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Article

Hydroxytyrosol Linoleoyl Ether Ameliorates Metabolic-Associated Fatty Liver Disease Symptoms in Obese Zucker Rats

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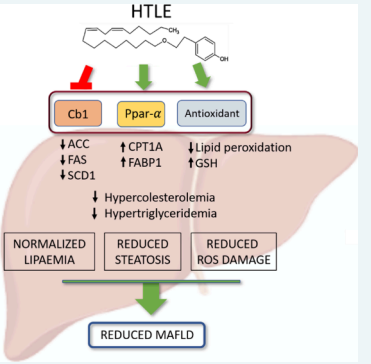
ABSTRACT: A main hepatic consequence of obesity is metabolic-associated fatty liver disease (MAFLD), currently treated by improving eating habits and administrating fibrates yet often yielding suboptimal outcomes. Searching for a new therapeutic approach, we aimed to evaluate the efficacy of hydroxytyrosol linoleoyl ether (HTLE), a dual Ppar-α agonist/Cb1 antagonist with inherent antioxidant properties, as an antisteatotic agent. Using lean and obese Zucker rats, they were administrated daily doses of HTLE (3 mg/kg) over a 15-day period, evaluating its safety profile, pharmacokinetics, impact on body weight, hepatic fat content, expression of key enzymes involved in lipogenesis/fatty acid oxidation, and antioxidant capacity. HTLE decreased the body weight and food intake in both rat genotypes. Biochemical analysis demonstrated a favorable safety profile for HTLE along with decreased concentrations of urea, total cholesterol, and aspartate aminotransferase AST transaminases in plasma. Notably, HTLE exhibited potent antisteatotic effects in obese rats, evidenced by a decrease in liver fat content and downregulation of lipogenesis-related enzymes, alongside increased expression of proteins controlling lipid oxidation. Moreover, HTLE successfully counteracted the redox imbalance associated with MAFLD in obese rats, attenuating lipid peroxidation and replenishing both glutathione levels and the overall antioxidant. Our findings highlight the effectiveness of triple-action strategies in managing MAFLD effectively. Based on our results in the Zucker rat model, HTLE emerges as a promising candidate with triple functionality as an anorexigenic, antisteatotic, and antioxidant agent, offering potential relief from MAFLD symptoms associated with obesity while exhibiting minimal side effects. In conclusion, our study positions HTLE as a highly promising compound for therapeutic intervention in MAFLD treatment, warranting further exploration in clinical trials.



KEYWORDS: *HTLE, MAFLD, Zucker rats, Cb1, Ppar-α, antioxidant, antisteatotic*

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In recent years, a silent epidemic of "metabolic-associated fatty liver disease" (MAFLD) represents a challenging disease that concerns both the Western and Asian worlds.[1] MAFLD is an alternative term recently proposed to redefine fatty liver disease as the chronic excess of fat deposition in hepatocytes (steatosis) focusing on the role of metabolic dysfunction.[2] Unlike MAFLD, the traditional terminology of nonalcoholic fatty liver disease refers to fat metabolism disorders that can coexist frequently with other conditions such as hepatotoxic agents or viral hepatitis among others, and therefore presenting a broader spectrum of factors that cause it.[3] Instead, the diagnosis of MAFLD requires the presence of steatosis combined with at least one of these three criteria: obesity, type 2 diabetes mellitus, or so-called metabolic syndrome. The latter implies a metabolic dysregulation involving oxidative stress, insulin resistance, dyslipidaemia (elevated circulating levels of cholesterol and triglycerides), and hypertension.[1,2,4,5] Long-term clinical consequences of fatty liver include inflammation (steatohepatitis), fibrosis and cirrhosis, and ultimately the development of hepatocellular carcinoma, making it the first cause of liver transplantation in the forthcoming years.[1,6,7] Therefore, the diagnosis of MAFLD, circumscribed to a metabolic origin, should allow first the identification of more homogeneous patient populations, second better definition of the progression of liver disease, and third better estimation of the likelihood of response to standard and innovative treatment programs by means of personalized medicine approaches.

To date, there are no pharmacological treatments effective enough that reverse or prevent the development of MAFLD, and so far, the most frequent medical indication against hepatic steatosis is the use of fibrates and a balanced diet follow-up.[8,9] The investigation for new therapeutic approaches for MAFLD starts with anti-obesity agents that induce considerable weight loss and reduction of fat tissue, with consequent beneficial effects such as the decrease in transaminase levels and hepatic steatosis.[10] Unfortunately, these types of drugs lack efficacy

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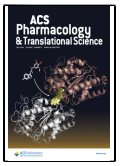

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against the potential worsening of steatosis toward steatohepatitis followed by advanced fibrosis and progression to cirrhosis and hepatocellular cancer. Even the newly designed GLP-1 receptor agonists, which have revolutionized the treatment of obesity, are limited and insufficient to improve MAFLD.[11] Then, there is an urgent need to find and develop effective therapies that can produce a clear effect on the MAFLD despite its complex physiopathology.

Currently, several candidates are in phase 3 clinical trials and they are about compounds that act either on nuclear receptors, membrane receptors, or on a particular kinase, all of them associated with a specific metabolic action such as the improvement of fatty acid β-oxidation or the suppression of steatohepatitis and fibrosis.[12] On one hand, some studies report the beneficial effects of the cannabinoid receptor 1 (Cb1) blockade. Thus, by using peripherally restricted antagonist appetite suppression, an increase in energy expenditure and a reduction of lipogenesis in both liver and adipose tissues can be achieved.[13−17] Indeed, Cb1 antagonists exhibit hepatoprotective activity by reversing the development of fatty liver in both animal and preclinical models of subjects with abdominal obesity.[15,18,19] However, clinical use of Cb1 antagonists has been refused since the Rimonabant case.[20] On the other hand, some agonists of the nuclear peroxisome proliferator-activated receptor alpha (Ppar-α), which is particularly abundant in the liver, have demonstrated its relevance in regulating the expression of a wide variety of target genes that encode proteins mainly involved in the transport of long chain fatty acid as the fatty acid-binding protein (FABP1) and in β-oxidation of fatty acids as acyl-CoA oxidase 1 (Acox1) and carnitine palmitoyltransferase 1 (CPT1A).[21] Administration of Ppar-α agonists increases hepatic fatty acid oxidation and decreases the levels of triglycerides in plasma, thereby reducing adipose cell hypertrophy and hyperplasia.[22,23] The use of fibrates as Ppar-α ligands has shown some improvement in hepatic steatosis and plasma lipid homeostasis. However, the undesirable adverse effects they cause (*e.g.*, increased creatinine and homocysteine) rule them out as suitable candidates.[24]

In addition to the aforementioned, the impact of the redox imbalance plays a crucial role as a pivotal component of the MAFLD pathophysiology as it promotes high toxicity on hepatocytes and liver inflammation.[25−27] Thus, a good candidate for MAFLD treatment should also exhibit an important antioxidant effect.

From all of the above, it can be inferred that to treat a pathology as complex as the MAFLD, new approaches should focus on designing drugs with a broader action to address not only the factors that cause the MAFLD (mainly increased appetite for high fatty and sugary diets) but also the consequence related to liver damages (increased lipogenesis, steatosis, and oxidative stress). An interesting pharmacological strategy is represented by a particular group of synthetic and natural molecules derived from fatty acids, capable of acting on more than one therapeutic target as potential anti-obesity drugs.[28] The main interest of this type of molecule lies in its concomitant ability to act as ligands capable of blocking the Cb1 while activating Ppar-α and reducing oxidative stress through the incorporation of a dihydroxy-phenol ring. Through this triple action, these compounds acquire anorectic properties alongside characteristics that enhance fatty acid catabolism and inhibit oxidative stress, ultimately resulting in reduced fat deposits in the liver.

