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Induction of Cd36 expression elicited by fish oil PUFA in spontaneously hypertensive rats

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Abstract

Cd36 is an integral membrane glycoprotein expressed on the surface of cells active in fatty acid metabolism (adipocytes, muscle cells, platelets, monocytes, heart and intestine cells). This protein plays diverse functions including uptake of long-chain fatty acids and oxidized low-density lipoproteins. A recent report demonstrates that Cd36 deficiency underlies insulin resistance, defective fatty acid metabolism and hypertriglyceridemia in spontaneously hypertensive rats (SHRs). Cd36 is a tightly regulated protein whose expression is modulated through peroxisome proliferator-activated receptor (PPAR) transcription factors, by conditions that alter lipid metabolism such as diabetes mellitus and high-fat feeding. The purpose of this study was to evaluate the effect of dietary fish oil, rich in n-3 polyunsaturated fatty acids (PUFAs), on metabolic parameters and on the expression levels of *Cd36* in adipose tissue in the SHR. Spontaneously hypertensive rats showed lower *Cd36* mRNA levels when compared to Kyoto-Wistar (KW) rats (control). After 6 weeks of fish oil (FO) administration, this group of SHRs (FO-SHR) presented increased levels of *Cd36* mRNA, concomitantly with decreased insulin, free fatty acids (FFAs), triglycerides, cholesterol, LDL, HDL, total lipids and blood pressure, in comparison to control rats that received a corn–canola oil diet. The study confirmed the beneficial effects of fish oil administration on the metabolic syndrome, suggesting that the induction of *Cd36* expression could be one of the molecular mechanisms elicited by fish oil PUFAs.
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Keywords: Fish oil; Cd36; Spontaneously hypertensive rats (SHRs); Metabolic syndrome

1. Introduction

The metabolic syndrome is considered as the major cause of morbidity and mortality in most of the world. It is a genetically complex disorder heavily influenced by environmental and lifestyle factors whose molecular basis is largely unknown [1]. It underlies a wide range of metabolic disorders including visceral adiposity, dysglycemia, hypertension, dyslipidemia, insulin resistance, oxidative stress, endothelial dysfunction and increased propensity for renal and macrovascular complications [2].

The spontaneously hypertensive rat (SHR) is the most widely used animal model of human essential hypertension. Under appropriate dietary conditions, the SHR exhibits multiple metabolic disorders similar to the human metabolic syndrome [3]. As in the human metabolic syndrome, a polygenic etiology has been considered in the SHR. Recently, a deletion variant in *Cd36*, which codes for a long-chain fatty acid transporter on cell membranes (Cd36), was detected in the SHR/NIH strain and was linked to the transmission of insulin resistance and disordered fatty acid metabolism [4]. Cd36 is an 88-kDa integral membrane glycoprotein expressed on the surface of several cell types active in fatty acid metabolism (adipocytes, muscle cells, platelets, monocytes, heart and intestine cells) playing diverse functions including uptake of long-chain fatty acids and oxidized low-density lipoproteins [5]. Cd36 is undetectable in SHR adipocyte plasma membrane, but overexpression of *Cd36* in transgenic mice has been associated with enhanced muscle fatty acid oxidation, alterations in body fat and in blood levels of fatty acids, triglycerides, cholesterol, glucose and insulin, and with significant amelioration of insulin

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resistance at the tissue level [4]. On the other hand, there are some reports that correlate insulin resistance with enhanced levels of Cd36 protein in other tissues and insulin resistance states, namely, in cardiac myocytes from obese Zucker rats [6], in skeletal muscle and macrophages from human subjects with type 2 diabetes [7,8] and in preadipocytes from individuals with familial combined hyperlipidemia [9], probably reflecting the genetic heterogeneity of the metabolic syndrome.

