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Basic nutritional investigation

Effects of resveratrol on obesity-related inflammation markers in adipose tissue of genetically obese rats

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ABSTRACT

Objective: The aim of this study was to examine whether resveratrol might represent a promising therapeutic tool with which to combat adipose tissue chronic inflammation in a model of genetic obesity and to link its anti-inflammatory activity with its effect on body fat reduction.
Methods: Twenty 6-wk-old male Zucker (*fa/fa*) rats were randomly distributed into two experimental groups. Resveratrol (RSV) was given orally (15 mg/kg body weight/d in RSV group) by means of an orogastric catheter for 6 wk. Enzyme activities were measured spectrophotometrically or fluorimetrically. Gene and protein expressions were analyzed by reverse transcriptase polymerase chain reaction and Western blot respectively. Cytokine concentrations and the activity of nuclear factor κ-light-chain-enhancer of activated β cells (NF-κB) were measured by using commercial kits.
Results: RSV reduced the weight of internal adipose tissues. In epididymal depot glucose-6P-dehydrogenase, acetyl-CoA carboxylase activities, as well as lipoprotein lipase expression and activity were reduced by RSV. The expression of hormone-sensitive lipase was increased, and that of the cluster of differentiation 36 was reduced. Serum concentrations of tumor necrosis factor-α, monocyte chemoattractant protein 1, and C-reactive protein were lower in the RSV-treated group than in the control group. Protein expression of interleukin-6 and the activity of NF-κB, were decreased by RSV.
Conclusion: The present results provide evidence that fatty acid uptake and lipolysis are metabolic pathways involved in the response of adipose tissue to RSV. This polyphenol modulates plasma cytokine levels partially by reducing macrophage infiltration in adipose tissue and inhibiting NF-κB activity.

Introduction

Adipose tissue plays a crucial role in energy homeostasis. In the normal physiological state, excess fuel substrate is partitioned to adipose tissue, where it is stored as triacylglycerols until its subsequent release as non-esterified fatty acids. Pathophysiologial disorders of adipose tissue, such as obesity, are associated with dysregulation of this process [1].

Particularly in the visceral area, adipose tissue acts as a critical endocrine organ that releases a variety of cytokines, involved in humoral control of metabolic homeostasis in adipose tissue and other tissues [2–4]. Under physiological conditions, macrophages account for 5% to 10% of cells within adipose tissue. However, when an excessive expansion of this tissue takes place, an intensified infiltration of macrophages can be observed [5,6]. In this situation, macrophages can represent up to 60% of all cells in adipose tissue [7]. Under obesogenic conditions, increased amounts of proinflammatory cytokines are produced either by adipocytes or by recruited macrophages [8], and this situation is

The contributions of each author were as follows: EH took care of animals during the experimental period; SGZ, AL, and AFQ carried out RNA quantification by real-time RT-PCR; SGZ performed Western blot analysis, enzyme activity assessment, and serum parameters; and MPP and LB supervised the results and wrote the manuscript.

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related to metabolic complications of obesity. Consequently, obesity has been considered to be a low-grade inflammatory state.

Resveratrol (RSV; *trans*-3,5,4′-trihydroxystilbene) is a polyphenolic compound occurring naturally in various plants, including grapes, berries, and peanuts. It is produced in response to biotic stress and against damage from exposure to ultraviolet radiation [9]. A remarkable range of biological functions has been ascribed to this molecule. It acts as a cancer chemopreventive agent and as an antioxidant [10,11]. It also has been shown to be a powerful anti-inflammatory molecule in several inflammation models including arthritis, asthma, encephalomyelitis, atherosclerosis, and intestinal inflammatory diseases, among others [12–15]. More recently, the preventive effect of RSV on body fat accumulation has been demonstrated in rodents [16–22], primates [23], and humans [24].

In the circumstances of this scientific panorama, and in view of the significant importance of inflammation on the development of obesity, the aim of the present study was to examine whether RSV could represent a promising therapeutic tool in combating adipose tissue chronic inflammation in a model of genetic obesity, and to link its anti-inflammatory activity to its action on body fat reduction.