Among them, we selected two compounds for study testing: the N-[1-(3,4-dihydroxyphenyl)propan-2-yl]oleamide (OLHHA) and the hydroxytyrosol linoleoyl ether (HTLE).[29,30] The molecular structure of both compounds includes a derivative of the unsaturated fatty acid omega-9 (OLHHA) and omega-6 (HTLE), which are commonly found in plant oil and able to bind and activate Ppar-α.[31−33] Also, the presence of a dihydroxyphenyl ring in the OLHHA's structure grants it the capacity to scavenge free radicals and mitigate oxidative stress, thereby exhibiting antioxidant activity.[30] Likewise, the HTLE's structure includes hydroxytyrosol, a nonflavonoid polyphenol, which is the most potent antioxidant bioactive compound present in olive oil.[34] Hydroxytyrosol by itself is known to exert benefits on body weight, adiposity, dyslipidaemia, hypertension, hyperglycaemia and insulin resistance, oxidative stress, and inflammation.[35] Indeed, this molecule is considered one of the most powerful natural antioxidants, being its consumption related to positive biological effects on health such as a decrease in the risk of suffering from cardiovascular diseases, prevention of certain types of cancers, and anti-inflammatory and antiviral actions.[36] Both OLHHA and HTLE compounds showed pharmacological effects as antiobesogenic, hypophagic, and antisteatotic agents, exerting particularly robust antioxidant activity on low-density lipoprotein.[29,30] However, compared with OLHHA, HTLE showed higher antioxidant activity and a strong anorectic effect in 24 h food-deprived rats.[29,37]

The aforementioned characteristics of the HTLE compound supported the rationale for testing it as a drug for the treatment of MAFLD in the scenario of obesity-related disorders and metabolic syndrome. Therefore, herein, we focused our interest in testing HTLE to alleviate obesity and some pathological aspects of lipid metabolism when administered chronically to fatty Zucker rats. This animal model of genetic obesity has a mutation in the leptin receptor leading to hyperphagia and severe obesity.[38,39] In the present study, we provide important evidence that HTLE is capable of acting simultaneously through Ppar-α activation while acting as a Cb1 antagonist and an antioxidant agent, particularly in an MAFLD context such as that developed in obese Zucker rats.

MATERIALS AND METHODS

Animal Protocol and Ethics Statement. The chronic experiments were performed on 28 4- to 5-week-old male Zucker rats [obese (*fa/fa*) and lean (+/?) rats] (Crl:ZUC-Lepr^{fa}; Charles River Laboratories, Barcelona, Spain), weighing 400 ± 40 g. For the time course analysis, 30 4- to 5-week-old male Wistar Han International Genetic Standard rats [Crl:WI-(Han] (Charles River Laboratories, Barcelona, Spain), weighing 350 ± 20 g, were used. All animals were housed individually with a 12 h−12 h light−dark cycle in a temperature- and humidity-controlled room. Water and rat chow pellets were available *ad libitum* throughout the course of the present studies.

Animal experimental procedures were carried out upon the European Communities Council Directives 2010/63/EU, Regulation (EC 86/609/ECC, 24 November 24, 1986), and Spanish National and Regional Guidelines for Animal Experimentation (*Real Decreto* 53/2013). Experimental animal protocols and procedures were approved by the Local Ethical Committee for Animal Research of the University of Malaga and performed in accordance with the ARRIVE guidelines (Animal Research: Reporting of *In Vivo* Experiments).[40]

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Preparation and Administration of HTLE for Chronic Treatment and Time Course. HTLE [(9Z,12Z)-1-(2-(3,4-dihydroxyphenyl)ethoxy)octadeca-9,12-diene] (Cayman Chemical, Ann Arbor, USA, #9002950) was dissolved in a 0.9% NaCl vehicle solution (VEH) and injected intraperitoneally (ip) at a dose of 3 mg/kg of body weight (mg kg^{1-} BW) for 15 days. This dose was selected based on previous experiments where the ability of HTLE to reduce food intake was observed.[29] Chronic treatments with HTLE or vehicle solution (VEH, control animals) were performed on 14 obese and 14 lean rats for 15 consecutive days (n = 7 animals per group). Food intake and body weight were measured daily.

For the evaluation of the plasma HTLE time course, the Wistar rats received a single ip dose of 5 mg/kg. A higher dose of HTLE was used in order to ensure a better detection of the compound on the high-performance liquid chromatography/mass spectrometry (HPLC/MS) system. The animals (n = 5 per group) were sacrificed at different times: 0, 15, 30, 120, 240, and 480 min after HTLE administration. Plasma was collected and evaluated for monitoring the time course of HTLE concentrations after a single parenteral dose.

Blood Sample Collection. The animals were anaesthetized with an ip overdose of sodium pentobarbital (50 mg/kg BW) and sacrificed 2 h after the last dose of the HTLE in a chronic treatment trial or at the time indicated for the time course study. Blood samples were collected in EDTA-2Na tubes and centrifuged (2000g, for 10 min at 4 °C), and the plasma samples were stored at −80 °C until biochemical or time course analyses.

Time Course of Plasma HTLE Concentrations after a Single ip Injection. The evaluation of circulating levels of HTLE in rat plasma was determined by HPLC/MS. Briefly, 250 μL of the plasma sample was spiked with an internal standard containing hydroxytyrosol linoleoyl D2 ether (1 μg/mL). Then, 1 mL of 0.1 M ammonium acetate buffer (pH 4) was added, and a liquid−liquid extraction was performed with 6 mL of *tert*-butyl methyl ether. The dry organic extracts were reconstituted with a 100 μL mixture of mobile phases (90% A/10% B, v/v), transferred to HPLC vials, and analyzed by HPLC/MS. Quantification was performed based on an external calibration curve within the range 1.0−75.0 ng/mL, and the limit of detection was set at 0.3 ng/mL.

The chromatographic separation of the lipid species was carried out using an Acquity Ultra Performance Liquid Chromatography (UPLC) instrument (Waters Associates, Milford, USA) controlled by the MassLynx 4.1 software. The LC system was equipped with an Acquity UPLC (BEH C18, 1.7 μm, 2.1 mm × 100 mm) column from Waters Associates. The flow rate was set to 0.3 mL/min, and the column temperature was maintained at 55 °C. The composition of the mobile phase was water (A) and methanol (B) with 1 mM ammonium formate (NH_4HCOO) and 0.01% formic acid (HCOOH) changing as follows: 0 min 85% of B, 3 min 100% of B for 30 s, 3.55 min 85% of B, maintained to 5.5 min for column equilibration. The injection volume was 5 μL. The detection was performed with a triple quadrupole (Waters, Xevo TQS-Micro MS, Millford, USA) mass spectrometer equipped with an orthogonal Z-spray electrospray ionization source (ESI) operated in the negative ion mode. Data were acquired in SRM mode, monitoring the following transitions: 401.2 > 134.9 (hydroxytyrosol linoleoyl ether) and 403.2 > 137 (hydroxytyrosol linoleoyl D2 ether).

Biochemical Analysis in Plasma. The following metabolites were measured in plasma: glucose, triglycerides, total cholesterol, creatinine, urea, uric acid, and bilirubin and the hepatic enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT). These metabolites were analyzed using a Hitachi 737 Automatic Analyzer (Hitachi Ltd., Tokyo, Japan). All serum samples were assayed in duplicate within each assay.

Extraction and Analysis of Liver Lipids. Total lipids from frozen liver samples (270−330 mg) were extracted using a mixture of chloroform−methanol (2:1 V/V) and butylated hydroxytoluene (0.025% W/V) as previously described.[30] Briefly, after two centrifugation steps (2800g, 4 °C for 10 min), the lower phase containing the lipids was separated and dried using N_2. The total lipid content in the liver was determined from the weight of the dry lipid extract and expressed as a percentage of the liver tissue weight.

Oil-red O (ORO) staining was used to analyze the fat depots in liver samples as described previously.[41] Briefly, frozen samples were cut into 30 μm-thick sections using a microtome (Leica SM200R, Wetzlar, Germany) and fixed in 10% paraformaldehyde. After washing with distilled water and rinsed with 60% isopropanol, liver sections were stained with freshly working ORO solution (Sigma, St Louis, USA, #O0625) for 20 min (ORO stock stain: 0.5 g of Oil-red/100 mL of isopropanol; working solution: 30 mL stock solution in 20 mL of distilled water) followed by nuclei staining with Mayer's hematoxylin as described.[41] An optical microscope (Olympus BX41, Allentown, USA) coupled with a digital camera (Olympus DP70, Allentown, USA) was used to acquire the liver section images. Three liver sections were selected per rat from each group to be analyzed. Red staining intensity was quantified by densitometry (image processing ImageJ software: Rasband W.S., ImageJ, U.S., NIH, http://imagej.nih.gov/ij, 1997−2012).

