Prostaglandins, Leukotrienes and Essential Fatty Acids 88 (2013) 171–177

Contents lists available at SciVerse ScienceDirect

Prostaglandins, Leukotrienes and Essential Fatty Acids

journal homepage: www.elsevier.com/locate/plefa

Fish oil reverses the altered glucose transporter, phosphorylation, insulin receptor substrate-1 protein level and lipid contents in the skeletal muscle of sucrose-rich diet fed rats

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ARTICLE INFO

Article history:
Received 21 August 2012
Received in revised form
25 October 2012
Accepted 6 November 2012

Keywords:
Insulin action
Dyslipidemia
Polyunsaturated fatty acids
Skeletal muscle

ABSTRACT

The role and underlying mechanisms by which $n-3$ polyunsaturated fatty acids (PUFA) prevent/reverse SRD-induced insulin resistance (IR) in the muscle are not completely understood. Therefore, we examined: triglyceride, diacylglycerol, PKCθ, Glut-4, enzymatic hexokinase activity, IRS-1 protein mass level, and fatty acid composition of muscle phospholipids. Rats were fed a SRD during 6 months. Thereafter, half the animals continued with SRD up to 8 months; the other half was fed a SRD in which CO (8% wt/wt) was replaced by FO (7%+1% CO) for 2 months. Results were compared with those obtained in rats fed a control diet (CD). In SRD-fed rats, FO oil normalized/improved lipid storage and PKCθ protein mass level. Effects of insulin were comparable with those of CD-fed rats. FO reversed impaired glucose phosphorylation, IRS-1, and, under insulin stimulation, Glut-4 protein mass level. FO normalized insulin resistance and increased $n-3$ PUFAs in muscle phospholipids.

1. Introduction

An impairment of insulin action (insulin resistance) is involved in many diseases, including non-insulin-dependent diabetes mellitus, obesity and Syndrome X [1]. In addition to insulin resistance, disorders in lipid metabolism and the excess delivery of fat to peripheral tissues underlie most of these prevalent diseases affecting mankind.

Skeletal muscle is considered as one of the primary tissues in glucose homeostasis. Nowadays, it is well established that an increase of lipid intermediates such as fatty acyl-CoA, ceramides and diacylglycerol (DAG) in muscle not only correlates with insulin resistance, but also directly and indirectly alters insulin signaling [2]. The insulin receptor substrate-1 (IRS-1)/phosphatidyl inositol 3 kinase (PI3K) association induced by insulin is necessary to elicit many of the insulin effects on glucose and lipid metabolism.

In this regard, dietary factors play an important role in the development of insulin resistance. Defects in the early steps of insulin signaling (insulin receptor (IR), IRS-1 and PI3K activity) and glucose transporter 4 (Glut-4) activation in peripheral tissues have been reported during a high-fat diet [3,4].

Moreover, Bezerra et al. [5] reported a significant reduction of tyrosine phosphorylation of IRS-1 after insulin stimulation in rats fed a high fructose diet for a short period of time (4 weeks). Besides, D'Alessandro et al. [6] showed a substantial increase of triglyceride (TG), long-chain acyl-CoA (LC ACoA) and DAG concentrations in the gastrocnemius muscle of rats fed a sucrose-rich diet (SRD) for a long term (6 months). This was accompanied by an altered non oxidative and oxidative glucose pathway and an increase of novel protein kinase Cθ (*n*PKCθ) protein mass level in the membrane fraction.

On the other hand, the dietary intake of marine polyunsaturated fatty acids (PUFAs) [fish oil (FO) rich in 20:5 $n-3$ and 22:6 $n-3$ fatty acids] has proven effective in preventing the onset of dyslipidemia, insulin resistance, hepatic steatosis as well as impaired glucose tolerance in rats fed a high fat or sucrose/fructose diet [7]. In the skeletal muscle of rats fed a high fat diet, Taouis et al. [4] showed that the partial substitution of $n-6$ for $n-3$ PUFAs was able to maintain IR, IRS-1 tyrosine phosphorylation, PI3K activity and the total amount of Glut-4. However, Corporeau et al. [8] reported a decrease of muscle PI3K activity in rats fed a low amount of FO in a normolipidic diet. A previous investigation from our group [7] on rats chronically fed (6 months) a SRD demonstrated that the partial substitution in dietary fat (from corn oil (CO) to FO) led to a normalization of the preexisting state of dyslipidemia, and whole-body insulin insensitivity. In addition, dietary fish oil was able to reverse the increased lipid content and the protein mass level of PKCθ in the skeletal muscle of this dietary group [7]. Besides, increasing evidence suggests that the fatty acid composition of membrane phospholipids in the skeletal muscle and other target tissues is a

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http://dx.doi.org/10.1016/j.plefa.2012.11.003

critical factor that may induce changes in the structure and fluidity of cell membranes that could, in turn, directly affect insulin action [9].

