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Dietary fish oil positively regulates plasma leptin and adiponectin levels in sucrose-fed, insulin-resistant rats

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Rossi, Andrea S., Yolanda B. Lombardo, Jean-Marc Lacorte, Adriana G. Chicco, Christine Rouault, Gérard Slama, and Salwa W. Rizkalla. Dietary fish oil positively regulates plasma leptin and adiponectin levels in sucrose-fed, insulin-resistant rats. *Am J Physiol Regul Integr Comp Physiol* 289: R486–R494, 2005; doi:10.1152/ajpregu.00846.2004.—Insulin resistance and adiposity induced by a long-term sucrose-rich diet (SRD) in rats could be reversed by fish oil (FO). Regulation of plasma leptin and adiponectin levels, as well as their gene expression, by FO might be implicated in these findings. This study was designed to evaluate the long-term regulation of leptin and adiponectin by dietary FO in a dietary model of insulin resistance induced by long-term SRD in rats and to determine their impact on adiposity and insulin sensitivity. Rats were randomized to consume a control diet (CD; $n = 25$) or an SRD ($n = 50$) for 7 mo. Subsequently, the SRD-fed rats were randomized to consume SRD+FO or to continue on SRD for an additional 2 mo. Long-term SRD induced overweight and decreased both plasma leptin and adiponectin levels without change in gene expression. Dyslipidemia, adiposity, and insulin resistance accompanied these modifications. Shifting the source of fat to FO for 2 mo increased plasma levels of both adipokines, reversed insulin resistance and dyslipidemia, and improved adiposity. These results were not associated with modifications in gene expression. These results suggest that increasing both adipokines by dietary FO might play an essential role in the normalization of insulin resistance and adiposity in dietary-induced, insulin-resistant models.

adipokines; insulin resistance; sucrose-rich diet

ADIPOSE TISSUE SYNTHESIZES and secretes a large number of biologically active molecules [hormone-like peptides called adipokines (50): e.g., leptin, adiponectin, TNF-α, etc.]. Although leptin levels are often positively correlated with adiposity, adiponectin concentration is paradoxically decreased in obesity (2). Both adiponectin and leptin modulate various biological functions and could play an important role in lipid and glucose metabolism (4). Adiponectin is an insulin-sensitizing adipocyte-derived protein (21). The decrease of plasma adiponectin level in obesity and type 2 diabetes is involved in the development of insulin resistance (48). Decreased expression of adiponectin was shown to correlate with insulin resistance in rodents (53). Moreover, adiponectin is stimulated by thiazolidinedione agonists of peroxisome proliferator-activated receptor γ (PPARγ) and may contribute to increased insulin sensitivity (27, 20).

Several studies demonstrated that leptin regulates body weight by its central effect on food intake and energy expenditure (19). Peripheral effects of leptin have also been shown on insulin action in target organs, as well as on skeletal muscle free fatty acid (FFA) oxidation (56). Some investigators have documented dietary regulation of plasma leptin levels. Restriction and refeeding inversely regulate plasma leptin levels and ob gene expression in rodents and humans (28, 24). A high-fat diet increased ob gene expression in fat tissue of male Sprague-Dawley rats (29). This does not, however, prevent hyperphagia and obesity, suggesting that high-fat-fed rodents became resistant to leptin. In addition, Cha et al. (8) showed that fatty acid composition, independent of adipose tissue mass, is an important determinant of circulating leptin levels in diet-induced obesity.

Plasma leptin was also found to be influenced by the nature of dietary fatty acids. One of our groups recently (36) demonstrated that the presence of n-3 polyunsaturated fatty acids (in the form of fish oil) in the diet of SRD-fed rats prevented adiposity induced by 3 wk of sucrose feeding and increased plasma leptin levels. Short-term feeding (3 wk) with SRD was demonstrated to induce rapid hypertriglyceridemia and hyperinsulinemia, with slight hyperglycemia, but with no detected constant rise in body weight (36). When the SRD diet was extended to 30–40 wk, different morphological and metabolic changes emerged. The insulin-resistant rats became frankly heavy with high plasma glucose but without any change in circulating insulin levels. The biphasic pattern of glucose-stimulated insulin secretion from perfused islets was progressively deteriorated with a complete absence of the first peak and an increase in the second phase of insulin secretion by the end of 40 wk (9). This was accompanied by increased fat accumulation and decreased pyruvate dehydrogenase complex activity within pancreatic β cells (37). Interestingly, the addition of dietary fish oil for 2 mo normalized the long-term, preexistent, altered glucose-stimulated insulin secretion, as well as adiposity (37). There was also a normalization of intracellular lipids in the pancreatic cells that would have improved pancreatic function. The possible implication of leptin and adiponectin in the normalization of these long-term modifications, different from short-term modifications, are questioned. Therefore, the present study was designed to evaluate: *1)* whether leptin and adiponectin regulation (plasma levels and gene expression) contribute to modifications in

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whole body insulin resistance and adiposity induced by a 9-mo sucrose diet, and *2)* whether these changes could be either improved or corrected by 2 mo of dietary fish oil.