Material and methods

Animals, diets, and experimental design

Twenty male Zucker (*fa/fa*) rats at the age of 6 wk (213 ± 4 g), purchased from Harlan Ibérica (Barcelona, Spain), were individually housed in polycarbonate metabolic cages and placed in an air-conditioned room (22 ± 2°C) with a 12-h light–dark cycle. Rats were randomly distributed in two experimental groups (n = 10), and fed on a standard diet (Panlab, Barcelona, Spain). *Trans*-Resveratrol (purity ≥99%, Sigma, Missouri, USA) was given orally (15 mg/kg body weight daily in the RSV group) through an orogastric catheter for 6 wk. RSV was diluted in 1 mL of ethanolic solution (20%). Rats from the control group received only the vehicle. Animals had free access to food and water. Food intake and body weight were measured daily.

At the end of the experimental period, and after a fasting period of 6 to 8 h, animals were sacrificed by cardiac exsanguination under anaesthesia (sodium pentobarbital). White adipose tissue from different anatomical locations (epididymal, peri-renal, mesenteric, subcutaneous) was dissected, weighed, and immediately frozen. Serum was obtained from blood samples after centrifugation (1000 g, 10 min, 4°C). All samples were stored at −80°C until analysis. The experiment was designed according to the institution's guide for the care and use of laboratory animals (CUEID CEBA/30/2010).

Serum parameters

Commercial enzyme-linked immunosorbent assay kits were used for the assessment of serum cytokine concentrations: tumor necrosis factor-alpha (TNF-α; Invitrogen, USA), monocyte chemoattractant protein 1 (MCP1; Bender Medsystems Co, Austria), C-reactive protein (CRP; Helica Biosystems, USA), interleukin-6 (IL-6; Quantikine R&D Systems, USA), and adiponectin (Millipore Iberica, Spain).

Enzyme activities

To determine total lipoprotein lipase (LPL) activity, homogenates of epididymal adipose tissue (500 mg) were prepared in 1.5 mL of Krebs-Ringer-Phosphate (KRP) buffer (pH 7.5). Then the homogenates were centrifuged (670 *g*, 10 min) and filtered. For heparin-releasable LPL (HR-LPL) activity determination, 500 mg of adipose tissue were incubated in 400 μL of KRP buffer (37°C, 45 min). 100 μL of tissue homogenate (total LPL) or incubation medium (HR-LPL) were incubated (5 min, 37°C) with a buffer containing dibutyril fluorescein, with and without NaCl. Finally, fluorescence was measured. Total LPL and HR-LPL activities were calculated by substracting non-LPL lipolytic activity in the presence of NaCl from the total lipolytic activity, determined without NaCl.

To carry out lipogenic enzyme analysis, samples of epididymal adipose tissue (1 g) were homogenized in 5 mL of buffer (pH 7.6) containing KCl, $MgCl_2$, *N*-acetyl-cysteine and dithiothreitol. After centrifugation (100 000 *g*, 40 min, 4°C), the supernatant fraction was used for quantitation of fatty acid synthase (FASN), glucose-6-phosphate dehydrogenase (G6PDH) and malic enzyme (ME), as previously described [25].

Extraction and analysis of RNA and quantification by reverse transcriptase polymerase chain reaction

Total RNA was isolated from 100 mg of epididymal adipose tissue using Trizol (Invitrogen, USA). RNA samples were then treated with DNA-free kit (Ambion, USA). 1.5 μg of total RNA of each sample was reverse-transcribed to first-strand complementary DNA (cDNA) using iScript™cDNA Synthesis Kit (Bio-Rad, USA).

Relative mRNA levels of acetyl-CoA carboxylase (ACC), FASN, adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), LPL, cluster of differentiation 36 (CD36), NAD^+ dependent deacetylase sirtuin1 (SIRT1), peroxisome proliferator-activated receptor γ and α (PPARγ, PPARα), carnitine palmitoyl transferase 1b (CPT1 b), TNF-α, MCP1, homologue in mouse to epidermal growth factor-like 1 in humans (F4/80), macrophage mannose receptor 1 (CD206), CD11c (complement component 3 receptor 4 subunit), IL-6, adiponectin and toll-like receptor 2 and 4 (TLR2, TLR4) were quantified using real-time polymerase chain reaction with an iCycler™-MyiQ™ real-time PCR Detection System (Bio-Rad, USA). SYBR® Green probes (Applied Biosystems) were used for ACC, FASN, ATGL, HSL, LPL, CD36, SIRT1, PPARα, CPT1 b, TNF-α, TLR2, TLR4, MCP1, F4/80, CD206, and CD11c, and TaqMan probes for HSL and adiponectin. β-actin mRNA levels were measured and they served as the reference gene.