The underlying mechanisms sustaining the possible beneficial effects of $n-3$ PUFAs on insulin action in the skeletal muscle still remain unclear. Therefore, from the above information we hypothesized that the partial replacement of CO by FO as a dietary source of fat in rats fed a SRD could affect some mechanisms in their skeletal muscle, namely glucose transport and phosphorylation, changes in lipid content, the early proximal insulin signaling step, and the fatty acid composition of muscle phospholipids. These mechanisms could collectively contribute to the normalization of the whole body insulin insensitivity observed in this dyslipidemic insulin resistant rat model. To test this hypothesis we analyzed the effect of FO on: (i) TG and DAG concentrations and the protein mass level of PKCθ, (ii) glucose uptake and phosphorylation, estimated by the protein mass level of GLUT-4 and the enzymatic activity of hexokinase, respectively, (iii) the protein mass level of IRS-1. This analysis was performed in the gastrocnemius muscle of rats chronically (8 months) fed a SRD, at basal state and under a euglycemic-hyperinsulinemic clamp. Besides, since $n-3$ PUFA might improve insulin sensitivity through a relative increase in the unsaturation of membrane phospholipids, the fatty acid composition of muscle phospholipids was also examined.

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 under controlled temperature (22 °C ± 1), humidity, and airflow conditions, with a fixed 12-hour light-dark cycle (light, 0700 h–1900 h). They were initially fed a standard non-purified diet (Ralston Purina, St Louis, Mo). After 1 week of acclimatization, they were randomly divided into 2 groups: control and experimental. The experimental group received a SRD containing by weight (g/100 g): 62.5 sucrose, 8 CO. The control group received the same semisynthetic diet but with sucrose replaced by corn starch (62.5%, high-starch diet, control diet (CD)). Details of the diet composition are shown in Table 1. The experimental protocol was approved by the Human and Animal Research Investigation Committee of the School of Biochemistry, University of Litoral (Santa Fe, Argentina).

2.2. Experimental design

Rats in the experimental group received the SRD for 6 months, period after which the animals were randomly divided into two subgroups. The first subgroup continued on the SRD up to 8 months. The second subgroup (SRD + FO) received a SRD in which the source of fat (CO 8% w/w) had been replaced by FO (7% of cod liver oil containing approximately 850 U/g vitamin A and 85 U/g vitamin D and 0.6 mg/g cholesterol) plus CO 1% w/w from month 6 to 8. The control group received the CD throughout the experimental 8-month period (Table 1). The SRD without the addition of FO used from month 6 to 8 and the CD were balanced for cholesterol and vitamins D and A present in the FO. Diets were isoenergetic (3.89 kcal/g of food) and were available ad libitum. Diets were prepared every day as previously described [10]. Details of the dietary fatty acid composition of each diet are shown in Table 2. The weight of each animal was recorded twice a week throughout the experimental period. 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 8-month dietary period, except as otherwise indicated, the experiments were performed at basal state (between 800 and 1000 h).

2.3. Analytical methods

Rats were anesthetized with intraperitoneal pentobarbital sodium (60 mg/kg body weight). Blood samples were obtained from the jugular vein, rapidly centrifuged, and serum was either immediately assayed or stored at −20 °C. Serum TG, free fatty acids (FFA) and glucose levels were measured by spectrophotometric methods as previously described [11]. The immunoreactive insulin assay was calibrated against a rat insulin standard (Novo, Nordisk, Copenhagen, Denmark) and the serum levels were determined, as previously described [12]. The gastrocnemius muscle was rapidly removed, frozen, clamped in liquid nitrogen and stored at −80 °C. The homogenates of frozen muscle powder were used for the determination of hexokinase activity, as previously described [6] and the IRS-1 protein mass level as described below. Gastrocnemius lipids were extracted according to the procedure described by Folch et al. [13] and total phospholipids separated by thin layer chromatography. Samples were esterified with boron trifluoride at 64 °C for 3 h and the fatty acid composition of total phospholipids was determined by gas liquid chromatography of their methyl esters as previously described [14].

Table 1
Composition of experimental diets[a].

Diet ingredients	Control diet (CD)		Sucrose-rich diet (SRD)		SRD + fish oil (FO)	
	% By weight	% Of calories	% By weight	% Of calories	% By weight	% Of calories
Casein free vitamin	17.0	17.5	17.0	17.5	17.0	17.5
Salt mix[b]	3.5		3.5		3.5	
Vitamin mix[c]	1.0		1.0		1.0	
Choline chloride	0.2		0.2		0.2	
Methionine	0.3		0.3		0.3	
Cellulose	7.5		7.5		7.5	
Corn-starch	62.5	64.0				
Sucrose			62.5	64.0	62.5	64.0
Corn oil	8.0	18.5	8.0	18.5	1.0	2.3
Fish oil					7.0	16.2

[a] Diets are based on AIN-93M diet.
[b] Salt mix is based on salt mix AIN-93M (in g/kg of mix): calcium carbonate, 357.0; potassium phosphate (monobasic) 250.0; sodium chloride, 74.0; potassium sulfate, 46.6; potassium citrate, tri-potassium (monohydrate) 28.0; magnesium oxide, 24.0; ferric citrate, 6.06; zinc carbonate, 1.65; manganese carbonate, 0.63; cupric carbonate, 0.30; potassium iodate, 0.01; sodium selenate, 0.01025; ammonium paramolybdate, 0.00795; chromium potassium sulfate, 0.2174.
[c] Vitamin mix is based on vitamin mix AIN-93M (in g/kg of mix): niacin 3.00; calcium pantothenate, 1.60; pyridoxine HCl, 0.70; thiamin HCl, 0.60; riboflavin, 0.60; folic acid, 0.20; D-biotin, 0,02; vitamin B-12, 2.5; vitamin E (500 IU/g), 15.00; vitamin A (500,000 IU/g) 0.80; vitamin D3 (400,000 IU/g), 0.25; vitamin K, 0.075.