RNA Isolation and RT-qPCR Analysis. Total RNA was extracted from liver tissue sections (30−50 mg) using a Trizol reagent (Invitrogen, Carlsbad, USA, # 15596026) according to the manufacturer's instructions. The RNA was quantified using a Nanodrop TM ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltman, USA). RNA (1 μg) was reverse-transcribed using a Transcriptor Reverse transcriptase kit and random hexamer primers (Transcriptor RT, Roche Applied Science, Penzberg, Germany, #3531295001). The cDNA from samples was amplified in 10 μL of reaction volume containing 4.5 μL of cDNA (previously diluted 1/100) and 5.5 μL of PerfeCTa qPCR ToughMix (Quantabio, Beverly, USA, # 733−2091) containing the 3-hydroxy-3-methylglutaryl-CoA reductase (*Hgmcr*) rat primer probe (Rn00565598_m1) from TaqMan Gene Expression Assays (Thermo Fisher Scientific, Waltman, USA). Real-time qPCR reactions and relative quantification were carried out in a CFX96TM Real-Time PCR Detection System (Bio-Rad, Hercules, USA) as previously reported.[42]

Liver Protein Extraction and Western Blot Analysis. For protein extraction, liver samples (10−20 mg) were homogenized in 1 mL of cold RIPA 1× buffer (50 mM Tris−HCl pH 7.4, 150 mM NaCl, 0.5% NaDOC, 1 mM EDTA, 1% Triton, 0.1% SDS, 1 mM Na3VO4, 1 mM NaF), supplemented with a mix of protease inhibitors (Roche complete Protease Inhibitor Cocktail, Penzberg, Germany) and a phosphatase inhibitor (Inhibitor Cocktail Set III, Millipore, Burlington, USA, # 539134) during 2 h at 4 °C.

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ACS Pharmacol. Transl. Sci. 2024, 7, 1571−1583

After centrifugation, the concentration of the total protein in each sample was determined by using a Bradford colorimetric method. The protein concentration was adjusted to 2 mg/mL, and protein extracts were diluted 1:1 in loading buffer containing dithiothreitol (DTT), heated at 99 °C for 5 min, and subjected to electrophoresis on 4−12% Criterion XT Precast Bis-Tris gels (Bio-Rad, USA). Separated proteins were blotted onto a nitrocellulose membrane (Bio-Rad, Hercules, USA, #77010) in wet transfer equipment (Trans-Blot Turbo system, Bio-Rad, Hercules, USA). Blotted membranes were stained with Ponceau S solution to check the transfer quality followed by blocking in TBST buffer (10 mM Tris−HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.6) containing 2% bovine serum albumin (TBST-BSA). Sequential incubations with primary and secondary antibodies (see the Supporting Information, Table S1 for primary and secondary antibodies information) were performed in TBST-BSA buffer and following conventional protocol (Bio-Rad Laboratories, Hercules, USA). For imaging analysis, the membrane was exposed to a chemiluminescent reagent (Santa Cruz Biotechnology Inc., Dallas, USA) according to the manufacturer's recommendation. Chemiluminescent protein bands were visualized by using a camera-based imager (ChemiDoc Imaging System, Bio-Rad, Hercules, USA). When phosphorylated proteins were detected, the specific antiphospho antibody was removed from the membrane by incubation with stripping buffer (2% SDS, 62.5 mM Tris HCL pH 6.8, 0.8% β-mercaptoethanol) 30 min at 50 °C. Membranes were extensively washed in ultrapure water and then reincubated with the corresponding antibody specific for total protein detection. The target band intensity was analyzed and quantified by densitometry using the ImageJ software. Detection of γ-adaptin protein was used as a protein reference and as a loading control for each sample. The amount of a specific protein in each sample was expressed as the ratio of the signal obtained with the specific antibody relative to the corresponding signal obtained with the anti-γ-adaptin antibody. The phosphorylation state of a protein was expressed as the ratio of the signal obtained with the antiphospho-specific antibody relative to the corresponding signal obtained with the specific antibody against the total protein.

Evaluation of Oxidative Stress Status and Antioxidant Capacity of HTLE in the Liver Samples. The thiobarbituric acid reactive substance (TBAR) assay was used to measure the malondialdehyde (MDA) levels as the end-product of lipid peroxidation in hepatic samples following the commercial assay kit instructions (Cell Biolabs Inc., San Diego, USA, #STA-330). Quantification of the reduced form of glutathione (GSH) in the liver was measured using a commercial kit according to the manufacturer's instructions (Invitrogen, Waltham, USA, #EIAGSHC). The determination of the antioxidative capacity of liver extracts was performed by the 2,2′-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) assay, also referred to as vitamin C equivalent antioxidant capacity (CEAC) using a commercial kit (BQC Redox Technology, Oviedo, Spain, # KF-01−002). This method is used to evaluate the relative antioxidant ability of a sample based on its faculty to convert the oxidized form $ABTS^{+}$ (strongly colored radical cation) to the reduced ABTS form (decolorized).

Statistical Analysis. GraphPad Prism version 9.01 software (GraphPad Software, Inc., San Diego, USA) was used for statistical analysis. All data are expressed as the mean ± SEM (standard error of the mean). Statistical analysis was taken on for studies where each group size was 8 (n = 8). One- and two-way (treatment vs genetic conditions) analysis of variance (ANOVA) was assessed followed by Bonferroni's *post hoc* multiple comparisons test. The *post hoc* tests were conducted only if F in ANOVA achieved a p value less than 0.05 ($p < 0.05$) and there was no statistically significant variance inhomogeneity. The results were considered statistically significant at $p < 0.05$.

RESULTS

Parenteral HTLE Administration Resulted in Sustained Plasma HTLE Levels for Several Hours. We performed preliminary studies in plasma to examine the clearance of HTLE after ip administration in Wistar rats. Pharmacokinetics analysis of HTLE administration was obtained by monitoring the plasma concentration of HTLE at 0, 15, 30, 120, 240, and 480 min after the administration of 5 mg/kg in 18 h food-deprived male rats. As shown in Figure 1,

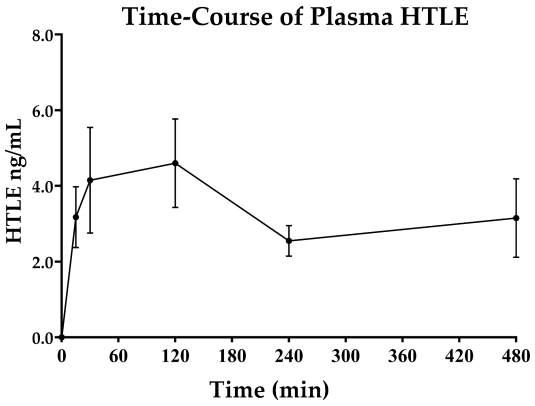

Figure 1. Time course of plasma HTLE. Plasma concentration of HTLE (ng/mL) after ip administration of a single dose (5 mg/kg). Sampling was done at 0, 15, 30, 120, 240, and 480 min post-injection in 18 h food-deprived male Wistar rats. The values are means ± (standard error of mean) SEM (n = 5 animals per treated group).

plasma HTLE concentrations became detectable as soon as 15 min and peaked at 120 min (T_{max}) showing permanent sustained levels for at least 480 min. Thus, HTLE seems to be a long half-life, long-acting compound when given parenterally, although further research is needed to establish its real half-life and the potential absorption through the oral route.