MATERIALS AND METHODS

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 ± 1°C), humidity and air flow condition, with a fixed 12:12-h light-dark cycle (light 0700 to 1900). They were initially fed a standard nonpurified diet containing by weight (g/100 g): 63 starch (corn, sorghum, wheat, oats, and barley), 22.5 protein, 3.5 fat, 6 fiber, 1 vitamin mixture and 4 salt mixture (Ralston Purina, St. Louis, MO). After 1 wk of acclimation, the rats were randomly divided into two groups. The experimental group ($n = 50$) received a purified SRD (62.5 g/100 g), while the control group ($n = 25$) received the same purified diet but with sucrose replaced by cornstarch (62.5 g/100 g) [high-starch diet (CD)]. The experimental group received the SRD for 7 mo after which the rats were randomly subdivided into two groups. The rats of the first subgroup continued the SRD up to 9 mo of feeding. The second subgroup (SRD+FO) received the SRD in which the source of fat (corn oil, 8 g/100 g) had been replaced by FO (7 g of cod liver oil/100 g plus 1 g/100 g of corn oil) from *months 7–9*; the control group received the CD throughout the experiment. Cod liver oil was purchased from ICN Biomedicals, Inc., Biomedical Research Products (Costa Mesa, CA). The composition of the diets is given in Table 1. The SRD without the addition of FO used from *months 7–9* and the CD were balanced for the cholesterol and vitamins D and A present in the FO. Diets were isoenergetic, providing ~16.3 kJ/g of food and were consumed by the rats ad libitum. Diets were prepared every day by adding the oils and base mixture containing the other nutrients. The oils and the base mixture were separately stored at 4°C until preparation of the diet. Dietary fats were analyzed by capillary GC, as previously described (45). The weight of each rat was recorded twice each week during the experimental period. In a separate experiment, the individual energy intakes and weight gains of eight rats in each group and subgroup were assessed twice each week. At the end of the 9-mo dietary period, except as otherwise indicated, food was removed at the end of the dark period (0700), and experiments were performed between 0900 and 1200. Rats were anesthetized with intraperitoneal pentobarbital sodium (60 mg/kg body wt). Blood samples were obtained from the jugular vein and were rapidly centrifuged; plasma was either immediately assayed or stored at −20°C. Epididymal and retroperitoneal adipose tissue were totally removed, weighed, immediately minced (to mix different parts of a pad), frozen in liquid nitrogen, and stored at −80°C. Frozen plasma and adipose tissue samples were then sent under special, strict frozen conditions to the French group of the study to perform the biological analysis of leptin and adiponectin in plasma, as well as the determination of gene expression.

The experimental protocol was approved by the Human and Animal Research Committee of the School of Biochemistry, University of Litoral, Santa Fe, Argentina.

Euglycemic clamp studies. Whole body peripheral insulin sensitivity was measured using the euglycemic hyperinsulinemic clamp technique, as previously described (9). Briefly, after 5 h of food deprivation, six rats from each dietary group were anesthetized with pentobarbital sodium (60 mg/kg body wt ip), and a blood sample was taken from which glucose and insulin levels were assessed. Afterward, an infusion of highly purified porcine neutral insulin (Actrapid; Novo Nordisk, Copenhagen, Denmark) was administered at 5.69 $nmol \cdot kg^{-1} \cdot h^{-1}$ for 2 h. Glycemia was maintained at an euglycemic level by infusing 200g/l of glucose at a variable rate. The blood glucose concentration was measured using a glucometer analyzer (Böhringer Mannheim, Indianapolis, IN) within 2 min after the samples were obtained. The glucose infusion rate (GIR) during the second hour of the clamp study was taken as the net steady state of the whole body glucose utilization. In all studies, blood samples (0.3 ml) for insulin determination were obtained at 60, 90, and 120 min. as previously described (9).

Preparation of isolated adipocytes. For the determination of fat cell size and number, the epididymal fat pads were rinsed in isotonic saline at 37°C. Adipocytes were isolated according to the method of Rodbell (40), with minor modifications as previously described (45, 46).

Determination of fat cell size and fat cell number. The microscopic method of Di Girolamo et al. (14) was used to measure cell diameters, as previously described (45, 46). For the estimation of the fat cell number, the lipid content of 100–200 mg of fat tissues was extracted by the method of Folch et al. (16). Total cell number in fat pad was calculated by dividing the fat pad lipid content by the mean cell lipid weight. The lipid weight of the average fat cell was calculated from the mean cell volume assuming a lipid density of 0.915 (triolein density).

Carcass composition. Six rats from each dietary group were anesthetized as mentioned above. Anesthetized rats were shaved, and the visceral organs were removed. Carcasses were weighed, placed in plastic bags, and frozen at −20°C. Each frozen carcass was ground to a homogeneous mixture with a mill cooled with liquid nitrogen, and the ground carcass was stored individually at −20°C. Carcass water was determined by drying ~10 g of sample in a drying oven (75–80°C) for 24 h (10). The weight differences before and after drying was used for its calculation. The dehydrated carcass sample was subsequently used for determination of ether-extractable fat. Nitrogen determination on 2 g of nondehydrated ground carcass was used to estimate protein content. Ash content was estimated by

Table 1. *Composition of experimental diets*

	CD		SRD		SRD+FO*	
Diet Ingredients	% 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†	3.5		3.5		3.5	
Vitamin mix‡	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

Diets are based on the AIN-93M diet. *The fish oil (FO) used is cod liver oil; †Salt mix (AIN-93M-Mx; Ref. 9), ‡Vitamin mix (AIN-93-Vx; Ref. 9). CD, control diet; SRD, sucrose-rich diet.

combustion of ~1 g of dehydrated ground carcass in a muffle furnace (10). All measurements were done in duplicate.

