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Dietary fish oil ameliorates adipose tissue dysfunction in insulin-resistant rats fed a sucrose-rich diet improving oxidative stress, peroxisome proliferator-activated receptor γ and uncoupling protein 2.

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Abstract

This work aimed to assess the possible beneficial effects of dietary fish oil (FO) on the preexisting adipose tissue dysfunction through the improvement or reversion of the mechanisms underlying oxidative stress and pro-inflammatory cytokines in dyslipemic insulin–resistant rats. Wistar rats were fed a sucrose rich diet (SRD) for 6 months. After that half of the animals continued with the SRD until month 8 while in the other half corn oil was replaced by FO for 2 months (SRD+FO). A reference group consumed a control diet all the time. In epididymal fat pad we analyzed: antioxidant and oxidant enzymes activities; ROS content and glutathione redox state; the protein level of peroxisome proliferator-activated receptor gamma (PPARγ) and the expression and protein levels of uncoupling protein 2 (UCP2) as well as oxidative stress biomarkers and TNF-α and IL-6 plasma levels. Besides, insulin sensitivity and the composition of fatty acid phospholipids of adipose tissue were measured. Compared with the SRD the SRD+FO fed group showed a decrease of fat pad weight and the antioxidant and oxidant enzyme activities and ROS content returned to control values as well as plasma TNF-α and IL-6 levels. FO normalized both the decrease of PPARγ protein and the increase of protein and expression of UCP2. Furthermore, FO increased *n-3/n-6* fatty acids ratio in the adipose tissue phospholipids and normalized dyslipidemia and insulin resistance. Finally, these findings reinforce the view that dietary FO may exert a beneficial effect in ameliorating the dyslipidemia and insulin resistance in this animal model.

Introduction

Adipose Tissue is considered as a leading site affecting systemic insulin resistance, especially visceral adiposity which plays a key role in the Metabolic Syndrome (MS).[1,2] MS is nowadays considered as one of the important health problems of both developed and undeveloped countries. MS involves the clusters of metabolic abnormalities including among them: obesity, insulin resistance, abnormal glucose homeostasis, dyslipidemia, and hypertension, all major risk factors of cardiovascular disease and type 2 diabetes, associated with high rates of morbidity and mortality.[3] The hypertrophied adipocytes are accompanied by low-grade chronic inflammation and increased oxidative stress [4,5] since they are a significant source of reactive oxygen species (ROS).

Experimentally, our group and others have demonstrated that rats chronically fed a sucrose-/fructose-rich diet develop metabolic and physiological alterations mimicking several aspects of the phenotype of MS in humans.[6–8] In this regard, we have recently demonstrated that the depletion of antioxidant defenses and redox state, the susceptibility to lipid peroxidation, changes in inflammatory cytokines and visceral adiposity induced by a sucrose-rich diet (SRD) contribute to the dysregulation of adipose tissue function and insulin resistance in an animal model. [9,10]

In recent years, numerous research studies have focused on the study of nutrients with potential beneficial effects upon the adverse signs of the MS, among them the *n-3* polyunsaturated fatty acids (*n-3* PUFAs) from marine source (EPA 20:5,*n-3* and DHA 22:6,*n-3*) which are major modulators of many gene expressions of several key proteins involved in the lipid metabolism energy utilization and inflammation.[11,12]

Animal dietary intervention trials have demonstrated that *n-3* PUFAs limit the development of obesity, prevent insulin resistance, reduce hypertrophy and cellularity of adipose tissue and improve lipid and glucose metabolism in high-fat diet-fed rodents. [8,13–17] On the other hand, Da Silva *et. al.*[18] indicated that fish oils (rich in EPA and DHA) were effective to enhance the anti-inflammatory response and exert an antioxidant activity in rats fed a high-fat high-sucrose diet. Han *et al*.[19] showed that *n-3* PUFAS can modulate ROS production by impacting NADPH oxidase in differentiated 3T3-L1 adipocytes culture in high glucose and palmitate.

Rossi *et al*.[20,21] demonstrated that fish oil (FO) markedly reduced epididymal fat pad mass and restored the altered capacity of insulin-stimulated glucose uptake. Moreover, FO improved the antilipolytic action of insulin normalizing the whole body insulin resistance in dyslipemic insulin rats chronically fed a SRD.

However, only few studies have been published analyzing if dietary cod liver oil exerts their beneficial effects in the dysfunctional adipose tissue of this experimental rat model by improving/reversing the mechanisms underlying the oxidative stress and inflammatory cytokines. In an attempt to answer this question, this study aims to determine the following aspects in the epididymal fat pad: i) Antioxidant and oxidant enzymes activities, ROS content and glutathione redox state. ii) The protein levels of peroxisome proliferator-activated receptor gamma (PPARγ) -a nuclear factor that regulates adipose tissue lipid metabolism and inflammatory gene expression. iii) The expression and protein levels of uncoupling protein 2 (UCP2) which is highly expressed in white adipose tissue and function as a sensor of mitochondrial oxidative stress. iv) The plasma levels of oxidative stress biomarkers and pro-inflammatory cytokines (IL-6, TNF-α). Additionally, insulin sensitivity and the composition of fatty acid phospholipids of adipose tissue were measured.

This study was conducted in rats fed a SRD during eight months, in which a stable dyslipidemia, insulin resistance and disrupted adipose tissue function were present before the source of dietary fat, corn oil, was replaced by an isocaloric amount of cod liver oil for the last two months of the experimental period in half of the animals.

Methods

Animals

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 ± 1ºC), humidity and air flow conditions, with a fixed 12-h light/dark cycle (light on from 07.00 h to 19.00 h). Adequate measures were taken to minimize the pain or discomfort of the rats and we used the smallest number of animals possible. Animal experiments complied with the Guide for the Care and Use of Laboratory Animals (National Research Council of the National Academies, USA, 2011) and were evaluated and approved by the Human and Animal Research Investigation Committee of the School of Biochemistry, University of Litoral, Argentina (CONICET-PIP #0105/2010).

