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Dietary Salba (Salvia *hispanica* L) seed rich in α-linolenic acid improves adipose tissue dysfunction and the altered skeletal muscle glucose and lipid metabolism in dyslipidemic insulin-resistant rats ☆

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ABSTRACT

This work reports the effect of dietary Salba (chia) seed rich in *n*-3 α-linolenic acid on the morphological and metabolic aspects involved in adipose tissue dysfunction and the mechanisms underlying the impaired glucose and lipid metabolism in the skeletal muscle of rats fed a sucrose-rich diet (SRD). Rats were fed a SRD for 3 months. Thereafter, half the rats continued with SRD while in the other half, corn oil (CO) was replaced by chia seed for 3 months (SRD+chia). In control group, corn starch replaced sucrose. The replacement of CO by chia seed in the SRD reduced adipocyte hypertrophy, cell volume and size distribution, improved lipogenic enzyme activities, lipolysis and the anti-lipolytic action of insulin. In the skeletal muscle lipid storage, glucose phosphorylation and oxidation were normalized. Chia seed reversed the impaired insulin stimulated glycogen synthase activity, glycogen, glucose-6-phosphate and GLUT-4 protein levels as well as insulin resistance and dyslipidemia.

1. Introduction

Changes in human diet and life style have been closely related to the growing epidemic of chronic diseases including the "so called" metabolic syndrome affecting both developed and undeveloped regions of the world [1]. The metabolic syndrome is defined as a cluster of risk factors including among them: type 2 diabetes, hypertension, dyslipidemia, insulin resistance (IR), central obesity and cardiovascular disease (CVD) [2]. Several features of the metabolic syndrome may be improved by nutritional manipulations such as increase of the dietary intake of marine polyunsaturated fatty acids (PUFAs) 20:5 *n*-3 (EPA) and 22:6 *n*-3 (DHA). These fatty acids act as potent hypolipidemic agents in both rodents and humans [3]. Moreover, they prevent the development of dyslipidemia, liver steatosis, impaired glucose homeostasis, IR and adiposity in rodents fed high-fat or sucrose/fructose diets [3].

Another important source of *n*-3 PUFAs is α-linolenic acid (ALA, 18:3 *n*-3) which derives from plant sources. Different epidemiological and clinical studies have suggested that a higher concentration of ALA is associated with a reduced risk of CVD [4,5]. In rats, ALA administration decreased plasma lipid concentration [6]. Ghafoorunissa and Natarajan [7] showed that the substitution of one-third of dietary 18:2 *n*-6 with 18:3 *n*-3 in sucrose-fed rats resulted in lowered blood lipid levels and increased peripheral insulin sensitivity.

The seed of Salvia *hispanica* L commonly named chia seed, which is rich in fiber and minerals, contains the richest botanical oil source of ALA known to date. Along with corn, beans and amaranth, it was a core component in the diet of many pre-Columbian civilizations in America including the Mayan and Aztec populations. A recent study by Poudyal et al. [8] in rats fed a high fat–carbohydrate diet showed that the administration of dietary chia seed during 16 weeks induced lipid redistribution and attenuated the abnormal metabolic cardiovascular and hepatic signs developed in this experimental model. In addition, the beneficial effects of feeding either chia seed or chia oil on rats plasma cholesterol, LDL cholesterol, HDL cholesterol and triglyceride (TG) contents were recently reported in two controlled studies [9,10].

In a previous work, we demonstrated that feeding rats for 3 weeks with a sucrose-rich diet (SRD), in which a white variety of chia seed called Salba was the source of dietary fat prevents the onset of IR without changes in plasma glucose levels. Furthermore, dyslipidemia and IR in rats fed the SRD for a long term (5 months) were reversed without changes in plasma insulin levels when chia seed instead of corn oil (CO) became the dietary source of fat for the last 2 months of the feeding period [11]. Moreover, we recently reported that both hepatic key enzymes' activities involved in

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lipogenesis and oxidative mitochondrial fatty acid oxidation are coordinately decreased and increased by dietary chia seed. This was accompanied by a parallel decrease and increase in the protein mass levels of mature sterol regulatory element-binding protein-1 (SREBP-1) and peroxisome proliferator-activated receptor-α (PPARα), respectively [12]. These findings suggest that the above changes could be involved in the mechanisms leading to a reduction in liver TG synthesis normalizing or improving liver steatosis and dyslipidemia induced in rats chronically fed a SRD. Besides, the increased visceral adiposity recorded in the long-term SRD-fed rats was significantly reduced by chia seed [11,12].

Dysfunctional adipose tissue is a risk factor for IR. Despite the only minimal contribution of this tissue to whole body glucose uptake, impairment of glucose transport in adipocytes results in IR in skeletal muscle and liver [13]. However, to the best of our knowledge no studies have been published focusing on the mechanisms by which chia seed improves visceral adiposity in the dyslipidemic insulin-resistant rats fed a SRD. On the other hand, the skeletal muscle is quantitatively the most important site of whole body glucose utilization and lipid accumulation in skeletal muscle fibers has been linked to IR and directly or indirectly alters insulin signaling. Chicco et al. [11] reported that chia seed reduced the increased TG content in the gastrocnemius muscle of SRD-fed rats.

In view of the above, this investigation has two goals: (i) to investigate if dietary chia seed (Salba) could be able to improve or even revert the morphological changes and metabolic abnormalities (lipogenic enzyme activities, lipolysis and the effect of insulin on lipolysis) underlying adipose tissue dysfunction in rats fed a SRD for a long term and (ii) to analyze whether or not the administration of dietary chia seed could improve the altered glucose and lipid metabolism in the skeletal muscle of SRD fed rats. Along this way information concerning the possible mechanisms involved can obtained. This study was conducted in rats fed a SRD during 6 months in which a permanent dyslipidemia, IR, visceral adiposity and abnormal glucose homeostasis was present before the source of dietary fat CO was replaced by an isocaloric amount of chia seed for the last 3 months of the experimental period in half the animals [11].

2. Materials and methods

2.1. Animals and diets

Male Wistar rats initially weighing 180–190 g and purchased from the National Institute of Pharmacology (Buenos Aires, Argentina) 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 on from 0700 h to1900 h). They were initially fed a standard non-purified diet (Ralston Purina, St. Louis, MO, USA).

2.2. Dietary manipulations

After 1 week of the acclimatization, the rats were randomly divided into two groups (control and experimental) and were housed individually. The control group received a semisynthetic diet containing corn starch (60% energy), protein (17% energy) and corn oil (CO) as a source of fat (23% energy) (control diet: CD). The experimental group received the same semisynthetic diet with sucrose as the carbohydrate and fat provided by CO (SRD). Both groups received each diet for 3 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 6 months of feeding. The second subgroup received the Salba seed (chia) 36.2 g% of food as the source of dietary fat for the next 3 months (SRD + chia). The control group was fed with the CD throughout the experimental period. The fiber, vitamin and salt mix contents of each diet were similar. The carbohydrate, protein, fiber, mineral and vitamin contents in the feed of the SRD + chia group were balanced with the CD and SRD groups taking into account the amount of these nutrients present in the chia seed. Details on the composition of the diets are given in Table 1. The fatty acid composition of each experimental diet (g/kg of food) is shown in Table 2. The preparation and handling of the diets have been reported elsewhere [11]. All diets provided approximately 17.00 kJ/g of food. The weight of each animal was recorded twice per week throughout the experimental period in all groups and subgroups of rats. In a separate experiment, the individual caloric intake and weight gain of eight animals in each group and subgroup were assessed twice a week. At the end of the experimental period food was removed at 0700 h (end of the dark period) and unless otherwise indicated experiments were performed between 0700 h and 0900 h.

