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ORIGINAL CONTRIBUTION

Nerea Pérez-Echarri
Patricia Pérez-Matute
Beatriz Marcos-Gómez
Maria J. Baena
Amelia Marti
J. Alfredo Martínez
María Jesus Moreno-Aliaga

Differential inflammatory status in rats susceptible or resistant to diet-induced obesity: effects of EPA ethyl ester treatment

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N. Pérez-Echarri · P. Pérez-Matute
B. Marcos-Gómez · M.J. Baena
A. Marti · J.A. Martínez
M.J. Moreno-Aliaga (✉)
Dept. of Nutrition and Food Sciences,
Physiology and Toxicology
University of Navarra
31008 Pamplona, Spain
Tel.: +34-948/425-600
Fax: +34-948/425-649 ext. -6558
E-Mail: mjmoreno@unav.es

Abstract *Background* Obesity has been associated with a chronic low degree inflammatory response, characterized by an increase of inflammatory adipocytokines like tumoral necrosis factor-α (TNF-α), interleukin-6 (IL-6) as well as the synthesis of acute phase reactants such as haptoglobin. *Aim of the study* To evaluate if impairments in the inflammatory response at the white adipose tissue (WAT) level could be involved in the mechanisms conferring susceptibility or resistance to high-fat diet-induced obesity (DIO). *Methods* The expression levels of WAT genes and systemic markers related to inflammation were evaluated in two groups of rats fed with a high-fat diet during 15 days that showed either an early susceptibility (DIO) or resistance (DR) to develop obesity. We also tested the efficacy of the eicosapentaenoic (EPA) ω-3 fatty acid treatment (35 days) to potentially counteract the obesity-associated inflammatory features in DIO rats. *Results* This trial showed that high-fat diet induces an increase on mRNA levels on TNF-α and haptoglobin in DIO animals ($P < 0.05$), while no significant changes were observed on DR rats. Furthermore, a significant increase in IL-6 mRNA ($P < 0.05$) was found in both DR and DIO rats. EPA-treatment caused a significant decrease in IL-6 mRNA ($P < 0.05$), without significant changes in haptoglobin mRNA levels in adipose tissue. An unexpected decrease was observed in haptoglobin serum levels ($P < 0.05$) in DIO rats, which was reverted to control values in EPA-treated animals. *Conclusions* Our data suggest that obesity susceptibility or resistance may depend on the genetic make up related to inflammatory features, and support a role for ω-3 fatty acids in the prevention of obesity-associated inflammation in adipose tissue. In addition, our data do not support the hypothesis that serum haptoglobin is an acute phase protein expected to be positively related to increased adiposity in rats, at least in early and medium stages of DIO.

Key words inflammation – high-fat diet – susceptibility – resistance – eicosapentaenoic fatty acid (EPA) – white adipose tissue (WAT)

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Introduction

Various products of white adipose tissue (WAT), linked specifically to inflammation [35], have been characterized including cytokines such as tumor necrosis factor α (TNF-α) [14], interleukin-6 (IL-6) [38], and acute phase proteins like haptoglobin [12], suggesting the association between obesity and chronic inflammatory status[13].

However, even though inflammation is mainly considered as an effect of obesity or weight gain increase [11], it has been also suggested that inflammation could be a causal event in obesity development [3]. Furthermore, several reports have suggested that some inflammation-sensitive plasma proteins are associated with future weight gain [7, 8] and other obesity-related pathologies in humans, like hypercholesterolemia [10] and diabetes [9]. Moreover, it is well known that some individuals are more susceptible to develop overweight when eating high-fat diets, while others are resistant to increase their body weight under similar conditions [27]. For this purpose, the first aim of the present work was to determine if alterations in the regulation of adipose tissue inflammatory genes could be involved in the mechanisms conferring susceptibility or resistance to obesity development in rats fed during 15 days with a high-fat diet.