Cd36 is a tightly regulated protein whose expression is modulated by conditions that alter lipid metabolism, such as diabetes mellitus and high-fat feeding. Regulatory mechanisms at the level of mRNA are mediated by transcription factors that belong to the peroxisome proliferator-activated receptor (PPAR) family [10].

Amongst the dietary interventions used to reduce metabolic syndrome disorders, fish oil [rich in n-3 polyunsaturated fatty acids (n-3 PUFA)] administration has proven to lower serum levels of triglycerides, cholesterol, free fatty acids (FFAs), insulin, blood pressure; to elevate high-density lipoprotein (HDL) cholesterol; and to improve insulin sensitivity [11–14].

It has been suggested that some of the beneficial effects of n-3 PUFA are due to changes in membrane fatty acid composition and subsequent alterations in hormone signaling. However, fatty acids, their CoA derivatives and their metabolites are able to regulate gene expression, up-regulating the expression of genes encoding proteins involved in fatty acid oxidation while simultaneously down-regulating genes encoding proteins of lipid synthesis, by activating PPAR transcription factors [15]. Particularly, PPARγ can be activated by a number of mono- and PUFAs. Besides, PPARγ is also the receptor for the thiazolidinedione class of antidiabetic drugs known to improve insulin resistance. Its activation leads to the expression of *Cd36* in adipose tissue, increasing the ability of mature adipose cells to take up circulating fatty acids [16].

The purpose of this study was to investigate whether Cd36 gene expression could mediate some of the beneficial effects of dietary fish oil in the metabolic syndrome. Therefore, the effect of fish oil administration on metabolic parameters and on the expression levels of *Cd36* in adipose tissue in the SHR was studied.

2. Materials and methods

2.1. Materials

All chemicals were of analytical grade, obtained from Sigma (St. Louis, MO, USA), Bayer (Mexico City, Mexico) and J.T. Baker (Mexico City, Mexico). Dietary components were purchased from Harlan Teklad (Madison, WI, USA). Insulin radioimmunoassay kit was obtained from Diagnostic xProducts Corporation (Los Angeles, CA, USA) and the nonesterified fatty acids kit was bought from Wako (Neuss, Germany).

2.2. Experimental design

Ten male Kyoto-Wistar (KW) and 15 SHRs, 21 days old, were purchased from Harlan Teklad. The animals were individually housed and maintained in a 12-h light/dark cycle at 25°C. Animal maintenance and handling were in accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" [17]. The animals were divided into two groups: the KW rats as the normotensive control group ($n=10$) and the SHR as the metabolic syndrome group ($n=15$). Both groups received a standard diet (Lab Diet 2001, Harlan Teklad) during 1 week. Body weight and systolic blood pressure were measured. Blood samples from 18-hour-fasted animals were carefully collected from the tail tip. Blood was centrifuged at 1086×g for 10 min, and serum was kept at −20°C until analysis of insulin, glucose, triglycerides and FFA concentrations.

Spontaneously hypertensive rats were divided into two groups: a corn–canola group (CC-SHR; $n=5$) and a fish oil group (FO-SHR; $n=5$). The CC-SHR group received a diet with 7.5% corn–canola oil mixture (Patrona, from the local market) as lipid source. The FO-SHR group received a diet with 7.5% sardine oil (Sonora University, Mexico) as lipid source [13]. Sardine oil contained 11% eicosapentaenoic acid (EPA, 20:5 n-3) and 7% docosahexaenoic acid (DHA, 22:6 n-3) (Table 1). The control group (KW; $n=10$) received the corn–canola oil diet. The three groups received the experimental diets during 6 weeks. Diets were prepared with 0.02% butylated hydroxytoluene as antioxidant and stored under refrigeration until the end of the study.

At the end of the experimental diet period, body weight and blood pressure were measured. Fasted animals (18 h) were killed by decapitation without anesthesia to avoid interference with insulin measurements. Serum, adipose tissue and organs were obtained, weighted, frozen under liquid nitrogen and kept at −70°C until analysis.