At least six rats from the three dietary groups were used in each procedure. They were anesthetized with intraperitoneal sodium pentobarbital (60 mg/kg body weight). Blood samples were obtained from the jugular vein, collected in tubes containing sodium EDTA as anticoagulant and rapidly centrifuged. Plasma was either immediately assayed or stored at −20 °C. Retroperitoneal and omental adipose tissues were totally removed and weighed. Epididymal adipose tissue was totally removed, weighed and immediately used for the preparation of adipocytes as mentioned below or frozen and stored at the temperature of liquid N_2. The visceral adiposity index (%) was calculated as: [retroperitoneal fat (g) + omental fat (g) + epididymal fat (g)]/body weight (g) × 100 and expressed as adiposity percent. The skeletal muscle (gastrocnemius) was removed, frozen and stored at the temperature of liquid N_2. This tissue contained both oxidative and glycolytic fibers. Insulin binding to the gastrocnemius membrane reflected insulin binding to the mixture of the whole muscle of the upper and lower extremities [14]. The experimental protocols were approved by the Human and Animal Research Committee of the School of Biochemistry, University of Litoral, Argentina.

2.3. Analytical methods

Plasma TG, free fatty acids (FFA) and glucose levels were determined by spectrophotometric methods as previously described [15]. The immunoreactive insulin was measured by the method proposed by Herbert et al. [16]. The immunoreactive insulin assays were calibrated against rat insulin standard (Novo Nordisk, Copenhagen, Denmark).

2.4. Preparation of isolated adipocytes and determination of fat cell volume and number

Epididymal fat pads were removed, weighed and rinsed in isotonic saline solution at 37 °C. Adipocytes were isolated according to the method of Rodbell with minor modifications as previously described [17]. One fraction of washed cells was used for the determination of fat cell size and number as previously described [17,18]. The lipid weight of the average fat cell was calculated from mean cell volume assuming a lipid density of 0.95 (triolein density).

2.5. Determination of basal lipolysis and stimulated lipolysis

For the study of basal lipolysis, aliquots of diluted isolated epididymal fat cells (1×10^5 cells/ml) were incubated in a Krebs–Henseleit phosphate buffer (pH 7.4) containing 1.25 mM Ca^{++}, 4% of bovine serum albumin essentially free of fatty acids, 5.5 mM glucose in a shaking Dubnoff water-bath (60 cycles/min) at 37 °C

Table 1
Composition of experimental diets.[a]

Diet ingredients	Control diet (CD)		Sucrose-rich diet (SRD)		SRD+chia seed[b] (SRD+chia)	
	(%) by weight	(%) of energy	(%) by weight	(%) of energy	(%) by weight	(%) of energy
Carbohydrates						
Corn starch	58.0	60.0	2.5	2.6	–	–
Sucrose	–	–	55.5	57.4	55.5	57.4
Chia seed	–	–	–	–	2.5	2.6
Fat						
Corn oil	10.5	23.0	10.5	23.0	0.1	0.2
Chia seed	–	–	–	–	10.4	22.8
Protein						
Casein (vitamin free)	16.3	17.0	16.3	17.0	8.6	9.0
Chia seed	–	–	–	–	7.7	8.0

[a] The composition of experimental diets are based on AIN-93M diet. All diets contain by weight: salt mix, 3.5% (AIN-93M-MX); vitamin mix, 1% (AIN-93VX); choline chloride, 0.2%; methionine, 0.3%; fiber, 10–11%. The SRD+chia was balanced in salt mix according to the amount of each one in the chia seed provided by the manufacturer.
[b] Chia seed (Salba; Salvia *hispanica* L): 362 g/kg diet. Chia composition (g/100 g chia seed): carbohydrate, 37.45; insoluble fiber, 81% of total carbohydrate; fat, 30.23; protein, 21.19. Mineral composition (mg/100 g chia seed): Na, 103.15; K, 826.15; Ca, 589.60; Fe, 11.90; Mg, 77.0; P, 604.0; Zn, 5.32; Cu, 1.66; Mn, 1.36.

Table 2
Total fatty acid composition of the experimental diets (g/kg of diet).

Fatty acids[a]	CD and SRD	SRD+chia seed
16:0	10.92	6.96
18:0	2.73	2.42
18:1 *n*-9	33.71	7.39
18:2 *n*-6	54.10	19.85
18:3 *n*-3	0.80	67.26
20:1 *n*-9	0.47	0.36
Total saturated	13.65	9.38
Monounsaturated	34.18	7.75
Polyunsaturated		
n-6	54.10	19.85
n-3	0.80	67.26
n-6:*n*-3	67.62	0.295

[a] Other minor fatty acids have been excluded.

for 1 h under an atmosphere of 95% O_2–5% CO_2 as previously described [17]. Under these conditions, there was a time-dependent increase in lipolysis for 60 min. Therefore, this time period was chosen for the incubation. A pure β-agonist, isoproterenol (10^{-6} M), was used to stimulate lipolysis. Lipolysis was determined by measuring glycerol release over 1 h at 15 min intervals in aliquots of infranatant from each incubation mixture by the enzymatic method of Wieland [19] both in the basal state and in the presence of isoproterenol so that the maximal lipolytic responsiveness could be examined, as previously described [17].

2.6. Assay of the anti-lipolytic action of insulin

The anti-lipolytic action of insulin was performed in epididymal isolated adipocytes as previously described by Soria et al. [17]. Briefly, isoproterenol (10^{-7} M) and adenosine deaminase (10 U/ml) were added to the fat cell suspensions (1×10^5 cell/ml), and incubations were conducted at 37 °C during 1 h under an atmosphere of 95%O_2–5%CO_2, both in the absence or in the presence of insulin (purified porcine insulin; NOVO laboratory, Burien, WA, USA) at a final concentration of 2.0 nM. At the end of the incubation period, three aliquots of the infranatant were removed from each incubation mixture and glycerol release was measured as described above. The anti-lipolytic action of insulin was expressed as the ratio of the value of insulin-inhibited lipolysis to that of isoproterenol-stimulated lipolysis in the absence of the hormone as a percentage.

2.7. Enzymatic activity assays in epididymal fat tissue

The activities of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) were assayed as previously described [20]. Malic enzyme (ME) activity was measured in the aqueous supernatant fraction of adipose tissue samples by the spectrophotometric method of Wise and Ball [21]. Adipose tissue glucose-6-phosphate dehydrogenase (G-6-PDH) activity was measured according to Cohen et al. [22], as described elsewhere [20].