Table 2
Fatty acid composition of the fat source included in each experimental diet.

Fatty acids	CO[a]	CO + FO[b]	FO[c]
	g/100 g Total fatty acids		
14:0	Traces	4.1	4.7
16:0	10.4	12.8	12.8
16:1 $n-7$		9.5	10.8
18:0	2.6	2.8	3.0
18:1 $n-9$	32.1	26.0	25.0
18:2 $n-6$	51.5	9.0	2.6
18:3 $n-3$	0.4		Traces
20:0	0.4	0.9	0.9
20:1 $n-9$	1.6	12.0	13.7
20:4 $n-3$		3.1	3.5
20:5 $n-3$		9.0	10.3
22:5 $n-3$		0.5	0.6
22:6 $n-3$		8.1	9.3
Total			
Saturated	13.4	20.6	21.4
Monounsaturated	33.7	47.5	49.5
Polyunsaturated			
$n-6$	51.50	9.00	2.60
$n-3$	0.40	20.70	23.70
P/S	3.87	1.44	1.23
$n-3/n-6$	0.008	2.30	9.11

Other minor fatty acids have been excluded.

[a] Corn oil (CO), mazola (Best Foods Canada Starch, Montreal, Quebec, Canada).
[b] Includes 1% CO plus 7% FO.
[c] FO: cod liver oil (ICN Biomedical, Costa Mesa, CA).

2.4. Determination of Glut-4 and PKCθ protein levels, TG and DAG contents (clamp studies)

Whole body peripheral insulin sensitivity was measured using the euglycemic hyperinsulinemic clamp technique as described elsewhere [15]. Briefly, after 5 h of food deprivation, 12 rats from each dietary group were anesthetized. The gastrocnemius muscle of 6 rats from each group was rapidly removed (starting clamp values), clamped in liquid nitrogen and stored at −80 °C. In the other 6 rats from each dietary group, an infusion of highly purified porcine neutral insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark) was administered at 0.8 units/kgxh for 120 min. Glycemia was maintained at euglucemic level by infusing glucose (200 g/L) at a variable time. 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 the gastrocnemius muscle was rapidly removed. The protein mass levels of Glut-4 and PKCθ as well as TG and DAG levels were determined at the beginning and at the end of the clamp. Briefly, gastrocnemius muscle homogenates were prepared for the isolation of cytosol and membrane fraction of PKCθ and Glut-4 protein mass levels, as previously described [6]. Total protein samples were resolved on SDS–PAGE and transferred to PVDF membranes. The membranes were probed with specific antibody (polyclonal goat anti Glut-4 and anti-rabbit anti specific PKCθ from Santa Cruz Biotechnology, Inc, Santa Cruz, CA). The blots were incubated with horseradish peroxidase linked secondary antibody followed by chemiluminiscence detection according to the manufacturer's instructions (SuperSignal West Pico Chemiluminescent 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 PKCθ density was corrected for variation in extraction between samples using a ratio of dry weight post-extraction to wet weight pre-extraction. The relationship between the amount of sample subjected to immunoblotting and the signal intensity observed was linear under the conditions described above. TG and DAG were measured as described elsewhere [6].

2.5. Western blot analysis of IRS-1

Animals were killed before 1000. To minimize differences in euthanization time between treatments, three rats were randomly chosen in the three different dietary groups and were killed simultaneously. Frozen gastrocnemius muscle powder was homogenized and centrifuged at 16000 g for 20 min to remove insoluble materials [5]. Total protein samples (100 μg) (Bio-Rad Protein Assay) were resolved on SDS-PAGE for IRS-1 and transferred to PVDF membranes. The membranes were probed with specified antibodies (rabbit polyclonal antibody anti-IRS-1 from Santa Cruz Biotechnology, Inc, Santa Cruz, CA). The blots were then incubated with horseradish peroxidase-linked secondary antibody followed by chemiluminescence detection according to the manufacturer's instructions mentioned above. The IRS-1 was normalized to β actin. The intensity of the bands was quantified by the NIH imaging software. The relationship between the amount of sample subjected to immunoblotting and the signal intensity observed was linear under the conditions described above.

2.6. Statistical analyses

Sample size was calculated on the basis of measurements previously made in our laboratory with rats fed either a control diet or a SRD considering an 80% power [10,11,15] as described by Glantz [16]. Results are expressed as mean ± SEM. The statistical comparison was 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 the Newman–Keuls' test [17]. Differences having values lower than 0.05 were considered to be statistically significant. In all cases, the interclass correlation coefficients were at least 0.73 (SPSS 15.0 for Windows, SPSS INC. Chicago, Illinois).

