]. Nuclear and microsomal membrane extracts of SREBP-1 from the liver of rats were assayed according to described by Rossi et al. [22]. The protein concentration of the extracts was determined by the Bradford assay. Protein samples were resolved on SDS–PAGE according to Laemmli and were transferred to polyvinyldifluoride (PVDF) membranes and were probed with specific antibodies (rabbit anti-PPAR-α antibody and rabbit anti-SREBP-1 antibody; Santa Cruz Biotechnology, Inc. CA, USA), respectively. The blots were then incubated with horseradish peroxidase-linked secondary antibody

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219 followed by chemiluminescence detection according to the
220 manufacturer's instructions (Super Signal West Pico
221 chemiluminescence detection; Pierce Biotechnology,
222 Rackford, IL). β-actin was used as a loading control. The
223 intensity of bands was quantified by NIH imaging software
224 (http://rsb.info.nih.gov/nih-image/). The relationship
225 between the amount of samples subjected to immunoblot-
226 ting and the signal intensity observed was linear under the
227 aforementioned conditions. The liver SREBP-1 analysis
228 showed one electrophoretic band with an approximate
229 molecular weight of 125 kDa and another band of 68 kDa;
230 these bands represent the precursor and mature forms of
231 SREBP-1, respectively [28].

232 Gastrocnemius muscle assays

233 Triglyceride, long-chain acyl CoA, diacylglycerol, glyco-
234 gen and glucose-6-phosphate content as well as the activ-
235 ities of hexokinase and pyruvate dehydrogenase complex
236 (PDHc) were analyzed in muscle homogenate at the
237 beginning of the clamp studies, as recently described by
238 D'Alessandro et al. [29–31]. The protein mass level of
239 protein kinase C theta (nPKCθ) in the cytosol and the
240 membrane fraction of the gastrocnemius muscle were
241 measured as described by D'Alessandro et al. [29, 30].
242 Briefly, total protein samples were resolved on SDS-
243 PAGE, transferred to PVDF membranes and probed with
244 specific antibody (polyclonal anti–rabbit anti-specific
245 nPKCθ from Santa Cruz, Biotechnology, Inc, Santa Cruz,
246 CA, USA). The blot was incubated with horseradish per-
247 oxidase-linked secondary antibody followed by chemilu-
248 minescence, and the intensities of the bands were
249 quantified using the NIH imaging software as described
250 above. β-actin was used as a loading control.

251 Glycogen, glucose-6-phosphate content and Glut-4
252 protein mass levels in the gastrocnemius muscle (clamp
253 studies)

254 Whole body peripheral insulin sensitivity was measured
255 using the euglycemic-hyperinsulinemic clamp technique as
256 previously described elsewhere [31]. Briefly, after 5 h of
257 food deprivation, twelve rats from each dietary group were
258 anesthetized, a blood sample was withdrawn, and glucose
259 and insulin levels were assessed. The gastrocnemius mus-
260 cle of six rats from each group was rapidly removed
261 (starting clamp values), clamped in liquid N_2 and stored at
262 −80 °C. In the other six rats from each dietary group, an
263 infusion of highly purified porcine neutral insulin (Act-
264 rapid, Novo Nordisk, Bagsvard, Denmark) was adminis-
265 tered at 0.8 units/(kg × h) for 2 h. Glycemia was
266 maintained at a euglycemic level by infusing glucose at a
267 variable rate. The glucose infusion rate during the second
268 hour of the clamp study was taken as the net steady state of
269 the whole body glucose. At the end of the clamp period, the
270 gastrocnemius muscle was rapidly removed. The protein
271 mass levels of Glut-4, glycogen and glucose-6-phosphate
272 concentrations were determined at the beginning and at the
273 end of the clamp. The assay of the protein mass level of
274 Glut-4 was recently described by D'Alessandro et al. [29,
275 30]. Briefly, total protein samples were resolved on SDS-
276 PAGE, transferred to PVDF membranes and probed with
277 specific antibody (polyclonal goat anti-Glut-4 from Santa
278 Cruz Biotechnology, Inc, Santa Cruz, CA, USA). The blot
279 was incubated with horseradish peroxidase-linked second-
280 ary antibody followed by chemiluminesence, and the
281 intensities of the bands were quantified as described above.
282 β-actin was used as a loading control. Glycogen and glu-
283 cose-6-phosphate contents were measured as recently
284 described by D'Alessandro et al. [29, 30].

285 Statistical analysis

286 Sample sizes were calculated on the basis of measurements
287 previously made with rats fed either a control diet or a SRD
288 [16, 22, 29] considering an 80 % power [32]. Results were
289 expressed as mean values with their standard errors. Sta-
290 tistical comparisons were done transversely between dif-
291 ferent dietary groups. The statistical significance between
292 groups was determined by one-way ANOVA, with one
293 factor (diet) followed by the inspection of all differences
294 between pairs of means by the Newman Keuls' test [33].
295 Differences having *P* values lower than 0.05 were con-
296 sidered to be statistically significant (SPSS 15.0 for Win-
297 dows, SPSS INC, Chicago, Illinois). All reported *P* values
298 are two-sided.

299 Results

300 Body weight gain and energy intake

301 Body weight and energy intake were carefully monitored in
302 all groups of rats throughout the experimental period. As
303 shown in an early publication of our group [34] and con-
304 firmed in the present work, increases in body weight gain
305 and energy intake were comparable in rats fed the CD or
306 SRD during the first 4 months of the feeding period of their
307 respective diets (Fig. 1). However, a significant increase in
308 both parameters was observed in the rats that continued
309 with the SRD up to 8 months. Besides, in agreement with
310 previous results, when the source of dietary casein in the
311 SRD was replaced by soy for the last 4 months on diet
312 (SRD-S), both energy intake and weight gain remained
313 similar to those observed in the control diet fed rats
314 [18, 34].