In addition, it has been suggested that the improvement of obesity and its associated co-morbidities, are accompanying by some beneficial changes on inflammation parameters [1, 22]. Supplementation with ω-3 fatty acids has shown anti-inflammatory effects in both health populations [34] and in models of chronic inflammatory conditions [29, 37]. Furthermore, there are many evidences suggesting that the intake of ω-3 fatty acids, especially eicosapentaenoic fatty acid (EPA), produce some benefits in obesity features [2, 28]. However, it is unclear if the beneficial effects of ω-3 fatty acids on obesity and insulin sensitivity are mediated through anti-inflammatory mechanisms. For this reason, the second aim of our study was to determine the effects of the EPA ethyl ester on inflammation-related genes in DIO rats.

Methods

Animals and treatments

Effects of high fat on outbreed obesity-prone and resistant Wistar rats

Six-week-old male Wistar rats were housed in cages, in a temperature-controlled room (22 ± 2°C) with a 12-h light dark cycle. All experimental procedures were performed under protocols approved by the University Ethics committee for the use of laboratory animals, according to the National and Institutional Guidelines for Animal Care and Use. A control group (n = 6) was fed a standard pelleted diet during 15 days (Rodent Toxicology Diet, B&K Universal) containing 76% of carbohydrates, 6% of lipids and 18% of proteins (362 kcal/100 g) whereas the cafeteria diet group (n = 18) was fed on a high-fat diet [28] containing 29% of carbohydrates, 62% of lipids and 9% of proteins (467 kcal/100 g as energy). The animals had ad libitum access to water and food. After 15 days, two groups of rats with different sensitivity to the cafeteria diet were clearly identified. The six animals from the cafeteria diet group, with lower body weight gain were designated as diet resistance (DR) group, whereas the six heaviest rodents from cafeteria-fed group were assigned as diet-induced obesity (DIO) group. After the feeding period of 15 days and an overnight fast, animals were euthanized by decapitation. Serum was obtained to analyze haptoglobin and IL-6 circulating levels. Visceral white adipose depots were frozen in liquid nitrogen and stored at −80°C [27].

Effects of EPA ethyl ester on outbreed obesity-prone (DIO) Wistar rats

Male Wistar rats (6 weeks old), supplied from the Centre of Applied Pharmacology (CIFA, Pamplona, Spain) were housed similarly as in the first experiment. The animals were distributed in three experimental groups: Control, DIO and DIO + EPA. The control group was fed chow diet whereas DIO and DIO + EPA groups were fed on a high-fat (cafeteria) diet, during 35 days. Compositions of diets are the same as described on experiment 1. All animals had ad libitum access to water and food during 5 weeks. Furthermore, DIO + EPA group was daily treated by oral gavage with 1 g/kg animal weight of highly purified eicosapentaenoic (EPA) acid ethyl ester (supplied by Brudy S.L., Spain) during 35 days, as previously described [28]. After 35 days, animals were submitted to an overnight fasting and then were euthanized by decapitation. Serum was obtained to analyze haptoglobin and IL-6 circulating levels. Visceral WAT was frozen in liquid nitrogen before stored at −80°C.

Gene expression analysis

Northern-blot analysis was performed as previously described [27, 28]. cDNAs were synthesized from total RNA using the following primers: TNF-α (sense: 5′-CCCCATTACTCTGACCCCTT-3′; antisense: 5′-AGGC CTGAGACATCTTCAGC-3′ a 320 pb fragment with

Table 1 Body weight gain and metabolic state in DR and DIO rats after 15 days of high fat feeding, and in DIO rats treated with EPA ethyl ester during 35 days