In the case of ACC, FASN, ATGL, HSL, LPL, CD36, SIRT1, PPARα, CPT1 b, IL6, TNF-α, TLR2, TLR4, MCP1, F4/80, CD206, and CD11c, 0.1 μL of each cDNA were added to PCR reagent mixture, SYBR® Green Master Mix, with the sense and antisense primers (300 nM each, except 600 nM for CD36 and IL6, and 900 nM for TNF-α and CD11c). Specific primers were synthesized commercially (Eurogentec, Belgium for ACC, FASN, ATGL, LPL, PPARα, PPARγ and β-actin, IDT, Belgium for TLR, MCP1, IL-6, TNF-α, CD36, F4/80, CD206, and CD11c Tib-Bolmiol, Germany for SIRT1). Specific sequence of sense/antisense primers and their annealing temperatures is given in Table 1. The PCR parameters were as follows: initial 2 min at 50°C, denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 sec, annealing for 30 sec and extension at 60°C for 30 sec.

In the case of TaqMan HSL and adiponectin gene expression assays, 2 μL of each cDNA were added to the PCR reagent mixture (TaqMan® Universal PCR Master Mix; Applied Biosystems, USA), with 20X TaqMan® Gene Expression Assay Mix containing specific primers and probes (HSL: Rn 00563444; adiponectin: Rn 00490758; Applied Biosystems, USA).

For β-actin mRNA levels, 1 μL of each cDNA was added to PCR reagent mixture, Premix Ex TaqTM® (Takara, USA), with sense and antisense primers (300 nM) and probe (0.5 μM). Specific primers were designed and synthesized commercially as previously described, and the probe sequence was as follows:

β-actin: 5′-FAM-CCTGCGTCTGGACCTGGCTGGC-TAMRA-3′ (probe).

The PCR parameters were as follows: denaturation at 95°C for 5 sec for β-actin and for 10 min for HSL and adiponectin followed by 40 cycles of denaturation at 95°C for 5 sec for β-actin and 15 sec for HSL and adiponectin and combined annealing and extension at 60°C for 30 sec for β-actin and for 60 sec for HSL and adiponectin.

In all cases, mRNA levels were normalized to their values of β-actin and the results were expressed as fold changes of threshold cycle (Ct) value relative to controls using the $2^{-\Delta\Delta Ct}$ method [26].

Enzyme measurements by Western blot

Epididymal adipose tissue (100 mg) was homogenated in 400 μL PBS buffer (pH 7.4), centrifuged (40 *g*, 10 min, 4°C) and 15 μg of tissue extracts (ACC, TNF-α) and 10 μg (IL-6) were separated by electrophoresis in a SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes. Then the membranes were blocked with caseine PBS-Tween buffer for 2 h.

The membranes were divided into two parts (one for β-actin determination and the other for ACC, IL-6, and TNF-α) and incubated overnight at 4°C with appropriate antibodies: ACC, TNF-α (Cell Signaling Technology, USA); IL-6 (SantaCruz Biotechnology, USA). Antibody dilutions were as follows: 1:5000 for β-actin; 1:1000 for ACC and IL-6; 1:700 for TNF-α. Bound antibodies were visualized by using a chemiluminescent substrate (Thermo Scientific, USA) and quantified by a ChemiDoc MP imaging system (Biorad, USA). The measurements were normalized by β-actin, except in the case of ACC, in which a stripping was carried out by blocking and incubating the membranes with a phosphorylated-ACC antibody (1:1000) (Cell Signaling Technology, USA).

NF-κB activation

The NF-κB/p65 TRANS AM Kit (Active-Motif Europe, Belgium,) was used to quantify NF-κB/p65 in the nuclear fraction. Nuclear extract (2.5 μg) was assayed for the DNA-binding activity of NF-κB.