Original Article
OBESITY BIOLOGY AND INTEGRATED PHYSIOLOGY

Obesity

Vitamin E Reduces Adipose Tissue Fibrosis, Inflammation, and Oxidative Stress and Improves Metabolic Profile in Obesity

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Objective: To test whether enhancing the capability of adipose tissue to store lipids using antioxidant supplementation may prevent the lipotoxic effects and improve the metabolic profile of long-term obesity.
Methods: C57BL/6J mice were randomized into three experimental groups for 28 weeks: control group ($n = 10$) fed chow diet (10% kcal from fat), obese group (O, $n = 12$) fed high-fat (HF) diet (45% kcal from fat), and obese group fed HF diet and supplemented twice a week with 150 mg of α-tocopherol (vitamin E) by oral gavage (OE, $n = 12$).
Results: HF diet resulted in an obese phenotype with a marked insulin resistance, hypertriglyceridemia, and hepatic steatosis in O mice. Histological analysis of obese visceral adipose tissue (VAT) revealed smaller adipocytes surrounded by a fibrotic extracellular matrix and an increased macrophage infiltration, with the consequent release of proinflammatory cytokines. Vitamin E supplementation decreased oxidative stress and reduced collagen deposition in the VAT of OE mice, allowing a further expansion of the adipocytes and increasing the storage capability. As a result, circulating cytokines were reduced and hepatic steasosis, hypertriglyceridemia, and insulin sensitivity were improved.
Conclusions: Our results suggest that oxidative stress is implicated in extracellular matrix remodeling and may play an important role in metabolic regulation.

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Introduction

The hypothesis of adipose tissue expansion suggests the existence of an individual's limit for the accumulation of fat within the adipocyte (1). Once that limit has been exceeded, circulating free fatty acids would be then redirected to other tissues, such as liver or muscle, where they accumulate and induce lipotoxicity, characterized by inflammation, oxidative stress, and insulin resistance (1).

The expansion of adipose tissue involves several modifications. In obesity, changes have been observed both in the number and in the size of adipocytes. Furthermore, the infiltration and activation of macrophages to a pro-inflammatory phenotype is also a common feature of adipose tissue in obesity (2,3). As a result, a further increase in the release of cytokines occurs, generating a pro-inflammatory environment (4). Finally, changes have also been described in the extracellular matrix (ECM), in order to promote a fibrotic process that limits adipocyte growth (5).

ECM remodeling in obesity reflects the balance between the increased rate of synthesis of matrix proteins and their degradation. It is mediated by fibrinolytic factors, such as plasminogen and plasmin, matrix metalloproteases (MMP), and antifibrinolytic systems like the tissue inhibitors of MMP (TIMPs), among others (6). Studies in individuals with obesity have reported the relation between body mass index, ECM components, and metabolic disorders in adipose tissue (5-9). Experiments in mice lacking collagen VI, the most abundant type in adipose tissue, in an *ob*/*ob* background, showed larger adipocytes and improved metabolic parameters, suggesting that collagen VI may constraint adipocytes, provoking metabolic alterations (10).

Despite the increasing efforts to understand the ECM remodeling process in adipose tissue, it remains unclear what is the relation between the pathophysiological processes observed in obesity and the remodeling mediators. While tissue hypoxia (11) and inflammatory cytokines (12) play an inductive role in the fibrotic process, the effects of oxidative stress are yet to be defined.

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In other conditions, such as hepatic or pulmonary fibrosis, it has been suggested that reactive oxygen species (ROS) play a role in the synthesis of collagen (13,14). Therefore, our hypothesis suggests that in a mouse model of diet-induced obesity (DIO), the blockade of obesity-related oxidative process through supplementation with α-tocopherol (vitamin E, a potent lipid-soluble antioxidant) would be able to reduce collagen deposition in adipose tissue, allowing its further expansion and alleviating the lipotoxic effects associated with obesity.

Methods

Animals and diets

Four weeks old male C57BL/6 mice were purchased from Harlan Laboratories (UK). After 2 weeks of acclimatization, mice were randomized into three groups. The control group (C, $n = 10$) received a low-fat diet (10% calories from fat; Purina TestDiet 58V8, Testdiet, USA). The obese group (O, $n = 12$) received a high-fat diet (HF; 45% calories from fat; Purina TestDiet 58Y2, Testdiet, USA). The supplemented group (OE, $n = 12$) was fed the same HF diet, and received 150 mg of vitamin E (DL-α Tocopherol acetate; Sigma, Spain) twice a week by oral gavage (15). All the animals had free access to food and water. Animal procedures were performed in accordance with the USP-CEU Ethical Committee for Animal Research.

The dietary and antioxidant treatment lasted 28 weeks, when mice were sacrificed by decapitation after 8 h fasting. Then, liver and visceral adipose tissue (VAT) were immediately dissected and stored in formaldehyde, RNA later, or snap frozen in liquid nitrogen and stored at −80°C. Blood was collected using tubes containing Na_2EDTA, plasma was obtained and stored at −20°C until used.

Plasma analysis and estimation of insulin resistance

Glucose and triglycerides were determined by enzymatic colorimetric test (GOD-PAP and LPL/GOP-Trinder, Roche Diagnostics, Barcelona, Spain). Plasma levels of insulin, adiponectin, leptin, resistin, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and plasminogen activator inhibitor-1 (PAI-1) and tisular levels of monocyte chemoattractant protein-1 (MCP-1) were measured using a Milliplex MADPK-71K adipokine kit according to manufacturer's description (Millipore). For estimation of insulin resistance, HOMA index was calculated as previously described (16).