Total RNA preparation. Adipose tissue total RNA was prepared from fat tissue in the first and second set of experiments. Total RNA was extracted from adipose tissue samples after homogenization according to the manufacturer's instruction (RNA plus kit, Bioprobe system, Montreuil Sous-Bois, France). Concentration of each sample was assessed by absorbance measurement and its purity by the 260/280 ratio. Total RNA samples were stored at −80°C until quantification of the target mRNAs.

Relative-quantitative RT-PCR analysis. A real-time, two-step RT-PCR assay was developed for mRNA relative quantification. Gene-specific primers were designed by TIB Mol-Biol Syntheselabor (Berlin, Germany). The sequences of specific rat primers and probes used were as follows: ob forward, ATTTCACACACgCAgTCggTAT; ob reverse, gAAgCCCgggAATgAAgT; ob probe, 6FAM-CgCCAggCA-gAgggTCACCg XT—PH; Adiponectin (APM) forward, CCAAg-gAAACTTgTgCAggTT; APM reverse, CgCTCCTgTCATTC-CAgCA; APM probe, 6FAM-ATACCgggCCgTgATggCAgAgAT—TMR. First-strand cDNA was synthesized from 1 μg adipose tissue total RNA in 10-μl reaction volume using random hexamers and murine Maloney leukemia virus (M-MLV) reverse transcriptase, according to the M-MLV first-strand cDNA synthesis system protocol (Invitrogen Life Technology, Cergy Pontoise, France). Using LightCycler instrument (Roche, Indianapolis, IN), the cDNA was amplified using TaqMan probe approach in a glass capillary in a final volume of 10-μl reaction mix containing 2.5-μl reverse-transcribed RNA, 1x LightCycler-FastStart DNA Master Hybridization probes (Roche Diagnostics, Meylan, France), 1-μM forward and reverse primers, 0.2 μM of specific probe and 3 mM of $MgCl_2$. PCR was performed in 40 cycles with 480 s at 95°C (denaturation), 10 s at 95°C followed by 40 s at 60°C (amplification) and 60 s at 60°C (extension). The specificity of amplification was determined by melting curve analysis, and the amplification of both adiponectin and leptin showed only a peak in the analysis.

The quantification of 18S rRNA was used for sample normalization. 18S rRNA was quantified using also TaqMan approach. Briefly, 10 μl of PCR mix contain 2.5 μl of reverse-transcribed RNA, 1x LightCycler-FastStart DNA Master Hybridization probes (Roche), 1x 18 S primers and probe mix (part no. 4333760F, Applied Biosystems, Courtaboeuf, France), and 3 mM of $MgCl_2$. PCR was performed in 40 cycles with 10 s at 95°C (denaturation) and 40 s at 60°C (annealing and extension).

A pooled adipose tissue total RNA from the different experimental groups was used to make the standard curves. In summary, after RT, the cDNA was diluted into 10-fold serial dilution ($10^{-1} - 10^{-6}$) and amplified along with samples. By plotting, in arbitrary units, the 10-fold serial dilutions, quantitative data of samples were obtained with LightCycler software. The standard curve was also used to assess PCR efficiency by examining its slope that was consistently around −3.3 for each gene in the study. Each DNA was quantified in duplicate and sometimes in triplicate to increase accuracy. The average value for each sample was used for quantification. The variation in measurements for a target gene in each sample generally ranged from 1–10% in the study. The relative-quantitative data were expressed as the ratio of the level of adiponectin or leptin mRNA to that of 18S rRNA in arbitary units.

Analytical methods. Plasma triglycerides (25), FFA (51), and glucose (5) levels were determined by spectrophotometric methods. The immunoreactive insulin was measured by the method of Herbert et al. (22) The immunoreactive insulin assays were calibrated against rat insulin standard (Novo Nordisk). Plasma leptin and adiponectin were determined by radioimmunoassay (Linco, Clinisciencies, Montrouge, France). Triglyceride content was determined in homogenates of frozen liver powder by the method of Laurell (25).

Statistical analysis. Samples sizes were calculated on the basis of measurements made previously in our laboratory with rats fed either a CD or a SRD (9, 37, 45, 46), considering an 80% power. Results were expressed as means ± SE. Statistical comparisons were done transversely between different dietary groups at each time of the study (*month 7* and then at *month 9*). 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 mean by the Newman-Keuls test (44). Differences having P values lower than 0.05 were considered to be statistically significant. In all cases the intraclass correlation coefficients were at least 0.73.

RESULTS

Body weight gain and energy intake. Energy intake and body weight were carefully monitored in all groups of rats throughout the experimental period. As we have previously shown (9), a significant increase ($P < 0.05$) in body weight and energy intake occurred in rats fed a SRD during 7 mo compared with rats fed a CD. These differences were still present when the SRD was fed for 9 mo. The presence of FO in the SRD diet did not modify body weight of the SRD-fed rats. In spite of a similar energy intake between the SRD and SRD+FO-fed rats at 9 mo, the gain of weight was slightly less in the FO-fed rats (Δ: 9 − 7 mo) (Table 2).