Experimental design

The rats (n= 60) were initially fed a standard non-purified diet (Ralston Purina, St Louis, MO, USA). After 1 week of acclimation period, the rats were randomly divided into two groups: control (n= 20) and experimental (n= 40) and were housed individually. The experimental group received a sucrose- rich diet (SRD) containing by weight (g/100 g): 62.5 sucrose, 8 corn oil (CO), 17casein. The control group received the same semisynthetic diet but with sucrose replaced by cornstarch (62.5 g/100g) [control diet (CD)]. Details of the diet composition are shown in Table 1. 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 g/100g) had been replaced by FO (7 g of cod liver oil per 100g) plus 1g/100g CO from months 6 to 8 (Table 1). The control group received the CD throughout the experimental 8-month period. 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 prepared every day by adding the oils to the base mixture containing the other nutrients. The food in the animal cages was shaded from light. The base mixture and FO were separately stored at 4°C and -20°C respectively until preparation of the diet. FO was kept under nitrogen atmosphere during storage. The fatty acid composition

of the fat source was analyzed by capillary GC, as previously described.[22] Details of the dietary fatty acid composition of each experimental diet are shown in Table 1. Diets were isoenergetic (16.3kJ/g of food) and were available ad libitum. The preparation and handling of the diets have been reported elsewhere.[23] The body 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 8-month dietary period, food was removed at 07.00 h (end of the dark period) and experiments were performed between 07.00 and 09.00 h. Rats from the three dietary groups were euthanized by decapitation. Blood samples were collected and rapidly centrifuged. Plasma was either immediately assayed or stored at -80°C. Retroperitoneal and omental adipose tissue was totally removed and weighed. The epididymal fat pad was totally removed, weighed and immediately frozen and stored at the temperature of liquid N_2. The visceral adiposity index (%) was calculated as previously described.[24] Epididymal adipose tissue lipids were extracted according to the procedure described by Folch *et al*.[25] and the fatty acid composition of total phospholipids were determined by gas liquid chromatography of their methyl esters, as previously described. [22]

Analytical methods

Plasma triglyceride, free fatty acids (FFA), uric acid and glucose levels were determined by spectrophotometric methods and insulin levels by immunoreactive assays as previously described.[10,22] The immunoreactive insulin assays were calibrated against rat insulin standard (Novo Nordisk, Copenhagen, Denmark). Plasma IL-6 and TNF-α and the TNF-α content of the cytosolic phase of adipose tissue were measured using commercial ELISA kits (Thermo Scientific, Rockford, USA; Legendmax Biolegend Inc., San Diego, USA; and Thermo Scientific rat TNF-α ELISA kit, Rockford, USA, respectively) All determinations were performed in triplicate as previously described.[10] Plasma thiobarbituric acid reactive substances (TBARS) levels, as an estimation of lipid peroxidation, were measured according to the method of Lee and Csallany as previously described[10] and protein carbonyl formation was determined spectrophotometrically by the method of Reznick and Packer with minor modifications as described by Ferreira *et al*.[26] Whole-body peripheral insulin sensitivity was measured using the euglycemic–hyperinsulinemic clamp technique as described elsewhere.[27] 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.

ROS and glutathione redox state determination in epididymal fat tissue

Intracellular reactive oxygen species (ROS) was measured using the dichlorodihydrofluorescein diacetate ($DCFH_2DA$) method according to Wang *et al.* as described by Ferreira *et al.*[26] Results were normalized by protein concentration (fluorescence intensity/mg protein) and expressed relative to the control group. Protein concentrations were quantified by the Bradford assay (Bio-Rad reagent). Total glutathione [reduced (GSH) + oxidized form (GSSG)] and GSSG were assayed as previously described [10] and expressed as redox state [(oxidized form/total form) x 100].

Antioxidant and oxidant enzyme activities in epididymal fat tissue

Epididymal adipose tissue catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) activities and xanthine oxidase (XO) activity - as a source of ROS - were determined as recently described.[10] The protein content in all enzyme assays was measured by the Bradford assay (Bio-Rad reagent).

RNA isolation and semi-quantitative RT-PCR analysis

MnSOD, NAD(P)H oxidase p47phox subunit (p47NOX) and UCP2 mRNA levels of epididymal fat tissue were determined by RT-PCR as previously described.[26] PCR amplification was carried out using specific oligonucleotide primers (Table 2). Relative amounts of mRNA were expressed as the ratio of band intensity for the target genes relative to that for 28S rRNA.

Determination of PPARγ and UCP2 protein mass levels

The assay of PPARγ protein level in the epididymal fat pad homogenate and UCP2 in the mitochondrial fraction were recently described by Ferreira *et al.*[26] and Creus *et al.*[28] The protein content was measured by the Bradford assay (Bio-Rad reagent). Total protein samples were resolved on SDS-PAGE, transferred to PVDF membranes and probed with specific antibodies (rabbit polyclonal anti-PPARγ or goat polyclonal anti-UCP2 from Santa Cruz Biotechnology, Inc.). The blots were incubated with horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology, Inc.) followed by chemiluminescent detection according to the manufacturer`s instructions (Pierce

Biotechnology, Rockford, Il, USA). β-actin was used as a loading control. The intensity of the bands was quantified by the National Institute of Health (NIH) imaging software (Bethesda, MD, USA). After the densitometry of immunoblots, the PPARγ and UCP2 from each CD group were normalized to 100%, and the SRD and SRF+FO groups were expressed relative to this. Preliminary studies showed linearity of Western blot assays from 10 to 100 ug for PPARγ and 25 to 100 ug of proteins for UCP2 respectively. The correlation coefficient between the amount of protein and the enhanced chemiluminescence image intensity was for 0.98 for PPARγ and 0.97 for UCP2 fractions. The relationship between the amount of the sample subjected to immunoblotting and the signal intensity observed was linear under the conditions described above.

Drugs and chemicals

FO was purchased from MP Biomedicals Inc. (Ohio, USA). Enzymes, substrates, coenzymes and all other chemicals used for experiments were of analytical grade and were purchased from Sigma- Aldrich Co. (St. Louis, MO, USA).

Statistical analysis

Sample sizes were calculated on the basis of measurements previously made with rats fed either a CD or SRD[10,26,29] considering 80% power as described by Glantz.[30] Results were expressed as mean values with their standard errors. Statistical comparisons were made transversely between different dietary groups. Data were tested for variance using Levene´s test and normality by Shapiro-Wilk´s test. Variables that were not normally distributed were transformed (using log 10 function) prior to the statistical analyses. 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.[31] Differences having *P* values lower than 0.05 were considered to be statistically significant (SPSS 17.0 for Windows, SPSS INC. Chicago, Illinois). All *P* values reported were 2-sided.

