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## Eicosapentaenoic acid actions on adiposity and insulin resistance in control and high-fat-fed rats: role of apoptosis, adiponectin and tumour necrosis factor- $\alpha$

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*n*-3 PUFA have shown potential anti-obesity and insulin-sensitising properties. However, the mechanisms involved are not clearly established. The aim of the present study was to assess the effects of EPA administration, one of the *n*-3 PUFA, on body-weight gain and adiposity in rats fed on a standard or a high-fat (cafeteria) diet. The actions on white adipose tissue lipolysis, apoptosis and on several genes related to obesity and insulin resistance were also studied. Control and cafeteria-induced overweight male Wistar rats were assigned into two subgroups, one of them daily received EPA ethyl ester (1 g/kg) for 5 weeks by oral administration. The high-fat diet induced a very significant increase in both body weight and fat mass. Rats fed with the cafeteria diet and orally treated with EPA showed a marginally lower body-weight gain ( $P=0.09$ ), a decrease in food intake ( $P<0.01$ ) and an increase in leptin production ( $P<0.05$ ). EPA administration reduced retroperitoneal adipose tissue weight ( $P<0.05$ ) which could be secondary to the inhibition of the adipogenic transcription factor PPAR $\gamma$  gene expression ( $P<0.001$ ), and also to the increase in apoptosis ( $P<0.05$ ) found in rats fed with a control diet. TNF $\alpha$  gene expression was significantly increased ( $P<0.05$ ) by the cafeteria diet, while EPA treatment was able to prevent ( $P<0.01$ ) the rise in this inflammatory cytokine. Adiposity-corrected adiponectin plasma levels were increased by EPA. These actions on both TNF $\alpha$  and adiponectin could explain the beneficial effects of EPA on insulin resistance induced by the cafeteria diet.

### Eicosapentaenoic acid: High-fat diets: White adipose tissue: Insulin resistance

*n*-3 PUFA present in fish oil are known to have numerous beneficial effects on health (Connor, 2000; Ruxton, 2004), suggesting important anti-adipogenic properties of EPA-enriched diets (Raclot *et al.* 1997). However, other trials have not observed any effects on body-weight gain and visceral fat after EPA treatment (Kusunoki *et al.* 2003). In this context, previous studies by our group in primary cultured rat adipocytes (Perez-Matute *et al.* 2005) and by others in 3T3-L1 cells (Murata *et al.* 2000) demonstrated that EPA was able to stimulate leptin gene expression and protein secretion. These data suggest potential anti-obesity effects of EPA *in vivo* since leptin is a hormone primarily secreted by white adipose tissue (WAT) involved in the regulation of body weight and body fat stores via its central actions on food intake and energy expenditure (Marti & Martinez, 1999).

In order to better elucidate the potential EPA anti-obesity actions, we analysed the effects of its administration in rats fed on a standard diet or a high-fat (cafeteria) diet (Moreno-Aliaga *et al.* 2002; Perez-Echarri *et al.* 2005). Our cafeteria-fed rats constitute a useful model for obesity studies because a high fat intake is a key factor in the development of obesity (Lopez *et al.* 2003).

The mechanisms (alterations of adipogenesis, lipolysis, apoptosis) involved in these potential anti-obesity actions of

EPA remain unclear. Some studies have suggested that an inhibition of PPAR $\gamma$  gene expression, a transcription factor with a key role as master regulator of adipogenesis, could be involved in the potential anti-obesity actions of EPA (Reseland *et al.* 2001). However, controversial data are described (Chambrier *et al.* 2002). Furthermore, contradictory results regarding the lipolytic action of *n*-3 fatty acids have also been shown (Rustan *et al.* 1993; Raclot *et al.* 1997). On the other hand, several authors suggested that EPA limits fat cell trophic growth without modifying the cell number (Parrish *et al.* 1990). However, others have observed a reduction in cellularity of mice epididymal adipose tissue after treatment with *n*-3 PUFA-enriched diets (Ruzickova *et al.* 2004), suggesting a possible apoptotic effect of EPA, although this fact has not been yet addressed. Therefore, the aim of the present study was to assess for the first time the direct potential apoptotic effects of EPA in WAT. In addition, we also tested EPA effects on WAT lipolysis *ex vivo* and on adipogenic PPAR $\gamma$  gene expression.

EPA treatment has also been suggested to improve insulin resistance in several models of obesity and diabetes in rodents (Mori *et al.* 1997, 1999; Nobukata *et al.* 2000). Obesity has been associated with an inflammatory state which has been proposed to be the link with several obesity-associated

**Abbreviations:** HOMA, homeostasis model assessment; OEPA, overweight EPA; WAT, white adipose tissue.

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disorders such as insulin resistance, CVD and metabolic syndrome (Dandona *et al.* 2004; Moreno-Aliaga *et al.* 2005). This inflammatory state associated with obesity appears to be predominantly triggered and to reside in adipose tissue (Wellen & Hotamisligil, 2003). The expression and release of inflammation-related adipokines generally rises as adipose tissue expands, except for adiponectin, which has an anti-inflammatory action (Trayhurn & Wood, 2005). Thus, plasma adiponectin concentrations are decreased in obesity and are positively associated with whole-body insulin sensitivity (Stefan *et al.* 2002). EPA and DHA are well known to have anti-inflammatory properties (Mori & Beilin, 2004), and the ability of fish oil to alter cytokine production from several cell types and reduce pro-inflammatory cytokines has been described (Calder, 2001; Browning, 2003). However, to our knowledge there are no data available regarding the potential anti-inflammatory actions of *n*-3 fatty acids in adipose tissue. In order to address this question, in the present study we analysed the effects of EPA supplementation on the pro-inflammatory cytokine TNF $\alpha$  gene expression in WAT. In addition, adiponectin gene expression and protein production were also studied.