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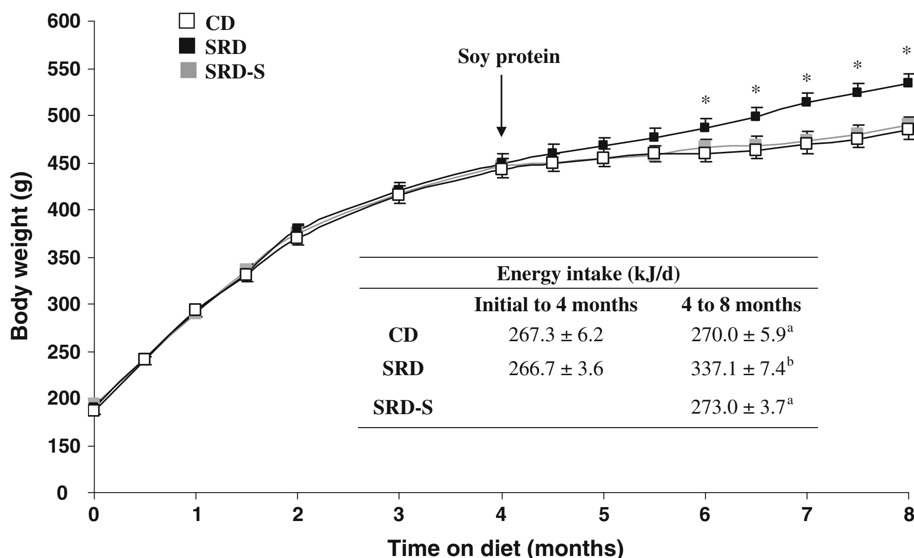

Fig. 1 Body weight and energy intake in rats fed a control diet (CD *square*), sucrose-rich diet (SRD *black square*) or SRD with soy protein (SRD-S *gray square*). Values are expressed as mean ± SEM (8 animals per group) *$P < 0.05$ SRD versus CD and SRD-S at each time point. In the table, values in a column that do not share the same superscript letter differ ($P < 0.05$) when one variable at a time was compared by the Newman Keuls' test. For more details, see animals and diets in section "Materials and methods"

Table 3 Plasma metabolites and insulin levels in rats fed a control diet (CD), sucrose-rich diet (SRD) or the SRD with soy protein (SRD-S)

Diet	Time-on diet (months)	Triglyceride (mM)	Cholesterol (mM)	Free fatty acids (μM)	Glucose (mM)	Insulin (pM)
CD	4	0.61 ± 0.05^{a}	2.04 ± 0.10^{a}	325.0 ± 20.2^{a}	6.3 ± 0.1^{a}	370.0 ± 26.0
CD	8	0.66 ± 0.04^{a}	2.15 ± 0.08^{a}	315.0 ± 10.5^{a}	6.2 ± 0.2^{a}	375.0 ± 30.3
SRD	4	1.60 ± 0.09^{b}	3.22 ± 0.08^{b}	705.0 ± 46.0^{b}	8.1 ± 0.3^{b}	360.0 ± 28.0
SRD	8	1.70 ± 0.12^{b}	3.18 ± 0.06^{b}	734.0 ± 32.8^{b}	8.2 ± 0.2^{b}	369.0 ± 33.0
SRD+	4+					
SRD-S	4	0.69 ± 0.05^{a}	2.05 ± 0.05^{a}	323.0 ± 34.0^{a}	6.7 ± 0.3^{a}	370.0 ± 27.0

Values are expressed as mean ± SEM, $n = 6$. Values in a column that do not share the same superscript letter are significantly different ($P < 0.05$) when one variable at a time was compared by the Newman Keuls' test

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315 Plasma metabolites and insulin levels

316 Both at 4 months and at the end of the experimental period
317 (8 months), plasma triglyceride, cholesterol, free fatty acid
318 and glucose levels were significantly higher ($P < 0.05$) in
319 the SRD-fed rats compared to control rats fed a cornstarch
320 diet (Table 3). However, when soy protein replaced casein
321 for the last 4 months of the diet, a return of all the above
322 parameters was observed reaching values similar to those
323 recorded in the control group. No statistically significant
324 differences in plasma insulin levels were observed either at
325 4 months or at the end of the experimental period between
326 all dietary groups (Table 3).

327 Liver triglyceride, cholesterol and lipogenic enzyme
328 activities

329 Table 4 shows how the significant increases of liver tri-
330 glyceride storage and cholesterol concentrations recorded
331 in rats fed a SRD during 8 months were completely

Table 4 Triglyceride and cholesterol contents and acetyl CoA carboxylase, fatty acid synthase, glucose-6-phosphate dehydrogenase and malic enzyme activities in the liver of rats fed a control diet (CD), sucrose-rich diet (SRD) or the SRD with soy protein (SRD-S)

	CD	SRD	SRD-S
Metabolites (μmol/g wet tissue)			
Triglyceride	11.80 ± 0.53^{a}	20.40 ± 0.90^{b}	10.45 ± 0.70^{a}
Cholesterol	3.42 ± 0.10^{a}	4.88 ± 0.16^{b}	3.48 ± 0.15^{a}
Lipogenic enzyme activities (pKtal/mg protein)			
Acetyl CoA carboxylase	728.4 ± 36.6^{a}	$1,536.6 \pm 31.6^{b}$	863.1 ± 38.2^{c}
Fatty acid synthase	118.1 ± 3.3^{a}	301.0 ± 14.9^{b}	134.7 ± 4.9^{a}
Glucose-6-phosphate dehydrogenase	375.8 ± 29.9^{a}	606.9 ± 23.3^{b}	429.0 ± 9.9^{a}
Malic enzyme	114.7 ± 4.9^{a}	211.2 ± 9.9^{b}	181.3 ± 6.6^{c}

Values are expressed as mean ± SEM, $n = 6$. Values in a line that do not share the same superscript letter are significantly different ($P < 0.05$) when one variable at a time was compared by the Newman Keuls' test