2.8. Gastrocnemius muscle assays

TG, long-chain acyl-CoA (LCA-CoA), diacylglycerol (DAG), glycogen and glucose-6-phosphate (G-6-P) contents as well as the activities of hexokinase and pyruvate dehydrogenase complex (PDHc) were analyzed in muscle homogenate as described elsewhere [23–25]. The in vitro glycogen synthase (GSa) activity was determined by the method of Golden et al. [26] as previously described [24]. Briefly, the GSa-independent activity was the activity measured at low G-6-P concentration and the total GSa activity was the activity measured at high G-6-P concentration. The fractional velocity of GSa was calculated as the rate of incorporation of labeled uridine-diphosphoglucose (uridine 5′ diphospho [$U^{14}C$] glucose, New England Nuclear, Boston, MA) into glycogen at 0.1 mM G-6-P divided by the rate at 10 mM and expressed as a percentage [27]. Frozen gastrocnemius muscle homogenates were prepared for the isolation of cytosol and membrane fraction of protein kinase C theta (nPKCθ) protein mass levels, as previously described by D'Alessandro et al. [23,24]. Total protein samples were resolved on SDS–PAGE and transferred to PVDF membranes. The membranes were probed with specific antibody (anti-rabbit anti specific nPKCθ from Santa Cruz Biotechnology, Inc, Santa Cruz, CA). The blots were incubated with horseradish peroxidase linked to secondary antibody followed by chemiluminiscence detection according to the manufacturer's instruction (Super Signal West Pico Chemiluminiscent Detection, Pierce Biotechnology, Rockford, IL). The protein levels were normalized to β-actin. The intensity of the bands was quantified by the NIH imaging software. The relationship between the amount of the sample subjected to immunoblotting and the signal intensity observed was linear under the conditions described above. Protein concentrations were quantified with the Bio-Rad protein assay.

2.9. Determination of TG, glycogen, G-6-P content, GSa activity and GLUT-4 protein mass levels in gastrocnemius muscle (clamp studies)

Whole-body peripheral insulin sensitivity was measured using the euglycemic–hyperinsulinemic clamp technique as described elsewhere [25]. Briefly, after 5 h of food deprivation, 12 rats from each dietary group were anesthetized, a blood sample was withdrawn, and glucose and insulin levels were assessed. The gastrocnemius muscle of six rats from each group was rapidly removed (starting clamp studies), frozen, clamped in liquid N_2 and stored at −80 °C. In the other six rats from each dietary group, an infusion of highly purified porcine neutral insulin (Actrapid, Novo Nordisk, Bagsvard, Denmark) was administered at 0.8 units/(kg × h) for 2 h. Glycemia was maintained at a euglycemic level by infusing glucose (200 g/L) at a variable rate. The glucose infusion rate during the second hour of the clamp study was taken as the net steady state of the whole body glucose. At the end of the clamp period, the gastrocnemius muscle was rapidly removed. TG, glycogen, G-6-P levels, GSa activity and GLUT-4 protein mass level were determined at the beginning and at the end of the clamp as previously described [23–25]. The assay of the protein mass level of GLUT-4 was described elsewhere [23,24]. Briefly, frozen gastrocnemius muscle tissue powder was homogenized and total protein samples (40 μg) were resolved on 10% SDS–PAGE (Laemmli) and transferred to PVDF membranes. The membranes were probed with specific antibody (polyclonal goat anti GLUT-4 from Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA). The blot was incubated at 4 °C with horseradish peroxidase linked secondary antibody followed by chemiluminiscence detection according to the manufacturer's instruction (Super Signal West Pico Chemiluminiscent Detection, Pierce Biotechnology, Rockford, IL). The protein levels were normalized to β-actin. The intensity of the bands was quantified by NIH imaging software. The relationship between the amount of the sample subjected to immunoblotting and the signal intensity observed was linear under the conditions described above. Protein concentrations were quantified with the Bio-Rad protein assay.

2.10. Statistical analysis

Sample sizes were calculated on the basis of measurements previously made with rats fed either a CD or a SRD [15,18,24,25] considering an 80% power as described by Glantz [28]. Results were expressed as means with their standard errors. Statistical comparisons were done transversely between different dietary groups. The statistical significance between groups was determined by one-way ANOVA, with one factor (diet) followed by the inspection of all differences between pairs of means by Newman Keuls' test [29]. Differences having P values lower than 0.05 were considered to be statistically significant (SPSS 15.0 for Windows, SPSS, Inc., Chicago, IL). All reported P values are 2-sided.

3. Results

3.1. Body weight gain, energy intake, visceral adiposity index, plasma metabolite levels and glucose infusion rate (GIR)

Body weight and energy intake were carefully monitored in all groups of rats throughout the experimental period. As previously shown [23], a significant increase in body weight and energy intake occurred in rats fed a SRD from 3 to 6 months compared to those fed a CD (Table 3). However, in spite of a similar energy intake recorded in both the SRD and the SRD+chia groups during the last 3 months of the experimental period (months 3–6), weight at 6 months was slightly lower without statistical difference in the latter group. Moreover, similar to our previous publications [11] SRD-fed rats showed a significant increase of the visceral adiposity index which was significantly reduced after chia seed administration (data no shown). Plasma TG, FFA and glucose levels were higher in rats fed a SRD for 6 months compared with the age-matched control fed the CD (Table 3). Similar values were observed in rats fed the SRD for 3 months (data not shown). All these parameters with the exception of body weight returned to control values in the SRD-fed rats when chia seed replaced CO for the last 3 months of the feeding period. No statistically significant differences in plasma insulin levels were observed at the end of the experimental period in the three dietary groups. Confirming previous reports the significant decrease of glucose infusion rate (euglycemic–hyperinsulinemic clamp study) recorded in the SRD-fed group returned to values similar to those obtained in the CD-fed rats in the SRD+chia group.

3.2. Fat pad morphology and TG content

As we have previously reported [30] an increase of epididymal tissue weight associated with a hypertrophy of the adipose cells was observed in rats chronically fed a SRD. The present results show that dietary chia seed reduced the increase of both the epididymal fat pad weight and the cell volume (hypertrophy) observed in adipocytes of SRD-fed rats, although values are still higher than those recorded in the CD-fed group. Besides, no differences in the total cell number expressed as total fat were observed in all dietary groups. The increase of the TG content within the adipocytes of SRD-fed rats was significantly reduced after chia seed administration (Table 4). Fig. 1 shows the histograms of epididymal adipose cell distribution (at 2.5 μm intervals) at the end of the experimental period. Confirming previous results [17], in the SRD group there was a clear differentiation in the cell size distribution with a significant increase (approximately 30%) of the mean cell diameter compared to the CD-fed animals. The addition of chia seed instead of CO as a dietary source of fat resulted in a significant reduction of adipocyte cell size diameter. In this group, cell size distribution approached that recorded in the CD-fed rats (Fig. 1).

3.3. Adipose tissue enzyme activities involved in "de novo" lipogenesis

As shown in Table 5 the activities of the enzymes related to "de novo" lipogenesis were significantly increased in the epididymal fat pad of rats chronically fed a SRD compared to age-matched control fed a CD. A significant reduction of FAS and G-6-PDH activities was observed in the SRD+chia group, which reached values similar to those recorded in the CD-fed rats. However, although no changes in ACC activity were observed when the enzyme was expressed as pkat/mg of protein, a behavior similar to that of the other enzymes was observed when the enzyme activity was expressed as pkat/total fat weight. Values were as follows: mean ± SEM; $n=6$, pkat/total fat weight: CD: 36.67 ± 5.00; SRD: 56.68 ± 4.10; SRD+chia: 43.34 ± 4.20 ($P<0.05$, SRD vs CD and SRD+chia). Besides, a significant decrease of ME activity was recorded in the adipose tissue in the SRD+chia group although values are still higher than the control group.