Oxidative damage determination in VAT

VAT homogenates with 5 mM butyl hydroxytoluene (BHT) were used for the determination of oxidation to lipids and proteins. Lipid peroxidation was determined using a commercial kit (Bioxytech LPO-586) from OxisResearch (USA). Advanced oxidation protein products (AOPP) were determined according to Witko-Sarsat's method (17) with modifications.

Antioxidant enzymes in VAT

Catalase (CAT) specific activity was measured by monitoring the disappearance of hydrogen peroxide at 240 nm. Glutathione peroxidase (GPx) specific activity assay is based on the oxidation of glutathione by GPx. Oxidized glutathione is regenerated by glutathione reductase using $NADPH + H^+$ as a cofactor. The reaction rate was measured following the disappearance of $NADPH + H^+$ at 340 nm. Glutathione S-transferase (GST) specific activity was measured using a commercial kit following manufacturer's instructions (Glutathione S-transferase Assay Kit, Cayman Chemical, Ann Arbor, MI).

Vitamin E determination in VAT

α-Tocopherol was detected by a HPLC using a routine method in our laboratory (18). The chromatograph system was a Beckman Mod. 126 coupled to a UV detector (Beckman Mod. 168) in line with a fluorescence detector (Waters 474). All the solvents used were purchased from Scharlau (Spain).

Immunohistochemistry

VAT was fixed in 4% paraformaldehyde, embedded in paraffin, and cut in sections of 5 μm. To analyze the size of the adipocytes, sections from four mice per group were hematoxylin–eosin stained. At least 250 cells per sample were measured with the MRI adipocyte tool in ImageJ software (NIH, USA).

Macrophages were detected using an anti-CD68 rabbit polyclonal antibody (Abbiotec, USA) after heat-induced antigen retrieval. Diaminobenzidine detection was performed using the Histostain-SP Broad Spectrum HRP Kit (Invitrogen). Slides were counterstained with hematoxylin. A total number of four mice were used for the analysis. Samples were analyzed using a Leica DM2700 P microscope. Snap shots were taken (Leica DFC495 Camera) using MetaMorph 6.1 software, and quantification of stained areas was determined through ImageJ software (NIH, USA).

Collagen analysis

Paraffin embedded slides of VAT were used for the detection of total collagen. After deparaffination and rehydration, samples of four mice per group were stained with 1% picrosirius red in a saturated solution of picric acid and quantified using ImageJ software. Types I, III, IV, and VI collagen expression were determined by real-time PCR (qPCR) as further described.

Western blot

VAT was homogenized in a lysis buffer and disrupted in a Tissuelyser (Qiagen, Spain) in two cycles of 2 min at 40 Hz. Samples were placed on ice for 15 min to achieve a complete cellular lysis, and then centrifuged at 12,000 rpm for 30 min. Supernatant was stored at −80°C until further use.

About 25 micrograms of each sample ($n = 4$) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After transference to PVDF membrane, blocking was performed with 10% milk-TBST (tris buffer saline tween). Rabbit anti-p38 (M-0800 Sigma, Spain), rabbit anti-phospho p38 (MABS64, Millipore, Spain) were used. Secondary antibodies conjugated to horseradish peroxidase were obtained from Sigma (Spain). Protein bands were observed by addition of ECL Western blotting detection system (GE Healthcare, Spain). For quantification of band intensities, ImageJ (NIH, USA) was used.

RNA extraction

Total RNA was isolated from VAT using Trizol Reagent (Invitrogen, Spain). The samples were processed using an RNeasy Mini Kit

TABLE 1 Sequences of the forward and reverse primers used in qPCR analysis

Gene	Forward	Reverse
Col1a1	5′-CATGTTCAGCTTTGTGGACCT-3′	5′-GCAGCTGACTTCAGGGATGT-3′
Col3a1	5′-TCCCCTGGAATCTGTGAATC-3′	5′-TGAGTCGAATTGGGGAGAAT-3′
Col4a1	5′-TTAAAGGACTCCAGGGACCAC-3′	5′-CCCACTGAGCCTGTCACAC-3′
Col6a1	5′-GCAAGGATGAGCTGGTCAA-3	5′-GTCCACGTGCTCTTGCATC-3′
Mmp2	5′-TAACCTGGATGCCGTCGT-3′	5′-TTCAGGTAATAAGCACCCTTGAA-3′
Cebpa	5′-AAACAACGCAACGTGGAGA-3′	5′-GCGGTCATTGTCACTGGTC-3′
Nrf2	5′-CATGATGGACTTGGAGTTGC-3′	5′-CCTCCAAAGGATGTCAATCAA-3′
Fabp4	5′-GGATGGAAAGTCGACCACAA-3′	5′-TGGAAGTCACGCCTTTCATA-3′
Cd36	5′-TTGTACCTATACTGTGGTAAATGAGA-3′	5′-CTTGTGTTTTGAACATTTCTGCTT-3′
Srebp-1c	5′-CGGAGGCTGTCGGGGTAG-3′	5′-GGCCAGAGAAGCAGAAGAGA-3′
Fas	5′-CAGATGATGACAGGAGATGGAA-3′	5′-CACTCACACCCACCCAGA-3′
Ppara	5′-CACGCATGTGAAGGCTGTAA-3′	5′-CAGCTCCGATCACACTTGTC-3′
Cpt1a	5′-GACTCCGCTCGCTCATTC-3′	5′-AAGGCCACAGCTTGGTGA-3′
Pgc-1a	5′-GAAAGGGCCAAACAGAGAGA-3′	5′-GTAAATCACACGGCGCTCTT-3′
Chop	5′-CCCTGCCTTTCACCTTGG-3′	5′-CCGCTCGTTCTCCTGCTC-3′
Bip	5′-ACTTGGGGACCACCTATTCCT-3′	5′-ATCGCCAATCAGACGCTCC-3′
Cd86	5′-GAAGCCGAATCAGCCTAGC-3′	5′-CAGCGTTACTATCCCGCTCT-3′
Cd105	5′-GACCCCTGCACAACCATT-3′	5′-TGTGGTGGGGTTTGGTTC-3′
Mgl1	5′-TGAGAAAGGCTTTAAGAACTGGG-3′	5′-GACCACCTGTAGTGATGTGGG-3′
Tbp	5′-ACCCTTCACCAATGACTCCTATG-3′	5′-TGACTGCAGCAAATCGCTTGG-3′