3. Results

3.1. Body weight, weight gain, energy intake, serum metabolites, insulin levels and glucose infusion rate (GIR)

Body weight and energy intake were carefully monitored in all groups of rats throughout the experimental period. Similarly, as previously shown [18], an increase ($P < .05$) in body weight (18%) and energy intake was present in rats chronically fed a SRD for 8 months compared with CD. The presence of FO from month 6 to 8 in the SRD diet did not modify body weight. Despite a similar energy intake between SRD and SRD + FO fed rats at 8 months, the body weight gain was moderately decreased in the latter group (Table 3).

In agreement with previous reports [18] and confirmed by the present findings, the high serum levels of TG, FFA, and glucose reported in rats fed the SRD returned to control values when FO replaced CO in the diet during the last 2 months of the experimental period. Moreover, insulin levels did not differ in any of the animal groups (Table 3). Besides, whole body peripheral insulin sensitivity determined by the euglycemic-hyperinsulinemic clamp (GIR) that was significantly lower in the SRD fed rats returned to normal after FO administration (Table 3).

3.2. Muscle TG, DAG levels and PKCθ protein mass level at the beginning and end of clamp studies

Compared to CD-fed rats, the gastrocnemius muscle of SRD-fed rats showed a significant increase ($P < .05$) of TG and DAG levels

Table 3
Body weight, energy intake, serum metabolites, insulin levels and glucose infusion rate (GIR) in rats fed a control (CD), sucrose-rich (SRD) or a SRD+fish oil (FO)[a].

Diets		CD	SRD	SRD+FO
Body weight at 8 months (g)	(8)	442.5 ± 10.0[b]	513.1 ± 19.0[a]	487.3 ± 6.4[a]
Body weight gain (month 6 to 8) (g)	(8)	34.9 ± 5.5[a]	33.5 ± 6.1[a]	15.0 ± 5.1[b]
Energy intake (month 6–8) (kcal/d)	(8)	66.3 ± 2.5[b]	82.0 ± 4.4[a]	77.8 ± 2.7[a]
Triglyceride[b] (mmol/L)	(6)	0.64 ± 0.04[b]	2.23 ± 0.10[a]	0.60 ± 0.06[b]
FFA[b] (μmol/L)	(6)	287.2 ± 18.0[b]	794.0 ± 30.0[a]	295.1 ± 38.0[b]
Glucose[b] (mmol/L)	(6)	6.40 ± 0.12[b]	8.18 ± 0.10[a]	6.52 ± 0.20[b]
Insulin[b] (pmol/L)	(6)	373.0 ± 21.0	365.0 ± 27.0	372.0 ± 28.0
GIR[c] (mg/kgxmin)	(6)	10.0 ± 0.15[a]	4.5 ± 0.40[b]	9.2 ± 0.53[a]

[a] Values are expressed as mean ± SEM, () number of rats. Values in a line that do not share the same superscript letter are significantly different $P < .05$ when one variable at a time was compared by the Newman Keuls' test.
[b] Values corresponding to the basal state (see Section 2).
[c] Values corresponding to the euglucemic-hyperinsulinemic clamp study (see Section 2).

Table 4
Triglyceride (TG) and diacylglycerol (DAG) contents and protein mass levels of PKCθ in the gastrocnemius muscle of rats fed a control (CD), sucrose-rich (SRD) or SRD+fish oil (FO) at the beginning (0 min) and at the end (120 min) of the euglycemic-hyperinsulinemic clamp[a].

Diets		CD	SRD	SRD+FO
TG (μmol/g wet weight)	Beginning End	3.26 ± 0.46[b] 6.98 ± 0.50[a]	7.03 ± 0.40[a] 7.80 ± 0.45[a]	3.22 ± 0.30[b] 7.40 ± 0.50[a]
DAG (nmol/g wet weight)	Beginning End	112.0 ± 7.7[c] 99.0 ± 8.3[b]	178.4 ± 8.5[a] 199.2 ± 22.3[a]	131.0 ± 3.6[b] 118.0 ± 5.1[b]
PKCθ membrane[b]	Beginning End	100.0 ± 3.0[c] 91.2 ± 3.2[b]	172.4 ± 8.0[a] 126.4 ± 2.6[a]	127.3 ± 4.2[b] 97.1 ± 6.2[b]

[a] Values are expressed as mean ± SEM, $n = 6$. Values in a line that do not share the same superscript letter are significantly different $P < .05$ when one variable at a time was compared by the Newman Keuls' test.
[b] After densitometry of immunoblots, the PKCθ of CD group at the beginning of the clamp was normalized at 100% and both SRD and SRD+FO 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. Dietary FO in the SRD was able to normalize the TG concentration and significantly reduce the DAG levels (Table 4).

At the end of the clamp study, muscle TG levels reached similar values in all groups but the increases of TG in the CD and SRD+FO groups were approximately 114% and 130%, respectively, compared with the values recorded at the beginning of the clamp, whereas in the SRD group the increase was only 11%. At the end of the clamp, muscle DAG levels decreased similarly (10%) in both the CD and SRD+FO groups while in the SRD fed rats DAG levels increased 12% as compared with levels recorded at the beginning of the clamp (Table 4).