	15 days of treatment			35 days of treatment		
	Control ($n = 6$) Mean ± SE	DR ($n = 6$) Mean ± SE	DIO ($n = 6$) Mean ± SE	Control ($n = 6$) Mean ± SE	DIO ($n = 6$) Mean ± SE	DIO + EPA ($n = 6$) Mean ± SE
Body weight gain (g)	85.9 ± 5.3	69.1 ± 4.6*,§§§	113 ± 8.5*,§§§	127.9 ± 4.5	178.3 ± 7.3***	148.8 ± 11.5
Retroperitoneal fat (g)	2.5 ± 0.4	3.5 ± 0.5	6.1 ± 0.7***,§	6.1 ± 0.7	11.3 ± 1.4**	9.2 ± 0.9*
Epididymal fat (g)	2.6 ± 0.2	2.9 ± 0.3	5.9 ± 0.5***,§§§	4.9 ± 0.3	8.6 ± 1.0**	7.7 ± 1.0*
Triglycerides (mg/dl)	74.5 ± 11.9	68.3 ± 12.5	80.5 ± 12.0	83.0 ± 10.2	94.2 ± 14.9	66.3 ± 7.3
Cholesterol (mg/dl)	64.8 ± 2.9	61.6 ± 6.3	69.9 ± 4.2	47.3 ± 1.4	56.6 ± 3.4*	42.4 ± 2.6††
Glucose (mg/dl)	98.2 ± 3.9	110.9 ± 8.1	112.6 ± 6.9	94.7 ± 3.6	104.8 ± 4.2	96.1 ± 3.9
Insulin (pmol/l)	70.5 ± 17.4	139.3 ± 50.8	92.5 ± 20.3	124.1 ± 20.1	233.3 ± 63.4*	138.8 ± 16.5†

Data (mean ± SE) were analyzed by one-way ANOVA
*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ versus respective Control group; §$P < 0.05$ and §§§$P < 0.001$ DR versus DIO; †$P < 0.05$ and ††$P < 0.01$ DIO versus DIO+EPA

Genebank number: AF329987 [17]) and IL-6 (sense: 5′-GACTGATGTTGTTGACAGCCACTGC-3′; antisense: 5′-TAGCCACTCCTTCTGTGACTCTAACT-3′; resulting a 509 pb fragment with Genebank number: NM_012589). The 18S ribosomal probe, used as a housekeeping gene, was obtained from Ambion (Ambion, Austin, TX, USA).

For the real-time PCR, 5 μg of RNA of each tissue sample were treated with DNA free (Ambion, Inc., Canada, US) in a total reaction volume of 24 μl during 30 min at 37°C; 13 μl of this product was retrotranscripted to cDNA. Reaction conditions of retrotranscription were as follows: 60 min at 37°C and 5 min at 95°C and for real-time PCR amplification, 9 μl of cDNA per reaction was used, being reaction conditions as follows: 10 min at 25°C and 120 min at 37°C. TaqMan probes for haptoglobin (Rn00561393_m1) and 18S (Hs99999901_s1) were Assay-on-Demand gene expression products. Reagents for real-time PCR analysis (Assays-on-Demand, TaqMan Reverse Transcriptase reagents and TaqMan Universal PCR Master mix) were purchased from Applied Biosystems (Foster City, CA, USA) and the conditions were used according to the manufacturer's protocol. Amplification and detection of specific products were performed with the ABI PRISM 7000HT Sequence Detection System (Applied Biosystems). Data were obtained as *Ct* values (the cycle at where the fluorescence signal emitted is significantly above background levels and is inversely proportional to the initial template copy number) according to the manufacturer's guidelines, and used to determine ΔCT values ($\Delta CT = Ct$ of the target gene $- Ct$ of the housekeeping gene) of each sample. Fold changes of gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method [23].

Serum analysis

Circulating levels of glucose, triglycerides and cholesterol were measured using a COBAS-Mira autoanalyzer (Roche Diagnostic, Basel, Switzerland). Insulin circulating levels were determined by by radioimmunoassay using a commercially available RIA kit (Linco Research, St. Charles, MO, USA), according to the manufacturer's instructions [27, 28]. Furthermore, serum levels of haptoglobin and IL-6 were measured by ELISA: Rat Haptoglobin ELISA test kit (Life Diagnostics, West Chester, PA, USA), Quantikine® Rat IL-6 ELISA kit (R&D Systems, MN, USA), respectively.

Statistical analysis

Results are given as mean ± standard error (SE). Statistical differences were evaluated through one-way ANOVA or two-tail *t* Student test analysis. Statistical analysis were performed using the *GraphPad Prism* version 4.00 (GraphPad Software Inc, San Diego, CA, USA). Differences were considered as statistically significant at $P < 0.05$.