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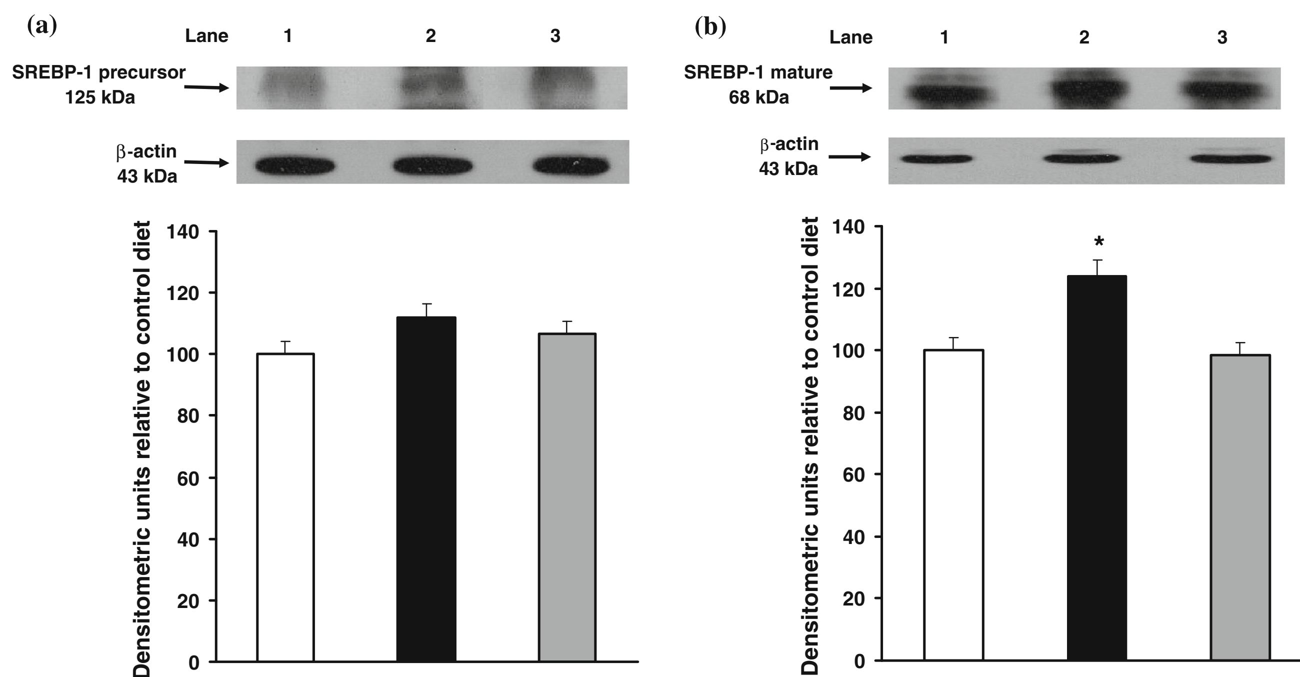

Fig. 2 Liver protein mass levels of **a** the precursor and **b** the mature forms of sterol regulatory element-binding protein-1 (SREBP-1) of rats fed a control diet (CD *square*), a sucrose-rich diet (SRD *black square*) or a SRD with soy protein (SRD-S *gray square*). *Top* a representative immunoblot of the liver precursor and mature form of SREBP-1 from the CD, SRD and SRD-S fed rats. Molecular marker is shown on the right. *Lane 1* CD; *lane 2* SRD; *lane 3* SRD-S. *Bottom* Densitometric immunoblot analysis of the precursor form of SREBP-1 protein mass levels in the liver tissue of rats fed a CD, SRD or SRD-S, and densitometric immunoblot analysis of the mature form of SREBP-1 protein mass levels in the liver tissue of rats fed a CD, SRD or SRD-S. Values are expressed as mean ± SEM (six animals per group), with their standard errors represented by *vertical bars*, and expressed as a percentage relative to each CD, respectively. *Mean values were significantly different from those of the CD and SRD-S groups ($P < 0.05$)

normalized in the group of rats fed SRD-S. Furthermore, the activities of the key enzymes involved in de novo lipogenesis, which reached an almost twofold increase in the liver of the SRD-fed groups, were significantly improved, reaching values similar to those recorded in the CD-fed group, after soy protein administration.

Liver SREBP-1 protein mass levels in the membrane fraction (precursor form) and nuclear extracts (mature form)

Figure 2 shows the Western blot analysis of the precursor and mature forms of SREBP-1 protein mass levels in the liver of CD, SRD and SRD-S-fed rats. The antibody of SREBP-1 reacted with both SREBP-1a and SREBP-1c forms; therefore, we could not distinguish these two forms and thus used the noncommittal term SREBP-1 (in the mouse liver, the ratio of SREBP-1c:1a transcriptors was 9:1) [28]. The 125 and 68 kDa proteins observed represent the precursor and mature forms of SREBP-1. Each gel contained an equal number of samples from the CD, SRD and SRD-S groups (Fig. 2a, b). After the densitometry of immunoblots, the precursor and mature forms of SREBP-1 of the CD group were normalized to 100 % and both SRD and SRD-S were expressed relative to this. The qualitative and quantitative analyses of Western blots showed no changes in the relative abundance of the precursor form of SREBP-1 in the three dietary groups (Fig. 2a). As previously shown, the relative abundance of the mature form of the SREBP-1 protein was significantly increased ($P < 0.05$) in SRD-fed rats (Fig. 2b). The present results show that when casein was replaced by soy protein in the SRD group, the relative abundance of the mature form of SREBP-1 protein mass levels returned to values similar to those observed in the CD group (Fig. 2b). The ratios of mature: precursor relative protein levels of SREBP-1 (six animals per group) were 1.2 ± 0.05 in the SRD versus 0.95 ± 0.04 in SRD-S ($P < 0.05$).