In summary, the present trial provides novel insights into the mechanisms involved in the anti-adipogenic and insulin-sensitising actions of EPA on WAT, especially regarding its effects on apoptosis, TNF $\alpha$  and adiponectin production.

## Material and methods

### Animals and treatment

Twenty-nine male Wistar rats weighing about 180 g (6 weeks old) were obtained from the Centre of Applied Pharmacology (CIFA, Pamplona, Spain). Animals were housed (two or three per cage) in polycarbonate cages in temperature-controlled rooms ( $22 \pm 2^\circ\text{C}$ ) with a 12 h light–dark cycle, fed a pelleted chow diet (Rodent Toxicology Diet; B&K Universal, Aldbrough, Hull, UK) and given deionised water *ad libitum* for an adaptation period of 4 d. Rats were then assigned to two dietary groups for 5 weeks. The control group (*n* 15) was fed a standard pelleted chow laboratory diet containing 18% of energy as protein, 76% of energy as carbohydrate and 6% of energy as lipid by dry weight. The overweight group (*n* 14) was fed on a fat-rich hyperenergetic ‘cafeteria’ diet composed of paté, chips, chocolate, bacon, biscuits and pelleted diet in a proportion of 2:1:1:1:1:1 as previously described (Moreno-Aliaga *et al.* 2002; Perez-Echarri *et al.* 2005). The composition was 9% of energy as protein, 29% as carbohydrate and 62% as lipid by dry weight. The fatty acid composition of both diets (control and cafeteria) is showed in Table 1. Both dietary groups were divided into two new subgroups. One subgroup from each dietary group received by oral administration EPA ethyl ester (1 g/kg per d) daily for 5 weeks (control EPA and overweight EPA (CEPA and OEPA) groups). The other subgroups of rats (control and overweight) received by oral administration the same volume of water as previously shown in other studies (Mori *et al.* 1997; Kusunoki *et al.* 2003). These groups of rats without treatment with any other type of fatty acid (such as saturated fatty acids with equal chain length as EPA or oleic acid) are more likely to be considered as control groups in

**Table 1.** Fatty acid composition (%) of control and cafeteria diets

Fatty acid	Control diet	Cafeteria diet
12:0 (Lauric)	0.00	0.00
14:0 (Myristic)	0.76	0.70
16:0 (Palmitic)	14.42	29.82
16:1 <i>n</i> -7 (Palmitoleic)	0.76	1.75
18:0 (Stearic)	3.19	13.45
18:1 <i>n</i> -9 (Oleic)	22.91	40.80
18:2 <i>n</i> -6 (Linoleic)	51.12	12.48
18:3 <i>n</i> -3 (Linolenic)	5.92	0.22
20:0 (Arachidic)	0.00	0.65
20:4 <i>n</i> -6 (Arachidonic)	0.00	0.11
20:5 <i>n</i> -3 (Eicosapentaenoic)	0.00	0.00
22:1 <i>n</i> -9 (Erucic)	0.83	0.03
22:6 <i>n</i> -3 (Docosahexaenoic)	0.09	0.00

our study design. This is because it has been demonstrated that supplementation with some other fatty acids is able to modify adiposity and the circulating levels of biochemical and hormonal markers planned to be determined in the present study (Mori *et al.* 1997).

Body weight and food intake were daily recorded. At the end of the experimental period and after an overnight fast, rats were killed by decapitation and blood and tissue samples were collected and kept at  $-80^\circ\text{C}$  for subsequent analysis. All procedures were conducted under protocols approved by the University Ethics Committee for the use of laboratory animals.

### Biochemical measurements

Serum glucose was measured using an autoanalyser (Cobas Roche Diagnostic, Basel, Switzerland). Plasma insulin and leptin were assayed by RIA (Linco Research, St Charles, MO, USA). Adiponectin concentrations in serum were determined by a Mouse/Rat Adiponectin ELISA kit (B-Bridge International, Inc., Sunnyvale, CA, USA).

### Adipocyte isolation and ex vivo lipolysis

Adipocytes were isolated from retroperitoneal fat pads of male Wistar rats. The fat pads were minced into pieces in Krebs–Ringer HEPES buffer (pH 7.4; containing 5 mM-D-glucose, 2% bovine serum albumin, 135 mM-NaCl, 2.2 mM-CaCl $_2$ ·2H $_2$ O, 1.25 mM-MgSO $_4$ ·7H $_2$ O, 0.45 mM-KH $_2$ PO $_4$ , 2.17 mM-Na $_2$ HPO $_4$  and 10 mM-HEPES). Adipose tissue fragments were digested in the same buffer with type I collagenase (1.25 mg/ml per 0.5 g tissue; Worthington, Lakewood, NJ, USA) at 37°C with gentle shaking at sixty cycles/min for 30 min. The resulting cell suspension was diluted in HEPES-phosphate buffer and the isolated adipocytes were then separated from the undigested tissue by filtration through a 400  $\mu\text{m}$  nylon mesh and washed three times. *Ex vivo* lipolysis was performed in isolated adipocytes according to Campion & Martínez (2004). Results were expressed as  $\mu\text{mol}$  glycerol released per 100 mg total lipids after 90 min of incubation in Krebs–Ringer buffer.

### Detection of white adipose tissue apoptosis

Apoptotic cell death was measured by using the Cell Death Detection ELISA<sup>PLUS</sup> kit (Boehringer Mannheim

Biochemicals, Indianapolis, IN, USA). This is a photometric enzyme-immunoassay for quantitative *in vitro* determination of cytoplasmic histone-associated DNA fragments, which are generated during apoptosis, using monoclonal antibodies directed against DNA and histones, respectively. All experimental procedures were carried out on ice. The assay was performed according to the manufacturer's instructions, reading absorbance at 405 nm and subtracting absorbance at 490 nm for each well ( $A_{405} - A_{490}$ ). Data represent average values from double absorbance measurements of each sample and were calculated subtracting the background value of the immunoassay from each value (Margareto *et al.* 2000).