The quantitative and qualitative analysis of Western blots showed that the relative abundance of the PKCθ isozyme was significantly increased ($P < .05$) in the membrane fraction of the gastrocnemius muscle of the SRD fed rats at the beginning of the clamp. However, the protein mass of PKCθ (membrane fraction) of rats fed a SRD+FO significantly decreased ($P < .05$), although values did not reach those of the rats fed a CD. The relative abundance of PKCθ in the membrane fraction at the end of the clamp study was significantly reduced ($P < .05$) although values in the SRD group were still above those observed in the control (CD) and SRD+FO groups. The relative abundance of PKCθ in the cytosol fraction was as follows: mean ± SEM, $n = 6$, at the beginning of the clamp: CD: 100 ± 3; SRD: 85 ± 7; SRD+FO: 94.85 ± 1.7, p NS, at the end of the clamp: CD: 100 ± 2.5; SRD: 129 ± 6.4; SRD+FO: 117.5 ± 3.1 ($P < .05$, CD vs SRD and SRD+FO).

3.3. Protein mass level of Glut-4 at the beginning and end of clamp studies

The immunoblotting of the gastrocnemius muscle revealed a single 45 kDa band consistent with Glut-4. Each gel contained equal number of samples from rats fed a CD, SRD and SRD+FO at the beginning (0 min) and at the end (120 min) of the euglycemic–hyperinsulinemic clamp (Fig. 1A). 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+FO at the start as well as the three dietary groups at the end of the study were expressed relative to this. At the start of the clamp, the qualitative and quantitative analysis of the Western blot showed no differences in the relative abundance of the total plasma membrane of Glut-4 protein between the three dietary groups. As expected, 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 protein mass levels was lower (22%) in the SRD fed group under the same experimental conditions. By shifting the source of fat in the diet to FO, the Glut-4 protein mass significantly increased (70%), reaching values above those recorded in the rats fed a CD (Fig. 1B).

On the other hand, the hexokinase activity, which was significantly lower in the skeletal muscle of SRD fed rats, returned to values similar to those observed in the CD fed group after FO administration. Values were as follows: (mean ± SEM; $n = 6$, pkat/mg protein: CD: 771.82 ± 45.01; SRD: 568.44 ± 35.00; SRD+FO: 708.50 ± 48.00 ($P < .05$, SRD vs CD and SRD+FO).

3.4. Protein mass level of IRS-1

The immunoblotting of the gastrocnemius muscle revealed a single 175 kDa band consistent with IRS-1. Each gel contained an equal number of samples from the CD, SRD and SRD+FO groups (Fig. 2A). After the densitometry of immunoblots, the IRS-1 of the CD group was normalized to 100% and the level of IRS-1 from the SRD and SRD+FO was expressed relative to this. The qualitative and quantitative analysis of the Western blot showed that the relative abundance of IRS-1 at basal conditions (without insulin stimulation) was significantly decreased ($P < .05$) in the muscle of the SRD group compared with rats fed a CD (Fig. 2B). The addition of FO to the SRD-fed rats significantly increased ($P < .05$) the protein mass level of IRS-1, which reached values similar to those recorded in the CD fed rats.