Chronic HTLE Administration Reduces Body Weight Gain and Food Intake. The body weight gain of the animals and the cumulative food intake were recorded daily throughout the 15 days of HTLE (3 mg/kg) chronic treatment. Weight gain of the lean Zucker rats was significantly lower from day 11 to day 15 of the chronic HTLE treatment as compared with the rats that areVEH-treated (Figure 2A). In obese Zucker rats, the HTLE chronic administration caused inhibition of body weight gain as soon as from day 2 and this effect lasted along the following days of treatment, with statistical differences between HTLE and VEH groups being significant for each day of the treatment (Figure 2B). Of note was that this inhibitory effect on weight gain appeared to reflect some tolerance after day 9 of the treatment in obese rats. Indeed, the weight gain inhibition in obese rats treated with HTLE was more evident at the beginning of the treatment than at the end. Even so, analysis of the relative final weight gain showed that HTLE

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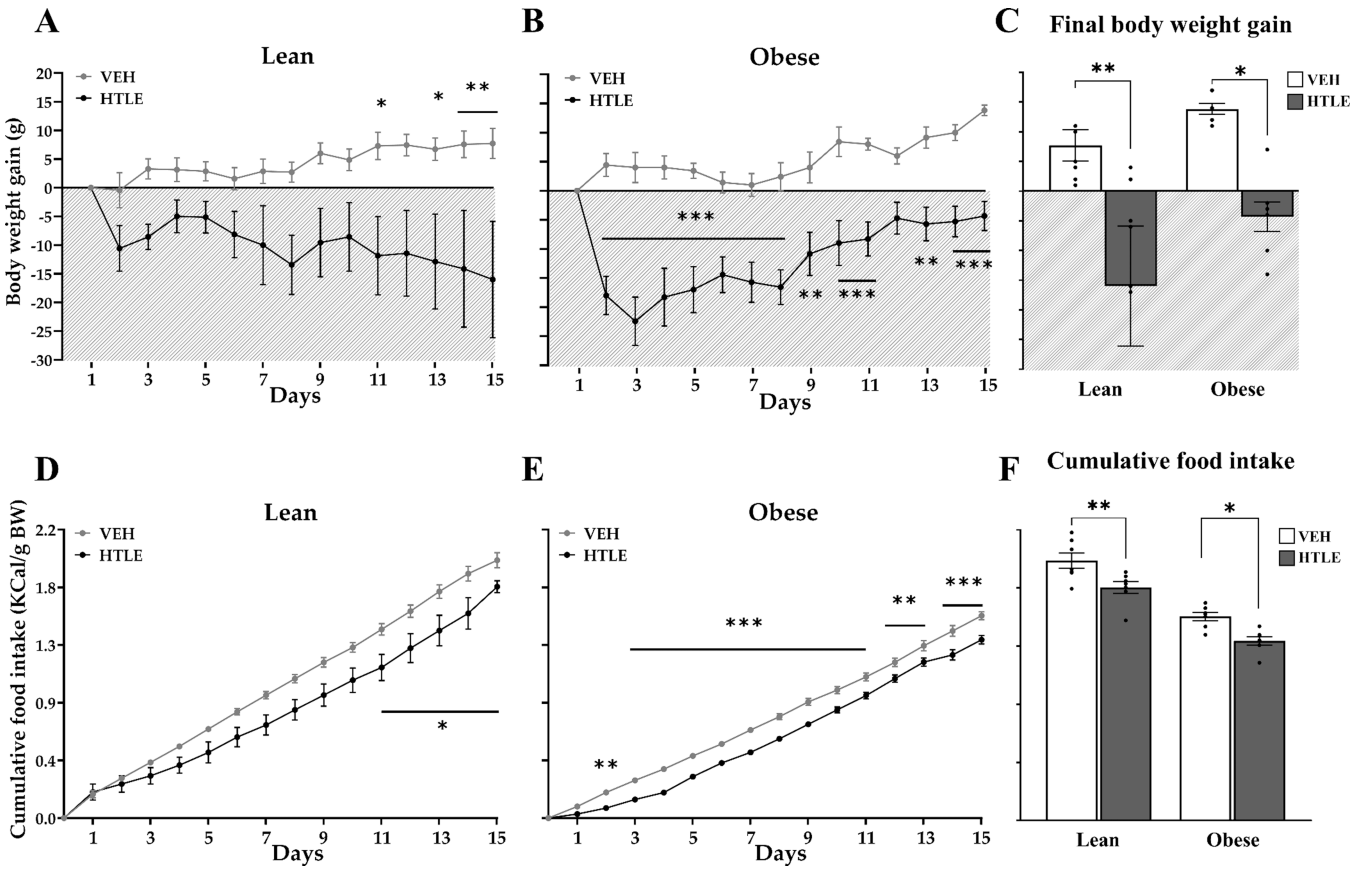

Figure 2. Hypophagic effect of chronic HTLE treatment. (A) Daily body weight gain measured during 15 days with HTLE (3 mg/kg) or VEH treatment in lean Zucker rats. (B) Daily body weight gain measured during 15 days with HTLE or VEH treatment in obese Zucker rats. (C) Final body weight (BW) gain after 15 days of HTLE or VEH treatment in both lean and obese Zucker rats. (D) Effect of chronic HTLE versus VEH administration on cumulative kilocalories (kcal) intake in lean rats. (E) Effect of chronic HTLE versus VEH administration on cumulative kilocalories (kcal) intake in obese Zucker rats. (F) Final cumulative kcal intake after 15 days of chronic HTLE versus VEH administration in both lean and obese Zucker rats. Data are expressed as means ± (standard error of mean) SEM (n = 7 animals per group). Data were analyzed by two-way ANOVA (genotype and treatment) for each parameter and with Bonferroni's *post hoc* for multiple comparisons. (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ denote significant differences compared with the corresponding VEH-treated group.

Table 1. Plasma biochemical parameters in plasma of Zucker rats treated with HTLE[a]

	lean		obese	
plasma biochemical parameters	VEH	HTLE	VEH	HTLE
glucose (mg/dL)	141.57 ± 6.58	143.29 ± 9.72	133.71 ± 3.15	137.57 ± 5.28
renal function				
creatinine (mg/dL)	0.47 ± 0.08	0.39 ± 0.06	0.42 ± 0.03	0.30 ± 0.07
urea (mg/dL)	45.71 ± 0.963	41.33 ± 0.68	52.83 ± 3.41$^{\#}$	45.58 ± 0.54*
uric Acid (mg/dL)	1.61 ± 0.10	1.49 ± 0.11	1.64 ± 0.13	1.49 ± 0.10
hepatic function				
bilirubin (mg/dL)	0.16 ± 0.01	0.14 ± 0.01	0.17 ± 0.00	0.16 ± 0.01*
AST (IU)	102.50 ± 1.57	92.57 ± 1.90*	92.67 ± 2.57	78.43 ± 3.82**/$^{\#\#}$
ALT (IU)	51.28 ± 3.53	53.14 ± 4.42	52.28 ± 2.49	56.57 ± 1.86

[a]Metabolic biochemical parameters in plasma of Zucker rats treated with HTLE (3 mg/kg) or vehicle (VEH) daily for 15 days. Values are expressed as the mean ± SEM (standard error of mean) (n = 7 animals per group). Data were analyzed by two-way ANOVA (genotype and treatment) for each parameter and Bonferroni's *post hoc* test for multiple comparisons. (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ denote significant differences compared with the corresponding VEH-treated group. (#) $p < 0.05$, (##) $p < 0.01$, and (###) $p < 0.001$ denote significant differences compared with its lean-treated group. AST: aspartate aminotransferase and ALT: alanine aminotransferase.

administration significantly reduced body weight gain in both lean and obese genotypes as compared with the corresponding rat groups that are VEH-treated (Figure 2C).

Concerning food consumption and as compared with the control groups of rats receiving VEH, the HTLE administration significantly reduced food intake in lean rats from days 11 to 15 (Figure 2D) and in obese rats from days 2 to 15 (Figure 2E), which correlated with the delay in body weight gain. Finally, the cumulative food intake remained significantly lower in both lean and obese animals that are HTLE-treated than in their corresponding control groups that are VEH-treated (Figure 2F). These observations indicate that HTLE

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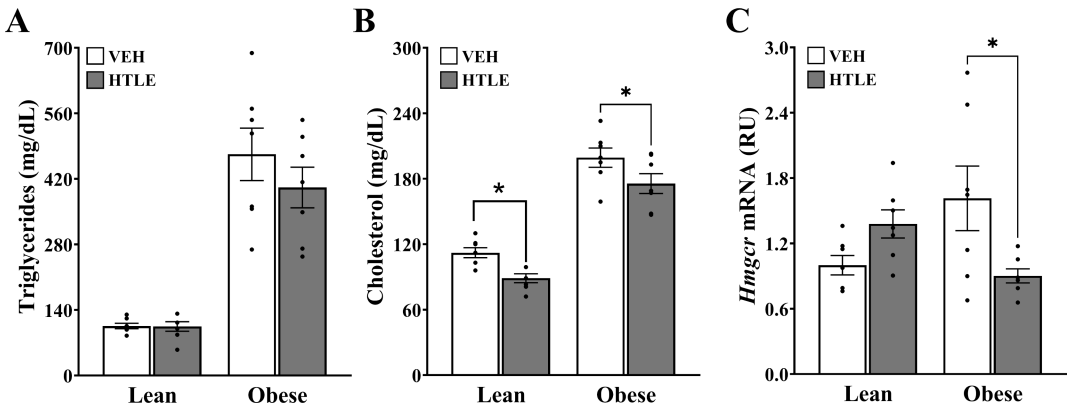