#### Gene expression levels in white adipose tissue

Epididymal adipose tissue mRNA expression levels were determined by Northern blotting as described by Pérez-Matute *et al.* (2003). The leptin cDNA probe was a 388 bp fragment whereas adiponectin cDNA probe was a 497 bp fragment. They were kindly provided by Dr Charles Mobbs (Mount Sinai School of Medicine, New York), which is gratefully acknowledged. The PPAR $\gamma$ 2 cDNA probe (Genbank accession number Y12882) was a 570 bp fragment of rat cDNA, and the primers used were 5'-TCTGATTATGGGTG-AACTC-3' (sense 43–62) and 5'-TTTCTACTCTTTTGTGGATC-3' (antisense 592–612). A 320 bp TNF $\alpha$  DNA fragment was prepared by the PCR with primers corresponding to the nucleotides 923–942 (5'-CCCCATTACTCTGACC-CCTT-3') and 1223–1242 (5'-AGGCCTGAGACATCTT-CAGC-3') of rat TNF $\alpha$  cDNA (Genbank accession number AF329987) (Kawaguchi *et al.* 2003). The 18S ribosomal probe was obtained from Ambion (Austin, TX, USA). mRNA expression levels were normalised with respect to this 18S ribosomal signal. Signals were quantified using a Sharp JX-325 densitometric scanner (Sharp Electric, Tokyo, Japan) and the analysis was carried out with the software ImageMaster™ (Pharmacia Biotech, Uppsala, Sweden).

#### Statistical analysis

Results are given as mean values with their standard errors. Statistical differences and interactions were evaluated with a factorial two-way ANOVA (diet  $\times$  EPA treatment). When statistically significant differences resulted at the interaction level, a Student's *t* test was carried out to compare the effects of each treatment (GraphPad Prism; GraphPad Software Inc., San Diego, CA, USA). Differences were considered as statistically significant at  $P < 0.05$ .

## Results

#### Effects on body-weight gain and food intake

High-fat-fed rats showed a significant increase in both final body weight ( $P < 0.001$ ) and body-weight gain ( $P < 0.001$ ) at the end of the experimental trial as compared with control animals (Fig. 1). The administration of EPA (1 g/kg) did not significantly alter body-weight gain, although a marginal decrease ( $P = 0.09$ ) could be observed after EPA treatment in the group of rats fed with the cafeteria diet (OEPA).

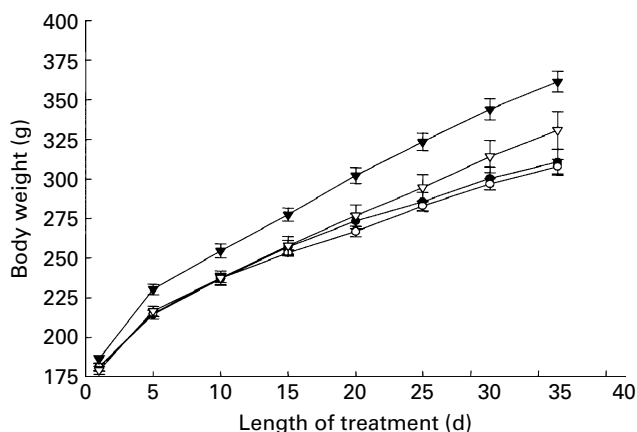


Fig. 1. Effects of EPA (35 d treatment) on the growth curve in rats: control rats ( $n$  8;  $-●-$ ), control rats treated with EPA (1 g/kg;  $n$  7;  $-○-$ ), overweight rats (high-fat-diet;  $n$  7;  $-▼-$ ) and overweight rats treated with EPA (1 g/kg;  $n$  7;  $-▽-$ ). Body weight was daily recorded. Data are means, with standard errors represented by vertical bars. Statistical analysis was by two-way ANOVA; the effect of diet was significant ( $P < 0.001$ ).

Energy intake was measured in order to find out if the differences showed in body-weight evolution could be related to differences in food intake. Overweight rats (fed with a high-fat diet) showed a significant increase in their food intake ( $P < 0.001$ ), which was reduced in part by EPA treatment ( $P < 0.01$ ). Feed efficiency, the weight gain:energy consumed ratio, was significantly decreased ( $P < 0.001$ ) in all groups receiving the high-fat diet as compared with the control group. However, EPA treatment did not modify this parameter (Table 2).

#### Effects on white adipose tissue weights

Wet weights (g) of WAT from overweight rats were significantly higher than in lean animals ( $P < 0.001$ ). Daily administration of EPA produced a significant reduction ( $P < 0.05$ ) in retroperitoneal adipose tissue weight. Indeed, EPA treatment showed a tendency (non-significant) to decrease the weight of all the fat pads, but only in the group of animals fed with a standard diet (Table 2).

Regarding brown adipose tissue, animals fed with the cafeteria diet showed an increase in brown fat ( $P < 0.001$ ) as compared with control rats, while the administration of EPA did not modify the size of this fat depot (Table 2).

#### Effects on plasma biochemical parameters

Neither the cafeteria diet nor EPA treatment modified glucose plasma levels; however, a slight increase was observed after the consumption of the high-fat diet ( $P = 0.08$ ). EPA treatment induced a significant reduction ( $P < 0.05$ ) in both insulin plasma levels and the homeostasis model assessment (HOMA) index, an index of insulin resistance, suggesting that EPA is able to partially prevent the development of insulin resistance induced in a non-significant manner by the cafeteria diet (Table 3).