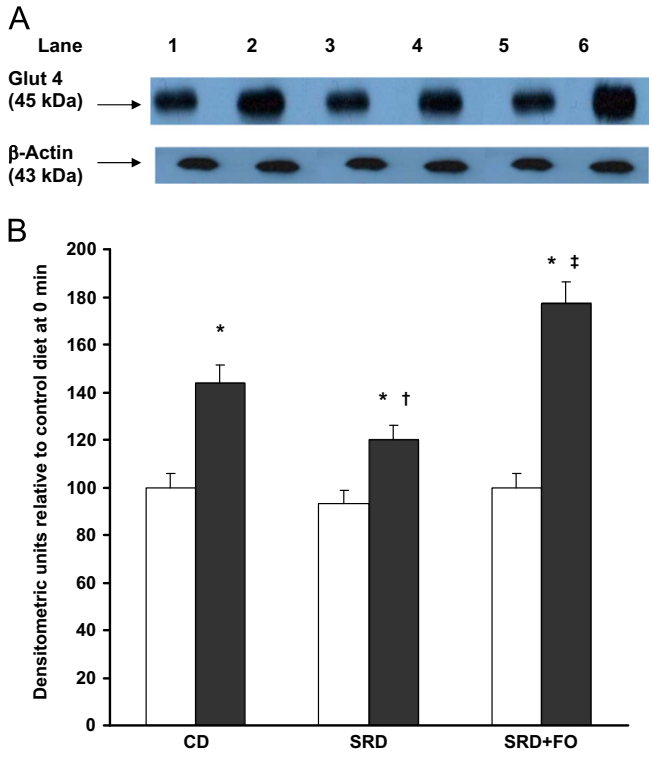

Fig. 1. . Skeletal muscle protein mass level of Glut-4 at the beginning (0 min) and under the insulin stimulation at the end (120 min) of the clamp studies in rats fed a control diet (CD), a sucrose-rich diet (SRD), or the SRD+fish oil (SRD+FO). (A) Immunoblot of Glut-4 of skeletal muscle from the CD, SRD and SRD+FO. 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+FO 0 min; lane 6, SRD+FO 120 min. (B) Densitometric immunoblot analysis of Glut 4 protein mass in skeletal muscle of rats fed a CD, SRD or SRD+FO at the beginning and at the end of clamp studies. Values are expressed as mean ± SEM, $n=6$, 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+FO rats at 120 min of the clamp vs CD, SRD and SRD+FO rats at 0 min of the clamp; $P<.05$.†Mean values were significantly different from those of the SRD rats at 120 min of the clamp vs CD and SRD+FO rats at 120 min of the clamp; $P<.05$.‡Mean values were significantly different from those of the SRD+FO rats at 120 min of the clamp vs CD at 120 min of the clamp; $P<.05$.

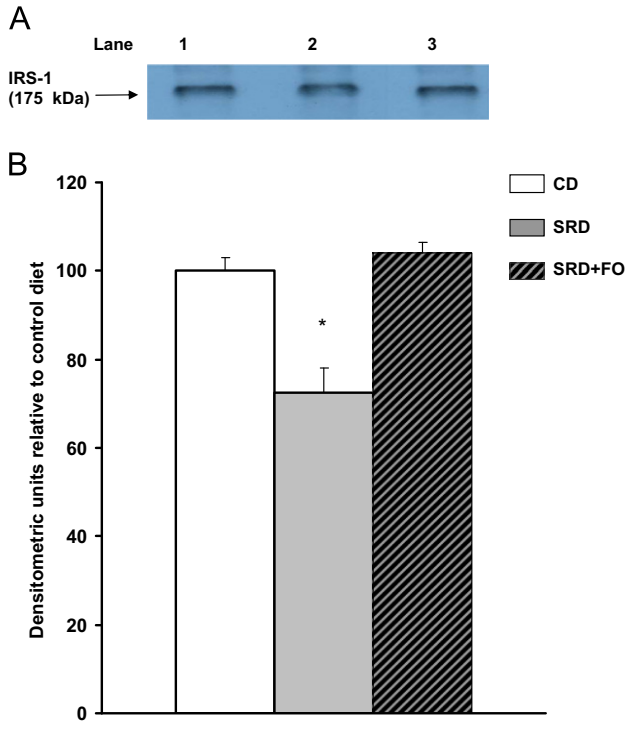

Fig. 2. Skeletal muscle protein mass level of IRS-1 of rats fed a control (CD), sucrose-rich (SRD) or SRD+fish oil (FO) diet. (A) Immnunoblots of gastrocnemius muscle of IRS-1 from CD, SRD and SRD+FO. Molecular marker is shown on the right. Lane 1, CD; lane 2, SRD and lane 3 SRD+FO. (B) Densitometric immunoblot analysis of IRS-1 protein mass in gastrocnemius muscle of rats fed a CD, SRD and SRD+FO. Values are expressed as mean ± SEM, $n=6$ and expressed as percentage relative to CD.*Mean values were significantly different to CD and SRD+FO; $P<.05$.

3.5. Fatty acid content of muscle membrane phospholipids

Table 5 shows the muscle fatty acid composition of phospholipids and the ratios of $n-3$ PUFAs to total fatty acids, $n-3$ to $n-6$ PUFAs and $n-3$ PUFAs to total saturated fatty acids. It can be noticed that SRD fed rats showed a decrease in the three ratios mentioned above as compared with the CD fed group. As expected, the muscle phospholipids of the SRD+FO group were enriched with $n-3$ PUFAs. In these rats, the ratios of $n-3$/total fatty acids; $n-3/n-6$ and $n-3$/total saturated fatty acids were significantly increased reaching values higher than those recorded in the CD fed group.

Table 5
Fatty acid composition of muscle phospholipids in rats fed a control (CD), sucrose-rich (SRD) or a SRD+fish oil (FO)[a].