Carcass composition. Rats fed 9 mo on SRD vs. CD showed an increase of carcass weight. An increase of fat content was found, while water content of the carcass was decreased. When the source of fat, corn oil, in the SRD was replaced by FO, carcass weight was slightly reduced, and the water content increased and reached values as in the CD-fed rats. Furthermore, the fat content was decreased ($P < 0.05$), although the lipid content was still above the values recorded in age-matched controls fed a CD (Table 3). Both the amount of protein and ash was similar in all dietary groups.

Plasma glucose, insulin and lipids, and liver triglyceride levels. At the end of the dark period (0700) plasma triglycerides, FFA, and glucose concentrations were higher in rats fed the SRD during 9 mo compared with age-matched controls fed a CD. However, a 9-mo SRD did not change plasma insulin

Table 2. *Body weight gain and energy intake in rats fed a control, sucrose-rich, or SRD+ fish oil diets*

Diet	Body Weight, g		Energy Intake, kJ/d	Diet	Body Weight, g	Weight gain, g	Energy Intake, kJ/d
	Initial	7 months	Initial to 7 months		9 months	*months 7–9*	*months 7–9*
CD(8)	183.2±6.1	408.2±20.1[b]	280.2±17.4[b]	CD (8)	442.5±10.2[b]	33.2±4.8[a]	278.6±10.4[b]
SRD(16)	187.5±7.5	475.4±9.8[a]	347.5±18.6[a]	SRD (8)	513.0±19.8[a]	31.0±4.7[a]	345.2±19.5[a]
				SRD+FO (8)	487.3±7.0[a]	16.3±4.9[b]	330.0±11.2[a]

Values are expressed as means ± SE. The numbers within parentheses indicate the number of animals included in each experimental group (see animals and diet in MATERIALS AND METHODS). Values in each 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. CD, control; SRD, sucrose-rich diet; SRD+FO, sucrose-rich diet + fish oil.

Table 3. *Carcass weight and composition of rats fed a CD, SRD, or SRD+FO*

	CD (9 months)	SRD (9 months)	SRD (6 months)+ FO (*months 7–9*)
Carcass, g	339.1±5.9[b]	410.0±19.9[a]	368.1±11.5[a]
Protein, % wet weight	20.6±0.3[a]	19.2±0.4[a]	19.2±0.5[a]
Fat, % wet weight	13.1±0.5[c]	24.6±1.7[a]	19.0±1.4[b]
Water, % wet weight	60.2±1.8[a]	54.5±1.4[b]	58.4±0.9[a]
Ash, % wet weight	4.1±0.2[a]	3.5±0.5[a]	3.5±0.2[a]

Values are expressed as means ± SE. Six animals were included in each experimental group. Values in each file 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.

Table 5. *Plasma leptin and adiponectin levels and glucose infusion rate in rats fed a CD, SRD, or SRD+ FO diets at the end of the nutritional period (9 months)*

Diet	Leptin, ng/ml	Adiponectin, μg/ml	GIR*, $mg \cdot kg^{-1} \cdot min^{-1}$
CD	18.70±3.39[a]	2.58±0.48[ab]	11.40±0.75[a]
SRD	9.04±1.01[b]	1.74±0.15[b]	4.52±0.80[b]
SRD+FO	17.56±3.13[a]	3.20±0.20[a]	12.45±0.84[a]

Values are expressed as means ± SE. Five animals were included in each experimental group. Values in each 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. *Steady state of blood glucose and insulin concentration during the last 60 min of the clamp were as follows (means ± SE, $n = 5$). Glucose (mM): CD, 5.70 ± 0.15; SRD, 5.80 ± 0.25; SRD+FO, 5.88 ± 0.20; insulin (pM): CD, 730 ± 45; SRD, 748 ± 30; SRD+FO, 735 ± 37. GIR, glucose infusion rate.

levels (Table 4), confirming recent reports from our laboratory (9). Plasma levels of the above metabolites were comparable in rats fed on SRD for 7 mo (data not shown). A complete normalization of all these variables occurred in rats fed a SRD+FO from the *month 7* to *month 9*. Furthermore, the enhanced liver triglyceride content in SRD-fed rats decreased when the diet was switched to SRD +FO (Table 4).

Plasma leptin and adiponectin levels. Plasma leptin significantly decreased ($P < 0.05$) and adiponectin slightly decreased ($P < 0.1$) after 9 mo on SRD diet. A significant increase in both plasma leptin ($P < 0.05$) and plasma adiponectin ($P < 0.05$) concentrations were recorded in the group of rats fed a SRD+FO compared with those fed a SRD for 9 mo (Table 5).

Whole body peripheral insulin sensitivity study. An euglycemic-hyperinsulinemic clamp was assessed at the end of the experimental period of 9 mo. Postprandial plasma glucose concentrations 5 h before the clamp were as follows: means ± SE ($n = 6$): CD-fed rats, 5.30 ± 0.08 mmol/l; SRD-fed rats, 7.85 ± 0.15 mmol/l; and SRD+FO fed rats, 5.38 ± 0.14 mmol/l. Plasma insulin levels were similar to those recorded at the end of the dark period (data not shown). The GIR was decreased ($P < 0.05$) in the SRD group compared with the 9-mo CD group. GIR returned to values similar to those recorded in rats fed a CD when FO replaced corn oil in the SRD from *months 7* to *9* (Table 5). There were no changes in hematocrit from the start to the end of the clamp (data not shown).