**Table 2.** Total food intake, feed efficiency and white adipose tissue weights in control and eicosapentaenoic acid-treated lean and overweight rats\* (Mean values with their standard errors)

	Control (n 8)		CEPA (n 7)		Overweight (n 7)		OEPA (n 7)		2 × 2 ANOVA		
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Diet	EPA	Diet × EPA
Total food intake (kJ)\$	9669	155.1	9794	173.8	18224†	172.2	17 046†‡	352.6	–	–	$P < 0.01$
Feed efficiency (g/kJ) × 100	1.33	0.06	1.29	0.05	0.96	0.03	0.88	0.05	$P < 0.001$	NS	NS
Epididymal fat (g)	5.1	0.3	3.4	0.2	8.2	0.9	7.9	0.9	$P < 0.001$	NS	NS
Retroperitoneal fat (g)	6.7	0.7	3.8	0.3	11.2	1.1	9.6	0.9	$P < 0.001$	$P < 0.05$	NS
Mesenteric fat (g)	2.7	0.4	2.1	0.2	4.4	0.3	4.7	0.6	$P < 0.001$	NS	NS
Subcutaneous fat (g)	5.0	0.7	3.2	0.4	7.5	0.9	9.0	1.3	$P < 0.001$	NS	NS
Brown adipose tissue (g)	0.2	0.01	0.2	0.02	0.3	0.04	0.3	0.04	$P < 0.001$	NS	NS

CEPA, control EPA; OEPA, overweight EPA.

\*Data were analysed by two-way ANOVA. When an interaction was found, comparisons between groups were analysed by a Student's *t* test.

†Mean value was significantly different from that of the control group ( $P < 0.001$ ).

‡Mean value was significantly different from that of the overweight group ( $P < 0.01$ ).

§Energy supplied by EPA was taken into account when total food intake was measured.

#### Effects on retroperitoneal white adipose tissue apoptosis

No differences in the relative apoptosis rate were observed in overweight rats as compared with the basal apoptosis of the control rats. EPA treatment induced a marked increase ( $P < 0.05$ ) in the levels of histone-associated DNA oligonucleosomal fragments, a characteristic marker of apoptosis in the group of animals fed with the standard diet but not in the high-fat-fed rats (Fig. 2).

#### Effects on ex vivo lipolysis

High-fat-diet feeding caused a significant decrease ( $P < 0.05$ ) in isoproterenol-stimulated lipolytic response in both EPA-treated and untreated rats (Fig. 3). EPA treatment, however, did not affect either basal lipolysis (data not shown) or isoproterenol-induced lipolysis (Fig. 3).

#### Effects on leptin circulating levels and gene expression

Regarding leptin circulating levels, a significant interaction ( $P < 0.01$ ) was obtained between diet and EPA treatment. As expected, leptin levels were significantly higher ( $P < 0.05$ ) in the cafeteria-fed group as compared with control animals. EPA treatment significantly decreased leptin circulating levels ( $P < 0.01$ ) in the group of animals fed on a standard diet while a significant increase ( $P < 0.05$ ) was observed in overweight rats treated with the fatty acid (Fig. 4 (A)). When leptin serum concentrations were corrected for adiposity (leptin per g WAT), which was estimated by the sum of all fat depots collected (see Table 2), a significant decrease ( $P < 0.05$ ) was observed in the group of control animals treated with EPA. On the contrary, EPA caused a significant elevation of leptin production levels ( $P < 0.01$ ) in the high-fat-fed rats (Fig. 4 (B)). Similarly, leptin gene expression in epididymal fat showed the same pattern as circulating levels (Fig. 4 (C)).

#### Effects on adiponectin circulating levels and gene expression

Regarding adiponectin, cafeteria-diet feeding significantly increased adiponectin circulating levels ( $P < 0.001$ ); however, EPA treatment did not affect the circulating levels of this adipokine (Fig. 5 (A)). When adiponectin concentrations were corrected for adiposity (adiponectin per g WAT), a marginally non-significant decrease ( $P = 0.07$ ) was observed in the cafeteria-fed group. Furthermore, EPA treatment induced a significant increase ( $P < 0.05$ ) in adiponectin concentrations per g WAT (Fig. 5 (B)).

Northern blot analysis revealed three mRNA bands for adiponectin in rats (Hu *et al.* 1996). In agreement with the observed levels of serum adiponectin, an increase in adiponectin gene expression in WAT was also observed after EPA treatment (Fig. 5 (C)).

Several studies have suggested that there is a strong relationship between low adiponectin levels and insulin resistance (Hotta *et al.* 2001). In fact, our data showed an inverse relationship between serum adiponectin levels corrected for adiposity and HOMA index ( $r = -0.43$ ;  $P < 0.05$ ).

**Table 3.** Serum glucose, plasma insulin and homeostasis model assessment (HOMA) index in control and eicosapentaenoic acid-treated lean and overweight rats\*

(Mean values with their standard errors)

	Control (n 8)		CEPA (n 7)		Overweight (n 7)		OEPA (n 7)		2 × 2 ANOVA		
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Diet	EPA	Diet × EPA
Glucose (mg/l)	948	27	933	37	1045	35	958	33	<i>P</i> =0.08	NS	NS
Insulin (pM)	161.5	27.0	78.0	5.3	195.8	46.6	138.8	16.4	NS	<i>P</i> <0.05	NS
HOMA†	5.4	0.9	2.5	0.08	7.1	1.6	4.8	0.5	NS	<i>P</i> <0.05	NS

CEPA, control EPA; OEPA, overweight EPA.

\*Data were analysed by two-way ANOVA.

†HOMA = insulin (μUnits) × glucose (mmol/l)/22.5.