2.3. Blood pressure measurement

Systolic blood pressure was estimated by a tail-cuff method (IITC noninvasive blood pressure system, model 29; Life Science Instruments, Woodland Hills, CA, USA) in conscious animals. The reported blood pressure value is the mean of five systolic measurements.

2.4. Analytical methods

Serum glucose concentration was measured by the glucose oxidase method [18]. Serum insulin concentration was determined by a commercial double-antibody solid-phase radioimmunoassay (Coat-A-Count, Diagnostic Products Corporation). Serum FFAs were determined by an enzymatic method (NEFA-C test, Wako). Total cholesterol was measured using an enzymatic assay [19]. Serum HDL was determined after precipitation of LDL and very low density lipoprotein (VLDL) cholesterol with phosphotungstic acid in the presence of Mg^+ [20]. In a similar way, LDL

Table 1
Fatty acid composition of experimental diets

Fatty acids	Chow diet[a]	Corn–canola diet[a]	Fish oil diet[a]
Saturated			
10:0	nd	0.05	nd
12:0	nd	nd	0.04
14:0	2.5	0.55	7.28
16:0	25.0	12.80	26.06
18:0	5.0	nd	8.04
20:0	nd	nd	0.73
22:0	nd	0.32	0.32
Total	35.5	12.8	42.47
Monounsaturated			
14:1	0.50	nd	0.26
16:1	nd	nd	6.08
18:1	52.0	50.7	15.1
20:1	3.0	nd	nd
Total	56.5	50.7	21.44
Polyunsaturated			
n-6			
18:2	6.5	32.8	0.48
20:4	nd	nd	0.36
Total	6.5	32.8	0.48
n-3			
18:3	1.5	3.5	2.85
20:5	nd	nd	20.0
22:6	nd	nd	13.0
Total	1.5	3.5	35.85
n-6/n-3	4.8	9.3	0.02

[a] Values are expressed as percentage of total fatty acids. nd, not detected.

cholesterol was determined after heparin precipitation of HDL and VLDL cholesterol [21]. A peroxide-coupled method for the colorimetric determination of serum triglycerides was used [22]. Total lipid measurement (color test method) was performed according to the 3321 Merck test.

2.5. Lipids extraction and fatty acid composition analysis

Lipids were extracted from diets according to Folch et al. [23]. Fatty acids were converted to methyl esters by H_2S-catalyzed transmethylation [24] and analyzed by gas chromatography. The gas chromatograph (HP model 6890) was equipped with a carbowax capillary column and a flame ionization detector. Injection and detector temperatures were 250°C, nitrogen was the carrier gas and the column temperature was programmed to rise from 100°C to 210°C at a rate of 2°C/min. Fatty acid methyl esters were identified by comparison with fatty acid standards (Sigma).

2.6. RT-PCR analysis of the Cd36 mRNA

Total RNA was prepared from abdominal fat by the acid guanidinium isothiocyanate–phenol–chloroform extraction method, according to manufacturer's instructions (Quantum Prep AquaPure Isolation Kit, BioRad) [25]. The cDNA was synthesized from 5 μg of total RNA using the One-Step RT-PCR kit from Quiagen and *Cd36*-specific primers. For the KW group, the upstream primer was 5′ -TCA AGG TGT GCT CAA CAG CC-3′, and the downstream primer was 5′-AGG ATA AAA CAC ACC AAC TGT-3′ [26], which gave a 107-bp PCR product. For the SHR group, the upstream primer was 5′-CAG AGA ATG ACA ACT TCA CAG-3′, and the downstream primer was 5′ -GGA ACA TAG AAG ACT TGG AC-3′ [4], which gave an 81-bp PCR product. These primers recognize the exon 6 region of the normal and mutated genes, respectively. The reverse transcription reaction was done at 50°C for 30 min. The PCR was performed for 30 cycles at 94°C for 1 min, followed by 1 min at 45°C and 1 min at 72°C. Reaction products were separated by electrophoresis (2.5% agarose gel in Tris–EDTA buffer) and visualized using ethidium bromide staining. *Cd36* mRNA levels were quantified by densitometry using a Kodak DS 1D 3.0 Digital Science image analyzer program (Kodak). 18S rRNA was used as an internal standard.