Results

Body weight gain, energy intake, epididymal adipose tissue weight, visceral adiposity index, plasma metabolites, insulin concentration, biomarkers of oxidative stress and inflammatory cytokine levels

As we have previously demonstrated[22] a significant increase ($P < 0.05$) in body weight and energy intake occurred in rats chronically fed a SRD during 6 and 8 months. The replaced of corn oil by FO in the SRD did not modify the body weight of SRD fed rats. However although a similar energy intake between the SRD and SRD+FO groups were recorded at 8 months of the feeding period, the weight gain was lower in the SRD+FO fed rats (months 6 to 8) (Table 3). Interestingly, in spite of a similar final body weight a significant reduction of the visceral adiposity index was recorded in the SRD+FO group compared to SRD fed rats, although values were still higher than those observed in the control group CD (Table 3). Moreover, as previously reported[8], the presence of FO as the principal source of dietary fat in the SRD normalized the moderate hyperglycemia and GIR values, as well as plasma triglyceride, FFA, cholesterol and uric acid levels without changes in insulinemia. The present results show that the increased plasma biomarkers of oxidative stress, TBARS and protein carbonyl groups – a marker of protein damage- were significantly decreased reaching values similar to those of the CD groups in the SRD+FO fed rats. Moreover, the increased plasma levels of inflammatory cytokines IL-6 and TNF-α in rats fed a SRD were reversed when dietary FO replaced corn oil during the last two months of the diet. Besides, dietary FO was able to normalize the increased TNF-α content in the adipose tissue of SRD fed rats; values were as follows: mean ± SEM (ng/g epididymal fat tissue) (n=6) 4.52 ± 0.27 in CD, 5.66± 0.38 in SRD and 3.62 ± 0.31 in SRD+FO (P <0.05 SRD vs CD and SRD+FO).

Epididymal fat pad enzymatic and non-enzymatic cellular antioxidant defenses

The effect of FO on the activities of the key enzymes of the cellular antioxidant defense system is shown in Table 4. FO was able to normalize the reduced enzymatic activities of CAT, GPx and GR recorded in the epididymal fat pad of the SRD-fed rats.

Moreover, the significant decreases ($P < 0.05$) of the total SOD activity that was not accompanied by a similar behavior of the mRNA SOD expression returned to values similar to those recorded in the CD-fed rats. Values for mRNA SOD expression were as follows: mean ± SEM (n=6) 1.00 ± 0.15 in CD, 1.54 ± 0.06 in SRD and 1.06 ± 0.01 in SRD+FO ($P < 0.05$ SRD vs CD and SRD+FO). Besides, total glutathione and GSSG - the components of the non-enzymatic cellular antioxidant defense system- as well as the redox state of glutathione that were depleted in the adipose tissue of SRD-fed rats, returned to control values when FO replaced corn oil as a source of dietary fat in the SRD from the last two months of the experimental period (Table 4).

Xanthine oxidase activity and ROS content in epididymal adipose tissue

Fig. 1 shows that the significant increase of both XO activity and ROS content in the epididymal fat pad of SRD fed groups were completely restored after FO administration. Interestingly, this was accompanied by no changes in the mRNA abundance of p47NOX subunit in the three dietary groups [mean ± SEM (n=6) 1.00 ± 0.03 in CD, 0.99 ± 0.05 in SRD and 0.95 ± 0.16 in SRD+FO].

Fatty acid composition of adipose tissue phospholipids

Table 5 depicts the adipose tissue fatty acid membrane phospholipids and the ratios of *n-3* PUFAs to total fatty acids, *n-3* to *n-6* and *n-3* to total saturated fatty acids. Compared with the CD-fed rats, a decrease in saturated and polyunsaturated fatty acids can be observed in the SRD-fed rats, while monounsaturated fatty acids significantly increased in the latter group. The *n-3*/*n-6* ratio was similar in both groups. The addition of FO to the SRD significantly decreased polyunsaturated fatty acids compared with both CD and SRD, while an enhancement of *n-3* PUFAs was observed due to an increase in EPA (*20:5 n-3*), DPA (*22:5 n-3*) and DHA (*22:6 n-3*). Besides, a greater increase in the *n-3*/*n-6* ratio was also recorded.

Protein mass level of PPARγ

The immunoblotting of adipose tissue revealed a single 67 kDa band consistent with PPARγ. Each gel contained an equal number of samples from the CD, SRD and SRD+FO group (Fig. 2a). After the densitometry of immunoblots, the PPARγ of the CD group was normalized to 100 % and both SRD and SRD+FO were expressed relative to this as previously reported. Qualitative and quantitative analyses of Western blot

showed that the relative abundance of PPARγ was significantly decreased ($P < 0.05$) in the epididymal fat pad of the SRD group compared with rats fed a CD (Fig. 2b). The present results show that replacement of corn oil by FO in the SRD during the last 2 months of the experimental period significantly increases the protein levels of PPARγ reached values even higher than those recorded on the control group CD.

Protein mass level and mRNA UCP2 expression

The immunoblotting of adipose tissue revealed a single 33 kDa band consistent with UCP2. Each gel contained an equal number of samples from the CD, SRD and SRD+FO groups (Fig. 3a). After the densitometry of immunoblots, the UCP2 of the CD group was normalized to 100 %, and both SRD and SRD+FO were expressed relative to this. Qualitative and quantitative analyses of Western blot showed that the relative abundance of UCP2 was significantly increased ($P < 0.05$) in the epididymal fat pad of the SRD group compared with rats fed a CD (Fig. 3b). Dietary FO decreased UCP2 protein mass level reaching values even lower than those observed in the CD fed rats. Moreover, dietary fish oil induced similar behaviors in the mRNA UCP2 expression (Table inserted in Fig.3).

Discussion

Adipose tissue plays an important role in modulated whole body insulin sensitivity through its endocrine function. This tissue is especially susceptible to the damage of oxidative stress which contributes to the development of adipose tissue dysfunction and insulin resistance. The present study provides new information about the effect of dietary cod liver oil on improving/reversing the biochemical and molecular mechanisms underlying the pre-existing deteriorated antioxidant defenses and oxidative stress involved in the dysregulation of adipose tissue of rats rendered dyslipemic insulin-resistant by feeding them a sucrose-rich diet for a long period of time.