### Effects on white adipose tissue peroxisome proliferator-activated receptor- $\gamma$ and tumour necrosis factor- $\alpha$ gene expression

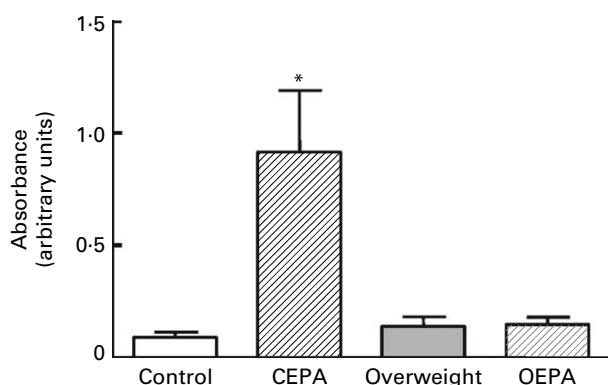
The expression of the adipogenic factor PPAR $\gamma$  was significantly ( $P$ <0.001) decreased by EPA treatment in both control and cafeteria-fed animals (Figs. 6 (A) and (B)).

As is observed in Figs. 6 (C) and (D), TNF $\alpha$  gene expression was significantly stimulated ( $P$ <0.05) by cafeteria-diet feeding. EPA treatment was able to prevent ( $P$ <0.01) the rise in the expression of this inflammatory cytokine induced by the high-fat diet.

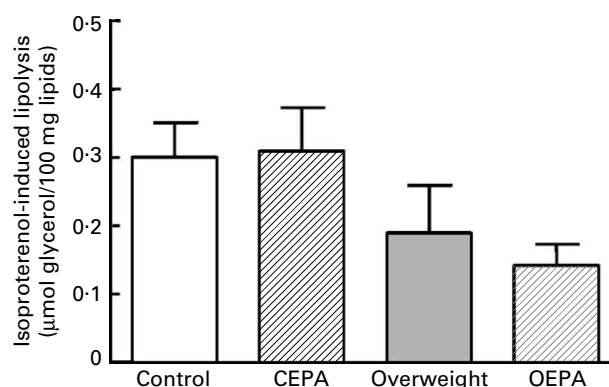
### Discussion

Animals fed with a high-energy-yielding diet during 35 d exhibited a significant increase in body-weight gain. EPA treatment, however, was not able to prevent this increase induced by the high-fat diet, although a moderate lower body-weight gain was observed in the group of animals fed with the high-fat diet and orally treated with EPA. These results are in agreement with those from Kusunoki *et al.* (2003) who did not observe any significant effect of EPA treatment (concentrations from 0.3 to 1 g/kg) on body-weight

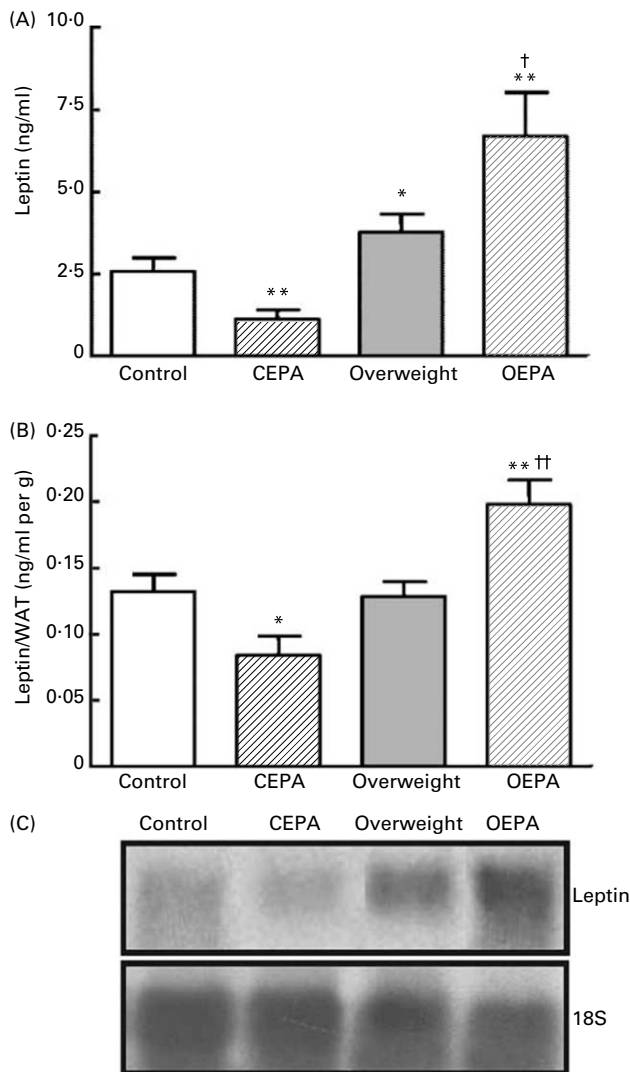
gain in Otsuka Long-Evans Tokushima Fatty (OLETF) rats. Energy intake was measured in order to find out if the moderate differences showed in body-weight evolution could be related to differences in food intake. As in previous studies, animals fed with the high-fat diet showed an increase in food intake in comparison with the control group, probably because palatability is the predominant force driving the hyperphagia in cafeteria-fed animals (Berraondo *et al.* 2000; Margareto *et al.* 2001). Interestingly, EPA treatment was able to decrease food intake, but only in the group of animals fed on the cafeteria diet (OEPA). It is well known that rising concentrations of leptin generate a signal to reduce food intake and increase energy expenditure (Havel, 2004). Taking into account this role of leptin in body-weight regulation, it could be suggested that the EPA-induced increase in leptin production specifically observed in the cafeteria-fed rats could explain the decrease in food intake observed in this group of animals. Peyron-Caso *et al.* (2002) also described an increase in leptin concentrations in rats fed an *n*-3 PUFA-enriched diet. In addition, our previous studies in primary cultured rat adipocytes also demonstrated a stimulatory action of EPA on leptin production (Perez-Matute *et al.* 2005). However, other studies showed an inhibitory action of this fatty acid on leptin (Raclot *et al.* 1997; Reseland *et al.* 2001). These conflicting results could be due to the observations that the effects of *n*-3 PUFA on leptin are depen-



**Fig. 2.** Effects of EPA on apoptosis in white adipose tissue of control and overweight rats. Data are means for at least seven independent animals per group, with standard errors represented by vertical bars. Statistical analysis was by two-way ANOVA; the diet × EPA interaction was significant ( $P$ <0.01). When an interaction was found, comparisons between groups were analysed by a Student's *t* test. \*Mean value was significantly different from that of the control group ( $P$ <0.05). CEPA, control EPA; OEPA, overweight EPA.