(Qiagen, Spain). The concentration, purity, and integrity of RNA were assessed. Reverse transcription was performed on 500 ng of RNA with iScript cDNA synthesis kit (Biorad, Spain) using random hexamer primers.

qPCR analyses

Optimal annealing temperature and amplicon size were checked. qPCR analyses were performed in a LightCycler 480 Instrument (Roche). Four samples of each group were run in triplicate, and the mRNA levels were determined using intron-skipping primers, *Tbp* as a housekeeping gene, and SYBR Green Master Mix (Applied Biosystems). All sequences are detailed in Table 1.

Statistical analysis

Results are presented as mean ± SEM. Statistical significance of differences between groups was assessed by one-way analysis of variance (ANOVA) followed by *post hoc* Tukey's multiple comparison tests using Graph-Pad Prism (version 5.03 for Windows, GraphPad Software, CA). Differences were considered statistically significant when $P < 0.05$.

Results

Vitamin E supplementation reduces steatosis in diet-induced obese animals

In this study, we used a DIO mouse model to test whether supplementation with a lipid-soluble antioxidant such as vitamin E exerts metabolic changes in DIO mice. As expected, mice fed on HF diet for 28 weeks (O group) significantly increased their body-weight compared to mice fed with a standard diet (C group, Figure 1A). Long-term supplementation with 150 mg twice a week of vitamin E (OE group) did not affect body weight gain (C: 14.6 ± 0.9 g vs. O: 26.7 ± 0.8 g and OE: 25.0 g ± 1.4 g, $P < 0.001$). O and OE animals showed a similar reduction in food intake compared to C, although these differences disappeared when adjusting the caloric content (Figure 1B,C). At sacrifice, HF diet caused hepatomegaly and steatosis in the O group. However, antioxidant supplementation was able to reduce the lipid storage in the liver (Figure 1D,E).

Vitamin E supplementation improves obesity-induced insulin resistance and reduces plasma triglycerides levels

Obese mice developed insulin resistance (as determined by HOMA index) whereas mice from the OE group have improved insulin sensitivity compared to the O group, despite no significant changes in fasting glucose levels among the experimental groups were observed (Table 2). Furthermore, circulating triglycerides were significantly reduced in the OE group. Thus, the marked insulin resistance in obese animals and the concomitant hypertriglyceridemia were efficiently counteracted by vitamin E supplementation.

Vitamin E supplementation suppresses obesity-induced oxidative stress

To assess the adequacy of the antioxidant treatment, the levels of vitamin E in adipose tissue were measured. We found an increase in vitamin E levels in the O group compared to C, probably because of the different content in the diets (60.6 IU/kg in the HF diet vs. 49.3 IU/kg in the LF diet) (Figure 2A). In any case, the adipose tissue of the OE group doubled the levels of vitamin E compared to the O group, confirming the enrichment of the tissue with the dose and frequency of administration chosen.