Liver oxidative enzyme activities and PPAR-α protein mass levels

The table inserted in Fig. 3 depicts the hepatic activities of key enzymes involved in mitochondrial (carnitine palmitoyltransferase-1) and peroxisomal (fatty acid oxidase) fatty acid oxidation in the three dietary groups. Soy protein was able to reverse the decreased activity of carnitine palmitoyltransferase-1 observed in rats fed the SRD, which

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achieved values similar to the CD-fed group. Compared with the CD-fed group, no changes in peroxisomal fatty acid oxidase activity were observed in rats fed the SRD. However, the activity of this enzyme was almost 50 % increased in the SRD-S group, reaching values above those observed in age-matched control fed rats.

From the above results, we examined the protein mass levels of the nuclear receptor PPAR-α. The immunoblotting of liver tissue revealed a single 55 kDa band consistent with PPAR-α. Each gel contained an equal number of samples from the CD, SRD and SRD-S groups (Fig. 3 Top panel). After the densitometry of immunoblots, the PPAR-α of the CD group was normalized to 100 %, and both SRD and SRD-S were expressed relative to this. The qualitative and quantitative abundances of the Western blot showed that the relative abundance of PPAR-α protein mass levels significantly decreased ($P < 0.05$) in the liver of the SRD group when compared with rats fed the CD (Fig. 3 Bottom panel). The replacement of casein by soy protein was able to significantly increase the PPAR-α protein mass levels, reaching values above those of the CD group ($P < 0.05$ SRD-S vs. CD).

	CD	SRD	SRD-S
Oxidative enzyme activities (pKat/mg protein)			
Carnitine palmitoyltransferase-1	19.12 ± 1.83[a]	11.97 ± 0.66[b]	23.78 ± 2.33[a]
Fatty acid oxidase	35.42 ± 1.66[a]	33.26 ± 2.66[a]	49.56 ± 5.15[b]

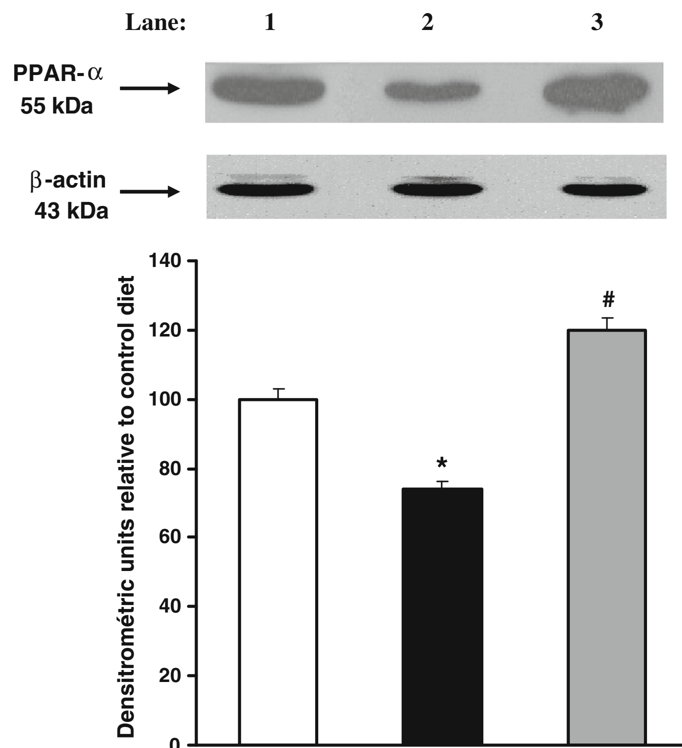

Fig. 3 Liver protein mass levels of PPAR-α of rats fed a control diet (CD *square*), a sucrose-rich diet (SRD *black square*) or a SRD with soy protein (SRD-S *gray square*). In the table values are expressed as mean ± SEM (six animals per group). Values in a line that do not share the same superscript letter are significantly different ($P < 0.05$) when one variable at a time was compared by the Newman Keuls' test. *Top* a representative immunoblot of liver PPAR-α from the CD, SRD and SRD-S-fed rats. Molecular marker is shown on the right. *Lane 1* CD; *lane 2* SRD; *lane 3* SRD-S. *Bottom* Densitometric immunoblot analysis of PPAR-α protein mass levels in the liver tissue of rats fed a CD, SRD or SRD-S. Values are expressed as mean ± SEM (six animals per group), with their standard errors represented by *vertical bars*, and expressed as a percentage relative to the CD. *Mean values were significantly different from those of the CD and SRD-S groups ($P < 0.05$). #Mean values were significantly different from those of the CD group ($P < 0.05$)

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398 Gastrocnemius muscle metabolite concentration,
399 enzyme activities and nPKCθ protein mass levels

400 Confirming previous results [29, 30], at the beginning of 401 the clamp study, the gastrocnemious muscle of SRD-fed 402 rats showed a significant increase of triglyceride, long-403 chain acyl CoA and diacylglycerol contents without 404 changes in glycogen and glucose-6-phosphate levels com-405 pared to that of CD-fed rats (Table 5). The present study 406 shows that dietary soy protein was able to decrease tri-407 glyceride storage and long-chain acyl CoA concentration 408 which reached values similar to those recorded in the CD-409 fed group. Besides, a significant reduction of diacylglyc-410 erol concentration was observed in the skeletal muscle of 411 the SRD-S-fed group. The administration of dietary soy 412 protein did not produce any changes in the glycogen and 413 glucose-6-phosphate levels. Moreover, the significant 414 reduction of both glucose phosphorylation and oxidation 415 estimated by hexokinase and pyruvate dehydrogenase 416 activities were improved or normalized when dietary soy 417 protein replaced casein in the SRD-fed group (Table 5). 418 Besides, the relative abundance of the nPKCθ isozyme in 419 the membrane fraction of the gastrocnemius muscle, which 420 was significantly increased ($P < 0.05$) in the SRD-fed rats, 421 returned to values similar to those recorded in the CD-fed 422 group in the SRD-S. No significant changes in the nPKCθ 423 protein mass levels in the cytosol fraction were observed 424 between the three dietary groups (Table 5).