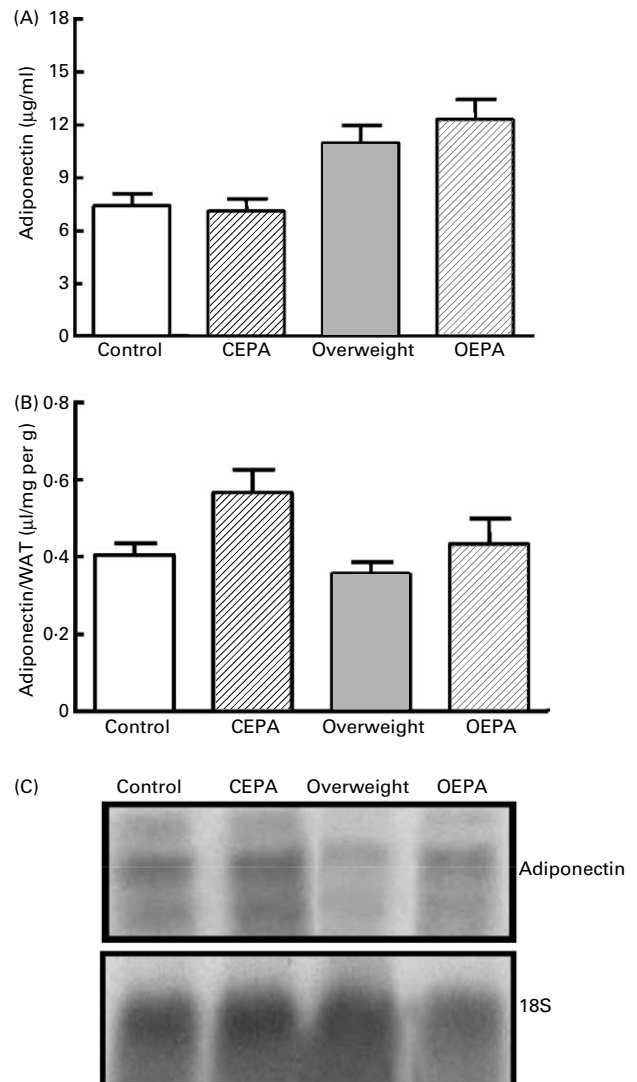
**Fig. 3.** Effects of EPA on isoproterenol-induced lipolysis in white adipose tissue from control and overweight-cafeeteria rats. Data are means for at least four independent animals per group, with standard errors represented by vertical bars. Statistical analysis was by two-way ANOVA; the effect of diet was significant ( $P$ <0.05). CEPA, control EPA; OEPA, overweight EPA.



**Fig. 4.** Effects of EPA on leptin circulating levels (A), leptin concentrations expressed per g white adipose tissue (WAT) (B) and mRNA expression in epididymal fat (C) in control and overweight-caféteria rats. The expression level of 18S ribosomal RNA was determined and used as an internal control to correct for minor variation in total RNA amount. Densitometric scanning was used to determine the relative amount of leptin mRNA and 18S RNA. Data are means for at least seven independent animals per group ( $n = 4$  in Northern blot), with standard errors represented by vertical bars. Statistical analysis was by two-way ANOVA; the diet  $\times$  EPA interaction was significant for leptin ( $P < 0.01$ ) and for leptin/WAT ( $P < 0.001$ ). When an interaction was found, comparisons between groups were analysed by Student's  $t$  test. Mean value was significantly different from that of the control group: \* $P < 0.05$ , \*\* $P < 0.01$ . Mean value was significantly different from that of the overweight group: † $P < 0.05$ , †† $P < 0.01$ . CEPA, control EPA; OEPA, overweight EPA.