3.4. Basal and stimulated lipolysis and insulin mediated inhibition of lipolysis of the fat cells

Basal lipolysis – as an estimation of both hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) activities – [31,32], is shown in Fig. 2A. A significant increase of basal lipolysis was recorded in enlarged fat cells of SRD-fed rats as compared to age-matched controls fed a CD. When the source of fat –CO– was

Table 3
Body weight, energy intake, plasma metabolite levels and glucose infusion rate (GIR) in rats fed a control diet (CD), a sucrose-rich diet (SRD) or a SRD with chia seed (SRD+chia).[1]

	CD	SRD	SRD+chia
Body weight (g)			
Initial	185.5 ± 2.7 (8)	187.0 ± 2.5 (16)	
3 months	367.0 ± 9.2 (8)	376.0 ± 8.5 (8)	382.3 ± 6.0 (8)
Final (6 months)	427.0 ± 6.2^{b} (8)	500.3 ± 5.6^{a} (8)	480.0 ± 15.9^{a} (8)
Energy intake (kJ/d)			
Initial – 3 months	280.4 ± 9.7 (8)	285.1 ± 10.0 (16)	
3–6 months	286.8 ± 12.6^{b} (8)	356.3 ± 10.8^{a} (8)	349.5 ± 8.4^{a} (8)
Plasma			
Triglyceride (mM)	0.68 ± 0.06^{b} (6)	1.90 ± 0.12^{a} (6)	0.63 ± 0.05^{b} (6)
FFA (μM)	315.0 ± 22.5^{b} (6)	703.0 ± 31.8^{a} (6)	325.0 ± 20.0^{b} (6)
Glucose (mM)	6.7 ± 0.1^{b} (6)	8.1 ± 0.2^{a} (6)	7.0 ± 0.1^{b} (6)
Insulin (pM)	495.0 ± 30.3 (6)	513.8 ± 39.4 (6)	568.0 ± 42.1 (6)
GIR [μmol/(kg × min)]	59.8 ± 1.7^{a} (6)	28.4 ± 1.8^{b} (6)	58.6 ± 4.1^{a} (6)

[1] Values are expressed as means ± SEM, ( ) number of rats. 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 Newman Keuls' test.

Table 4
Epididymal adipose tissue total and relative weights, cellularity and triglyceride content of rats fed a control diet (CD), a sucrose-rich diet (SRD) or a SRD with chia seed (SRD+chia).[1]

	Epididymal adipose tissue		Adipocyte		
	Total weight (g)	Relative weight (g/100 g body weight)	Triglyceride (nmol/cell)	Cell volume (pl)	Cell number × 10^{6} per total weight
CD	7.1 ± 0.5^{c}	1.61 ± 0.10^{c}	0.30 ± 0.05^{c}	289.8 ± 16.1^{c}	25.8 ± 1.8
SRD	12.3 ± 0.7^{a}	2.46 ± 0.15^{a}	0.72 ± 0.06^{a}	674.3 ± 12.3^{a}	24.9 ± 0.5
SRD+chia	9.4 ± 0.4^{b}	1.91 ± 0.09^{b}	0.49 ± 0.05^{b}	401.7 ± 37.9^{b}	22.3 ± 1.5

[1] Values are expressed as means ± 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 Newman Keuls' test.

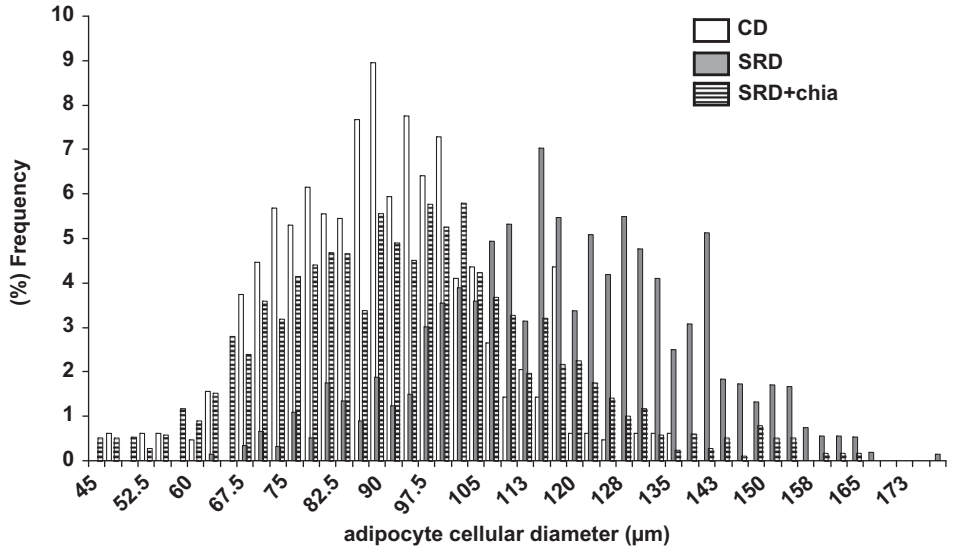

Fig. 1. Representative histogram showing the distribution of adipocytes cell diameters isolated from the epididymal depots of rats fed a control diet (CD), a sucrose-rich diet (SRD) or SRD+chia seed (SRD+chia). The histogram was constructed by sizing at intervals of 2.5 μm, 100 adipocytes from each individual rat. Six animals were included in each experimental group. Bars represent the mean of the cell measured (percent) that falls within a given size indicated.

replaced by chia seed in the SRD, basal lipolysis significantly decreased reaching values similar to those recorded in the control group. On the other hand, the isoproterenol stimulated rate of lipolysis was significantly greater than the basal rate in all dietary groups. The SRD-fed group exhibited a stimulated rate of lipolysis significantly higher than those recorded in adipocytes of rats fed either a CD or a SRD in which chia seed was added. However, the relative stimulation, fold increase over basal, was lower in the SRD group (2 fold) than in the other dietary groups (9 and 7 fold in CD and SRD+chia seed, respectively) (Fig. 2A). Insulin-mediated suppression of isoproterenol-stimulated lipolysis is shown in Fig. 2B. As previously demonstrated when compared with the CD-fed rats, animal fed a SRD showed a decreased adipocyte sensitivity to the anti-lipolytic action of insulin. The hormone failed to inhibit

Table 5
Lipogenic enzyme activities in epididymal adipose tissue of rats fed a control diet (CD), a sucrose-rich diet (SRD) or a SRD with chia seed (SRD+chia).[1]

	CD	SRD	SRD+chia
Fatty acid synthase (pkat/mg protein)	246.7 ± 17.0[b]	300.7 ± 14.0[a]	239.0 ± 10.2[b]
Glucose-6-phosphate dehydrogenase (pkat/mg protein)	509.3 ± 30.0[b]	823.5 ± 72.2[a]	555.1 ± 40.8[b]
Acetyl-CoA carboxylase (pkat/mg protein)	480.1 ± 51.7	441.7 ± 27.2	442.0 ± 29.5
Malic enzyme (nkat/total fat weight)	23.0 ± 1.8[c]	44.5 ± 2.8[a]	31.8 ± 1.2[b]

[1] Values are expressed as means ± 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 Newman Keuls' test.