Results and discussion

After 15 days of nutritional intervention, two groups of rats with different response to the high-fat diet were identified. In fact, the high-fat diet intake induced a significant increase in body weight gain and adipose tissue weights in DIO animals, but not in DR rats (Table 1). On the other hand, EPA ethyl ester treatment during 35 days was partially able to prevent the body weight gain ($P = 0.06$), the hyperinsulinemia ($P < 0.05$) and hypercholesterolemia ($P < 0.01$) induced by the intake of high-fat diet in DIO rats (Table 1).

The link between inflammation and obesity has been attracting growing attention in the recent years [4, 26]. Whilst the general assumption is that inflammation is subsequent to obesity [11], it has been suggested that obesity may be the result of a low

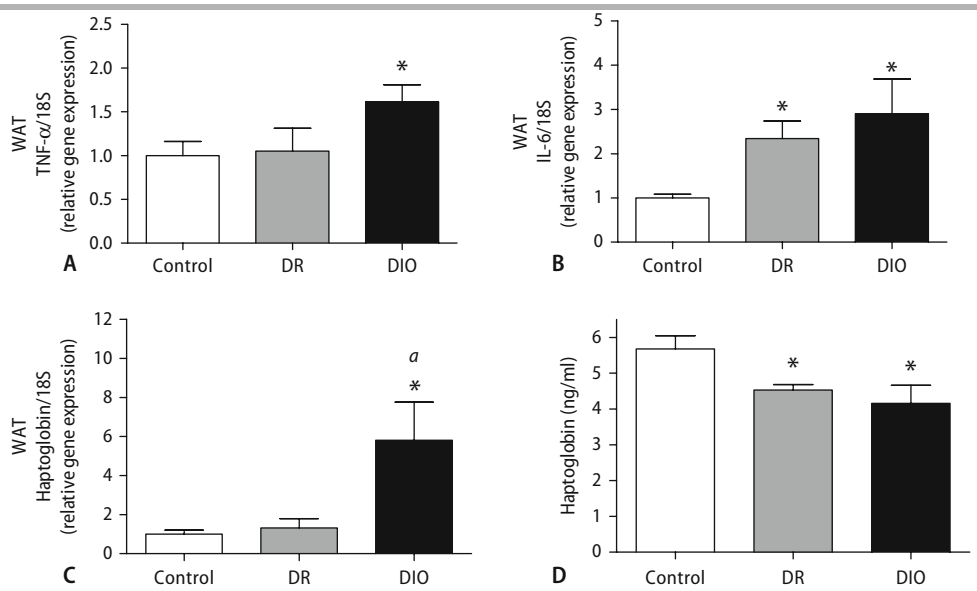

Fig. 1 Gene expression levels of TNF-α, IL-6 and haptoglobin in white adipose tissue (*WAT*) and haptoglobin circulating levels of DR and DIO rats fed with a high-fat diet during 15 days. Data are mean ± SE of six independent animals per group. The expression levels of TNF-α (**a**) and IL-6 (**b**) were determined by Northern-blot whereas haptoglobin (**c**) was determined by RT-PCR in visceral WAT of DIO and DR rats fed on a high fat diet for 15 days. The expression level of 18S RNA was determined and used as internal control to correct for minor variation in total RNA amount. Densitometric scanning was used to determine the relative amount of TNF-α, IL-6 and 18S RNA. On RT-PCR method data were calculated by the $2^{-\Delta\Delta Ct}$ method (mean value for control group was set at 1), and 18S RNA was used as reference to normalize the expression levels of haptoglobin. *$P < 0.05$ versus Control group; *a*: $P < 0.05$ DIO versus DR group

degree pro-inflammatory status [7]. In fact, elevated levels of inflammation-sensitive plasma proteins like haptoglobin, and complement factor 3 predict a large weight gain in middle-aged men [7, 8].

An induction of TNF-α mRNA expression and activity has been observed in adipose tissue from different genetic models of obesity in rodents [14, 25]. Furthermore, it was also found that a member of TNF-α induced gene family is upregulated in WAT at the onset of obesity [20]. Our results showed a significant increase on TNF-α mRNA levels in 15 days high-fat fed DIO, but not in DR rats, in comparison with control group (Fig. 1a) suggesting a progressive increase of animal's adiposity according to TNF-α mRNA rise [27] and body weight gain.