Studies in rodent models by our group and others have shown that sucrose and fructose rich diets have been associated with adipose tissue endocrine dysfunction including among others the up regulation of inflammatory markers, pro-oxidative effects and altered genes expressions and activities of anti-oxidant enzymes.[10,32,33] On the other hand, different investigations in rodents indicate a complex modulation of gene expression in white adipose tissue by *n-3* long-chain fatty acids, especially EPA and DHA.[17,34,35] The present data demonstrate that the replacement of dietary corn oil by FO in the SRD fed rats restores the activities of key enzymes involved in the antioxidant defenses (eg: GPx,CAT,GR,SOD) in the epididymal fat pad, as well as the expression of MnSOD. In agreement with our results Mellouk *et al.*[36] demonstrated an improvement in the decrease of SOD, CAT and GR activities in the adipose tissue when sunflower oil (5%w/w) was partially replaced (1.6%w/w) by salmon oil (rich in EPA and DHA fatty acids) as a dietary fat in rats fed a fructose-rich diet during 8 weeks. Interestingly, in the epididymal adipose tissue of SRD fed rats, the increased in mitochondrial mRNA MnSOD expression did not correlate with the observed reduction of total SOD activity. Similarly, a lack of correlation between activity and expression was also observed by Busserolles *et al.*[37] in the heart of rats fed a high sucrose diet. However, it is pointed out that the level of SOD activity measured in the present work does not discriminate between Cu-Zn-SOD and Mn-SOD isoforms. Both isoforms could have post-transcriptional modifications that could alter their enzymatic activities.[38] Thus, we do not rule out the possibility that changes in individual SOD activities could

participate in the absence of correlation between activity and gene expression in the adipose tissue of SRD fed rats.

On the other hand, our data show an increase in the enzymatic activity of XO and ROS production in parallel with fat accumulation in the epididymal fat pad of rats chronically fed a SRD. Both XO and NAD(P)H oxidase play a key role participating in the pathway involved in the generation of ROS production in mature adipocytes. In this regard, Fariña *et al.*[33] showed an increase of NAD(P)H oxidase activity and ROS production in the abdominal adipose tissue of rats fed a 10% of fructose in drinking water for 3 weeks. Besides, a significant increase of XO and XOR (XO+XDH) activities was also recorded by Tsushima *et al*.[39] in epididymal fat pad of ob/ob mice fed a high-fat high-sucrose diet when compared to control mice. Moreover, they reported that the fat pad of these mice can secrete uric acid increasing the levels of this metabolite in plasma. Along this line the present study shows that dietary FO was able to reverse both the increased XO activity and ROS concentration within the fat pad and normalized plasma uric acid levels in the SRD-fed rats. It is pointed out that although the expression of p47NOX subunit remained unchanged in the SRD+FO group (values are similar to those recorded in the SRD and CD groups) we cannot discard the possibility that modifications in their activities could also contribute to improved ROS levels within the adipose tissue. Besides, FO corrects the lower redox state which reached values similar to the CD group.

Fat accumulation in adipose tissue correlates with systemic oxidative stress in rodents and humans.[40,41] In this vein, our data show that dietary FO normalized the increased plasma levels of protein carbonyl groups and lipid peroxidation estimated by TBARS concentrations, associated with the reduction of epididymal fat pad in the SRD fed rats. In agreement with our results a normalization of the increased plasma levels of TBARS, carbonyls radicals, and hydroperoxides as well as the decrease of nitric oxide was recently demonstrated in rats fed during 8 weeks a fructose rich diet when salmon oil (1.6%w/w) partially replaced sunflower oil (5%) as a dietary fat.[42]

Confirming our previous studies,[20] as well as other investigations,[43,44] the capacity of dietary FO to decrease adipose tissue hypertrophy normalizing visceral adiposity index without changes in body weight and energy intake is shown in the SRD fed rats. It is known that the effect of *n-3* PUFAs in the abdominal fat is associated with increased expression of genes engaged in mitochondrial biogenesis and oxidative metabolism contributing to the shrinkage of the adipocyte.[45] In this regard, we previously

demonstrated a significant reduction of adipocyte size diameter and improved cell size distribution in the SRD-fed rats. This was accompanied by the normalization of the increased key lipogenic enzyme activities which reached levels that approached those observed in the control group when FO replaced corn oil as a dietary source of fat.[21]

The expression and release of inflammation related adipocytokines increase in adipose tissue enlargement which leads to oxidative stress. The present results demonstrated that dietary FO was able to normalize the increased plasma levels of TNF-α and IL-6 as well as the TNF-α contents in the fat pad of SRD fed rats. This again could be connected with the reduction of visceral adiposity. It is well known that EPA and DHA have anti-inflammatory properties and dietary FO has the ability to alter cytokine production and reduced pro-inflammatory cytokines such as TNF-α and IL-6, among others, from the adipose tissue.[46,47] In this regard in animal studies, the anti-inflammatory effects of *n-3* PUFA have been demonstrated by the measurement of the cytokines TNF-α and IL-6 levels and expression.[48] Pérez-Matute *et al.*[49] showed in rats fed a high-fat (cafeteria) diet, that the administration of EPA ethyl ester (1 g/kg/day) for 5 weeks prevents the rise of TNF-α gene expression increasing adiponectin plasma levels. The suppression of the expression of TNF-α by EPA is partially attributed to its inhibitory effect on the activation of nuclear factor NF-kB. EPA appears to prevent NF-kB activation by preventing the phosphorylation of IkB-α, the inhibitory subunit of NF-kB.[50] EPA and DHA act through a variety of mechanisms including via cell surface GPR 120 receptor pathway -expressed among others in adipose tissue –controlling inflammatory cell signaling and gene expression patterns. In this regard, Yamada *et al.* [51] demonstrated that administration of EPA suppress palmitate-induced inflammation via GPR120 by inhibiting TAK1/TAB1 complex interaction in 3T3-L1 adipocytes. On the other hand, EPA generates E-series resolvins that show a potent anti-inflammatory and pro resolving actions decreasing the production of the classic inflammatory cytokines TNF-α, IL-6, IL-1b and decreased T cell activity. [12,52]

Additionally, Zhao *et al.*[53] showed in THP-1cells that DHA could bring its anti-inflammatory effects mediated PPARγ activation via inhibition of nuclear NF-kB activation. *n-3* LC PUFAs are natural ligands of PPARγ. This nuclear receptor directly regulates inflammatory genes expression interfering with the activation of NF-kB -preventing its nuclear translocation- creating an intriguing interaction between these two transcription factors. PPARγ repress gene transcription by negatively interfering with