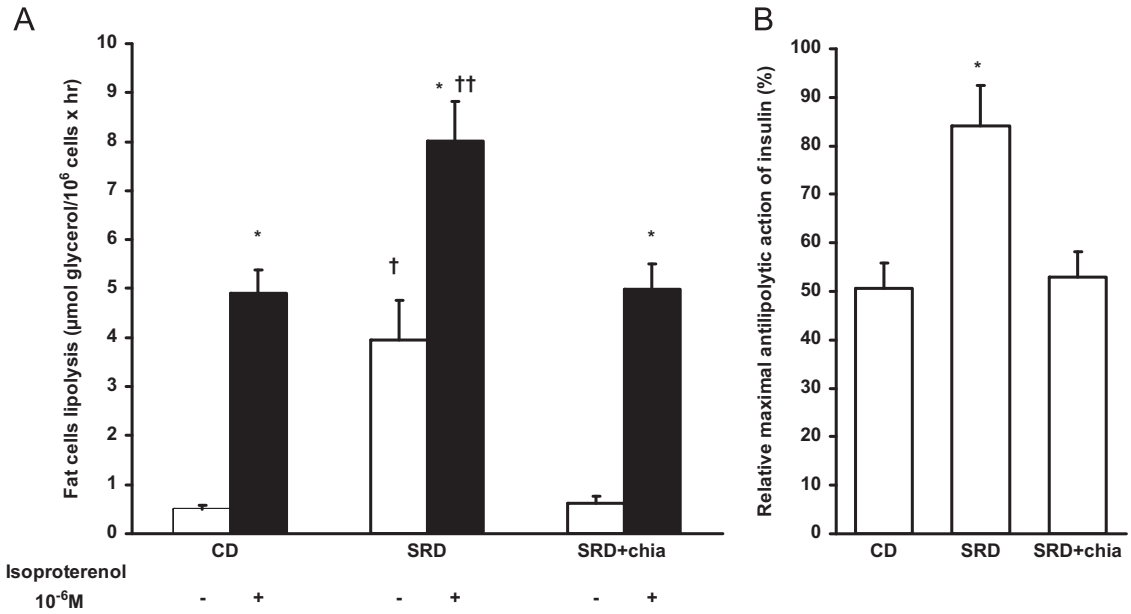

Fig. 2. (A) Basal and isoproterenol-stimulated lipolysis in isolated adipocytes from epididymal fat tissue of rats fed a control diet (CD), a sucrose-rich diet (SRD) or SRD+chia seed (SRD+chia). Values are expressed as mean ± SEM; six animals were included in each experimental group. Lipolysis was estimated as the glycerol release from the isolated adipocytes. For further details on methodology see Section 2. *$P<0.05$ isoproterenol (10^{-6} M) vs basal in each experimental group; †$P<0.05$ SRD basal vs CD and SRD+chia basal; ††$P<0.05$ SRD isoproterenol (10^{-6} M) vs CD and SRD+chia isoproterenol (10^{-6} M). (B) Relative maximal anti-lipolytic action of insulin in isolated adipocytes from epididymal fat tissue of rats fed a control diet (CD), a sucrose-rich diet (SRD) or SRD+chia seed (SRD+chia). Values are expressed as mean ± SEM; six animals were included in each experimental group. Adipocytes were incubated in triplicate with 10^{-7} M isoproterenol either in the presence or absence of 2 nM insulin. The results are expressed as the ratio of the values of the insulin inhibited-lipolysis to that of the isoproterenol-stimulated lipolysis in the absence of insulin. *$P<0.05$ SRD vs CD and SRD+chia.

Table 6
Metabolites and enzyme activities in gastrocnemius muscle of rats fed a control diet (CD), a sucrose-rich diet (SRD) or a SRD with chia seed (SRD+chia).[1]

Diets	CD	SRD	SRD+chia
Metabolites			
Triglyceride (µmol/g wet tissue)	3.4 ± 0.1[b]	6.7 ± 0.3[a]	3.5 ± 0.2[b]
Long-chain acyl CoA (nmol/g wet tissue)	6.2 ± 0.4[b]	12.8 ± 0.8[a]	5.6 ± 0.4[b]
Diacylglycerol (nmol/g wet tissue)	108.3 ± 13.2[c]	184.0 ± 8.9[a]	135.5 ± 4.0[b]
Glycogen (µmol/g wet tissue)	21.8 ± 1.7	22.3 ± 0.8	19.4 ± 0.4
Glucose-6-phosphate (µmol/g wet tissue)	0.42 ± 0.02	0.43 ± 0.03	0.39 ± 0.01
Enzyme activities			
Hexokinase (pkat/mg protein)	746.0 ± 39.7[a]	561.8 ± 23.3[b]	656.8 ± 35.0[a]
Glycogen synthase[2] (% fractional activity)	36.1 ± 3.1	35.3 ± 2.9	33.8 ± 3.6
PDHa[3] (% of total PDHc)	33.9 ± 0.7[a]	21.0 ± 1.6[b]	37.3 ± 1.0[a]

[1] Values are expressed as means ± 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 Newman Keuls' test.
[2] Glycogen synthase was expressed as percentage of fractional activity (see Section 2).
[3] PDHa: active form of PDH complex, expressed as percentage of total PDHc activity (PDHa: basal activity × 100/total activity).

β-agonist stimulated lipolysis in isolated epididymal fat cells of the SRD fed rats (16% inhibition in SRD vs 50% in the CD fed group). The addition of chia seed to the SRD completely restored the sensitivity of adipocyte to the anti-lipolytic action of insulin reaching values similar to those recorded in the CD group (Fig. 2B).

3.5. *Effect of dietary chia seed on metabolite concentration, enzyme activities and nPKCθ protein mass levels in the gastrocnemius muscle of SRD-fed rats*

As previously demonstrated [23], at the basal state (beginning of the clamp study) the gastrocnemious muscle of SRD-fed rats showed a significant increase of TG, LCA-CoA and DAG contents without changes in glycogen and G-6-P levels compared to CD-fed rats (Table 6). The present results show that dietary chia seed was able to decrease TG, and LCA-CoA which reached values similar to those recorded in the CD-fed group. Besides, a significant decrease of DAG concentration was observed in the SRD+chia group (values were similar to those observed in the control group). No changes in glycogen, G-6-P levels and the GSa activity were observed in the skeletal muscle of SRD fed rats. Moreover, the administration of dietary chia seed did not produce any changes in either the above mentioned metabolites or the GSa activity. Moreover, the significant reduction of both hexokinase and the active form of PDHc activities were completely normalized when dietary chia seed replaced CO as a source of fat in the SRD-fed group (Table 6). Besides, immunoblotting of muscle cytosol and membrane fractions revealed a single 79 kDa band consistent with nPKCθ. Each muscle fraction was run on separate gels, with each gel containing equal number of samples from CD, SRD and SRD+chia rats (Fig. 3). After densitometry of immunoblots, both the mean cytosolic and membrane nPKCθ content of the CD-fed rats group were normalized to 100% and the cytosolic as well as membrane levels of nPKCθ from SRD and SRD+chia were expressed relative to this. The qualitative and quantitative analyses of Western blot showed that the relative abundance of nPKCθ isozyme that was significantly increased ($P < 0.05$) in the membrane fraction of the gastrocnemius muscle of SRD returned to values similar to those recorded in the CD group