Fat pad morphology and triglyceride content. Table 6 shows that at the end of the 9 mo, epididymal and retroperitoneal adipose tissue weight of SRD-fed rats were increased compared with age-matched controls fed a CD. Epididymal adipocytes of the SRD-fed rats were almost twofold more voluminous, and a significant reduction of the adipose cell number, expressed by gram of tissue was observed. The triglyceride content in the SRD fat pads was higher than in rats fed the CD. The presence of FO in the diet decreased cell volume and triglyceride content, as well as epididymal total fat weight, and increased adipose cell number. These values, however, did not reach those recorded in CD-fed rats. Besides, a significant reduction of retroperitoneal total fat weight was also observed after fish oil administration.

Adipose tissue cell size distribution. Figure 1 shows the histograms of epididymal fat cell size distribution (at 2.5-μm intervals) at the end of the experimental period of 9 mo. In the SRD group, there was a clear differentiation in the cell size distribution with a significant increase (40%) of the mean cell diameter compared with the CD-fed animals. The addition of FO to the SRD under the present experimental conditions resulted in a reduction of the cell size diameter. In this group the cell size distribution approached that recorded in the CD group (Fig. 1). There were no differences in total cell number when expressed as total fat pads in all the dietary groups. Values were as follows: means ± SE ($n = 6$), number × 10^6/total weight: CD, 28.4 ± 2.7; SRD, 30.5 ± 2.9; and SRD+FO, 30.9 ± 3.2.

Quantification of gene expression. At a 10-fold serial dilution, all of the tested genes showed good amplification plots starting from 20 pg and yielded standard curves with a correlation coefficient higher than 0.99. Figure 2*A* demonstrates ob mRNA in adipose tissue (epididymal plus retroperitoneal) of the various dietary groups. Ob mRNA in both adipose tissues was not changed between the different groups. Figure 2*A* represents the mRNA levels of both adipose tissue sites. Adiponectin mRNA showed similar profile in both in epididymal and retroperitoneal adipose tissues. Thus there was no inhibitory effect of SRD on ob or adiponectin mRNA. The

Table 4. *Plasma metabolites and insulin concentration and liver triglyceride content in rats fed a CD, SRD, or SRD+ FO diets at the end of the nutritional period (9 months)*

Diet	Triglyceride, mmol/l	Free fatty acid, μmol/l	Glucose, mmol/l	Insulin, pmol/l	Liver triglyceride, μmol/g wet tissue
CD	0.51±0.09[b]	344.0±31.2[b]	6.50±0.08[b]	380.2±26.8[a]	12.0±0.7[b]
SRD	2.57±0.04[a]	884.5±52.4[a]	8.80±0.15[a]	368.0±40.5[a]	23.2±2.2[a]
SRD+FO	0.60±0.03[b]	330.0±44.3[b]	6.60±0.20[b]	375.0±29[a]	9.4±0.6[b]

Values are expressed as means ± SE. Six animals were included in each experimental group. Values in each 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.

Table 6. *Adipose tissue weight, cell volume and number, and triglyceride content of rats fed a CD, SRD, or SRD+ FO diets at the end of the nutritional period (9 months)*

	CD	SRD	SRD+FO
Epididymal fat			
Total weight, g	7.35±0.79[c]	14.40±0.92[a]	10.83±0.44[b]
Relative weight, g/100 g body weight	1.67±0.12[c]	2.91±0.19[a]	2.15±0.06[b]
Cell volume, pl	263.5±16.6[c]	497.2±11.2[a]	358.5±20.3[b]
Cell number, × 10^6/g tissue	4.00±0.20[a]	2.24±0.15[c]	3.09±0.12[b]
Triglyceride, nmol/cell	0.30±0.02[c]	0.59±0.04[a]	0.44±0.02[b]
Retroperitoneal fat			
Total weight, g	6.17±0.35[c]	13.75±1.03[a]	10.01±0.53[b]
Relative weight, g/100 g body weight	1.25±0.15[c]	2.77±0.20[a]	2.09±0.07[b]

Values are expressed as means ± SE. Six animals were included in each experimental group. 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.

presence of FO after the installation of insulin resistance induced by SRD (Fig.. 2*B*) had no additional effects. Therefore, Fig. 2*B* represents the adiponectin mRNA levels of both adipose tissues.

DISCUSSION

The present study demonstrated for the first time that long-term (9 mo) administration of a SRD in rats induced a decrease of plasma leptin and adiponectin levels. Moderate obesity evidenced by increased visceral fat mass, as well as carcass fat content, dyslipidemia, and insulin resistance accompanied these modifications. Moreover, shifting the source of fat in the SRD from corn oil to fish oil for 2 mo led to increased plasma adiponectin and leptin levels, improved the signs of adiposity, and reversed dyslipidemia and whole body peripheral insulin resistance. No changes in the gene expression of leptin and adiponectin in both epididymal and retroperitoneal fat tissues were recorded with either the absence or presence of fish oil in the diet.