NFkB and AP-1 signalling pathways in DNA –binding independent manner. Molecular studies have shown that PPARγ can interfere in vitro with inflammatory pathways, such as NFkB by physically interacting with p50 and p65. PPARγ inhibits AP-1 transcriptional activity by reducing AP-1 DNA binding. This inhibition is likely due to a direct interaction between PPARs and c-jun. This trans-repression activity likely constitutes the mechanistic basis for the anti-inflammatory properties of PPARs.[12,54,55] Moreover, in 3T3-L1 adipocytes incubated for 24 h in the presence of TNF-α a significant decrease in PPARγ protein mass levels was observed.[56] Higher TNF-α levels have also been associated with increased insulin resistance in adipose tissue by reducing the phosphorylation of IRS-1.[57] Moreover, the increase of both TNF-α and IL-6 levels that are potent inhibitors of adiponectin expression and secretion favors insulin resistance.[58] Recently, Oster *et al.*[59] in 3T3-L1 adipocytes incubated with DHA demonstrated that this fatty acid increases both cellular adiponectin mRNA and protein secretion by a possible mechanism involving PPARγ, since DHA and EPA are potential ligands of PPARγ a key transcription factor for the adiponectin gene. Along this line our results show that FO induced a significant increase in the reduced adipose tissue protein level of PPARγ which reached values similar to those observed in the CD group. As mentioned above these findings were accompanied by a normalization of both the key antioxidant enzymes activities and the biomarkers of oxidative stress. In addition to these results and using the same experimental model, we previously showed that FO was able to reverse the lower plasma levels of adiponectin restoring whole body peripheral insulin sensitivity.[20] Therefore, these findings suggest that the anti-inflammatory actions of FO play a major role in the mechanisms involved in the amelioration of the pre-existing alteration of dysfunctional adipose tissue contributing to improved insulin sensitivity effects in SRD fed rats.

UCP2 is broadly expressed in various tissues and cells, functions as a sensor of mitochondrial oxidative stress. Studies “in vivo” indicate that physiological and pathological elevations of plasma FFA (2-3 folds) induce up regulation of UCP2 expression in the white adipose tissue of rodents.[60] The present findings show that the increases of both the gene expression and the protein mass levels of UCP2 in the adipose tissue of SRD-fed rats are associated with an increased availability of circulating FFA. The link between the levels of fatty acids and the expression of UCP2 in the white adipose tissue was confirmed by Reilly *et al.*[61] in cultured cells (3T3-L1

pre-adipocytes) in vitro suggesting that fatty acids themselves are the molecule signals that bring about the changes in UCP2 at least in this tissue. Castrejón-Tellez *et al.*[62] demonstrated an increase of UCP2 expression without changes in the protein mass levels in the visceral white adipose tissue of rats fed 30 % of sugar in drinking water for 20 weeks, in these animals plasma lipids were significantly higher. Furthermore, an increase of UCP2 expression in adipose tissue was also shown in Wistar rats fed a high caloric diet for 2 months.[63] It was demonstrated that the overall molecular mechanisms of UCP2 regulation which can occur at different stages, including genetic, protein expression /degradation and protein activity level (transcriptional, translational, post-translation and proton conductance regulation) involved among others nutritional and hormonal regulation (eg: fatty acids, ROS, nuclear receptors: PPARs ,SREBPs etc).In the present experimental protocol we do not analyze most of the mechanisms mentioned above. However the chronically increased of plasma FFA, and ROS and as previously demonstrated leptin levels in adipose tissue could contribute to the up regulation of UCP2 in SRD fed rats. [23,64]

On the other hand, Tsuboyama *et al.*[65] demonstrated that dietary FO compared to safflower oil down regulated the expression of UCP2 in the adipose tissue of C57BL/6j mice fed a high fat diet. In this line, our data show that FO down regulated the UCP2 expression with reduction of their protein mass levels in the epididymal tissue of SRD fed rats, reaching values even lower than those recorded in the CD-fed group. It was accompanied by normalization of plasma FFA levels. Interestingly, we previously demonstrated in this animal model that FO reduced the availability of plasma FFA by mechanisms that include the reversal of the increased basal and stimulated lipolysis and the altered action of insulin in the fat pad of SRD-fed rats.[9] At present we are unaware of other studies that evaluate the effects of FO in the underlying mechanisms involved in reversing the altered levels of UCP2 in the fat pads of rats chronically fed a SRD. However, under the present experimental conditions this study suggests that changes in circulating FFA availability could probably be at least one of the possible mechanisms related to dietary FO on the regulation of adipose tissue UCP2. Moreover, recently Kim *et al.*[66] showed that fish oil intake induced a high UCP1 mRNA and protein expression in white adipose tissue via de sympathetic nervous system in C57BL/6 mice as a browning effect that could be involved in the beneficial effects of dietary *n-3* PUFAs. Further studies are needed to evaluate this issue.

Another possible pathway by which dietary FO might exert its beneficial effects on adipose tissue dysfunction is the incorporation of EPA and DHA into the lipid membrane phospholipids fraction of adipocytes. Our results show that the consumption of dietary FO during the last two months of the experimental period induces an increase in the incorporation of 20:5, 22:5 and 22:6 *n-3* fatty acids into the phospholipids of the epididymal tissue of SRD-fed rats. Moreover, a significant increase of *n-3/n-6* ratio was also observed after FO administration. The incorporation of *n-3* PUFA into the membrane phospholipids of adipocytes contributes to its anti- inflammatory effects by inhibiting the production of inflammatory mediators. In this regard, arachidonic acid (AA) derived eicosanoids such as PGE_2 and thromboxane A_2 are pro-inflammatory whereas EPA derived ones such as PGE_3 are less inflammatory. The increased incorporation of EPA and DHA into membrane phospholipids altering the relative proportions of *n-3* and *n-6* PUFA could reduce the production of AA derived eicosanoids that modulates the overall inflammatory environment.[35,47] Besides, the present data demonstrate that the increased *n-3* PUFA in the adipose tissue phospholipids induce a significant rise in membrane unsaturation (*n-3* PUFA / saturate fatty acids ratio was higher in SRD+FO compared to SRD : 0.074 vs 0.036 respectively) suggesting an increase in membrane fluidity that is associated with increased insulin sensitivity and insulin stimulated glucose uptake.[15] Thus, the changes observed in the fatty acids profile in the adipose tissue phospholipids of the SRD+FO group might be another possible mechanism involved in ameliorating the altered adipose tissue function by modulating the inflammatory products and improving the impaired insulin sensitivity.

Finally, expanding our previous research work, the present study provides new information indicating that dietary FO is able to decrease visceral adiposity and improve/reverse the oxidative stress by normalizing the antioxidant and oxidant enzymes activities and ROS content. Besides, FO increases PPARγ protein mass levels and this was accompanied by a normalization of plasma pro-inflammatory cytokines levels as well as the protein mass and expression of adipose tissue UCP2. Moreover, FO increases the amount of *n-3* over *n-6* fatty acids in membrane phospholipids and reversed dyslipidemia and insulin resistance contributing all these changes to amend preexistent adipose tissue dysregulation in the fat pad of high sucrose-fed rat model. Although care must be taken when extrapolating these results from rats to humans,

these findings reinforce the view that dietary FO may exert a beneficial effect improving the undesirable features prevalent in the metabolic syndrome.