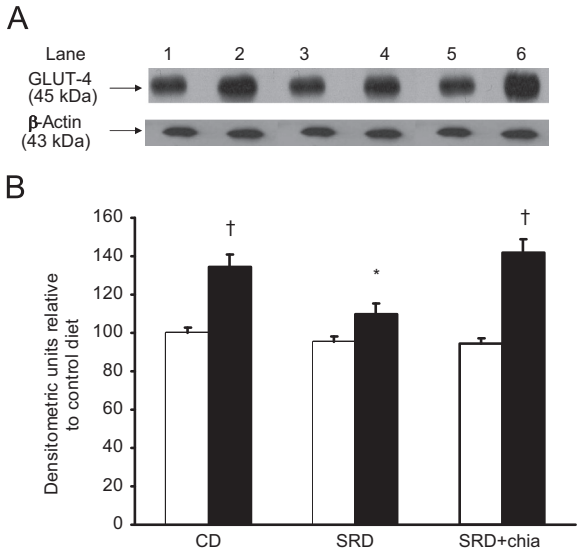

Fig. 4. Skeletal muscle protein mass level of GLUT-4 at the beginning (0 min) and under the insulin stimulation at the end (120 min) of the clamp studies in rats fed a control diet (CD), a sucrose-rich diet (SRD) or SRD+chia seed (SRD+chia). (A) Immunoblot of GLUT-4 of gastrocnemius muscle from CD, SRD and SRD+chia. 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+chia 0 min; lane 6, SRD+chia 120 min. (B) Densitometric immunoblot analysis of GLUT-4 protein mass in gastrocnemius muscle of rats fed a CD, a SRD or SRD+chia at the beginning (0 min □) and at the end (120 min ■) of clamp studies. Values are mean, with their standard errors depicted by vertical bars (six animals per group) and expressed as percentage relative to the control diet at 0 min of the clamp. *$P < 0.05$ SRD at 120 min vs CD and SRD+chia at 120 min of the clamp. †$P < 0.05$ CD and SRD+chia at 120 min vs CD and SRD+chia al 0 min of the clamp.

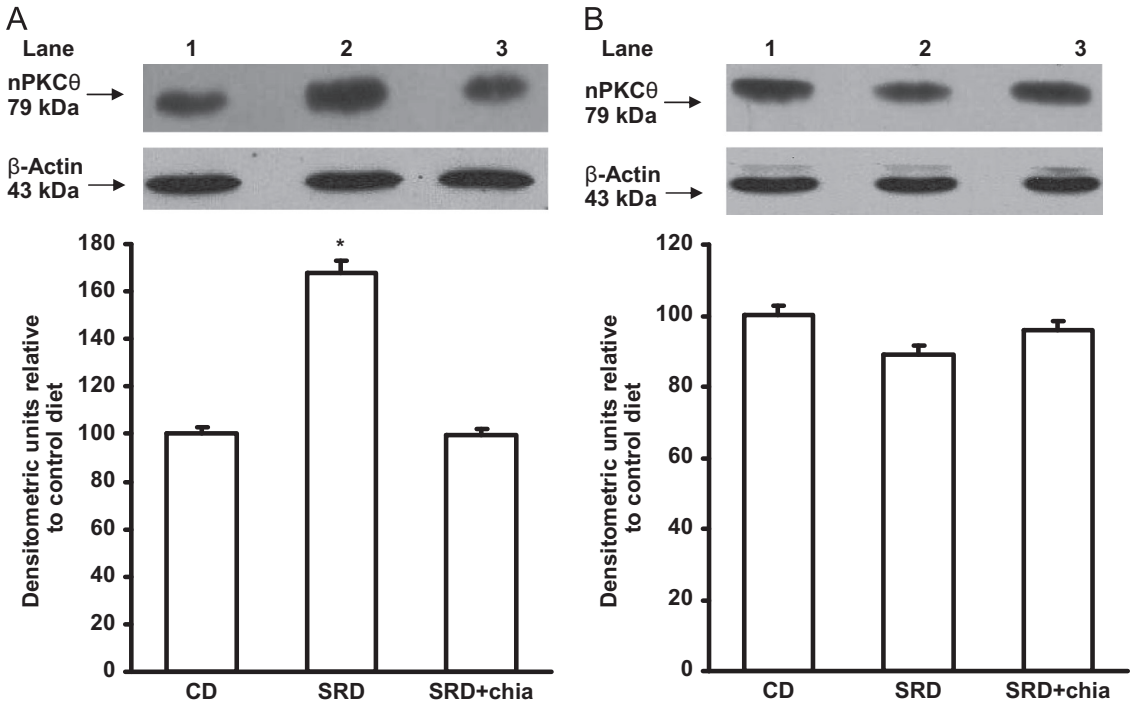

Fig. 3. Skeletal muscle protein mass level of nPKCθ membrane fraction (A) and cytosol fraction (B) in rats fed a control diet (CD), a sucrose-rich diet (SRD) or SRD+chia seed (SRD+chia). Upper part: immunoblots of nPKCθ membrane fraction (A) and cytosol fraction (B) in gastrocnemius muscle from CD, SRD and SRD+chia. Molecular marker is shown on the right. Lane 1, CD; lane 2, SRD; lane 3, SRD+chia. Lower part: densitometric immunoblots analysis of nPKCθ protein mass membrane fraction (A) and cytosol fraction (B) in gastrocnemius muscle of rats fed a CD, a SRD and SRD+chia. Values are mean, with their standard errors depicted by vertical bars (six animals per group) and expressed as percentage relative to the control diet. *$P < 0.05$ SRD vs CD and SRD+chia.