Plasma leptin levels that reflect the sum of all peripheral leptin production correlates with body fat mass and adipocyte cell size in both lean and obese mice (17). White adipose tissue size increased with age and contributed to increased adiposity and elevation of plasma leptin levels (1). In the present study, however, the moderate increase of body weight, as well as adiposity and fat cell hypertrophy in the SRD-fed rats, did not

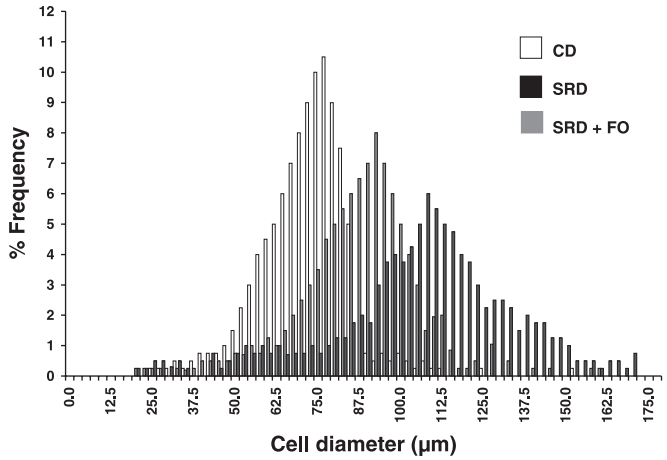

Fig. 1. Representative histogram distribution of adipocytes' mean diameters: Adipocytes were isolated from the epididymal depots of rats fed a control (CD), sucrose-rich (SRD), or sucrose-rich + fish oil (SRD+FO) diets at the end of the nutritional period (*month 9*). The histograms were constructed by sizing, at intervals of 2.5 μm, 100 adipocytes from each individual rat. Six animals were included in each experimental group. Columns represent the mean of the cells measured (in percentage) that falls within a given size indicated.

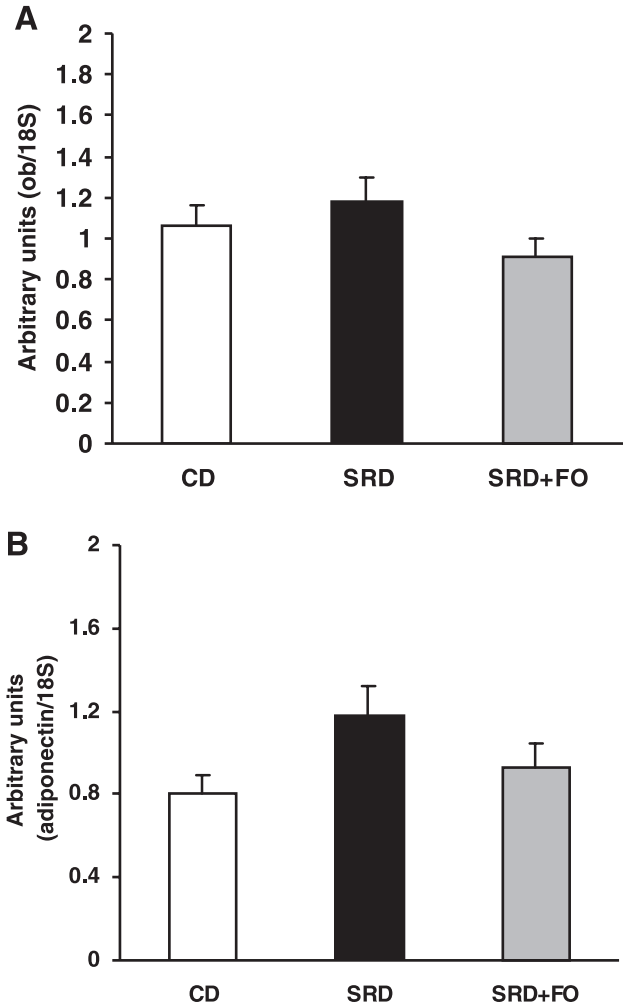

Fig. 2. White adipose tissue (WAT) (retroperitoneal and epididymal) ob and adiponectin mRNAs expression of rats fed a CD, SRD, or SRD+ FO diets at the end of the nutritional period (*month 9*). *A*: WAT ob mRNA expression. *B*: WAT adiponectin mRNA expression. Levels of mRNA were determined by real-time quantitative RT-PCR. The values represent means ± SE and are expressed as arbitrary units (ob/18S; adiponectin/18S). Five individual determinations were included in each experimental group.