Fatty acids	CD	SRD	SRD+FO
16:0	23.75 ± 1.24	29.22 ± 3.23	26.52 ± 1.37
16:1 $n-7$	0.44 ± 0.15[b]	1.47 ± 0.28[a]	1.40 ± 0.31[a]
18:0	15.60 ± 1.08[a]	14.14 ± 1.13[a b]	12.54 ± 0.27[b]
18:1 $n-9$	5.35 ± 0.94	5.53 ± 0.29	5.00 ± 0.28
18:1 $n-7$	2.05 ± 0.11[b]	3.52 ± 0.40[a]	3.13 ± 0.76[a]
18:2 $n-6$	18.59 ± 1.08[a]	14.78 ± 1.18[b]	15.49 ± 0.27[b]
20:4 $n-6$	18.83 ± 2.09[a]	20.14 ± 1.45[a]	10.79 ± 0.61[b]
20:5 $n-3$	–	–	2.08 ± 0.06
22:4 $n-6$	0.74 ± 0.05[b]	1.28 ± 0.14[a]	0.09 ± 0.08[c]
22:5 $n-6$	0.52 ± 0.06[b]	3.81 ± 0.62[a]	0.56 ± 0.05[b]
22:5 $n-3$	1.86 ± 0.23[b]	1.25 ± 0.27[b]	2.80 ± 0.17[a]
22:6 $n-3$	11.76 ± 0.60[b]	4.33 ± 0.89[c]	19.05 ± 1.59[a]
Total			
Saturated	39.33 ± 1.16	43.36 ± 2.18	39.06 ± 0.82
Monounsaturated	7.84 ± 0.40[b]	10.52 ± 0.32[a]	9.53 ± 0.49[a]
Polyunsaturated	52.30 ± 0.62[a]	45.59 ± 0.70[c]	50.86 ± 0.32[b]
$n-6$	38.68 ± 0.81[a]	40.01 ± 0.88[a]	26.93 ± 0.20[b]
$n-3$	13.62 ± 0.44[b]	5.58 ± 0.58[c]	23.93 ± 0.61[a]
$n-3$/Total fatty acids	0.14 ± 0.01[b]	0.06 ± 0.01[c]	0.24 ± 0.04[a]
$n-3/n-6$	0.35 ± 0.02[b]	0.14 ± 0.01[c]	0.88 ± 0.04[a]
$n-3$/Total saturated acid	0.35 ± 0.02[b]	0.13 ± 0.01[c]	0.61 ± 0.02[a]

Only the main fatty acids are tabulated. Other fatty acids make 100%.

[a] Values are expressed as means ± SEM, $n=6$. Values in a line that do not share the same superscript letter are significantly different $P<.05$ when one variable at a time was compared by the Newman Keuls' test.

4. Discussion and conclusion

The present study provides new information on the potential mechanisms involved in the beneficial effects of dietary $n-3$ PUFAs in improving insulin action in the skeletal muscle of rats rendered dyslipemic and insulin resistant by feeding them with a SRD for a long time (8 months). The major findings of this study were the following: (1) FO normalized or improved lipid storage as well as PKCθ protein mass level in the membrane fraction of the skeletal muscle of rats fed a SRD. In addition, under insulin stimulation (euglycemic-hyperinsulinemic clamp) the levels of TG, DAG and PKCθ reached values that did not differ from those observed in the control group fed a CD. (2) The impaired glucose phosphorylation (estimated by the activity of hexokinase) and

insulin stimulated glucose transporter (Glut-4) observed in the SRD fed rats were completely normalized by FO. (3) The presence of FO in the diet corrected the inhibitory effect of the high sucrose diet upon the relative abundance of IRS-1 without the stimulus of insulin (basal conditions). (4) FO reversed the impaired whole body insulin sensitivity observed in the SRD fed rats. This was accompanied by a significant incorporation of $n-3$ PUFAs into the phospholipids of skeletal muscle. All the changes mentioned above were obtained by shifting the source of fat in the sucrose-rich diet from CO to FO during two months.

Increased intramyocellular fat content and fatty acid metabolites (e.g., LC ACoA and DAG) are likely to play a pivotal role in the development of insulin resistance in the skeletal muscle. In this regard, DAG could have a detrimental effect on insulin signaling through its ability to activate PKC [19].

In the present work, the increase in intracellular TG, DAG and PKCθ protein mass level (membrane fraction) of rats fed a SRD was accompanied by a significant reduction of IRS-1 protein mass level, suggesting that a lower level of IRS-1 in the skeletal muscle could participate in the development of insulin resistance induced by high sucrose feeding. Bezerra et al. [5] reported no changes in basal (without the stimulus of insulin) IRS-1 levels in the skeletal muscle of rats fed a high fructose diet for a short period of time (4 weeks). However, the phosphorylation of IRS-1 induced by insulin was reduced. The present results extend those reported by Bezerra et al. [5] since when the SRD was administered for a long term (8 months) instead of 4 weeks, a reduction of skeletal muscle protein mass levels of IRS-1 was observed under basal conditions. This suggests that an increase in the length of time in which the SRD is consumed leads to a further deterioration in the early step of skeletal muscle insulin action. An increase in the proportion of membrane-localized PKCθ, which positively correlated with TG and DAG contents, was also found in red skeletal muscle from high-fat fed rats [20]. Moreover, decreased levels of IRS-1 in response to a high-fat-refined-sugar diet have been reported in rats [21].

Insulin stimulates the translocation of Glut-4 from an intracellular pool to the plasma membrane, increasing the rate of glucose uptake in insulin-responsive tissues, including skeletal muscle. In the present work, the basal level of the protein mass of Glut-4 in the skeletal muscle of SRD fed rats was similar to that recorded in the CD group, while an altered glucose phosphorylation was observed in this dietary group. Furthermore, insulin-stimulated cell surface recruitment of Glut-4 was reduced in the SRD fed rats as compared with control rats (22 vs 43% above basal level, respectively). In this regard, in mice fed a high-fat diet, Zierath et al. [22] reported that Glut-4 protein mass level in muscle was unaltered, while under insulin-stimulation the increase of Glut-4 protein mass was reduced by 50%. Moreover, a reduction of insulin-stimulated IRS-1-associated PI3K activity, protein kinase B activity and Glut-4 protein mass content has been reported after euglycemic-hyperinsulinemic clamp in rats fed a high-fat diet [23].