Figure 3. Effect of HTLE on lipid metabolism. Effect of chronic treatments of HTLE on (3 mg/kg) on triglyceride and total cholesterol serum levels and hepatic *Hmgcr* expression in lean and obese Zucker rats. (A) Bar histogram representing serum levels of triglycerides. (B) Bar histogram representing serum levels of total cholesterol. (C) Bar histogram representing relative mRNA levels of 3-hydroxy-3-methylglutaryl-CoA Reductase (*Hmgcr*) in the liver. The rat genotype and treatment are indicated in the corresponding histogram. Values are expressed as the mean ± SEM (standard error of mean) (n = 7 animals per group). Data were analyzed by two-way ANOVA (genotype and treatment) for each parameter and with Bonferroni *post hoc* for multiple comparisons. (*) $p < 0.05$ denotes significant differences compared with the corresponding VEH-treated group.

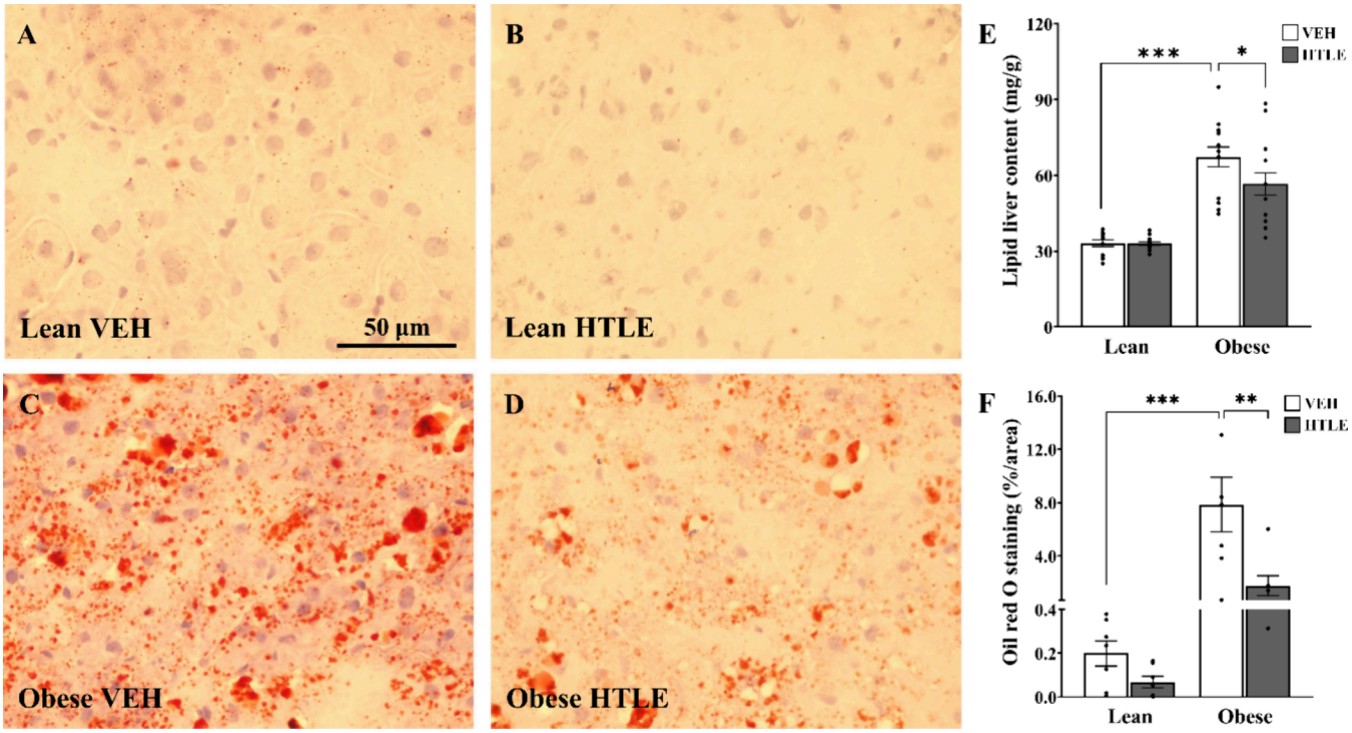

Figure 4. Effect of HTLE on fat liver content. Effect of chronic treatments of HTLE (3 mg/kg) on fat liver content of lean and obese Zucker rats. (A–D) Representative images of histological liver sections (40× magnification) stained with ORO. (E) Quantification of the content of the ORO staining for each group of treatment as indicated in the figure. (F) Total lipid content in the tissue liver of each group of treatment as indicated in the figure. Rat genotype and treatment are indicated in the corresponding image or histogram. Values are expressed as the mean ± SEM (standard error of mean) (n = 7 animals per group). Data were analyzed by two-way ANOVA (genotype and treatment) for each parameter and with Bonferroni post hoc for multiple comparisons. (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ denote significant differences compared with the corresponding VEH-treated group.

treatment is capable of modulating eating behavior, which may contribute to the observed body weight reduction. A complete description of the statistical analysis is shown in the Supporting Information, Table S2.

Chronic HTLE Administration Showed No Signs of Kidney or Liver Toxicities. Several biochemical parameters of the blood plasma evaluated after the experimental *in vivo* treatments are shown in Table 1. HTLE administration did not alter the concentration of hematoxylin in both lean and obese genotypes. Likewise, no significant variations in creatinine and uric acid concentrations were detected in response to the HTLE treatment in both genotypes, indicating no significant impact on renal function. Also, HTLE did not affect the urea levels in the lean genotype. Obese Zucker VEH-treated rats exhibited significantly higher levels of blood urea as compared with lean Zucker rats that are VEH-treated (Table 1). In this

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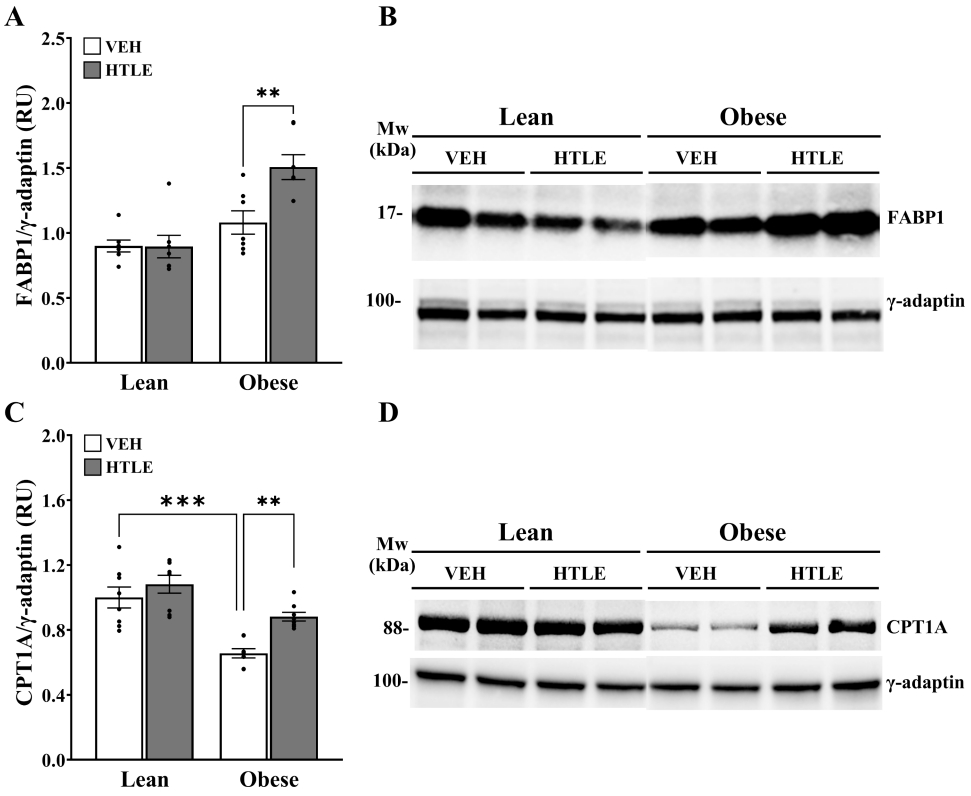