2.7. Data analysis

Data are presented as mean±S.D. Results of *Cd36* mRNA levels are expressed as percentage (means±S.D.) of ribosomal 18S rRNA levels determined as a control for each sample. Statistical significance was determined by analysis of variance and Tukey's multiple mean comparison test.

3. Results

3.1. Metabolic parameters

Before fish oil administration, some metabolic parameters were evaluated in KW rats and SHRs. Statistically significant differences were found between the KW and SHR groups in blood pressure (136.2±18.2 vs. 200±13.3 mm Hg, P<.001), serum levels of insulin (4.5±0.92 vs. 8.9±1.7 μUI/ml, P<.001), FFAs (0.77±0.09 vs. 1.04±0.15 mEq/L, P<.05) and triglycerides (94.2±3.9 vs. 109.4±8.4 mg/dl, P<.01). No differences were observed in glucose and body weight between the two groups.

After 6 weeks on the experimental diets, no differences in liquid consumption and in abdominal fat weight were

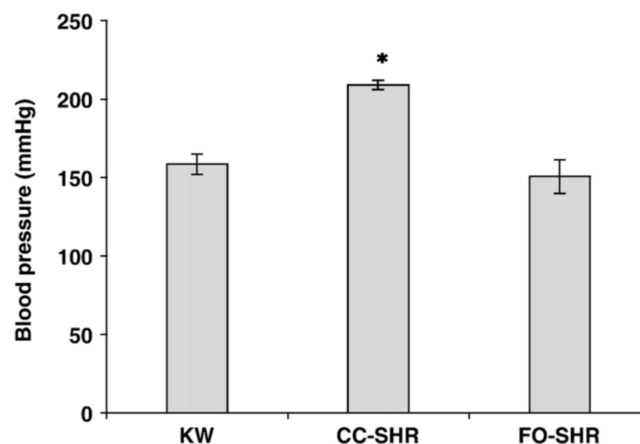

Fig. 1. Systolic blood pressure in control KW rats, CC-SHRs and FO-SHRs after 6 weeks of diet administration. *P<.001.

Table 2
Serum parameters in rats fed with experimental diets during 6 weeks

Parameter	KW	CC-SHR	FO-SHR
Insulin (μUI/ml)	3.6±0.83[a]	6.02±0.9[b]	3.3±0.9[a]
Glucose (mg/dl)	99.0±8.07[a]	88.8±4.8[a]	92.8±1.9[a]
Free fatty acids (mmol/L)	0.72±0.08[a]	1.01±0.1[b]	0.63±0.07[a]
Triglycerides (mg/dl)	88.6±3.6[a]	156.0±5.8[b]	69.0±7.5[a]
Cholesterol (mg/dl)	65.4±2.07[a]	85.4±6.3[b]	48.8±1.6[c]
LDL (mg/dl)	67.6±16.1[a]	104.7±18.07[b]	60.5±10.3[a]
HDL (mg/dl)	55.8±4.8[a]	70.5±4.9[b]	45.3±2.8[a]
Total lipids (mg/dl)	465.7±50.8[a]	651.3±66.9[b]	315.3±82.5[a]

Values are mean±S.D. Different letters mean statistical difference between groups (P<.01). Statistical significance was determined by analysis of variance and Tukey's multiple mean comparison test.

found among the three experimental groups. However, body weight, epididymal fat weight, and food and caloric consumption were similar in both SHR groups, but higher than in KW rats.