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Authors' contributions:

Conceived and designed the experiments: Y B L and AC

Performed the experiments: DS and M R F

Analyzed the data: DS, AC, MRF and YBL

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Figure Legends

Figure 1. Xanthine oxidase activity and ROS content in epididymal adipose tissue of rats fed a control diet (CD), sucrose rich diet (SRD) and sucrose rich diet + fish oil (SRD+FO). Values are expressed as mean ± SEM, at least 6 animals were included in each experimental group. Values that do not share the same superscript symbol are significantly different ($P < 0.05$).

Figure 2. Epididymal adipose tissue protein mass levels of PPAR-γ of rats fed a control diet (CD), sucrose rich diet (SRD) and sucrose rich diet + fish oil (SRD+FO). (a) Immunoblots of PPARγ of adipose tissue from the CD, SRD and SRD+FO rats. Molecular marker is shown on the right. Lane 1, CD; lane 2, SRD; lane 3, SRD+FO. (b) Densitometric immunoblot analysis of PPARγ protein mass levels in adipose tissue of rats fed a CD, SRD or SRD+FO. Values are mean ± SEM, at least 6 animals were included in each experimental group, with their standard errors represented by vertical bars and expressed as percentage relative to the CD. Values that do not share the same superscript symbol are significantly different ($P < 0.05$).

Figure 3. Epididymal adipose tissue protein mass levels of UCP2 of rats fed a control diet (CD), sucrose rich diet (SRD) and sucrose rich diet + fish oil (SRD+FO). (a) Immunoblots of UCP2 of adipose tissue from the CD, SRD and SRD+FO rats. Lane 1, CD; lane 2, SRD; lane 3, SRD+FO. (b) Densitometric immunoblot analysis of UCP2 protein mass levels in adipose tissue of rats fed a CD, SRD or SRD+FO. Values are mean ± SEM, at least 6 animals were included in each experimental group, with their standard errors represented by vertical bars, and expressed as percentage relative to the CD. In the table insert, values of UCP2 mRNA/18S abundance are expressed as mean ± SEM; at least six animals were included in each experimental group. Values that do not share the same superscript symbol are significantly different ($P < 0.05$).

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

Diet Ingredients (g/kg of food)	**Control diet [a] (CD)**	**Sucrose-rich diet [a] (SRD)**	**SRD + fish oil [b] (SRD+FO)**
Carbohydrates			
Corn-starch	625		
Sucrose		625	625
Protein			
Casein (vitamin free)	170	170	170
Fat			
Corn oil	80	80	10
Fish oil			70
Fatty acid profile (g/kg of food)			
14:0	traces	traces	3.28
16:0	8.32	8.32	10.00
18:0	2.08	2.08	2.36
20:0	0.32	0.32	0.67
16:1 *n*-7	-	-	7.60
18:1 *n-9*	25.68	25.68	20.71
20:1 *n-9*	1.28	1.28	9.75
18:2 *n-6*	41.20	41.20	6.97
18:3 *n-3*	0.32	-	-
20:4 *n-3*	-	-	2.45
20:5 *n-3*	-	-	7.20
22:5 *n-3*	-	-	0.42
22:6 n-3	-	-	6.51
Total Saturated	10.72	10.72	16.33
Total Monounsaturated	26.96	26.96	38.06
Total Polyunsaturated	41.52	41.52	23.55
n-6	41.20	41.20	6.97
n-3	0.32	0.32	16.58

n-3/n-6	0.008	0.008	2.378

The home-made experimental diets are based on the AIN-93M recommendations. [a]CD and SRD include corn oil (Mazola, Best Foods Canada Starch, Montreal, Quebec, Canada). [b]SRD+FO includes 1% corn oil plus 7% cod liver oil (MP Biomedicals, LLC, Ohio, USA-formerly ICN- Cat. Number: 901405). Cod liver oil fatty acids composition (g/100g of total fatty acids): 14:0 4,7; 16:0 12,8; 16:1 *n-7* 10,8; 18:0 3,0; 18:1 *n-9* 25,0; 18:2 *n-6* 2,6; 18:*3 n-3* traces; 20:0 0,9; 20:1 *n-9* 13,7; 20:4 *n-3* 3,5; 20:5 *n-3* 10,3; 22:5 *n-3* 0,6; 22:6 *n-3* 9,3. Diets contain by weight: salt mix (35g/kg) based on salt mix AIN-93M-MX, vitamin mix (10g/kg) based on vitamin mix AIN-93M-VX, choline chloride (2g/kg), methionine (3g/kg) and cellulose (75g/kg).

Table 2 Sequences of the primers used to amplify different genes by RT-PCR and sizes of the fragments generated

Gene name	Forward primer sequences (5′-3′)	Reverse primer sequences (5′-3′)	Size (bp)
SOD	AGCTGCACCACAGCAAGCAC	TCCACCACCCTTAGGGCTCA	191
p47NOX	AGGGAACGCTCACCGAGTACT	TCTTTGGCCGTCAGGTATGTC	160
UCP2	GAGAGTCAAGGGCTAGCGC	GCTTCGACAGTGCTCTGGTA	350
28S	GTGAAAGCGGGGCCTCACGATCC	TACTGAGCAGGATTACCATGGC	200

Table 3 Body weight gain, energy intake, epididymal adipose tissue weight, visceral adiposity index, plasma metabolites, insulin, glucose infusion rate (GIR), oxidative stress biomarkers and inflammatory cytokine levels in rats fed a CD, SRD or SRD+FO[1].