In the present study, dietary fish oil markedly reversed and or improved the increased lipid storage (TG and DAG) and the protein mass level of PKCθ recorded in the SRD fed rats. Values at basal level as well as under insulin stimulation were similar to those observed in the CD fed group. Interestingly, $n-3$ PUFAs increased fat oxidation in tissues including skeletal muscle [24]. In muscle homogenate of rats fed a 10% of FO, Herzberg et al. [25] showed that eicosapentaenoic acid (EPA) was oxidized faster than DHA. This explains the lower EPA relative to DHA content in their TG stores.

The replacement of CO by FO normalized Glut-4 transporter, glucose phosphorylation and the protein mass level of IRS-1. Moreover, as previously demonstrated [18], it reverses the low plasma adiponectin levels and the whole body peripheral insulin insensitivity. The possible mechanisms involved in the effect of dietary FO supplementation on insulin action in the skeletal muscle have not been completely clarified. In this regard, in rats fed a sucrose-rich diet containing 14% w/w of either FO or olive oil for a short period of time (3 weeks), Peyron-Caso et al. [26] showed that FO but not olive oil prevented hyperinsulinemia and hypertriglyceridemia and restored basal Glut-4 protein quantity in adipocyte. However, FO was unable to restore basal Glut-4 in muscle. It is possible that the discrepancy between our results and those of Peyron-Caso could be due at least in part to the length of the dietary interventions (3 weeks vs 2 months). On the other hand, Girón et al. [27] reported a lower plasma glucose level and an increase of Glut-4 protein and mRNA levels in the diaphragm of streptozotocin diabetic rats fed a 5% of FO as the only fat supplied during 5 weeks as compared with rats fed the same amount of either olive or sunflower oil. Moreover, insulin resistance as well as the decrease of Glut-4 content and PI3K activity could be prevented in rats fed a high fat diet when FO partially substituted safflower oil in the diet [4]. Besides, under the same experimental conditions, we have recently demonstrated that FO was able to inhibit hepatic lipogenesis through the up regulation of peroxisome proliferators -activated receptor-α and down regulation of liver X receptor-α, normalizing liver TG content and dyslipidemia as well as adipose tissue lipogenic enzyme activities and TG content, thus reducing adipocyte hypertrophy [28,11]. This metabolic switch induced by dietary FO leads to a reduction of the accumulation of fatty acid derivatives (e.g., TG, DAG, LC ACoA) and normalization of PKCθ protein mass level. Thus, in turn, it could play a role in the mechanisms involved in the improvement of the altered insulin signaling in the skeletal muscle of the SRD fed rats.

Several studies in rodents indicate that $n-3$ PUFAs potentially elicit a number of effects: they alter the gene expression, increase fatty acid oxidation, and reduce deposition in the adipose tissue. These effects can attenuate weight gain and decrease visceral fat without reduction of energy intake [24]. It is possible that the mechanisms mentioned above are involved in the reduction of body weight gain when FO replaces CO in rats chronically fed a SRD. This could also contribute to restoring insulin action.

The effects of dietary $n-3$ PUFAs could also be related to the subsequent changes in fatty acid content in membrane phospholipids of insulin target tissues. In this regard, Liu et al. [29] showed that dietary omega-3 and polyunsaturated fatty acids increase insulin binding to sarcolemma by changing the fatty acid composition of phospholipids surrounding the IR, suggesting that this might be the mechanism by which dietary fatty acids modify insulin action. In rats fed a high fat diet, Storlien et al. [9] showed a very close relationship between the percentage of long-chain $n-3$ fatty acids in skeletal muscle phospholipids and insulin-stimulated glucose metabolism, suggesting a role for this class of fatty acids in insulin action. In the present study, the consumption of dietary FO (16% of the total energy intake) during 2 months was sufficient to induce a marked increase in the incorporation of 20:5 $n-3$ and 22:6 $n-3$ fatty acids into the phospholipids of the skeletal muscle of SRD fed rats. These might be one of the mechanisms responsible for the improvement of insulin sensitivity since the alterations in membrane composition could affect IR and/or IRS-1 and PI3K expression and protein abundance.

In brief, the present results confirm our hypothesis showing that some of the possible mechanisms by which dietary FO was able to reverse the altered insulin action in the skeletal muscle of the dyslipemic insulin resistant rat model involved the improvement/normalization of glucose transport and phosphorylation, the early proximal insulin signaling IRS-1, lipid contents and a substantial incorporation of $n-3$ PUFAs in the fatty acid composition of skeletal muscle phospholipids. Finally, although caution is warranted before extrapolating from rodents to humans, this rat model proves to be useful to study the influence of nutrients

on the development and management of these metabolic diseases. Results from different recent human studies [30] suggest that the supplementation of $n-3$ PUFAs has clinical significance in the prevention and reversal of insulin resistance as well as in diminishing several risk factors of cardiovascular disease.

Acknowledgments

The authors are grateful to Prof. Rodolfo R. Brenner and Ana M. Bernasconi, INIBIOLP, Facultad de Ciencias Médicas, La Plata, Argentina for the evaluation of fatty acids composition of muscle phospholipids.

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