Figure 5. Effects of HTLE fatty acid oxidation-related protein expression. Effects of HTLE (3 mg/kg) treatment on the expression of proteins related to fatty acid oxidation in lean and obese Zucker rats. Western blot analysis of hepatic expression of (A) fatty acid-binding protein (FABP1) and (C) carnitine palmitoyltransferase 1 (CPT1A). For each analysis, the left panel represents the bar histogram depicting the protein levels determined through densitometry corrected for γ-adaptin; the right panels (B and D) show a representative immunoblot of each protein from two samples out of seven per group. The corresponding bands of γ-adaptin are shown as loading controls per lane. The rat genotype and treatment are indicated in the corresponding histograms or blot image. All samples were migrated into the same gel and processed in parallel. The adjustment to digital images did not alter the information contained therein. Data were analyzed by two-way ANOVA (genotype and treatment) for each parameter and Bonferroni's *post hoc* for multiple comparisons. Values are expressed as the mean ± SEM (standard error of mean) (n = 7 liver samples per group). (**) $p < 0.01$ and (***) $p < 0.001$ denote significant differences compared with the corresponding VEH-treated group.

case, HTLE administration in obese rats significantly restored the concentrations of urea to levels found in the lean genotype. These results indicated that at least HTLE was not detrimental to kidney function when chronically administered but rather showed beneficial properties under obese conditions.

Other parameters related to hepatic function, such as the plasma levels of transaminases ALT and AST or bilirubin, were also evaluated. As shown in Table 1, HTLE administration did not affect ALT levels but significantly reduced circulating AST levels in both lean and obese animals as compared with their respective VEH-treated groups. Also, HTLE reduced the bilirubin plasma concentrations in obese rats, whereas no such effect was observed in lean rats. These observations indicated not only the absence of hepatotoxic effects of HTLE during treatment but a certain hepatoprotective effect at least in obese rats (Table 1). A complete description of the statistical analysis is provided in the Supporting Information (Table S3).

Analysis of plasma lipid parameters showed that obese Zucker rats exhibited a significant increase in triglycerides and cholesterol compared with lean animals receiving VEH (Figure 3A,B). The chronic administration of HTLE did not significantly change the plasma triglyceride levels in lean or obese rats regarding their respective VEH-treated groups (Figure 3A). Noteworthy, the total cholesterol concentrations in plasma were significantly lowered upon HTLE treatment in both rat strains (Figure 3B).

Because the 3-hydroxymethylglutaryl-coenzyme-A reductase (HMGCR) is the rate-limiting enzyme for cholesterol biosynthesis in the liver,[43] we reasoned that levels of the *Hmgcr* gene expression may account for changes in blood cholesterol levels. So, we next examined the impact of HTLE administration on the gene expression of this enzyme in hepatic sections. As shown in Figure 3C, statistical analysis indicated that HTLE treatment did not affect hepatic *Hmgcr* transcription in lean rats; however, in obese rats, the gene expression of *Hmgcr* was markedly reduced in HTLE-treated rats as compared with its control group. The Supporting Information (Table S4) provides a complete description of the statistical analysis of Figure 3.

Chronic HTLE Administration Reduces Fatty Content in the Liver. One of the crucial consequences of obesity is the fatty acid overload in the liver. Hence, we next evaluated the ability of HTLE to reduce the liver fat deposits in obese rats. Analysis of the images from liver sections stained with ORO revealed a weak presence of fatty depots in lean rat-VEH- or -HTLE-treated (Figure 4A,B). Consistent with the high concentrations of triglycerides and cholesterol found in the plasma of obese rats treated with VEH, we detected a notable increase in fat deposits in their corresponding liver samples (Figure 4C). Liver sections of obese HTLE-treated rats showed a marked decrease in these fat deposits (Figure 4D). The statistical analysis of the percentage of ORO staining is

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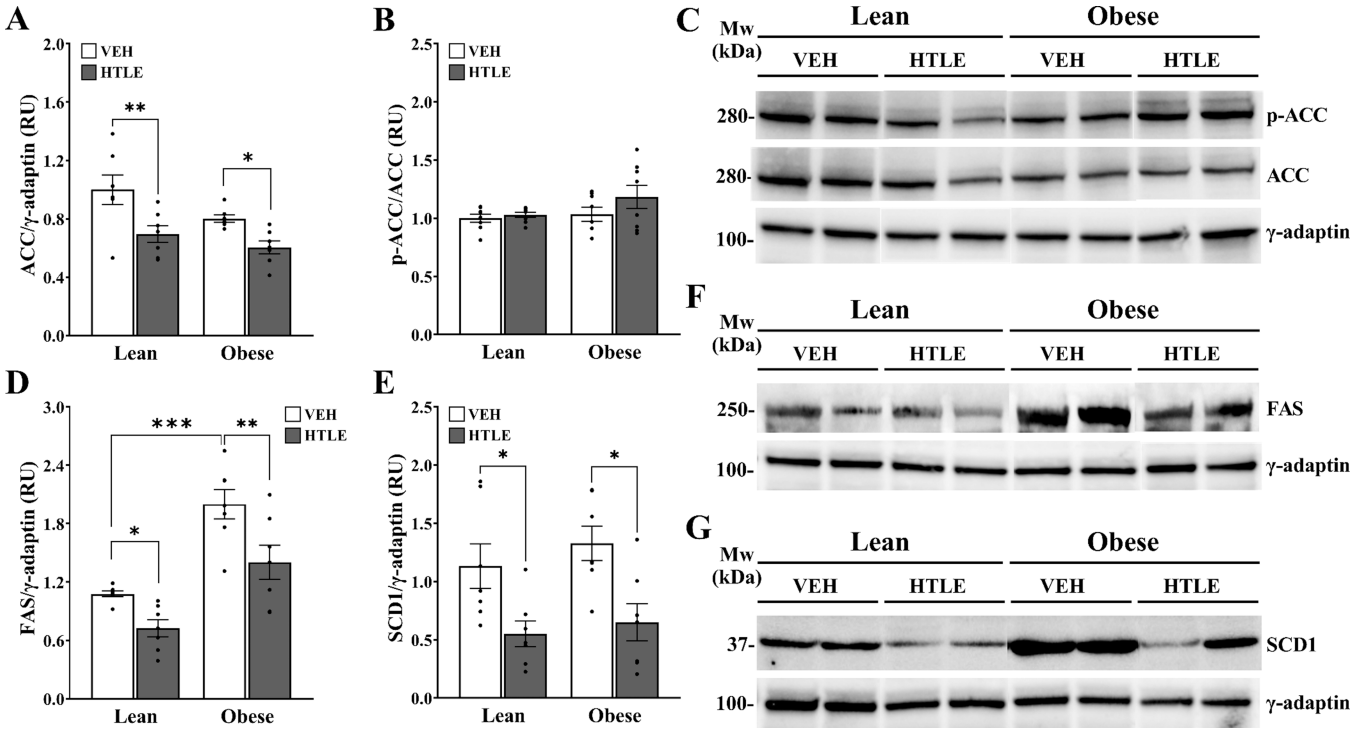

Figure 6. Effects of HTLE on the expression of liver lipogenesis proteins. Effects of HTLE (3 mg/kg) treatment on the expression of proteins related to lipogenesis in the liver of lean and obese Zucker rats: (A) bar histogram depicting the acetyl-CoA carboxylase protein (ACC) levels determined through densitometry corrected for γ-adaptin, (B) bar histogram depicting the phosphorylated-ACC (p-ACC) protein levels determined through densitometry corrected for ACC total protein, (D) bar histogram depicting the protein fatty acid synthase protein (FAS) levels determined through densitometry corrected for γ-adaptin, and (E) bar histogram depicting the stearoyl-CoA desaturase 1 protein (SCD1) levels determined through densitometry corrected for γ-adaptin. The right panels (C, F, and G) show a representative immunoblot of each protein from two samples out of seven per group. The corresponding bands of γ-adaptin are shown as a loading control per lane. The rat genotype and treatment are indicated in the corresponding histogram or blot image. All samples were migrated in the same gel and processed in parallel. The adjustment to digital images did not alter the information contained therein. Data were analyzed by two-way ANOVA (genotype and treatment) for each parameter and with Bonferroni's *post hoc* for multiple comparisons. Values are expressed as the mean ± SEM (standard error of mean) (n = 7 animals per group). (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ denote significant differences compared with the corresponding VEH-treated group.

shown in Figure 4E. Accordingly, the analysis of the lipid liver content showed significantly higher lipid content in the liver of obese rats than in lean rats (Figure 4F) and the chronic administration of HTLE reduced the quantity of lipids in the liver of obese rats but not in lean rats as compared with their respective VEH-treated group (Figure 4F). Based on these findings, it can be concluded that the chronic administration of HTLE exerts a clearly antisteatotic action. The Supporting Information (Table S5) provides a complete description of the statistical analysis of Figure 4.