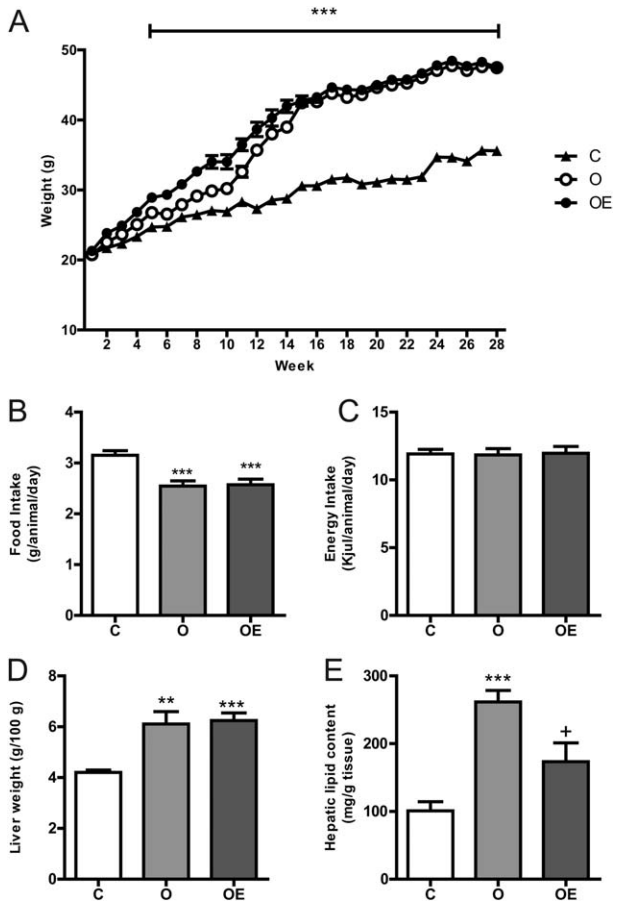

Figure 1 Vitamin E supplementation reduces liver steatosis in HF-fed fed mice. (**A**) Changes in body weight in response to HF diet (45% energy from fat) during 28 weeks in non-supplemented animals (O; $n = 12$) and animals supplemented with 150 mg of vitamin E twice a week by oral gavage (OE; $n = 12$). HF-fed mice were statistically heavier than the controls fed on a standard diet (C; $n = 10$) from week 5. (**B**) Mean food intake of each group. The intake of HF-fed fed mice was reduced compared to C group. (**C**) Mean caloric intake of each group. When adjusting the food intake by respective energetic content, no differences in the consumed calories were found between any of the three experimental groups. (**D**) Liver weight relative to 100 g of animal, increased in both groups fed on HF diet. (**E**) Lipid content in the liver. Vitamin E supplementation reduced HF-induced steatosis seen in O group. Results are represented as mean + SEM. $^{**}P < 0.01$; $^{***}P < 0.001$ (O, OE vs. C). $^{+}P < 0.05$ (OE vs. O).

TABLE 2 Metabolic parameters

	C	O	OE
Glucose (mg/dl)	153.6 ± 5.5	160.5 ± 8.1	159.6 ± 11.8
Triglycerides (mg/dl)	138.8 ± 7.2	165.0 ± 7.7*	119.8 ± 3.8†
Insulin (nmol/ml)	1.0 ± 0.1	7.0 ± 0.9***	3.2 ± 0.7‡
HOMA	9.9 ± 0.9	73.9 ± 11.2***	34.4 ± 7.4*

HOMA: Homeostasis Model Assessment.
Results are represented as mean ± SEM.
$^{*}P < 0.05$; $^{***}P < 0.001$ (O, OE vs. C).
$^{\dagger}P < 0.05$; $^{\ddagger}P < 0.01$ (OE vs. O).

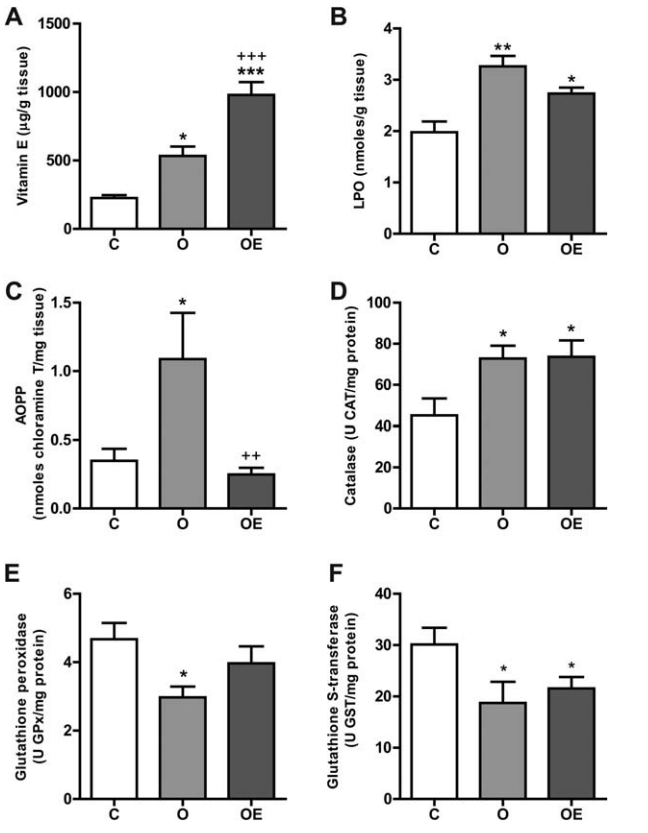

Figure 2 Vitamin E reduces HF diet-induced oxidative stress in VAT. (**A**) Vitamin E in VAT, assessing the proper enrichment of the tissue with the selected dosage. Reduced markers of oxidative damage to (**B**) lipids (lipoperoxides) and (**C**) proteins (advanced oxidation protein products) in vitamin E-supplemented DIO mice. Specific activity of the antioxidant enzymes (**D**) catalase, (**E**) glutathione peroxidase, and (**F**) glutathione reductase. Results are represented as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (O, OE vs. C). $^{++}P < 0.01$; $^{+++}P < 0.001$ (OE vs. O).

Oxidative stress damage was determined by measuring the products of the oxidation of macromolecules and the activity of several antioxidant enzymes in the VAT. HF diet produced an increase in the levels of LPO and AOPP in the O group compared to C (Figure 2B,C). Vitamin E supplementation was able to diminish the oxidative damage, although only AOPP reduction was significant. Concerning antioxidant enzymatic defense, catalase activity was increased in both groups fed on HF diet, suggesting an increased production of hydroxyl radical (Figure 2D). However, the activities of glutathione peroxidase and glutathione S-transferase were decreased in the O group, leading to a loss of capability to scavenge peroxides. Vitamin E action over peroxidation was able to enhance the antioxidant defense of both glutathione-related enzymes (Figure 2E,F). Taken together, these data support that antioxidant supplementation produces a protective effect on the oxidative damage induced by obesity.