Similarly to what was reported by other authors, 15 days intake of a high-fat diet induced an increase in visceral adipose tissue IL-6 gene expression levels [21]. However, in contrast to that observed for TNF-α, we found that the increase on IL-6 mRNA was not related to adiposity, as a significant increase on the expression levels of IL-6 was observed in both DR and DIO animals, without significant differences between them (Fig. 1b). This fact could be related to the observations of Sopasakis et al. [32], which demonstrated that adipose tissue from non-obese individuals releases substantial amounts of IL-6 but not of TNF-α. These results suggest that other adipose tissue cells different from the adipocytes are mainly responsible for the secretion of IL-6. In this sense, it was recently reported that adipose tissue macrophages contribute to enhance the inflammatory state related with obesity [21]. Furthermore, it has been demonstrated that the increase of some cytokines like IL-6 and TNF-α caused the suppression of insulin signal transduction [15, 31]. In this sense, a previous study of our group [27] showed that after 15 days on a high-fat diet, both groups of animals, DR and DIO, exhibited a tendency to increase glucose and insulin circulating levels (Table 1), suggesting that these rodents could be in early stages of insulin resistance development. Perhaps, the significant increase in IL-6 mRNA expression levels observed after 15 days on high-fat feeding could contribute to cause, at least in part, an early onset of insulin resistance in both DR and DIO animals. In this sense, it has been suggested that it is not required a disproportionate weight gain mediated by high-fat diet to produce insulin resistance [19].

Several in vivo and in vitro studies have evidenced that TNF-α and IL-6 induces the expression of haptoglobin [4, 26] showing an increase in haptoglobin expression levels in obesity [4, 7]. We observed a significant increase in haptoglobin gene expression after 15 (Fig. 1c) and 35 days (Fig. 2a) of high-fat feeding in DIO rats, but not in DR rats supporting that the induction of haptoglobin gene transcription takes place in adipose tissue during obesity onset [4]. The stimulatory effect of TNF-α and IL-6 does not occur in a similar range, being TNF-α (more than IL-6) the main regulator of haptoglobin expression in

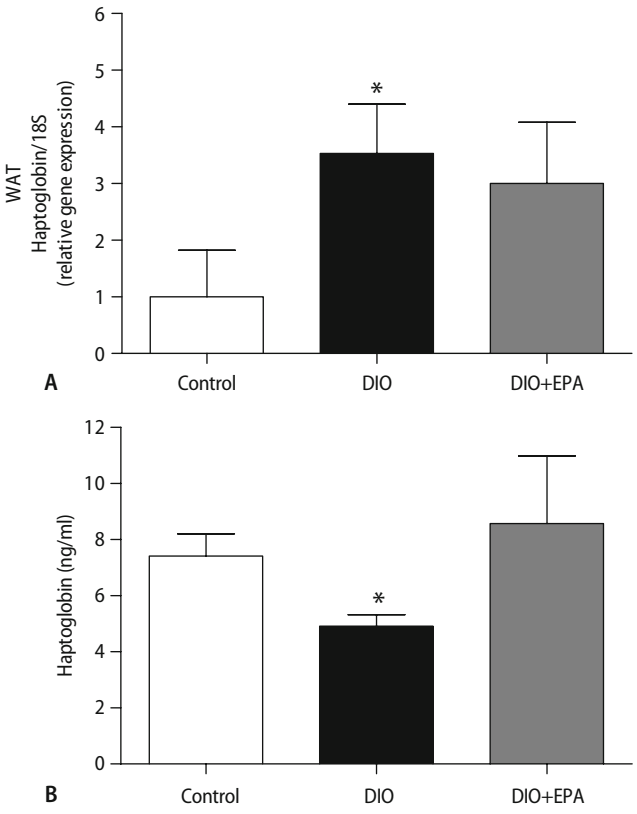

Fig. 2 **a** Effects of EPA ethyl ester on haptoglobin gene expression in WAT and **b** haptoglobin circulating levels in DIO rats fed with a high-fat diet during 35 days. The expression levels of haptoglobin were determined in visceral WAT by RT-PCR. Data were calculated by the $2^{-\Delta\Delta Ct}$ method (mean value for control group was set at 1), and 18S RNA was used as housekeeping gene to normalize the expression levels of haptoglobin. Gene expression data are mean ± SE of at least four independent animals per group. *$P < 0.05$ versus Control group