425 Protein mass levels of Glut-4 in the gastrocnemius
426 muscle at the beginning and end of the clamp studies

427 In Fig. 4, the immunoblotting of the gastrocnemius muscle 428 revealed a single 45 kDa band consistent with Glut-4. Each 429 gel contained an equal number of samples from rats fed a 430 CD, SRD and SRD-S at the beginning (0 min) and at the 431 end (120 min) of the euglycemic-hyperinsulinemic clamp. 432 After the densitometry of immunoblots, the Glut-4 of the 433 CD group at the beginning of the clamp was normalized to 434 100 % and both SRD and SRD-S at the beginning, and also 435 the three dietary groups at the end of the study, were 436 expressed relative to this. At the beginning of the clamp, 437 the qualitative and quantitative analyses of the Western 438 blot showed no differences in the relative abundance of the 439 total plasma membrane of Glut-4 protein between all die-440 tary groups. As expected, under insulin stimulation the 441 translocation of Glut-4 to the plasma membrane signifi-442 cantly increased in CD-fed rats, while the increase of 443 plasma membrane Glut-4 was lower in the SRD-fed group 444 under the same experimental conditions. By shifting the 445 source of protein in the diet to soy protein in the SRD-fed 446 group, the Glut-4 protein mass significantly increased, 447 although values are still lower than those recorded in the

Table 5 Metabolites, enzyme activities and protein mass levels of nPKCθ in gastrocnemius muscle of rats fed a control diet (CD), a sucrose-rich diet (SRD) or a SRD with soy protein (SRD-S)

	CD	SRD	SRD-S
Metabolites			
Triglyceride (μmol/g wet tissue)	3.6 ± 0.1^a	7.1 ± 0.3^b	4.0 ± 0.4^a
Long-chain acyl CoA (μmol/g wet tissue)	5.8 ± 0.5^a	11.9 ± 0.8^b	6.3 ± 0.6^a
Diacylglycerol (nmol/g wet tissue)	108.3 ± 13^a	203.3 ± 20^b	150.8 ± 10.9^c
Glycogen (mg/g wet tissue)	4.5 ± 0.2	4.6 ± 0.1	5.3 ± 1.4
Glucose-6-Phosphate (μmol/g wet tissue)	0.43 ± 0.02	0.42 ± 0.03	0.48 ± 0.02
Enzyme activities			
Hexokinase (pkat/mg protein)	750.0 ± 38.0^a	541.8 ± 31.3^b	653.4 ± 14.0^c
PDHa[a] (% of total PDHc)	35.9 ± 0.7^a	20.2 ± 1.6^b	37.3 ± 1.0^a
Protein mass levels (% of control diet)			
nPKCθ membrane fraction	100.0 ± 3.0^a	168.0 ± 9.8^b	121 ± 6.2^c
nPKCθ citosol fraction	100.0 ± 2.5	89.0 ± 8.1	91.2 ± 10.0

Values are expressed as mean ± SEM, $n = 6$ Values in each line that do not share the same superscript letter are significantly different ($P < 0.05$) when one variable at a time was compared by the Newman Keuls' test

[a] PDHa: active form of PDHcomplex, expressed as percentage of total PDHc activity (PDHa: basal activity × 100/total activity)

CD-fed group. This was accompanied by a significant 448 improvement of the glucose infusion rate (GIR) although 449 values in the rats fed a SRD-S were lower than the rats fed 450 a CD. Furthermore, under the stimulus of the hormone the 451 SRD-S group showed an increase of glucose-6-phosphate 452 and glycogen concentration, which reached values similar 453 to those obtained in the CD-fed group (Table inserted in 454 Fig. 4). 455

Discussion 456

The present study focuses on the effect of dietary soy 457 protein upon the mechanism/s involved in the reversion or 458 improvement of dyslipidemia, liver steatosis and the 459 impaired skeletal muscle lipid and glucose metabolism, 460

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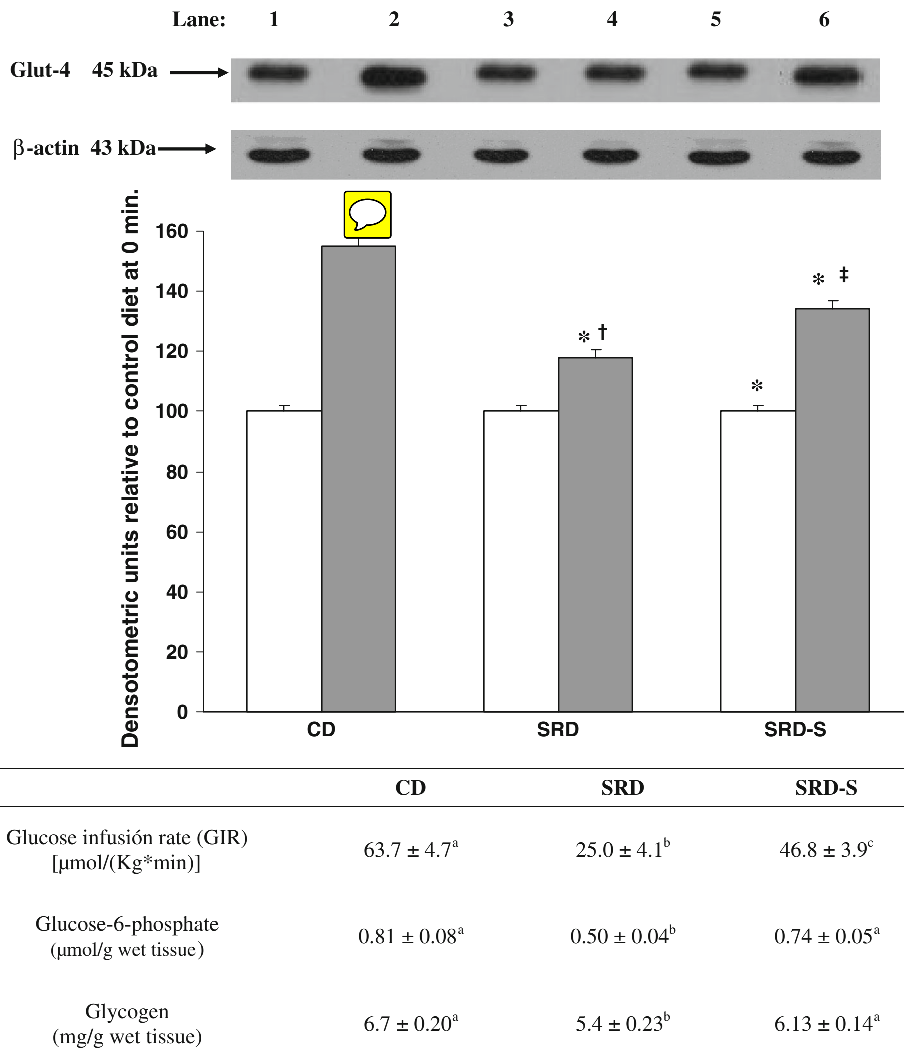