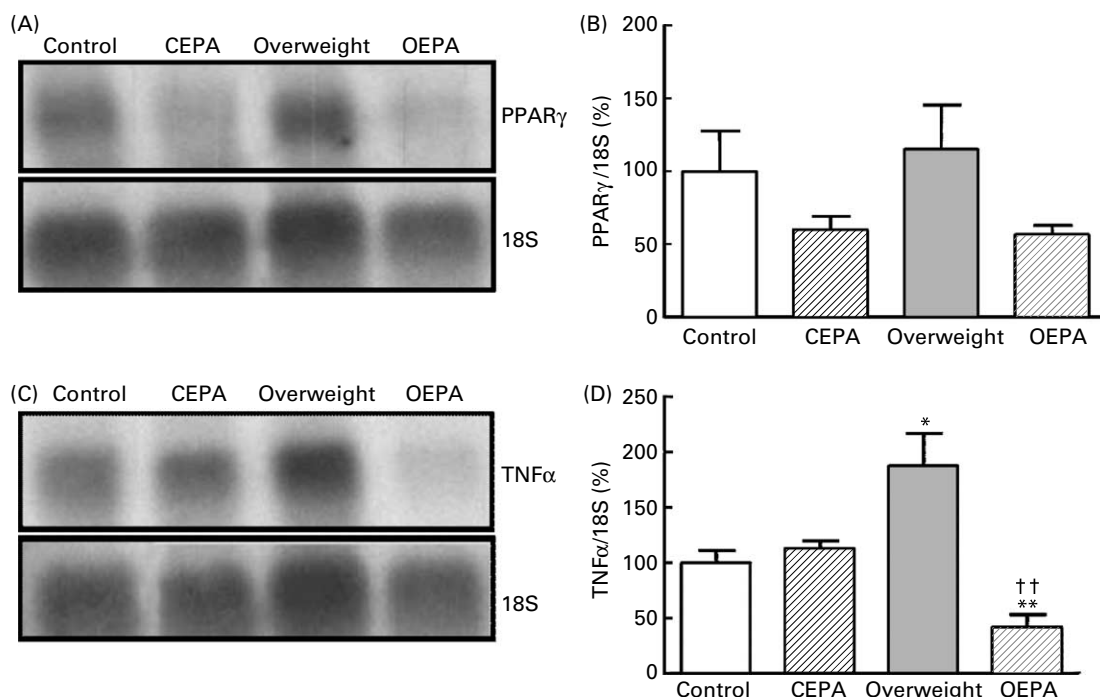
dependent on a number of factors such as diet composition and energy balance. In this way, the present results showed opposite effects of EPA treatment in control rats in comparison with the overweight group. Other studies have also observed differential actions of some anti-obesity treatments (such as  $\beta$ -adrenergic agonist) on genes related to energy homeostasis between control and obese animals (Berraondo *et al.* 2000).

With regard to adiposity, EPA treatment induced a depot-specific reduction in the weight of retroperitoneal fat, as previously observed by Raclot *et al.* (1997). Furthermore, EPA treatment also induced a moderate reduction in the weight



**Fig. 5.** Effects of EPA on adiponectin circulating levels (A), adiponectin concentrations expressed per g white adipose tissue (WAT) (B) and mRNA expression in epididymal fat (C) in control and overweight-caféteria rats. The expression level of 18S ribosomal RNA was determined and used as an internal control to correct for minor variation in total RNA amount. Densitometric scanning was used to determine the relative amount of adiponectin mRNA and 18S RNA. Data are means for at least seven independent animals per group ( $n = 4$  in Northern blot), with standard errors represented by vertical bars. Statistical analysis was by two-way ANOVA; the effect of diet was significant for adiponectin ( $P < 0.001$ ), the effect of EPA was significant for adiponectin/WAT ( $P < 0.05$ ) and the effect of diet was marginally significant for adiponectin/WAT ( $P = 0.07$ ). CEPA, control EPA; OEPA, overweight EPA.

of all fat depots but only in control animals. Hill *et al.* (1993) observed a decrease in all fat depots of adult male Wistar rats after 6 months of treatment with dietary  $n$ -3 PUFA, suggesting that the potential anti-adipogenic actions of  $n$ -3 PUFA are dependent on treatment duration and metabolic status. In this sense, if our EPA treatment lasted more than 5 weeks, it could be possible to observe a significant decrease in the weight of all fat depots and not only in the retroperitoneal fat pad. Paradoxically, the lowering effects of EPA on adiposity contrast with the lack of effects on body-weight gain observed in control rats. Previous studies have



**Fig. 6.** Effects of EPA on PPAR $\gamma$  mRNA (A and B) and TNF $\alpha$  mRNA (C and D) expression in epididymal fat in control and overweight-caféteria rats. The expression level of 18S ribosomal RNA was determined and used as an internal control to correct for minor variation in total RNA amount. Densitometric scanning was used to determine the relative amount of PPAR $\gamma$ , TNF $\alpha$  mRNA and 18S RNA. Data are means for at least four independent animals per group, with standard errors represented by vertical bars. Statistical analysis was by two-way ANOVA; the effect of EPA was significant for PPAR $\gamma$  mRNA ( $P < 0.001$ ) and the diet  $\times$  EPA interaction was significant for TNF $\alpha$  mRNA ( $P < 0.001$ ). When an interaction was found, comparisons between groups were analysed by a Student's *t* test. Mean value was significantly different from that of the control group: \* $P < 0.05$ , \*\* $P < 0.01$ . ††Mean value was significantly different from that of the overweight group ( $P < 0.01$ ). CEPA, control EPA; OEPA, overweight EPA.

found that a fish oil-enriched diet induces greater gain in lean body mass and a lower gain in fat in comparison with animals fed with other type of enriched diets (Su & Jones, 1993). Although we have not performed any body composition measurements, our data also suggest that EPA treatment could induce a body composition redistribution, which could explain that a reduction in adipose tissue weight did not always mean a decrease in total body weight, as observed in the present study.

An increase of lipolysis could be a potential mechanism responsible for the limitation of retroperitoneal fat weight after EPA treatment. The increase in fat pad weights in high-fat-fed animals was accompanied by a decrease in the isoproterenol-induced lipolytic response, as previously shown (Portillo *et al.* 1999). The present study demonstrated a lack of effect of EPA on both basal and isoproterenol-stimulated lipolysis, suggesting that the potential anti-adipogenic properties of this fatty acid are not mediated by a lipolytic action in WAT. In addition, our *in vitro* study in cultured adipocytes also argues against a direct lipolytic action of this fatty acid (Perez-Matute *et al.* 2005).

PPAR $\gamma$ , a member of the nuclear hormone receptor superfamily, is an important adipogenic transcription factor. Several articles have described an increase in the expression of this factor in WAT of high-fat-fed obese animals (Margareto *et al.* 2002; Lopez *et al.* 2003). In the present study, we have also observed a slight non-significant increase in the expression of this factor after the cafeteria-diet consumption

without statistical significance. Several studies have reported the ability of EPA to modulate the expression and activity of this adipogenic factor, although the results are controversial. Thus, some studies have observed an inhibitory action of EPA on PPAR $\gamma$  (Reseland *et al.* 2001) while others have observed an stimulatory effect (Chambrier *et al.* 2002). Our data revealed that EPA-treated animals exhibited a decrease in the adipogenic PPAR $\gamma$  gene expression levels, which could impair the adipogenic process, contributing to the decrease in WAT weight observed in these animals.