	CD	SRD	SRD+FO
Body weight at 6 months (g)	439.2 ± 16.1*	484.9 ± 13.4†	481.0 ± 10.9†
Body weight at 8 months (g)	469.1 ± 13.9*	527.8 ± 12.8†	506.2 ± 10.1†
Weight gain (g) (months 6-8)	31.5 ± 5.6*	38.7 ± 4.1*	18.5 ± 3.9†
Energy intake (kJ/d) (initial to 6 months)	268.7 ± 17.2*	367.8 ± 20.7†	332.5 ± 12.8†
Energy intake (kJ/d) (months 6-8)	268.7 ± 17.2*	367.8 ± 20.7†	332.5 ± 12.8†
Epididymal fat pad weight (g)	9.8 ± 0.4*	18.3 ± 0.1†	13.3 ± 0.7‡
Visceral adiposity index	4.13 ± 0.31*	8.51 ± 0.41†	6.30 ± 0.49‡
Plasma metabolites and insulin levels			
Triglyceride (mM)	1.00 ± 0.10*	2.30 ± 0.19†	1.10 ± 0.09*
Total Cholesterol (mM)	2.02 ± 0.07*	3.65 ± 0.03†	2.15 ± 0.13*
FFA (μM)	294 ± 17*	887 ± 20†	280 ± 38*
Uric acid (μM)	242 ± 33*	379 ± 33†	161 ± 12*
Glucose (mM)	6.4 ± 0.1*	8.2 ± 0.1†	6.6 ± 0.3*
Insulin (pM)	375 ± 30	369 ± 33	370 ± 27
GIR [mg/(kg x min)]	12.0 ± 0.5*	5.1 ± 0.4†	12.3 ± 0.8*
Plasma biomarkers of oxidative stress and inflammatory cytokines			
TBARS (nmol/ml)	2.51 ± 0.14*	4.39 ± 0.33†	3.35 ± 0.49*
Protein carbonyl groups (nmol/ml)	33.92 ± 0.87*	38.65 ± 1.01†	35.17 ± 1.32*
TNF-α (pg/ml)	5.64 ± 0.60*	14.69 ± 2.23†	5.37 ± 0.75*
IL-6 (pg/ml)	40.20 ± 11.13*	79.78 ± 15.93†	33.34 ± 8.07*

[1] Values are expressed as mean ± SEM, at least six animals were included in each experimental group. Values in a line that do not share the same superscript symbol are significantly different ($P < 0.05$) when one variable at a time was compared by one-way ANOVA followed by Newman-Keuls test.

Table 4 Epididymal fat pad enzymatic and non-enzymatic cellular antioxidant defenses in rats fed a CD, SRD or SRD+FO[1].

	CD	SRD	SRD+FO
CAT (U/mg protein)	2.37 ± 0.08[*]	1.81 ± 0.09[†]	2.27 ± 0.16[*]
GPx (U/mg protein)	0.37 ± 0.02[*]	0.26 ± 0.01[†]	0.36 ± 0.01[*]
GR (mU/mg protein)	20.64 ± 0.87[*]	14.66 ± 0.30[†]	18.42 ± 1.40[*]
SOD (U/mg protein)	4.57 ± 0.25[*]	3.08 ± 0.19[†]	4.08 ± 0.27[*]
Total Glutathione (nmol/g wet tissue)	34.05 ± 2.25[*]	26.92 ± 2.13[†]	39.09 ± 2.49[*]
GSSG (nmol/g wet tissue)	11.43 ± 0.67[*]	6.55 ± 0.52[†]	13.06 ± 0.59[*]
Redox State [(GSSG/total glutathione)x100]	40.46 ± 2.10[*]	25.20 ± 2.89[†]	34.44 ± 2.61[*]

[1] Values are expressed as mean ± SEM, at least six animals were included in each experimental group. Values in each line that do not share the same superscript symbol are significantly different ($P < 0.05$)) when one variable at a time was compared by one-way ANOVA followed by Newman-Keuls test.

Table 5 Fatty acid composition of epididymal adipose tissue phospholipids in rats fed a CD, SRD or SRD+FO[1].

Fatty acids	CD	SRD	SRD+FO
16:0	18.30 ± 0.80*	16.20 ± 0.90*	23.20 ± 1.10†
16:1	1.40 ± 0.10*	2.30 ± 0.10†	4.30 ± 0.10‡
18:0	4.50 ± 0.20*	3.20 ± 0.10†	3.80 ± 0.20†
18:1	34.30 ± 1.80*	42.40 ± 1.50†	40.10 ± 0.90†
18:2 *n-6*	38.30 ± 2.00*	34.20 ± 1.80*	25.40 ± 1.00†
18:3 *n-3*	0.60 ± 0.10	0.50 ± 0.20	0.60 ± 0.10
20:2 *n-6*	0.80 ± 0.10*	0.40 ± 0.10†	0.40 ± 0.10†
20:3 *n-6*	0.20 ± 0.02*	0.10 ± 0.02†	0.30 ± 0.02‡
20:4 *n-6*	1.60 ± 0.10*	0.50 ± 0.10†	0.50 ± 0.10†
20:5 *n-3*	Traces	Traces	0.30 ± 0.01
22:5 *n-3*	-	0.20 ± 0.01*	0.50 ± 0.10†
22:6 *n-3*	-	-	0.60 ± 0.10
Total			
Saturated	22.80 ± 0.50*	19.40 ± 0.50†	27.00 ± 0.65‡
Monounsaturated	35.70 ± 0.95*	44.70 ± 0.80†	44.40 ± 0.50†
Polyunsaturated	41.50 ± 0.46*	35.90 ± 1.10†	28.60 ± 0.20‡
n-6	40.90 ± 1.04 *	35.20 ± 0.94†	26.60 ± 0.30‡
n-3	0.60 ± 0.10*	0.70 ± 0.15*	2.00 ± 0.08†
n-3/total fatty acids	0.006 ± 0.001*	0.007 ± 0.001*	0.020 ± 0.009
n-3/*n-6*	0.015 ± 0.002*	0.020 ± 0.004*	0.081 ± 0.008†
n-3/ total saturated fatty acids	0.026 ± 0.004*	0.036 ± 0.006*	0.074 ± 0.004†

[1]Values are expressed as mean ± SEM, six animals were included in each experimental group. Values in a line that do not share the same superscript symbol are significantly different ($P < 0.05$) when one variable at a time was compared by one-way ANOVA followed by Newman-Keuls test.