Vitamin E supplementation reduces collagen deposition and improves adipose tissue expansion

Hematoxilyn and eosin stained sections were used to analyze the size of the adipocytes (Figure 3A,B). In the O group, we observed a higher frequency of both small and large adipocytes compared to C group

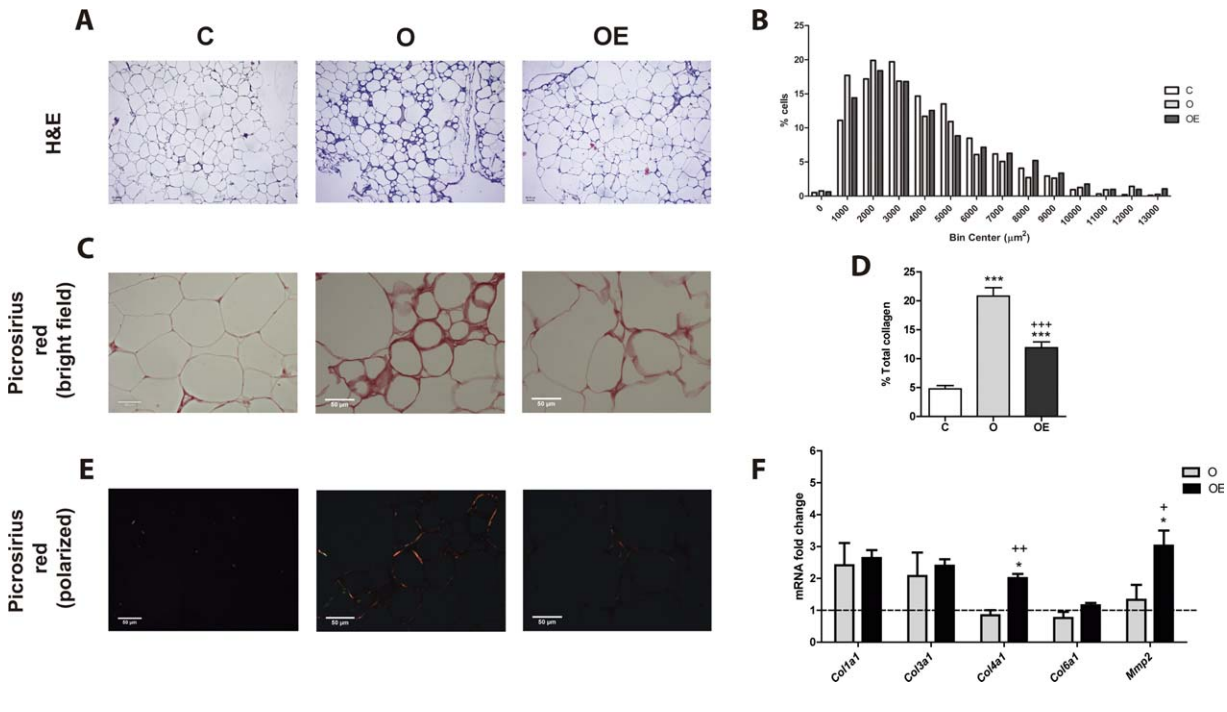

Figure 3 Vitamin E supplementation causes adipocyte enlargement and reduces collagen deposition in VAT of DIO mice. 5 μm paraffin sections of VAT were used. (**A**) Representative images of hematoxylin and eosin staining. Images were taken at 10× magnification. (**B**) Frequency distribution of adipocyte cell surface area, showing higher frequencies of small adipocytes in O group while in OE group intermediate and large adipocytes were more frequent. $n = 4$ per group. > 250 cells were measured for each mouse. (**C**) Representative images of picrosirius red staining in bright field showing collagen accumulation surrounding adipocytes. Images were taken at 20× magnification. (**D**) Relative collagen amount obtained from the surface stained by picrosirius red. (**E**) Same representative images of picrosirius red staining in polarized light showing the appearance of thicker fibers (orange, yellow, and green fibers). (**F**) *Col1a1, Col3a1, Col4a1, Col6a1*, *and Mmp2* expression in VAT. Expression values represent four biological replicates and are shown relative to *Tbp* expression as a housekeeping gene. The expression of C group for each gene was set as 1 and is represented by the dashed line. Results are represented as mean ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$ (O, OE vs. C). $^{+}P < 0.05$; $^{++}P < 0.01$; $^{+++}P < 0.001$ (OE vs. O).

(≤2,000 μm^2 C: 28.8% vs. O: 38.4%; ≥10,000 μm^2 C: 1.7% vs. O: 5.3%) while intermediate sizes were less frequent. Vitamin E supplementation reduced adipocyte size. We found less adipocytes with a small size (≤2,000 μm^2 OE: 33.40%), with the concomitant increase in the amount of adipocytes with an intermediate and large size (%; ≥10,000 μm^2 OE: 6.45%).

To elucidate the causes of the different patterns of adipose tissue expansion during weight gain, we measured the deposition of collagen in the ECM. Picrosirius red staining reflected a blunt increase in the amount of collagen deposited in the O group compared to the C group, while vitamin E reduced the fibrosis (Figure 3C,D). The expression profile of different types of collagen revealed an upregulation in mRNA levels of *Col1a1* and *Col3a1* in the O group, while no differences were observed in *col4a1* and *col6a1*. Surprisingly, vitamin E supplementation also induced an upregulation in the expression of *Col1a1, Col3a1*, *and Col4a1*. In this same sense, *Mmp2* expression, one of the metalloproteases in charge of degrading type IV collagen, was only upregulated in the OE group.