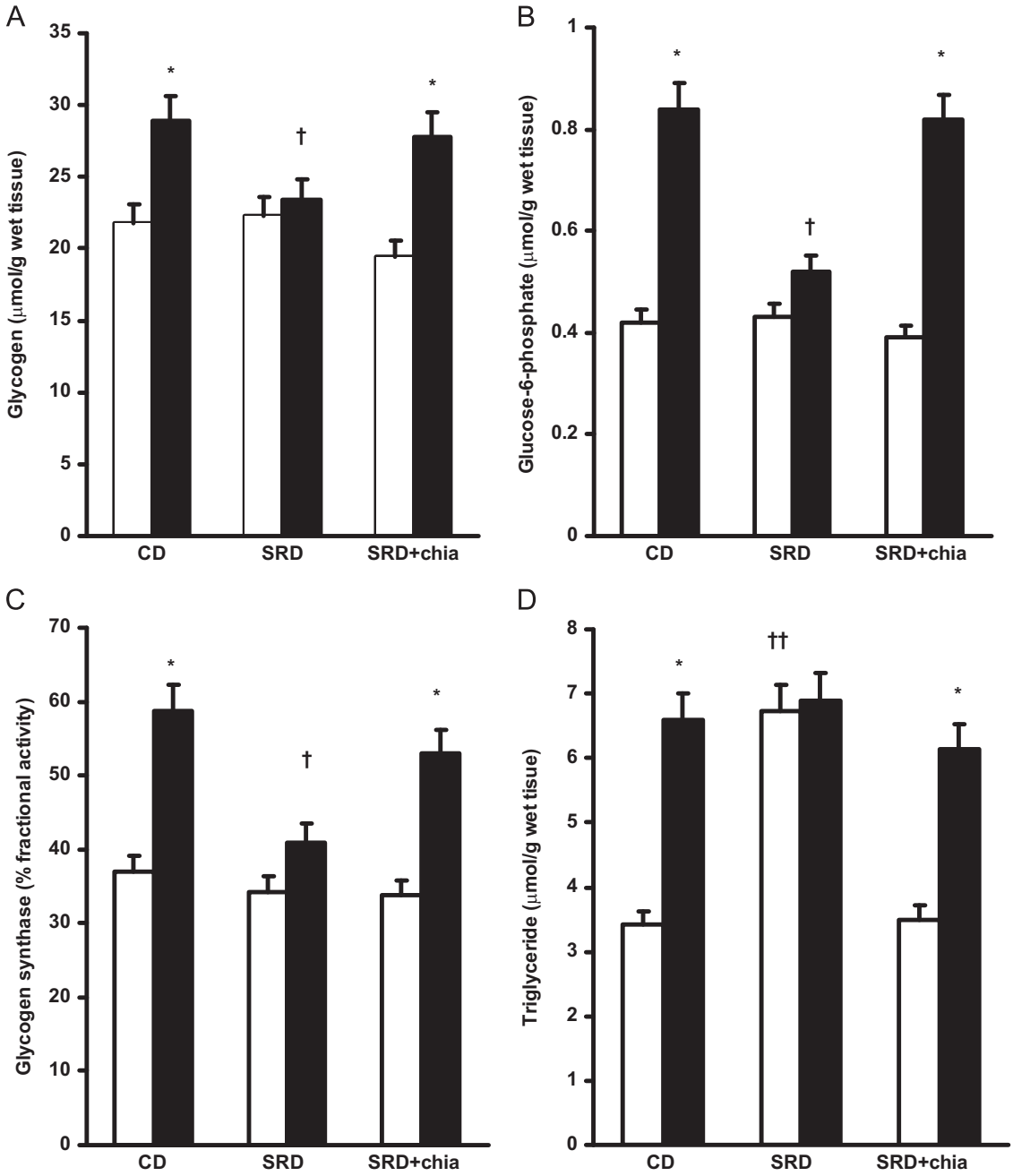

Fig. 5. Glycogen (A), glucose-6-phosphate (B) levels, glycogen synthase activity (C) and triglyceride (D) concentration in the gastrocnemius muscle at the beginning (0 min □) and the end (120 min ■) of the clamp studies in rats fed a control diet (CD), a sucrose-rich diet (SRD) or SRD+chia seed (SRD+chia). Values are expressed as mean, at the 0 min and at the 120 min of the clamp, with their standard errors depicted by vertical bars (six animals per group). *$P < 0.05$ CD and SRD+chia at 120 vs 0 min of the clamp, †$P < 0.05$ SRD at 120 min vs CD and SRD+chia at 120 min of the clamp, ††$P < 0.05$ SRD at 0 min vs CD and SRD+chia at 0 min of the clamp.

in rats fed a SRD+chia (Fig. 3A). No significant changes in the nPKCθ protein mass levels in the cytosol fraction were observed among the three dietary groups (Fig. 3B).

3.6. GLUT-4 protein mass level, glycogen, G-6-P, TG concentrations and GSa activity at the beginning and at the end of the clamp studies in the gastrocnemius muscle

The protein mass level of GLUT-4 at the beginning and the end of the clamp studies is depicted in Fig. 4. The immunoblotting of the gastrocnemius muscle revealed a single 45 kDa band consistent with GLUT-4. Each gel containing an equal number of samples from rats fed a CD, SRD and SRD+chia at the beginning (0 min) and at the end (120 min) of the euglycemic–hyperinsulinemic clamp (Fig. 4A). After the densitometry of immunoblots, the GLUT-4 of the CD group at the beginning of the clamp was normalized to 100% and both SRD and SRD+chia at the beginning as well as the three dietary groups at the end of the study were expressed relative to this. At the beginning of the clamp, the quantitative and qualitative analyses of the Western blot showed no differences in the relative abundance of the total plasma membrane of GLUT-4 protein among all dietary groups. As expected and previously demonstrated [24], under insulin stimulation the translocation of GLUT-4 to the plasma membrane significantly increased in CD-fed rats, while the increase of plasma membrane GLUT-4 was lower (16%) in the SRD-fed group under the same experimental conditions. The present results show that by shifting the source of fat in the diet to chia seed in the SRD-fed group, the GLUT-4 protein

mass significantly increased (50%), reaching values higher than those recorded in the rats fed a CD (Fig. 4B). Moreover, dietary chia seed reversed the impaired insulin stimulated glycogen storage and G-6-P concentration as well as GSa activity observed in the SRD-fed group during the euglycemic–hyperinsulinemic clamp (Fig. 5A–C). Besides, at the end of the clamp study, muscle TG levels reached similar values in all dietary groups but the increase of TG in the CD and SRD+chia groups was approximately 91% and 82%, respectively compared to the values recorded at the beginning of the clamp (Fig. 5D).

4. Discussion

The present study provides new information on the mechanisms behind the beneficial effects of dietary chia seed to reverse or improve the preexistent morphological and metabolical abnormalities of adipose tissue and the impaired skeletal muscle lipid and glucose metabolism, which developed in rats fed a SRD during six months. Expanding our previous findings the major new results from this investigation are the following: In adipose tissue dietary chia seed (i) markedly reduced visceral adiposity and the hypertrophy of epididymal fat cells, (ii) reduced the enhanced basal lipolysis and normalized both isoproterenol-stimulated lipolysis and the anti-lipolytic action of insulin, and (iii) normalized or improved the enhanced activities of the key enzymes involved in the "de novo" lipogenesis recorded in the fat pad of the SRD fed rats. In the gastrocnemius muscle, chia seed: normalized the lipid storage and both the altered glucose phosphorylation and glucose oxidation observed in the SRD-fed rats, decreasing the protein mass level of nPKCθ in the membrane fraction of the skeletal muscle of rats fed a SRD. In addition, chia seeds reversed the impaired insulin-stimulated glucose transporter (GLUT-4), GSa activity, G-6-P and glycogen levels during the euglycemic–hyperinsulinemic clamp and normalized dyslipidemia and peripheral insulin insensitivity. All the changes mentioned above were obtained by shifting the source of fat in the sucrose-rich diet from corn oil to chia seed during 3 months.

Several studies in rodents have shown that the consumption of diets rich in sucrose, fructose or fat induces adipocyte hypertrophy with increased TG storage and dysfunction [17,30,33,34]. Confirming previous results and in agreement with the aforementioned studies, the present work shows that rats fed a sucrose-rich diet for a long term developed accretion of fat pad weight, adipocyte hypertrophy with abnormal cell size distribution, and increased activities of several enzymes involved in "de novo" lipogenesis. In addition, isolated adipocyte from epididymal fat pad showed an increase of basal lipolysis and a substantial reduction of the anti-lipolytic action of insulin [17,20,30]. On the other hand, several studies [35–37] over the last decade were aimed to understanding the mechanisms of action of long-chain *n*-3 PUFA (mainly EPA and DHA) in reducing adiposity and the altered adipose tissue function. In this regard, studies in rodents indicate a complex modulation of gene expression in white adipose tissue by long-chain *n*-3 PUFA [38] suggesting a decrease in lipogenesis and fatty acid release from the adipocyte and enhanced mitochondrial oxidative capacity, glucose uptake and mitochondrial biogenesis [39]. An increase in cellular *n*-3 PUFA has also resulted from ingestion of vegetable sources rich in ALA, such as *S. hispanica* L (chia) seed and flax seed-oil [8,40]. However, the mechanism of action of ALA on adipose tissue fat deposition is not completely understood. Baranowski et al. [41] demonstrated that dietary interventions with flax seed oil in obese Zucker rats reduced adipocyte hypertrophy and the adipose tissue protein levels of several inflammatory markers and Okuno et al. [42] observed that perrilla oil prevents the excessive growth of adipose tissue in rats, at least in part suppressing the late phase of adipocyte differentiation. Regarding dietary chia seed, Poudyal et al. [8] showed that chia seed supplementation during eight weeks reduced the visceral adiposity index induced in rats fed a high fat–sucrose diet. Moreover, the fatty acid profile of retroperitoneal adipose tissue shows an increase of ALA and the *n*-3 to *n*-6 ratio [8].