Blood pressure in the FO-SHR group was lower than in the CC-SHR group (150.6±10.6 vs. 209.1±2.9 mm Hg, P<.001) but similar to that found in the normotensive KW rats (158.4±6.5 mm Hg) (Fig. 1). The FO-SHR group showed significantly lower concentrations of serum insulin (1.8-fold, P<.01), FFAs (1.6-fold, P<.01), triglycerides (2.3-fold, P<.01), cholesterol (1.7-fold, P<.01), LDL (1.7-fold, P<.01), HDL (1.5-fold, P<.01) and total lipids (2.06-fold, P<.01) than the CC-SHR group. These values, however, were similar to those found in the KW group (Table 2). Serum glucose levels were similar in the three groups.

The effect of fish oil administration on adipocyte *Cd36* mRNA levels was analyzed by RT-PCR (Fig. 2). The CC-SHR group had significantly lower levels of *Cd36* mRNA than the KW rats (117.4±1.7 vs. 140.1±5.2, P<.001). After fish oil administration, the FO-SHR group showed an increase in *Cd36* mRNA levels with respect to the CC-SHR group, reaching almost the same levels as the KW rats (133.7±6.7 vs. 140.1±5.2).

4. Discussion

The purpose of this study was to evaluate the effect of fish oil administration on metabolic parameters and on the expression levels of *Cd36* in a metabolic syndrome rat model, the SHR. As expected, before fish oil administration, the SHRs had significantly higher systolic blood pressure and circulating levels of insulin, FFAs and triglycerides than the KW rats. After 6 weeks on a fish oil-rich diet, the SHRs showed significantly lower concentrations of serum insulin, FFAs, triglycerides, cholesterol, LDL, HDL, total lipids and blood pressure, reaching levels similar to those found in the normotensive KW rats.

Long-chain (n-3) PUFAs have attracted considerable attention during the last decades. Indeed, dietary fish oil rich in EPA (20:5 n-3) and DHA (22:6 n-3) fatty acids has proven to play an important role in reducing hypertriglyceridemia, dyslipidemia and blood pressure, in improving insulin sensitivity and in lowering mortality rates due to coronary heart disease [11–14]. However, discrepancies exist regarding the effects on metabolic syndrome parameters after fish oil or n-3 fatty acid administration, as well as in the mechanisms proposed to explain such effects.

Recently, it has been proposed that Cd36 deficiency underlies insulin resistance, defective fatty acid metabolism and hypertriglyceridemia in SHRs and most likely in humans too [4]. In this study, *Cd36* mRNA levels were reduced in SHR adipocytes (16%) in comparison with those found in KW rats (Fig. 2). This result confirmed previous reports that showed down-regulation of *Cd36* mRNA in SHRs as well as other genes involved in lipid and glucose metabolism and in the insulin signal transduction pathway [2,4]. It has been suggested that the apparent reduced *Cd36* expression found in SHR may be a methodological artifact due to the divergent 3′ UTR observed in the SHR *Cd36* cDNA [1,4]. However, in this study we used primers that amplify the exon 6 region of SHR Cd36 gene, so we cannot rule out the possibility that the lower Cd36 gene mRNA levels are due to metabolic down-regulation or to mutations that affect the expression of the gene. Besides, it should be born in mind that alterations in the promoter region and in the PPARγ ligand-binding domain have been reported in Cd36-deficient patients and in subjects with severe insulin resistance, respectively [5,27]. These situations may also be present in SHRs.