Vitamin E supplementation enhances adipocyte differentiation, lipid transport, adipogenesis, and mitochondrial function

We next investigated whether the reduction of oxidative stress associated with vitamin E supplementation may improve obesity-induced impairment of lipid metabolism. HF diet produced a decrease in the expression of *Cebpa* and *Fabp4* compared to C mice, which implies a reduction in differentiation and intracellular fatty acid transport (Figure 4). In comparison to O mice, α-tocopherol supplementation was able to increase the mRNA levels of key genes implicated in several lipid metabolism processes such as differentiation (*Cebpa, Nrf2*), lipid transport (*Fabp4, Cd36*), lipogenesis (*Srebp-1c, Fas*), and mitochondrial biogenesis (*Pcg-1a*) and to reduce obesity-induced endoplasmic reticulum stress through the reduction of *Chip* expression (Figure 4).

Vitamin E supplementation exerts anti-inflammatory properties

Immunohistochemical analysis of CD68 in the VAT revealed that the presence of macrophages around adipocytes was threefold higher in both obese groups as compared to controls (Figure 5A,B). After 28 weeks of HF diet feeding, we found that the monocyte attractant MCP-1 was increased in the O group compared to the C group (Figure 5C). However, the differences between the OE and the C groups did not reach statistical significance.

Next, we analyzed the polarization of the macrophages within the VAT. M1 (*Cd86*) and M2c (*Cd150*) markers were not differentially expressed between the three groups. Although no statistical differences

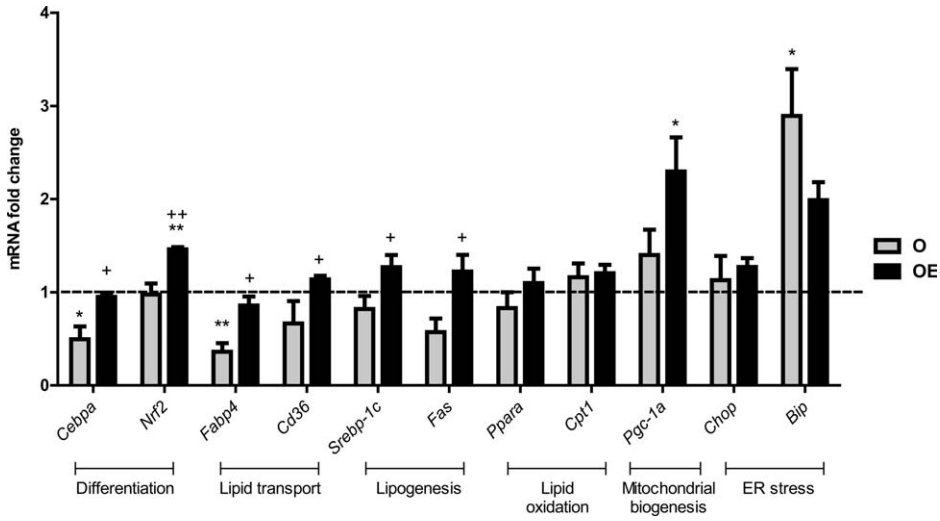

Figure 4 Vitamin E supplementation enhances adipocyte differentiation, lipid transport, and adipogenesis in VAT of DIO mice. qPCR assays were carried out for a range of white adipocyte genes: *Cebpa, Nrf2, Fabp4, Cd36, Srebp-1c, Fas, Pparα, Cpt1, Pgc-1a, Chop*, *and Bip*. Expression values represent four biological replicates and are shown relative to *Tbp* expression as a housekeeping gene. The expression of C group for each gene was set as 1 and is represented by the dashed line. Results are represented as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$ (O, OE vs. C). $^{+}P < 0.05$; $^{++}P < 0.01$ (OE vs. O).

were reached, our results may suggest a trend in obesity-induced polarization toward alternatively activated M2-macrophages according to the nearly twofold increase in the expression of *Mgl1* (Figure 5D).

Despite this apparent shift toward anti-inflammatory phenotype in VAT macrophages, at a protein level, we observed a blunt increase in p38 phosphorylation in the VAT of the O group, which has been previously reported as a mechanism of inflammation and insulin resistance induction. Antioxidant supplementation with vitamin E managed to reduce p38 phosphorylation to basal level.

This observation supports the systemic inflammation observed in response to HF diet and the anti-inflammatory effects of vitamin E. Plasma circulating levels of cytokines were determined to characterize the inflammatory status. Vitamin E supplementation was able to reduce obesity-induced increase in leptin, resistin, IL-6, TNF-α, and PAI-1 (Figure 5D-I).

Discussion

The alterations in the biology of the adipose tissue (inflammation, oxidative stress or lipotoxicity) play a key role in the onset of obesity-related insulin resistance. We hypothesize that the dysfunction of the adipose tissue is related to structural changes mediated by oxidative stress, such as fibrosis and collagen deposition (13,14), preventing its further healthy expansion. In order to achieve a reduction in adipose tissue fibrosis that may improve the metabolic profile of obese mice, we administered vitamin E, a potent lipid-soluble antioxidant.