Chronic HTLE Treatment Stimulates the Expression of Enzymes Related to the Fatty Acid β-Oxidation Process. To better understand the mechanism of action of the HTLE compound on lipid metabolism, further investigation was carried out with special emphasis on the analysis of the expression of liver fatty acid-binding proteins as FABP1 and hepatic enzymes regulating the fatty acid-β-oxidation process as CPT1A. The gene expression of FABP1 and CPT1A proteins is controlled by the transcription factor Ppar-α in the liver.[21] When comparing the hepatic levels of these proteins between lean and obese rat groups, it was noted that CPT1A levels were particularly downregulated in obese rats, in contrast to the amounts of FABP1 that showed no significant changes between the two genotypes (Figure 5A). Statistical analysis revealed no significant effects in the expression levels of these proteins after HTLE treatment in lean rats compared to the corresponding group receiving VEH alone (Figure 5A,B). Analysis of the obese rats showed that HTLE treatment specifically increased FABP1 and CPT1A protein levels above the basal levels found in the respective control group VEH-treated rats (Figure 5A,B). The Supporting Information (Table S6) provides a complete description of the corresponding statistical analysis.

Altogether, these results denote that HTLE plays a critical role in improving the expression of proteins like FABP1, which is involved in the long chain fatty acid (LCFA) shuttle to the nucleus, thereby increasing the viability of ligands to Ppar-α while favoring the Ppar-α transcriptional activity.[21] Likewise, since CPT1A catalyzes the transport of LCFA into mitochondria for β-oxidation, increasing the expression of this enzyme would enhance the β-oxidation of fatty acid process in the liver.[21]

Chronic HTLE Treatment Downregulates the Expression of Lipogenic Enzymes. In the next step, we determined whether HTLE could affect the expression of the enzymes involved in the lipogenesis pathway. The enzymes acetyl-CoA carboxylase (ACC), fatty acid synthetase (FAS), and stearoyl-CoA desaturase 1 (SCD1) act in consecutive steps to control *de novo* fatty acid synthesis in the liver and adipose tissue.[44] Under the experimental conditions herein, we found that the

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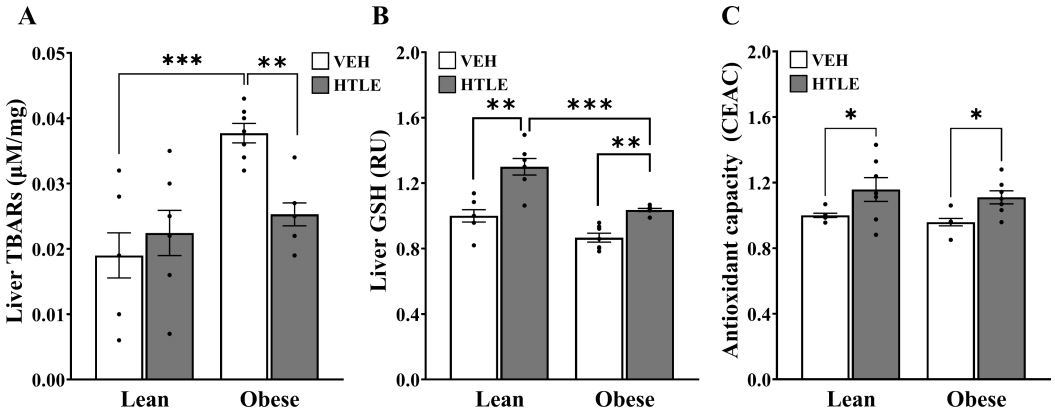

Figure 7. Effect of HTLE on oxidative stress in the liver. Effect of HTLE (3 mg/kg) treatment on the expression of oxidative stress parameters in the liver of lean and obese Zucker rats. The bar histogram represents the serum level of (A) TBARs, (B) reduced GSH, (C) antioxidant capacity expressed as CEAC. The rat genotype and treatment are indicated in the corresponding histogram. Values are expressed as the mean ± SEM (standard error of mean) (n = 7 animals per group). Data were analyzed by two-way ANOVA (genotype and treatment) for each parameter and Bonferroni *post hoc* for multiple comparisons. (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ denote significant differences compared with the corresponding VEH-treated group.

expression of FAS enzyme was significantly higher in obese rats versus lean rats, whereas no significant differences were observed for levels of ACC and SCD1 enzymes between both genotypes. Interestingly, after the HTLE chronic treatment, the expression of these three pro-lipogenic enzymes was decreased in both genotypes to levels significantly lower than those found in the corresponding VEH-treated rats (Figure 6A–D). It should be noted that the ACC enzyme is the main controller of lipogenesis in cells, which, in turn, is negatively regulated by phosphorylation at its serine 79 residue. The analysis of the ACC phosphorylation in Ser79 (Figure 6 right panel) showed no statistical differences between VEH- and HTLE-treated groups of both genotypes; however, it should be noted that 50% of the samples from obese rats treated with HTLE did show an increase in the ACC phosphorylation status. The Supporting Information (Table S6) provides a complete description of the corresponding statistical analysis.

HTLE Displays Antioxidative Stress Actions in the Liver. Oxidative stress was evaluated in the livers of lean and obese animals after the experimental *in vivo* treatments by means of several tests. We first evaluated the lipid peroxidation and the formation of malondialdehyde (MDA) in liver sections by the TBAR test. Consistent with an obesogenic status, TBAR levels were significantly higher in liver extracts from VEH-treated obese animals compared to the lean group receiving the same treatment (Figure 7A). The administration of HTLE exerted no apparent effect on the amounts of TBARs in liver extracts of lean rats. On the contrary, HTLE caused a significant decrease in the amounts of TBARs in obese animals, even restoring their normal levels, thus reflecting a reduction of the lipid peroxidation process in this rat genotype.

The liver samples from all animal groups were also examined in terms of the amounts of GSH, a tripeptide with scavenging activity of the reactive oxygen species (ROS) found in high concentrations in the liver.[45] No statistical differences in the amounts of GSH were observed between obese and lean rats that are VEH-treated, thus indicating *a priori* similar initial GSH levels (Figure 7B). After HTLE treatment, a significant rise in GSH quantities was observed in the lean and obese groups, although the increase in the GSH content was less noticeable in obese rats (Figure 7B). This result indicated that HTLE enhances the glutathione antioxidative response under normal conditions and in obesity.

Finally, the antioxidant capacity of HTLE in liver extracts was also evaluated through the vitamin C equivalent antioxidant capacity (CEAC) assay. In this assay, the oxidized $ABTS^{+}$ radical cation is reduced to ABTS by the presence of antioxidants in the liver extracts. The ABTS assay showed that the CEAC in the liver from animal groups receiving HTLE was higher compared with the corresponding control groups receiving VEH (Figure 8C), thus indicating that HTLE can enhance or recuperate the antioxidant capacity in response to metabolic complications.

Altogether, these data show that HTLE may exert important antioxidant benefits with interesting antiaging and anti-inflammatory potential. The Supporting Information (Table S7) provides a complete description of the statistical analysis.