Targeting apoptotic pathways in adipocytes has been proposed as a potential novel treatment for obesity (Nelson-Dooley *et al.* 2005). We tested if an increase in apoptosis could be contributing to the reduced WAT size. Ruzickova *et al.* (2004) demonstrated that *n*-3 PUFA limit diet-induced obesity in mice by reducing cellularity of adipose tissue and, although they did not directly measure apoptosis, their data suggest a potential apoptotic action of EPA. Our data showed for the first time that an increase in apoptosis is involved in the more efficient action of EPA on the reduction of WAT size observed in control rats. Thus, the rise in the apoptotic cell death rate would decrease adipocyte number and consequently adipose tissue weight. Finally, we also observed in the present study that the cafeteria diet strongly impaired this apoptotic action induced by EPA, suggesting that EPA-induced apoptosis depends on the nutritional and metabolic status of animals. Furthermore, the opposite effects of EPA observed in control and cafeteria-fed rats could be also

due to the different percentage of EPA in relation to total lipids supplied by both types of diets. In the present study, the EPA supplemented by oral administration in rats fed with the cafeteria diet (62% of energy as fat) accounts for 2.1% of the total lipids. However, EPA accounts for a 30.2% of the total lipids ingested by the rats fed with the control diet (6% of energy as fat) and orally treated with EPA. In this context, recent evidence suggests that the ratio between different types of fatty acids such as the *n*-6 and *n*-3 fatty acids is an important fact in determining their effects on health (Simopoulos, 2002; Weiss *et al.* 2005).

Several studies have suggested that obesity could be an inflammatory disease (Das, 2001; Ramos *et al.* 2003). In this context, several markers of chronic inflammation, such as TNF $\alpha$ , have been found to be associated with obesity and insulin resistance (Ramos *et al.* 2003). In fact, similarly to our present data, an induction of TNF $\alpha$  mRNA expression and activity was observed in adipose tissue from different rodent models of obesity (Hotamisligil *et al.* 1993; Morin *et al.* 1997). Here, we have demonstrated for the first time that EPA treatment was able to down regulate the increased adipose TNF $\alpha$  gene expression induced by a high-fat diet. Although the mechanisms involved in this inhibitory action have not been yet elucidated, it has been suggested that EPA could prevent the NF- $\kappa$ B activation (Zhao *et al.* 2004). The inflammatory state associated with obesity seems to be predominantly initiated and to reside in adipose tissue, although other metabolically key organs may also be involved during the course of the disease (Wellen & Hotamisligil, 2003). In addition, this inflammatory state associated with obesity has been proposed to be the link with several obesity-associated disorders such as insulin resistance (Dandona *et al.* 2004). Indeed, increased TNF $\alpha$  levels have been associated with increased insulin resistance in adipose tissue, by reducing the phosphorylation of insulin receptor substrate-1 (Hotamisligil, 1999). Therefore, our data lead us to suggest that the improvement in insulin sensitivity induced by EPA could be mediated, at least in part, by decreasing the inflammation in the adipose tissue of the overweight rats.

Adiponectin is an anti-inflammatory adipokine with an important role in insulin sensitivity (Combs *et al.* 2004). Thus, hypoadiponectinaemia has been related to the development of insulin resistance and cardiovascular risk associated with obesity (Hotta *et al.* 2001; Kubota *et al.* 2002; Kumada *et al.* 2003). However, our data showed that levels of circulating adiponectin were increased in overweight rats fed with the high-fat diet. Several studies have also shown an increase in both adiponectin circulating levels and gene expression in early stages of obesity (11, 15 and 21 d of high-fat-diet consumption) (Li *et al.* 2002; Lopez *et al.* 2004; Perez-Echarri *et al.* 2005) suggesting that this could be an initial mechanism in the adaptation to high energy intake. In the present study, when the levels of circulating adiponectin were corrected for adiposity, a moderate non-significant decrease was observed in the untreated cafeteria rats, accompanied by a decrease in the expression of this adipokine, suggesting an impairment in adiponectin production processes by the overweight adipocytes. These results suggest that when obesity is more established (for example, after 65 d of a high-fat diet as described by Lopez *et al.* (2005)) the ability of adipocytes to express and secrete adiponectin is more strongly impaired. Our data

also suggest that this ability of adipocytes to produce adiponectin was significantly increased by the administration of EPA, which could contribute to the insulin-sensitising properties of this fatty acid. Furthermore, a negative relationship was found in the present study between adiposity-corrected adiponectin plasma levels and the HOMA index which supports the beneficial effects of EPA supplementation on insulin sensitivity. A recently published study (Flachs *et al.* 2006) has also observed that feeding mice with a high-fat diet enriched with EPA and DHA leads to elevated systemic concentrations of adiponectin, and suggested that this increase could explain, to some extent, the *n*-3 EPA antidiabetic properties.

In summary, our data suggest that EPA supplementation could help to reduce adiposity through the down regulation of PPAR $\gamma$  and through the increase of apoptosis in WAT. EPA also exhibited antidiabetic properties which could be related to its reducing effects on the pro-inflammatory cytokine TNF $\alpha$  mRNA levels in adipose tissue and increasing the capacity of the adipose tissue to produce the insulin-sensitising adipokine, adiponectin. In addition, the present study suggests that some EPA actions seem to be dependent on diet composition and the physiological and metabolic status of animals, which could be important to take into account when considering supplementation with *n*-3-enriched-products.

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