	CD	SRD	SRD-S
Glucose infusión rate (GIR) [μmol/(Kg*min)]	63.7 ± 4.7[a]	25.0 ± 4.1[b]	46.8 ± 3.9[c]
Glucose-6-phosphate (μmol/g wet tissue)	0.81 ± 0.08[a]	0.50 ± 0.04[b]	0.74 ± 0.05[a]
Glycogen (mg/g wet tissue)	6.7 ± 0.20[a]	5.4 ± 0.23[b]	6.13 ± 0.14[a]

Fig. 4 Skeletal muscle protein mass level of Glut-4 at the beginning (*square* 0 min) and under the insulin stimulation at the end (*gray square* 120 min) of the clamp studies in rats fed a control diet (CD), a sucrose-rich diet (SRD) or the SRD with soy protein (SRD-S). *Top* immunoblot of Glut-4 of skeletal muscle from the CD, SRD and SRD-S. Molecular marker is shown on the right. *Lane 1* CD 0 min, *lane 2* CD 120 min; *lane 3* SRD 0 min; *lane 4* SRD 120 min; *lane 5* SRD-S 0 min; *lane 6* SRD-S 120 min. *Bottom* Densitometric immunoblot analysis of Glut-4 protein mass in the skeletal muscle of rats fed a CD, SRD or SRD-S at the beginning and at the end of clamp studies. Values are expressed as mean ± SEM (six animals per group) with their standard errors represented by *vertical bars* and expressed as percentage relative to the control diet at 0 min of the clamp. *Mean values were significantly different from those of the CD, SRD and SRD-S rats at 120 min of the clamp versus CD, SRD and SRD-S rats at 0 min of the clamp $P < 0.05$. †Mean values were significantly different from those of the SRD-rats at 120 min of the clamp versus CD and SRD-S rats at 120 min of the clamp $P < 0.05$. ‡Mean values were significantly different from those of the SRD-S rats at 120 min of the clamp versus CD at 120 min of the clamp $P < 0.05$. In the table, values are expressed as mean ± SEM (six animals per group). Values in a line that do not share the same superscript letter are significantly different ($P < 0.05$) when one variable at a time was compared by the Newman Keuls' test

which developed in rats chronically fed a SRD [22, 26, 29]. Expanding our previous findings, the new main results of this work are the following: In the liver of SRD-fed rats, the administration of dietary soy protein (i) reduced the increased triglyceride and cholesterol storage and decreased liver steatosis, (ii) decreased both the enhanced protein mass level of the mature form of SREBP-1 and the activities of key enzymes involved in "de novo" lipogenesis, (iii) increased the protein mass level of PPAR-α and the enzymatic activity of fatty acid oxidase. Both parameters reached values higher than those recorded in the CD-fed rats. In the gastrocnemious muscle, soy protein normalized the enhanced lipid storage and the altered glucose oxidation and improved the glucose phosphorylation observed in the SRD-fed rats. Moreover, soy protein was able to significantly decrease the protein mass level of

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nPKCθ in the membrane fraction of the skeletal muscle of rats fed a SRD, reaching values similar to those of the CD-fed rats. In addition, soy protein reversed the impaired insulin-stimulated glucose transporter Glut-4, glucose-6-phosphate and glycogen levels during the euglycemic-hyperinsulinemic clamp. Besides, as previously shown, this was accompanied by a reduction of body weight gain, improving of whole body peripheral insulin insensitivity and normalization of dyslipidemia and plasma glucose levels without changes in insulinemia. All the changes mentioned above were obtained by shifting the source of protein in the sucrose-rich diet from casein to soy during the last 4 of the 8-month experimental period.

Several studies with rodent models demonstrated that the administration of soy protein isolate reduces plasma lipid levels, hepatic lipogenic enzymes gene expression, hepatosteatosis and increases insulin sensitivity [4, 9, 17]. We previously showed that the increase of liver lipids storage during dyslipidemia and insulin resistance in the SRD-fed rats was the result of the enhanced uptake of free fatty acids released from adipose tissue and through an increase in endogenous fatty acid biosynthesis and decreased oxidation [22]. The present data show that in the SRD-fed rats the replacement of dietary casein by soy protein significantly reduced fatty liver decreasing triglyceride and cholesterol concentrations. In addition, soy protein normalized dyslipidemia and reduced the excessive availability of plasma-free fatty acid. Concerning this, we previously showed that soy protein normalized the enhanced lipolysis from adipose tissue of SRD-fed rats and limited the accretion of visceral adiposity [34]. Different transcription factors are involved in the expression of genes that could contribute to the accumulation of lipids in the liver. Our results show a significant decrease of the relative abundance of the protein mass level of the mature form of SREBP-1 without changes in their precursor form and an increase of the protein mass level of the nuclear receptor PPAR-α in the SRD-S group. Similar changes were observed regarding the activities of target enzymes involved in “de novo” lipogenesis (fatty acid synthase, acetyl CoA carboxylase, glucose-6-phosphate dehydrogenase and malic enzyme) and fatty acid oxidation (fatty acid oxidase and carnitine palmitoyltransferase-1). These findings suggest that changes in the direction of the metabolic fate of fatty acids from synthesis and storage of lipids toward oxidation could be at least a mechanism contributing to the lipid lowering effect of soy protein in the SRD-fed group. In addition to these results, we previously demonstrated [18] that soy protein was able to reduce the secretion of VLDL-TG from the liver limiting the formation of LDL particles and, therefore, reducing plasma triglyceride and cholesterol levels in rats chronically fed a SRD.