In this study, we developed an insulin resistant, hypertriglyceridemic DIO model after 28 weeks of HF diet feeding. The supplementation of vitamin E was able to improve this phenotype, reducing both insulin and triglycerides levels. HF-fed animals reached their maximum body weight after 28 weeks of feeding, similar to what has been observed in previous studies (19). At this point, lipid flux shifted toward ectopic organs such as the liver, where we found an increase in liver weight and lipid accumulation in the O group. Vitamin E diminished the accumulation of lipids in the liver, pointing out to a possible role of oxidative stress in tissue lipid distribution and adipogenesis (20) as it will be further discussed.

According to the adipose tissue expandability hypothesis, the adipose tissue capability to increase their storage limit is a critical step in the physiology of obesity (1,21,22). In our model, where maximum weight gain was achieved, we suggest that the key step that defines the expansion limit of the adipose tissue is collagen deposition. The fibrotic process observed in the ECM of obese VAT may be responsible for the higher frequency of smaller adipocytes, consequence of a blockade in the adipocyte growth and responsible of the blockade of further lipid accumulation. Furthermore, the differentiation of new adipocytes and the transport of lipids (*Cebpa, Fabp4, Fas*) were reduced in obese VAT, which represents further evidence for the lack of storage capacity. However, in the vitamin E-treated mice, we found a significant reduction in collagen deposition, which, together with an enlargement in the adipocyte size, may be responsible for their improved metabolic phenotype (22). In fact, adipocytes from the OE group showed enhanced differentiation capability, lipid transport, adipogenesis, and mitochondrial function.

Several authors have shown the relationship between ECM remodeling and metabolic disturbances in obesity. Previous studies have demonstrated that several components of ECM are increased in individuals with obesity (10,23,24). In this sense, collagen VI-null *ob*/*ob* mice showed better metabolic control when fed on HF diet (10)