positively correlate with plasma leptin level that even decreased. This dissociation between changes in plasma leptin, body weight, and adipose tissue mass, in this sucrose-fed insulin-resistant rat model, is present only after long-term sucrose feeding. Previously, we found that both 3- and 6-wk sucrose diets induced a parallel increase in both plasma leptin and adiposity (36). Contrary to these findings, increasing the length of sucrose feeding, as in the present study, gradually decreased the high leptin levels. Many factors are implicated in these results. This decrease in leptin levels could be the result of the chronic exposure to the elevated plasma FFA concentration, presented in this model. It has been recently shown that both protein and mRNA levels of leptin were decreased by triacin (42) and by long-chain monounsaturated and polyunsaturated fatty acids in cultured rats adipocytes (42, 7), suggesting that elevated plasma FFA could lead to a relative suppression of leptin by the adipose tissue. Cammisotto et al. (7) showed that an intracellular increase in fatty acids, generated because of activated lipolysis, inhibits leptin secretion. Consequently, they suggested that long-chain fatty acids might play an important role as messengers between the activation of lipolysis and the final inhibition of leptin secretion from white adipocytes. This is likely, as we previously found in younger rats that by increasing the length of sucrose feeding from 3 to 6 wk, leptin started to increase its accumulation within adipose tissue (36). Consequently, increasing the length of sucrose feeding might increase leptin accumulation within adipose tissue and decrease leptin secretion induced by insulin resistance of adipose tissue in this model (45, 34). An alternative mechanism of decreasing leptin levels in the long-term, sucrose-fed rats might be the marked hypertriglyceridemia in this model. Banks et al. (3) recently demonstrated that increasing plasma triglycerides inhibits leptin transport across the blood-brain barrier, thus inducing leptin resistance. Triglycerides might act directly on the leptin transporters or by binding leptin in the circulation. In the present study, leptin dosage in the circulation would be masked by the complex triglycerides/leptin and result, therefore, in low measured plasma leptin levels and leptin resistance. This leptin resistance might be the cause of increasing food intake and the subsequent increase in fat mass and body weight. Therefore, in the present study, long-term exposure of adipocytes to both triglycerides and FFA together with the long-term insulin resistance of these rats might be the main reasons for the low leptin levels in the long-term SRD-fed-rats.

Interestingly, the addition of dietary fish oil to the SRD for 2 mo increased plasma leptin levels but maintained a low amount total body fat. These data suggest, for the second time, a dissociation between changes in plasma leptin and adipose tissue mass. This is consistent with another study, done by our group, demonstrating increased plasma leptin levels after a 3-wk fish oil supplement in insulin resistant, sucrose-fed, rats (36). Increased plasma leptin concentration was similarly observed in normal rats fed 20% fish oil during 10 wk compared with rats fed beef tallow. These findings indicate that the nature of dietary fatty acids independent of adipose tissue mass, is an important determinant of circulating leptin levels in diet-induced obesity (8). Other studies (11, 39), however, found that fish oil feeding for either 3 wk or 3 mo might reduce leptin mRNA. These results do not contradict the present study, as only a slight, and nonsignificant, decrease in the leptin mRNA levels was found in the rats studied. These findings suggest that the increase in plasma leptin might be due to increased leptin secretion rather than increased synthesis.

Although sucrose-fed rats had leptin deficiency and possible leptin resistance, the increased plasma leptin levels (which remained within the range of the values of control rats) by fish oil administration, in the present study, could not be considered to be leptin resistance. These levels of leptin could help maintain body weight and limit the increase of some body fat stores, e.g., visceral fat pads and liver triglycerides, as well as total fat content in the SRD-fed rats. These data suggest that the effect of leptin on regulating body weight and fat mass cannot always be explained by food intake alone. Indeed, this regulation might be mediated by the ability of leptin to promote triglyceride hydrolysis and FFA oxidation and inhibits FFA synthesis (49, 38). Moreover, leptin effects on fat mass could also be mediated by increasing energy expenditure through enhanced thermogenesis in brown adipose tissue (13, 41).

The results of the present study emphasize that biological factors other than adipose tissue size are involved in determining leptin levels. Increasing whole body insulin sensitivity, as in the current study, as well as increasing glucose transport into adipocytes of SRD+FO-fed rats (34), might have a role in increasing plasma leptin levels. Nyholm et al. (33) demonstrated a positive relationship between glucose flux into adipocytes and circulating leptin levels. Moreover, lowering plasma triglycerides by FO administration might potentiate leptin secretion and leptin sensitivity by enhancing leptin transport across the blood-brain barrier (3). Similarly, the decrease in free fatty acids might relieve adipocytes from the inhibitory effects of free fatty acids (7). Another factor to be considered is a direct effect of the nature of plasma free fatty acids. Recently, we demonstrated that in vitro insulin-stimulated leptin secretion was increased by eicosapentaenoic acid (one of the constituents of FO) and inhibited by palmitoic acid in adipocyte cell culture (35). Therefore, fish oil could increase plasma leptin levels by a synergy between different possible direct and indirect mechanisms.

On the other hand, the increased leptin levels could be the cause and not the consequence of improving insulin resistance, as well as glucose and lipid homeostasis in the SRD+FO-fed rats. Recent reports suggest complex interactions between the leptin and insulin signaling pathways, which can potentially lead to differential modification of the metabolic effects of insulin exerted through insulin receptor substrates IRS1 and IRS2 (18). Consequently, this might further increase whole body insulin sensitivity via peripheral effects on insulin-responsive tissues such as the liver, muscle, and adipose tissue (56, 19). The increase of plasma leptin levels in the SRD+FO-fed rats might promote fatty acid oxidation and reduce ectopic fat accumulation in nonadipose tissues, thereby also increasing insulin sensitivity. Indeed, in the present study, this hypothesis is strengthened by the observed decrease in liver triglyceride content, carcass fat, as well as increased peripheral glucose utilization after switching to the FO diet. This effect might be mediated by activation of the AMP-activated kinase by leptin through a direct effect on certain skeletal muscles and indirectly through the hypothalamic-sympathetic nervous system axis (31). In this study, however, we could not ignore that FO could also increase fatty acid oxidation in the liver through its ability to function as a ligand activator of PPARα and thereby

induces the transcription of several gene-encoding proteins affiliated with fatty acid oxidation (32).