Extending the above observations the present work shows that the replacement of corn oil by chia seed in the SRD-fed rats decreases visceral fat pad mass and reduces epididymal adipocyte hypertrophy, improving their altered cell size distribution.

The balance between fat synthesis and breakdown determines adipocyte fat accumulation. The release of fatty acids from the adipocyte depends on the rate of lipolysis. This rate is mediated by both the activities of ATGL and HSL that are the major enzyme contributing to TG breakdown since they are responsible for more than 95% of the TG hydrolase activity present in murine white adipose tissue [31,32]. Although in the isolated adipocyte we only estimated the lipolytic activities of ATGL and HSL through the levels of glycerol release and not by the activity of these enzymes, our study shows that dietary chia seed completely normalized both basal and stimulated lipolysis. Both parameters reached values similar to those recorded in the CD group.

The administration of dietary chia seed significantly decreased the enhanced enzymatic activities of FAS, ACC, G-6-PDH and ME shown in the adipose tissue of SRD. Thus, reducing lipogenesis could contribute to the decrease of adipocyte cell volume and epididymal fat pad hypertrophy. Recently, in rats fed a diet containing 0.095–6.3% of ALA and constant *n*-6 PUFA levels Muhlhausler et al. [43] showed that increasing dietary ALA content resulted in altered expression of FAS and glycerol-3-phosphate dehydrogenase mRNA in adipose tissue depot. The mRNA expression of the key lipogenic transcription factor SREBP-1c was inversely related to the concentration of ALA, EPA and 22:5 *n*-3 docosapentaenoic acids and positively correlated to *n*-6 PUFA levels. Besides, adipose tissue ACC and FAS mRNA expression were suppressed by feeding rats a diet enriched in *n*-3 PUFA [44].

Confirming our previous studies [30], hypertrophy adipocyte from the SRD-fed rats are resistant to the anti-lipolytic effect of insulin. The present data shows that dietary chia seed was able to correct the inhibitory effect of high sucrose diet upon the anti-lipolytic action of insulin. This was associated with both a reduction of adipocyte cell size and the fatty acid influx through the circulation (plasma FFA and TG reached normal values). Moreover, as mentioned in the introduction, in the SRD-fed rats we have recently demonstrated [12] that dietary chia seed normalized hepatic lipogenesis and increased fatty acid oxidation by shifting the balance of fatty acid metabolism toward oxidation rather than storage. This in turn normalized hepatic steatosis, dyslipidemia and whole body peripheral insulin resistance. The above mechanisms could contribute to normalize/improve adipose tissue dysfunction.

Besides, whole chia seed also contains a high percentage of soluble fiber, proteins and minerals and we cannot discard the possibility of their contribution to both the decrease of dyslipidemia and improvement of insulin resistance.

Interestingly, the effect of chia seed on fat deposition does not result from reduction of food intake since the energy intake of either SRD or SRD+chia groups was similar. However, dietary chia seed significantly decreased the visceral adiposity index, reducing the epididymal, retroperitoneal and omental tissue weights. The reduction of retroperitoneal tissue weight would indicate an important reduction of body lipid. Newby et al. [45] demonstrated in Wistar rats a marked association between the growth of the retroperitoneal tissue and the accretion of body lipids. In this regard, carcass analysis of rats fed a SRD+chia shows a significant reduction of fat content and an increases of water content compared to those observed in the SRD fed rats (data not shown).

Moreover, we cannot discard the possibility that the oxidative capacity of adipose tissue could be increased after chia administration since we were unaware of data regarding the effect of dietary chia seed on protein content and /or gene expression of mitochondrial enzymes and uncoupling protein.

On the other hand, confirming previous publications [23,24], the present data show in the skeletal muscle of SRD-fed rats an increase of lipid storage and the protein mass level of nPKCθ in the membrane fraction. Besides, an altered glucose phosphorylation and oxidation was accompanied by impaired insulin-stimulated GSa activity, glycogen and G-6-P concentration and the translocation of GLUT-4 from the intracellular pool to the plasma membrane. In the present study, the metabolic shift induced by dietary chia seed is reflected in the skeletal muscle of the SRD group by a reduction of the accumulation of fatty acids derivatives (e.g. TG, LCA-CoA, DAG) and normalization of the increased nPKCθ protein mass levels in the membrane fraction. Recent studies have given strong evidence of the fact that the ability of fatty acids to interfere with insulin signaling and glucose transport into muscle correlates with the generation of fatty acid metabolites such as fatty acyl-CoA and DAG [46].

The present data shows that the significant reduction of lipid storage within the skeletal muscle of SRD-fed rats by dietary chia seed would allow both the reversal of the altered insulin-stimulated cell surface recruitment of GLUT-4, GSa activity and the increase of glycogen and G-6-P concentration under the stimulus of the hormone. Furthermore, the decrease capacity of glucose phosphorylation and glucose oxidation were normalized after chia seed administration. This is the first study to analyze the underlying possible mechanism/s involved in the effect of chia seed on reversing or improving insulin action in the skeletal muscle of the SRD-fed rat model. Our results suggest that this effect could be related to the reduction of the availability of plasma TG and FFA that in turn decreases lipotoxicity and normalizes glucose homeostasis. Besides, it could also be the result of a subsequent change in fatty acid content in membrane phospholipids in the skeletal muscle due to both the absolute and relative amount of LA (18:2 *n*-6) and ALA in the diet. Changes in membrane fluidity or in the DAG signaling function could influence insulin secretion and its biological activity [47]. On this regard, Poudyal et al. [48,49], in rats fed a high fat–sucrose diet supplemented with either chia oil or chia seed, showed that the fatty acid profile of skeletal muscle had a significant increase of *n*-3 PUFA (C18:3 *n*-3; C22:5 *n*-3 and C22:6 *n*-3) increasing the *n*-3/*n*-6 ratio. We have recently demonstrated significant increases of ALA, EPA, 22:5 *n*-3, and DHA levels and the *n*-3/*n*-6 ratio in the plasma of rats fed a SRD in which chia seed replaced corn oil as the source of dietary fat [11]. However, in the current study we did not evaluate the hepatic bioconversion of ALA to EPA, 22:5 *n*-3 and DHA, their accumulation in tissues and the fatty acid composition of adipose tissue and skeletal muscle phospholipids. This is a limitation of the present study since changes in their profile could also contribute to the normalization of peripheral insulin insensitivity.

In brief, this study provides new data regarding the beneficial effect of dietary chia seed on improving morphological and metabolic aspects involved in the adipose tissue dysfunction and the mechanism/s underlying the impaired glucose and lipid metabolism in the skeletal muscle of a dyslipidemic insulin-resistance rat model. Finally, these results warrant further human-subject research on the use of chia seed as a complementary nutrient for treating some signs of the metabolic syndrome, especially considering the few studies on humans published so far.

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