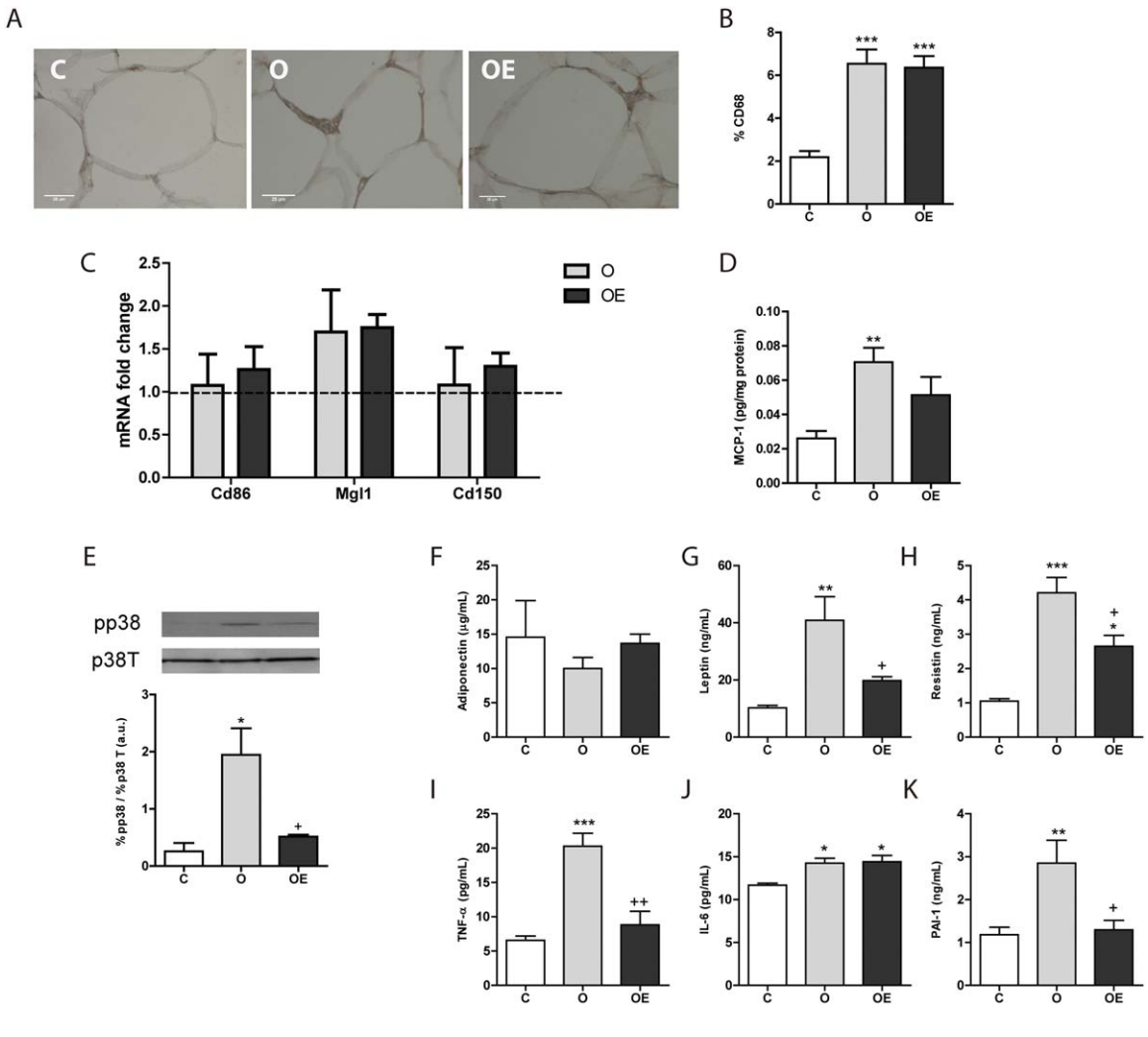

Figure 5 Vitamin E supplementation reduces systemic inflammation without improving macrophage infiltration in VAT. (**A**) Representative images of macrophage surface marker CD68 immunostaining, taken at 40× magnification. Arrows point to positively stained cells. (**B**) Quantification of the percentage of the surface stained by CD68 antibody showing increased infiltration in HF-fed animals. (**C**) Expression of *Cd86, Mgl1*, and *Cd150* as markers of M1, M2, and M2c phenotypes of macrophages in VAT. Expression values represent four biological replicates and are shown relative to *Tbp* expression as a housekeeping gene. The expression of C group for each gene was set as 1 and is represented by the dashed line. (**D**) Concentration of MCP-1 protein in adipose tissue as an indicator of monocyte chemotaxis. (**E**) Determination of p38 activation by phosphorylation in VAT homogenates by Western blot showing activated MAPK signaling in DIO. Systemic inflammation was evaluated according to the circulating levels of (**F**) adiponectin, (**G**) leptin, (**H**) resistin, (**I**) TNF-α, (**J**) IL-6, and (**K**) PAI-1 cytokines. Results are represented as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (O, OE vs. C). $^{+}P < 0.05$; $^{++}P < 0.01$ (OE vs. O).

allowing a better expansion of adipocytes. Furthermore, the inhibition of osteopontin, another ECM component that is upregulated in obesity, has recently been shown to improve insulin sensitivity in HF-fed animals (25). When we analyzed the expression of different types of collagen present in the VAT, we observed an upregulation in type I and III collagen in the obese, non-treated animals. However, the mRNA levels of type IV and VI collagens were similar to lean mice, even when the differences at a protein level were consistent. As some authors have reported, in long-term obesity there is a process of fibrosis resorption that may begin when maximum weight is reached (26). In the supplemented animals, that exhibited lower collagen deposition, the mRNA levels of type I, III, and IV were still increased, together with the matrix metalloprotease MMP2, suggesting that the remodeling process is still active and that vitamin E treatment delays the fibrotic process in VAT.

The mechanisms involved in the enhanced adipose tissue expansion achieved by vitamin E are still unrevealed. As an antioxidant, vitamin E acts as a chain-breaker of lipid peroxidation. In addition, it has been shown to inhibit the activity of ROS-generating systems such as iNOS or NADPH oxidase (27,28). ROS have been identified as mediators in pulmonary or hepatic fibrosis (13,14) and antioxidant supplementation has been used to reduce collagen deposition (29,30). For instance, Ibuki et al. showed how oral supplementation with vitamin E in diabetic, obese mice reduced oxidative stress, decreased the expression of some MMPs and improved skin tensile

strength and collagen fibers (31). Lipid peroxides and oxidized proteins may also play an important role in the activation of matrix degradation and collagen deposition (32,33). Supported by this evidence, our results suggest that reducing oxidative stress in adipose tissue may be one potential mechanism to reduce adipose tissue fibrosis.

But vitamin E also exhibits non-antioxidant properties. For instance, it inhibits signaling pathways, such as the family of mitogen-activated protein kinases (MAPK) (34,35). These MAPK (ERK, JNK, and p38), activated by ROS, modulate the synthesis of MMPs and the release of pro-inflammatory cytokines. For instance, p38 MAPK activation is involved in the activation of transforming growth factor-beta (TGF-β) (36), a potent inducer of collagen synthesis. Thus, the reduction in collagen deposition in the vitamin E-supplemented animals may be also related to the described reduction in p38 phosphorylation (37,38).

Since low-grade inflammation has been widely reported in obesity, we tested the anti-inflammatory properties of vitamin E. In the VAT of both groups of obese animals, we found an increase in MCP-1 protein as well as a marked macrophage infiltration surrounding adipocytes, forming crown-like structures (Figure 5A-C). In addition, both obese groups trend to a M2 polarization of their macrophages. Consistent with previous evidence, while short-term obesity enhances the expression of M1 pro-inflammatory markers, long-term obesity induces a shift in the polarization toward the anti-inflammatory M2 phenotype (3).

However, the inflammatory profile was substantially different between the two groups of obese animals. HF diet caused a process of systemic inflammation, characterized by the increase of cytokines with direct metabolic effects such as leptin, resistin, TNF-α, IL-6, or PAI-1. Vitamin E supplementation managed to restore cytokine secretion to a basal level. In fact, antioxidants have also been used to reduce the secretion of pro-inflammatory cytokines that interfere with insulin signaling (39,40). In this scenario, the reduction of fibrosis in the supplemented animals may lead to a reduced inflammation, with a direct effect on metabolic improvement and insulin sensitivity. This is supported by Halberg et al., who, using a time course of HF diet, described ECM remodeling as one of the first events, promoting inflammation that was later detected (11).

In summary, we have investigated the effect of obesity-induced oxidative stress in adipose tissue remodeling. We found that insulin resistant obese animals exhibited a fibrotic process that inhibited VAT expansion. As a consequence, we observed an increase in oxidative damage, macrophage infiltration and low-grade inflammation. Vitamin E supplementation managed to improve adipose tissue expansion through a reduction in the fibrotic process. This, in turn, reduced steatosis and inflammation, restoring insulin sensitivity. The mechanism may involve, although not exclusively, vitamin E antioxidant activity, reducing ROS-mediated collagen deposition and inflammation. Thus, our results point to a new potential mechanism in vitamin E-induced metabolic improvement in obesity. O

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