DISCUSSION

The current study demonstrates the remarkable efficacy of chronic HTLE administration in reducing obesity, liver steatosis, and hypercholesterolemia in the obese Zucker rat model. Unlike its related compound, OLHHA, which primarily affects the lean genotype, HTLE exhibits a distinct advantage by effectively addressing the complexities of obesity presented by the obese Zucker rat strain.

Consistent with previous findings demonstrating hypophagic effects of HTLE when administered acutely to non-overweight rats,[37] our study reveals that chronic treatment with HTLE resulted in a reduction in weight gain and food intake in both lean and obese Zucker rats from the beginning of the study. However, the kinetics of weight gain deceleration differed between the two genotypes. While lean rats showed a more noticeable decrease toward the end of treatment, obese rats experienced a pronounced effect early in the treatment, which waned toward the end, suggesting a potential tolerance development. This observation aligns with previous findings indicating tolerance to hypophagia and weight loss after prolonged treatment with Cb1 antagonists, which was also associated with an inhibitory impact on the expression of anorexigenic peptides in the hypothalamus.[46] The observed gradual plasma clearance of HTLE after its administration

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likely facilitates tolerance development, emphasizing the need for careful consideration of dosage and treatment duration.

Analysis of plasma biomarkers revealed a safe pharmacological profile for chronic HTLE administration in both lean and obese Zucker genotypes with no impact on glucose homeostasis. Notably, HTLE successfully normalized plasma levels of urea, AST, and bilirubin, indicative of liver damage, which is a common concern in obese individuals. Additionally, HTLE demonstrated efficacy in mitigating plasma triglyceride levels in obese rats. All these data are consistent with previous findings described for combined treatment with the Ppar-α ligand oleoylethanolamide (OEA) and the Cb1 antagonist Rimonabant in obese Zucker rats.[20] Hence, our results strongly support the notion that these beneficial effects stem from HTLE's dual capability to function as both a Ppar-α ligand and a Cb1 antagonist.

Hypercholesterolemia, a hallmark of the obese Zucker rat model,[47] was effectively alleviated by HTLE in both lean and obese strains. The observed similar effects in obese rats when co-administered with a Ppar-α ligand and a Cb1 antagonist reinforce that HTLE operates via these two mechanisms simultaneously.[20,48,49] Indeed, some other data in the literature also support this perspective. On the one hand, the HTLE structure contains the linoleic acid, which is not only a well-known Ppar-α ligand but also displays a potent effect in reducing hypercholesterolemia as well as the levels of the transaminases AST and ALT in an obesity rat model.[31,48,49] On the other hand, Cb1 blockade was also reported to decrease serum levels of total cholesterol in a mouse model.[50] Therefore, it is conceivable that the HTLE's impact on plasma cholesterol arises from its combined actions as both a Ppar-α ligand and a Cb1 antagonist. Additionally, this is further supported by the normalization of *Hmgcr* mRNA levels observed in the liver of obese rats following HTLE treatment, highlighting its role in regulating cholesterol biosynthesis.

In this study, we also examined hepatic expression levels of enzymes associated with β-oxidation such as CPT1A and the FABP1, whose gene expressions are regulated by the nuclear factor Ppar-α.[21] A deficiency in either the expression or function of CPT1A would lead to a decreased rate of fatty acid β-oxidation.[51,21] Consistent with previous studies,[52,53] we found a significant reduction in the hepatic expression of CPT1A in obese Zucker rats, likely contributing to liver steatosis. After HTLE treatment, no change in the expression of FABP1 and CPT1A proteins was noted in lean rats, whereas obese rats exhibited increased FABP1 expression and normalized CPT1A levels. Hence, this strongly suggests that HTLE diminishes steatosis in obese rats by reactivating Ppar-α, thereby promoting fatty acid catabolism.

Regarding the ability of HTLE to mitigate steatosis, the impact of this compound on lipogenesis was also examined. The ACC enzyme is known to play a pivotal role in lipogenesis by catalyzing the carboxylation of acetyl-CoA to form malonyl-CoA, a critical step in fatty acid biosynthesis.[54] Additionally, malonyl-CoA acts as a negative regulator of CPT1A, hampering the fatty acid β-oxidation in mitochondria. Hence, the balance of the malonyl-CoA concentrations governs lipid metabolism. The AMP-activated protein kinase phosphorylates and inactivates the ACC enzyme to decrease malonyl levels, thereby releasing CPT1A from inhibition and promoting fatty acid β-oxidation. Given the HTLE's capacity to alleviate liver steatosis in obese rats, we initially anticipated elevated levels of ACC phosphorylation in HTLE-treated groups. However, our results revealed no significant alteration of the ACC phosphorylation status. Instead, we observed a notable reduction in the total ACC hepatic levels following HTLE treatment in both rat strains. Moreover, the HTLE treatment also reduced the protein levels of the FAS and SCD1 lipogenic enzymes in lean and obese rats. These findings align with a Cb1 antagonist effect since the stimulation of Cb1 receptor typically promotes the transcription of genes encoding the lipogenic enzymes like *Acc*, *Fas*, and *Scd1*, which in turn stimulates hepatic lipogenesis and contributes to the development of steatosis.[55,56,20] The finding that Rimonabant, a well-established Cb1 inverse agonist, successfully blocks the Cb1 signaling pathway and thereby inhibits the transcription factors responsible for expressing genes encoding lipogenic enzyme was of considerable relevance in addressing steatosis and obesity.[57] In line with these data, our results showing that prolonged treatment with HTLE leads to a decrease in the levels of the lipogenic enzymes ACC, FAS, and SCD1 in liver rats strongly validate its role as a Cb1 antagonist. Furthermore, together with its ability to act as a Ppar ligand, this explains its powerful action in reversing steatosis.

Another important issue addressed herein is the evaluation of the HTLE compound as an antioxidant agent. The inclusion of a hydroxytyrosol group in HTLE was expected to confer an antioxidant capacity. Fatty acid overload in the liver, often associated with obesity, leads to mitochondrial hyperactivation and heightened oxidative stress in hepatocytes (reviewed in Delli Bovi et al.).[58] This oxidative stress stems from electron leakage in the electron transport chain, leading to overproduction of ROS, lipid peroxidation (manifested by increases in malondialdehyde; MDA), and endoplasmic reticulum oxidative stress.[59] HTLE administration significantly reduced lipid peroxidation and augmented the synthesis of free radical scavenger GSH in both lean and obese rats, indicating a role in improving redox imbalance. Assessment of HTLE's antioxidant capacity by CEAC assay in liver extracts further demonstrated considerable antioxidative potential in both lean and obese rats.

In addition to the above, it is noteworthy that while the hydroxytyrosol group in HTLE can contribute to its antioxidant properties, the HTLE's dual role as a Cb1 antagonist and as a Ppar-α ligand also plays a significant capacity in counteracting oxidative stress in fatty liver. This is achieved by concurrently promoting antilipogenic action and fatty acid β-oxidation. Furthermore, the HTLE treatment augmented the protein levels of FABP1, which is also recognized for its potent endogenous antioxidant properties.[60] Based on these findings, it seems that HTLE's antioxidant effect may arise directly from the compound itself. However, it is important to consider that the various metabolic actions that HTLE promotes *in vivo*, such as those secondary to hypophagia, the prevention of weight gain, and inhibition of lipogenesis, also contribute to reducing fatty liver, consequently diminishing oxidative stress.

In conclusion, HTLE emerges as a promising candidate for combating obesity-related metabolic disorders, offering a multifaceted approach in addressing obesity, hypercholesterolemia, hypertriglyceridemia, liver steatosis, and oxidative stress. Further research is warranted to explore its interactions with other pathways and its clinical applications in treating MAFLD.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsptsci.4c00105.

(Table S1) Primary and secondary antibodies used for protein detection in Western blot analysis; (Table S2) body weight and food intake: description of the statistical analysis shown in Figure 2; (Table S3) biochemical analysis of plasma: description of the statistical analysis shown in Table 1; (Table S4) triglycerides, cholesterol, and *Hmgcr* gene expression description of the statistical analysis of Figure 3; (Table S5) liver fat description of the statistical analysis of Figure 4; (Table S6) liver protein expression: description of the statistical analysis corresponding to Figures 5 and 6; and (Table S7) oxidative stress in liver: description of the statistical analysis corresponding to Figure 7 (PDF)

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Notes

The authors declare no competing financial interest.

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