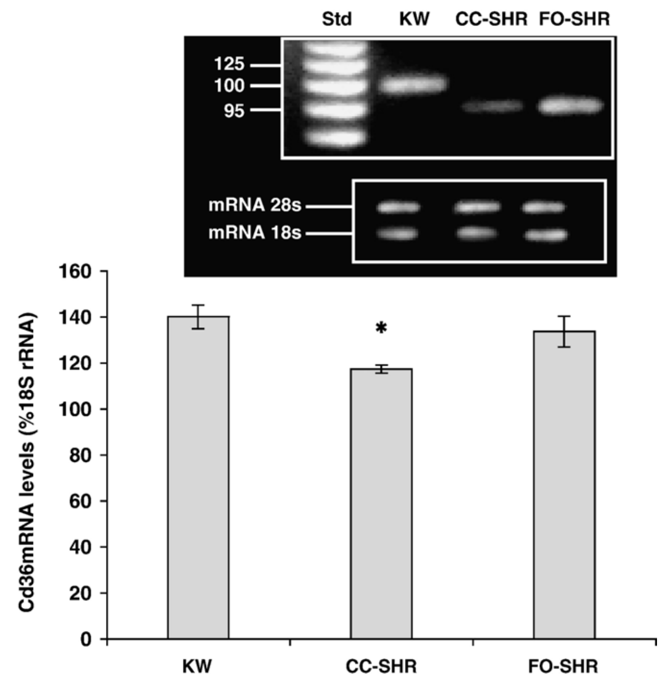

Fig. 2. *Cd36* mRNA levels on abdominal adipocytes in control KW rats, CC-SHRs and FO-SHRs after 6 weeks of diet administration. *Cd36* mRNA levels are expressed as a percentage (means±S.D.) of ribosomal 18S rRNA levels determined as a control for each sample. *P<.001.

After fish oil administration, *Cd36* mRNA levels were increased (14%), reaching the levels found in KW rats (Fig. 2). The ability of PUFAs to stimulate Cd36 gene transcription has been reported in the small intestine, in preadipocytes, in neonatal cardiomiocytes [28] and in mature adipose cells [16]. This effect may be mediated by the binding of PUFA or their metabolites to the nuclear transcription factors of the peroxisome proliferator-activated nuclear receptor family [15,16]. Increased Cd36 concentration in adipose cell's plasma membranes would produce diverse outputs. It would stimulate lipid uptake and storage, reducing the fatty acids available for hepatic triglyceride synthesis, and/or promote glucose uptake, reducing the metabolic burden in the liver and the muscle, and increasing insulin sensitivity [29]. In fact, the increase in *Cd36* mRNA levels observed in this study was associated with decreased levels of FFAs, triglycerides, insulin, cholesterol, LDL, HDL and total lipids in SHR fed the fish oil diet (Table 2). Overexpression of *Cd36* in transgenic mice and rats has also proven to reduce blood triglycerides, fatty acids and cholesterol, and to ameliorate insulin resistance [4,30]. However, it has been reported that SHRs probably carry a *Cd36* deletion variant that results in a Cd36 protein unable to be located in the plasma membrane [1,4], in which case an increase in Cd36 protein in adipocyte membranes could only be achieved if SHRs were heterozygous to the mutated gene. Additional experiments are required to evaluate Cd36 concentration and functionality in adipocyte plasma membranes in SHRs fed the fish oil diet. Nevertheless, activation of PPAR transcription factors by fish oil PUFA would elicit other insulin-sensitizing mechanisms that could account for the results obtained: in adipocytes, expression of genes involved in glucose uptake (c-Cbl-associated protein and glucose transporter 4), lipid uptake and storage (aP2, LPL, FATP, acyl-CoA synthetase, besides Cd36), and energy expenditure (glycerol kinase, uncoupling proteins 2 and 3); or in other tissues, inducing genes involved in lipid uptake and storage in liver, glucose oxidation in muscle and decreasing gluconeogenesis in liver [29].

In conclusion, this study confirmed previous reports regarding the beneficial effects of fish oil administration on the metabolic syndrome. The induction of *Cd36* expression by the activation of PPAR transcription factors could be one of the molecular mechanisms elicited by fish oil PUFA, as has been reported for many insulin-sensitizing drugs like fibrates and the antidiabetic thiazolidinediones [30]. Therapeutic strategies designed to manipulate Cd36 gene expression may be relevant in treating and preventing metabolic syndrome in humans. A nonpharmacological intervention, like fish oil administration, could be a sensible research area.

Acknowledgments

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