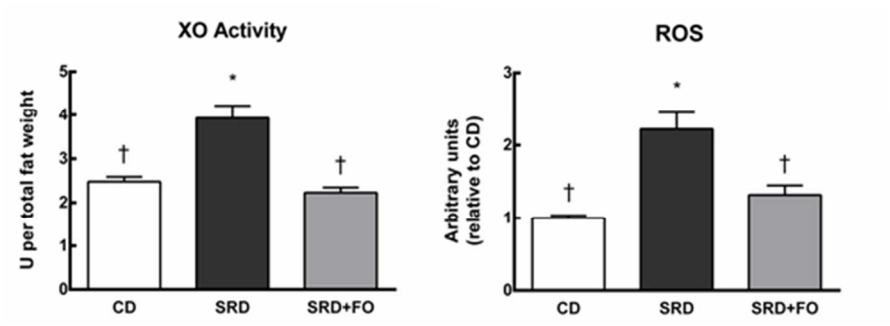

Figure 1

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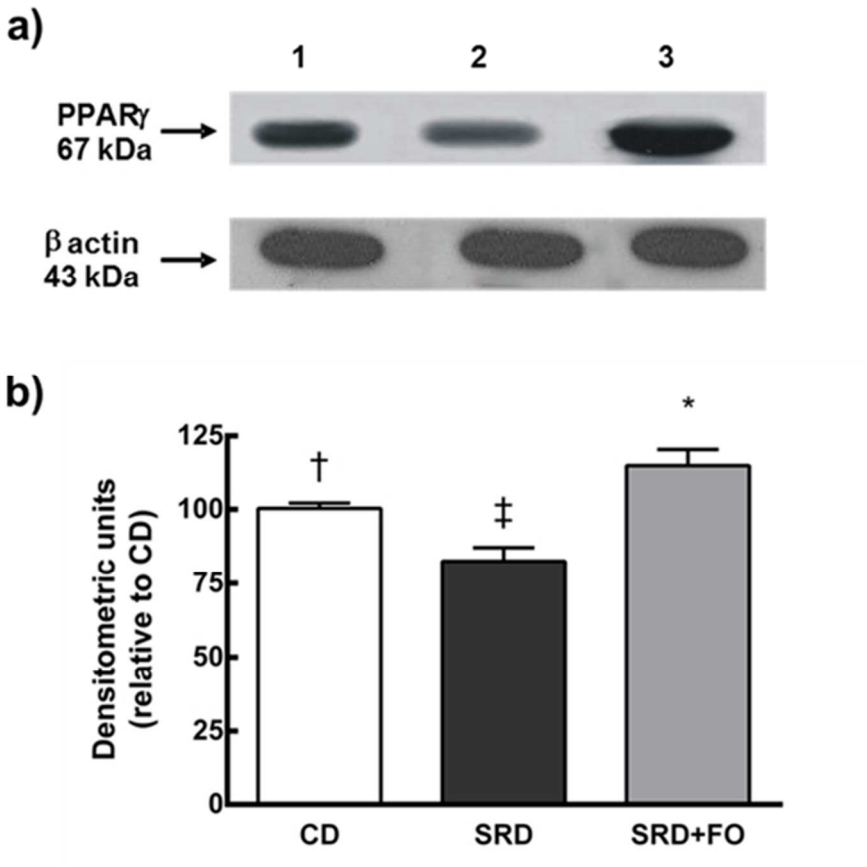

Figure 2

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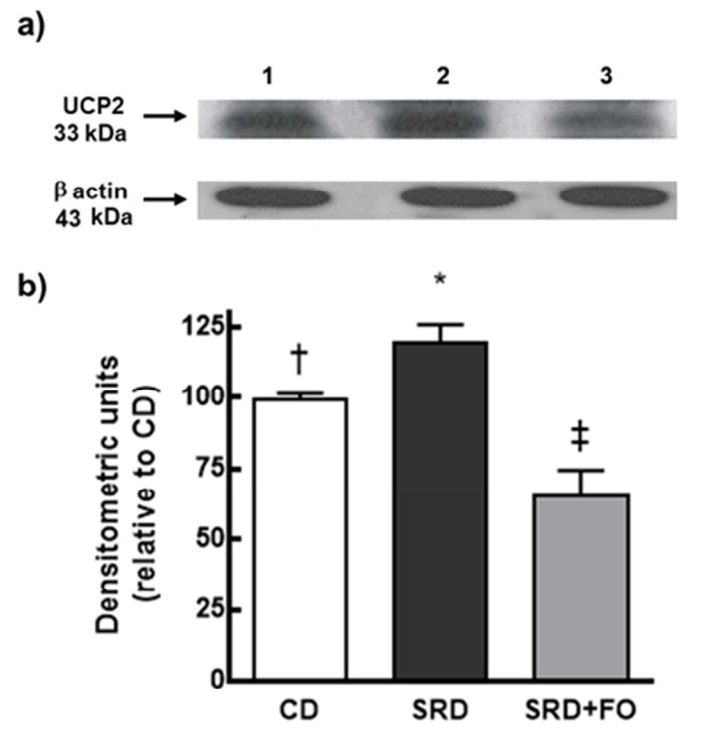
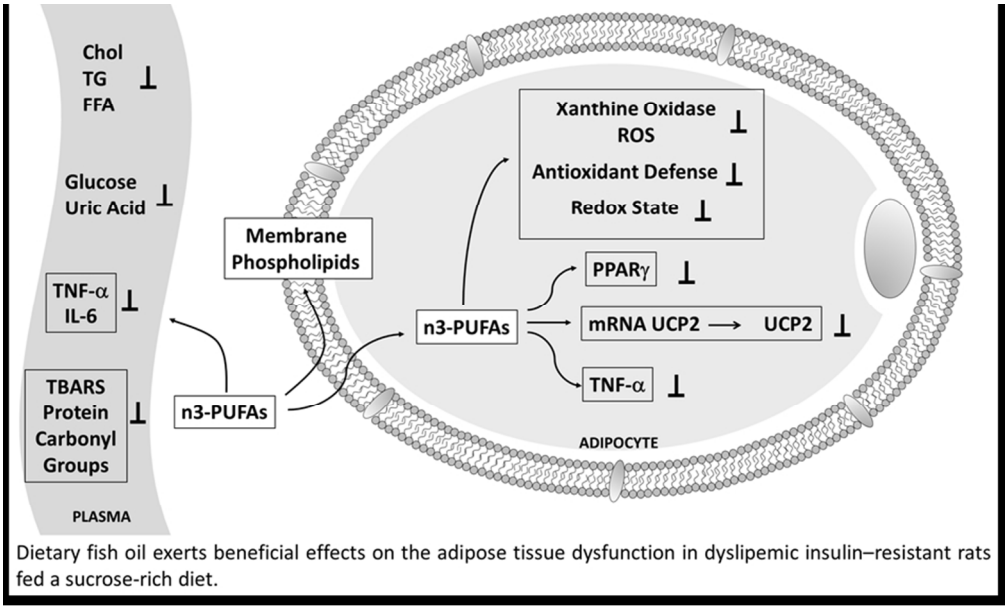


	CD	SRD	SRD+FO
Arbitrary units (UCP2/28S)	1.00 ± 0.07†	1.59 ± 0.12*	0.75 ± 0.14‡

Figure 3

169x151mm (96 x 96 DPI)



Dietary fish oil exerts beneficial effects on the adipose tissue dysfunction in dyslipemic insulin–resistant rats fed a sucrose-rich diet.

80x48mm (300 x 300 DPI)