3T3-L1 adipocyes [26]. According to this fact, the results observed in our study also suggest that IL-6 does not induced the expression of haptoglobin in a similar manner as TNF-α does, (a significant increase in IL-6 mRNA was observed in both DR and DIO rats, but haptoglobin mRNA was only upregulated in DIO rats). Besides, some resistance to increase haptoglobin expression levels mediated by IL-6 stimulation could be a mechanism that explained the decrease in haptoglobin mRNA levels observed in DR rats. Nevertheless, our data suggest that an early increase in inflammation-related adipocytokine genes in DIO rats, could contribute, at least in part, to the mechanism conferring differences in the susceptibility to obesity development. In addition, this does not contradict the assumption that obesity increases inflammatory markers: once obesity increases, rising adipose tissue development (as occurs in DIO group) it stimulates the production of inflammatory cytokines, creating a vicious circle of inflammation and obesity [7, 24]. In the present study, we have not determined the adipose tissue production or the protein levels of TNF-α or IL-6, and therefore a correlation with the changes observed on mRNA expression cannot be certainly concluded. However, the fact that previous studies have found similar changes in mRNA and in adipose protein levels as well as in the adipose production of some cytokines such as TNF-α during obesity [16, 18], lead us to suggest that a good correlation between both factors could be assumed.

In addition, it is well known that ω-3 fatty acids supplementation induces anti-inflammatory effects in health populations [34] and in models of chronic inflammatory conditions [29, 37], suggesting that the improvement of the metabolic status involves regulation of the adipose tissue gene expression profile an the prevention of macrophage infiltration into fat depots [36]. Data from a recent study of our group and from the present trial demonstrated the ability of EPA ethyl ester administration during 35 days to decrease TNF-α [28] and IL-6 gene expression (data not shown) in adipose tissue of diet-induced overweight rats. However, and in contrast to what was observed for TNF-α and IL-6, our present data revealed that treatment with EPA ethyl ester during 35 days did not caused any significant improvement on the increase of haptoglobin gene expression observed in adipose tissue of DIO rats (Fig. 2a). Although, some studies reported that serum haptoglobin concentration is a marker of fat mass and body mass index in humans [5], the relationship between circulating levels of haptoglobin and obesity is controversial. In fact, one trial that investigated the effects of several restriction diets on haptoglobin serum levels in overweight subjects described a significant increase on serum levels of haptoglobin after dietary intervention, together with a decrease in adiposity. This does not correspond to the fact that circulating haptoglobin is a positive acute phase protein expected to be decreased after weight loss [6]. In this context, we found that high-fat feeding induced a significant decrease on haptoglobin circulating levels both in DR and DIO groups, independently of their adiposity (Fig. 1d). In addition, EPA-treatment, which exhibited anti-inflammatory properties in adipose tissue, was able to reverse to control values the drop in haptoglobin observed in DIO rats (Fig. 2 b). Therefore, our results do not support the hypothesis that serum haptoglobin is an acute phase protein positively related to increased adiposity in rats, at least in early and medium stages (15 and 35 days) of DIO.

Our data also demonstrate a lack of relationship between haptoglobin gene expression in visceral adipose tissue and haptoglobin serum levels. In this regard, it has been suggested that the contribution of

haptoglobin synthesis in adipose tissue to the total serum haptoglobin concentration seems only marginal, being the liver the predominant site of haptoglobin synthesis [33]. However, a possible contribution of haptoglobin from adipose tissue to the systemic chronic inflammation associated with obesity could be of significant when higher percentages of fat are accumulated as obesity progresses by longer term of high-fat feeding [30].

In summary, our data show a differential regulation of some inflammation-related genes in adipose tissue between DIO and DR rats, and suggest a role for ω-3 fatty acids in the prevention of obesity-associated inflammation. In addition, our data do not support the hypothesis that serum haptoglobin is an acute phase protein expected to be positively related to increased adiposity in rats, at least in early and medium stages (15 and 35 days) of DIO progression.

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