Plasma adiponectin values demonstrated a parallel regulation to that found for leptin: insulin resistance induced by long-term SRD was found to decrease adiponectin levels. This is consistent with many studies demonstrating a decrease in plasma adiponectin concentrations in insulin resistance and obesity (23, 52). Moreover, in the present work and for the first time, we found that fish oil was able to reverse the altered plasma adiponectin levels. We recently observed that simultaneous administration of fish oil and sucrose during a short period of time (3 wk) was able to prevent the decrease of plasma adiponectin levels induced by sucrose feeding (CD, 2.55 ± 0.49 μg/ml; SRD, 1.59 ± 0.18 μg/ml; SRD+FO, 3.11 ± 0.32 μg/ml, unpresented data). These results are in agreement with the demonstrated increase in plasma adiponectin with increasing insulin sensitivity by other agents as rosiglitazone treatment, but over longer periods (6 mo) (55). Similarly, some studies found that adiponectin expression in fat cells increases in parallel to increased insulin sensitivity and weight loss (54). In contrast to other studies (54, 30), these modifications in plasma levels were not associated with an increase in adiponectin mRNA expression. The mechanisms implicated in changes of adiponectin levels are still not clear. Studies in vivo indicated that fasting plasma insulin levels are negatively correlated with adiponectin plasma concentrations (55, 6). These findings are in accordance with in vitro results demonstrating that chronic treatment with insulin reduces adiponectin expression in 3T3-L1 adipocytes (15). Still, long sucrose feeding, as in the present study, was accompanied by normoinsulinemia. Therefore, insulin was not always correlated with adiponectin levels.

Alternatively, adiponectin concentration was suggested to be negatively correlated with both increased lipolysis and released FFA (12). Conversely, short-term infusion of FFA up to 7 days does not alter adiponectin levels (17). In the present study, however, after 9 mo of sucrose feeding, plasma FFA modifications were opposite to changes in adiponectin levels: an increase after the sucrose feeding and a decrease in the presence of FO in the diet. It is possible that in sucrose-fed rats, high plasma FFA levels might exert an inhibitory effect on the release of adiponectin in plasma and that the normalization of plasma FFA by the addition of fish oil to the diet might eliminate this inhibitory effect.

The effect of sucrose or fish oil feeding on fat cell size and thereby on adipocyte insulin sensitivity could also be another possible mechanism involved in plasma adiponectin regulation. We observed that the large fat cells in the sucrose-fed rats are less sensitive to the antilipolytic action of insulin, whereas smaller adipocytes, as in the group of rats fed SRD + fish oil, might be more sensitive (data not shown). Moreover, in previous studies, we demonstrated that adipocytes of 3-wk FO-fed rats are smaller and more sensitive to insulin stimulation than adipocytes of insulin-resistant, sucrose-fed rats (26, 34).

The results of the present study suggest the following scenario: First, long-term sucrose feeding induced high triglyceride and FFA levels that induce both insulin resistance and leptin deficiency and or resistance. These modifications stimulated food intake and adiposity. Second, fish oil supplementation decreased plasma FFA and triglyceride levels and might induce a selective modification in some adipose tissue: e.g., modulate the size of adipocytes thereby increasing their insulin sensitivity by other mechanisms. Consequently, the insulin-sensitizing adipocytes, in the absence of high FFA and triglyceride levels, secreted more leptin and adiponectin. These adipokines were then involved in increasing whole body insulin sensitivity.

Therefore, the normalization of dyslipidemia (plasma FFA and triglycerides) and insulin resistance induced by dietary FO might be implicated in increasing plasma leptin and adiponectin levels. Both adipokines might contribute to increased whole body insulin sensitivity. This is strengthened by the recent finding demonstrating that insulin resistance in lipoatrophic mice was completely reversed by the combination of physiological doses of adiponectin and leptin, but only partially by either leptin or adiponectin alone (53). This suggests that adiponectin and leptin may work hand-in-hand to sensitize peripheral tissues to insulin.

When looking to the comparative physiology of these systems in reference to humans, the results might be more or less relevant. Caution is warranted before extrapolating these results to humans. The amount of fish oil used in animal studies is quite higher (1.4 to 1.8 g fish oil/day for a body weight of 400 to 500 g, as in the present study) than those in human studies (6 g fish oil/day for a body weight of 80–95 kg). Indeed, the hypotriglyceridemic effect of fish oil has been found in both animal and human studies. For the meantime, however, the positive effect of fish oil on improving insulin resistance in insulin-resistant animal models failed to be found after 2 mo of treatment compared with placebo in subjects with type 2 diabetes (43). This duration might be too short to demonstrate an effect on insulin resistance in humans. Recently, we found that 2 mo of treatment with fish oil is sufficient to induce a decrease in adiposity and adipocyte's size (43). Therefore, this is another point that is similar to results in animals. More studies are needed in humans with long-term treatment to identify and understand the relationship of fish oil intake to human physiology and health.

GRANTS

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