Similarly, different studies showed that the gene expression of liver SREBP-1 and the hepatic enzymes fatty acid synthase, malic enzyme, stearoyl CoA desaturase, delta 5 and 6 desaturases decreased while the gene expression of carnitine palmitoyltransferase-1 and PPAR-α increased when dietary soy protein was given to normal rats or obese Zucker fa/fa rats for different periods of time [7, 9, 10, 35]. Moreover, Torre-Villalvazo et al. [4] observed a decrease of liver gene expression of SREBP-1 and lipid deposition in Sprague–Dawley rats fed a high-fat diet during 6 months when soy protein instead of casein was administered. Tovar et al. [10] showed in Zucker obese fa/fa rats that soy protein reduced liver cholesterol levels; this reduction was associated with low expression of liver X receptor α and its target genes. Recently, Yamazaki et al. [36] showed in ddY mice that β conglycinin, a major protein of soy, significantly prevented increases in mRNAs of SREBP-1c and ChREBP and lipogenic genes such as fatty acid synthase, stearoyl CoA desaturase1, acetyl CoA carboxylase1 and liver-type pyruvate kinase in sucrose—supplemented mice contributing to the anti-fatty liver effects of β conglycinin.

Our data contribute to the studies mentioned above since its shows that soy protein isolate was able to improve/reverse the pre-existing severe dyslipidemia and liver steatosis which developed in rats fed a SRD when this macronutrient replaced casein as a source of protein.

On the other hand, soy protein also contains fiber, lipids and isoflavones, among other components, that directly or indirectly could interact with hepatic genes to modulate lipid metabolism. In cultured human hepatoma ($HepG_2$) cell line, Kim et al. [37] showed that genistein, a major isoflavone of soy protein, increased the gene expression of liver carnitine palmitoyltransferase-1 and PPAR-α. Borradaile et al. [38] found that genistein and daidzein inhibited the ApoB secretion in $HepG_2$ cell line through several mechanisms including, among others, inhibition of cholesterol synthesis and esterification and increased expression of LDL receptors. Besides, genistein added to a control diet lower serum triglyceride and affected mRNA levels of several genes involved in fatty acid, cholesterol and steroids metabolism, decreasing the activities of many lipogenic enzymes while those related to β oxidation in the liver of normal rats increased [39]. Moreover, a reduction of liver cholesterol and triglyceride contents was observed in obese fa/fa Zucker rats fed with isolate soy protein, low or high in isoflavones contents [40]. The isolated soy protein used in the present study contains a moderate

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577 amount of isoflavones (175.4 mg/Kg of diet). Therefore,
578 we cannot discard the possibility that a cooperative inter-
579 vention between soy protein and isoflavones as well as the
580 other components could contribute to our findings.

581 The metabolic shift of lipid fate in the liver induced by
582 dietary soy protein also ameliorated the impaired glucose
583 and lipid metabolism present in the skeletal muscle of
584 SRD-fed rats by a reduction of the accumulation of fatty
585 acid derivatives and normalized the increased protein mass
586 level of nPKCθ in the membrane fraction. It is well known
587 that an increase of nPKCθ could inhibit insulin signaling
588 and altered glucose metabolism [41]. Muscle nPKCθ can
589 be activated by an increase of intracellular fatty acids and
590 metabolites, such as fatty acid acyl CoA and diacylglycerol
591 as well as by chronic elevation of plasma-free fatty acids
592 levels, which has been associated with an increase in
593 expression and activity of this PKC isoform [42]. In this
594 regard, the substantial reduction of plasma-free fatty acid
595 levels induced by dietary soy protein might contribute to
596 the decrease of both lipid storage and the protein mass
597 levels of nPKCθ in the skeletal muscle of SRD-fed rats.

598 The normalization of dyslipidemia and lipid contents
599 within the skeletal muscle of SRD-rats fed soy protein was
600 accompanied by a significant increase of both hexokinase
601 and PDHc enzyme activities, which suggests an improve-
602 ment of glucose phosphorylation and glucose oxidation,
603 respectively. Insulin-stimulated glucose uptake by the
604 skeletal muscle plays an important role in maintaining
605 whole body glucose homeostasis. Concerning this, our data
606 showed both a significant increase of the protein mass level
607 of glucose transporter Glut-4 translocation to the cell
608 membrane and an improvement of the whole body
609 peripheral insulin sensitivity under insulin stimulation
610 when soy protein replaced casein in the SRD-fed rats.
611 Besides, the moderate hyperglycemia observed in the SRD-
612 fed group reached values similar to those of the CD-fed rats
613 after soy administration. In this vein, Lavigne et al. [17], in
614 normal rats fed soy protein, demonstrated an improvement
615 of fasting and postprandial insulin response when com-
616 pared to rats fed casein, suggesting that the effect of soy
617 protein on glucose and insulin dynamics appears to be an
618 increased insulin action in the skeletal muscle. Noriega
619 et al. [7] showed that soy protein attenuated insulin resis-
620 tance "in vivo" (clamp study) in rats fed a high-fat diet.
621 Besides, the administration of aglycin, a soybean peptide,
622 prevented or attenuated hyperglycemia by increasing the
623 insulin receptor signaling pathway in the skeletal muscle of
624 diabetic mice [14].

625 On the other hand, the effect of dietary soy protein on
626 the skeletal muscle of SRD-fed rats mentioned above could
627 also be related to the hypoglycemic effects of soy iso-
628 flavone genistein and daidzein [6, 43, 44]. A recent study of
629 Arunkumar et al. [45] on Swiss albino mice fed a high-fat
630 high-fructose diet showed that the administration of geni-
631 stein corrected the defects of insulin signaling pathways,
632 increasing Glut-4 protein levels in the cell membrane of
633 muscle, lowering blood glucose and improving insulin
634 action by targeting AMPK. Cederroth et al. [46], in CD1
635 mice fed with a soy diet containing genistein and daidzein,
636 showed a reduction of whole body adiposity, improving
637 insulin sensitivity at least in part by activating AMPK in
638 tissues including skeletal muscle and improving glucose
639 uptake.

640 At present, we are unaware of any study analyzing the
641 underlying possible mechanism/s involved in the effect of
642 dietary soy protein isolate on improving the altered glucose
643 metabolism and insulin sensitivity in the skeletal muscle of
644 a dyslipemic insulin resistant SRD-fed rat model. Our
645 results suggest that at least one possible mechanism
646 involved the lipid lowering effect of soy protein that con-
647 tributes to normalize the lipid fuel availability and the
648 accretion of lipid storage in the muscle. Thus, in turn, it
649 improves the translocation of Glut-4 and the oxidative and
650 nonoxidative pathways of glucose metabolism leading to
651 ameliorate the overall peripheral insulin sensitivity. How-
652 ever, since we do not evaluate the effect of the amount of
653 isoflavones present in the soy protein by itself, the sug-
654 gested mechanisms might include the known beneficial
655 effect of these phytoestrogens.

656 In conclusion, this work provides new information about
657 the possible mechanisms underlying the beneficial effect of
658 dietary soy protein on lipid liver metabolism and insulin
659 action on skeletal muscle in the presence of dyslipidemia
660 and insulin resistance induced by a SRD. Although caution
661 is warranted before the extrapolation results from rodents to
662 humans, this rat model proves to be useful to study the
663 influence of nutrients on the management of these metabolic
664 alterations susceptible to dietary manipulations (Fig. 5).

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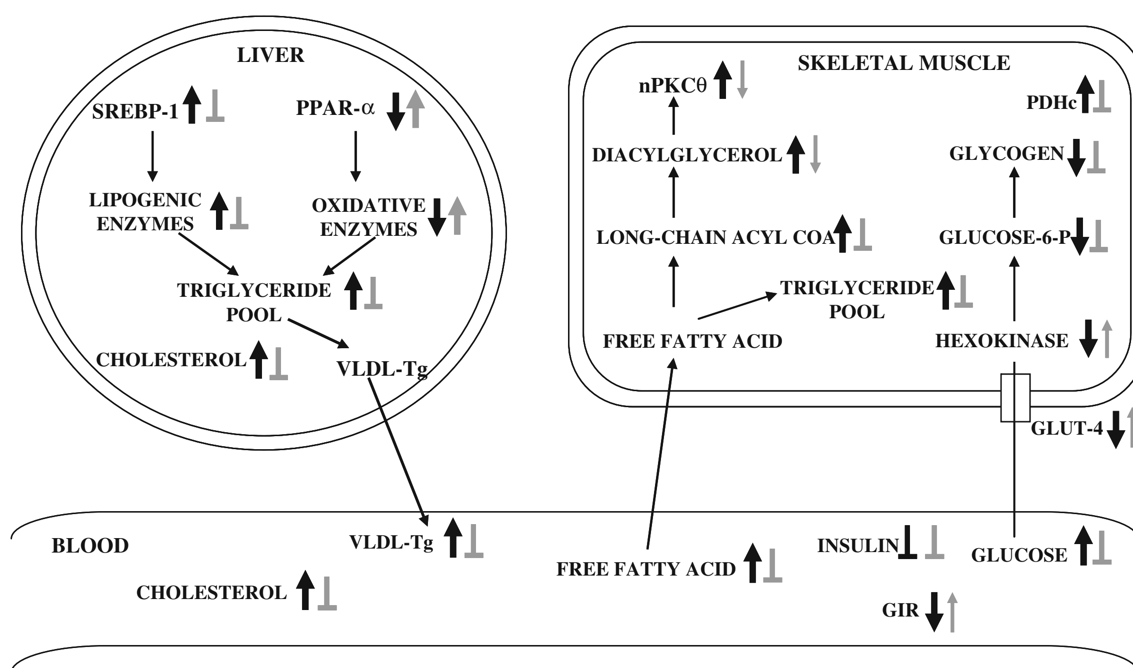

Fig. 5 Possible mechanisms through which the dietary soy protein reverses or improves in the liver and skeletal muscle the metabolic abnormalities induced by the intake of long-term a SRD. In the liver, the administration of dietary soy protein may exert their effects by regulating the expression of genes encoding proteins involved in the fatty acid oxidation (through PPARs) while simultaneously normalizing protein mass levels of SREBP-1 and the activities of the enzymes involved in "de novo" lipogenesis. Changes in the direction of the metabolic fate of fatty acids from synthesis and storage of lipids toward oxidation lead to a reduction of hepatic lipogenesis, the triglyceride pool size, the VLDL-Tg secretion and normalized plasma triglyceride and cholesterol levels. In muscle, dietary soy protein reduces the accumulation of fatty acid derivatives and significantly decreases protein mass level of PKCθ in the membrane fraction. Besides, it improves glucose phosphorylation while restoring the nonoxidative glucose pathway under insulin stimulation. All these effects could contribute to ameliorate the overall peripheral insulin insensitivity. *black color* SRD, *gray color* SRD-S: *thick black up arrow* increase; *thin gray up arrow* moderate increase; *thick black down arrow* decrease; *thin gray down arrow* moderate decrease; *perpendicular to* normal

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