Journal of Functional Foods 27 (2016) 69–83

Available online at www.sciencedirect.com

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journal homepage: www.elsevier.com/locate/jff

Extracts of Chilean native fruits inhibit oxidative stress, inflammation and insulin-resistance linked to the pathogenic interaction between adipocytes and macrophages

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ARTICLE INFO

Article history:
Received 21 March 2016
Received in revised form 24 August 2016
Accepted 29 August 2016
Available online 16 September 2016

Keywords:
Obesity
Adipocytes
Macrophages
Inflammation
Oxidative stress
Insulin-resistance

ABSTRACT

Obesity-associated insulin-resistance is set by a chronic inflammatory state established in the adipose tissue. Chilean native fruits calafate (CA) and maqui (MA) berries present remarkable anti-inflammatory features. Here, we evaluated antioxidant, anti-inflammatory and insulin-sensitizer effects of these fruits in an *in vitro* inflammatory setting. Differentiated 3T3-L1 cells exposed to conditioned media (CM) from activated macrophages were treated with CA and MA extracts. MA increased metalloproteinase (MMP)-2 activity on day 3, and both CA and MA modulated MMP-9 activity on day 10 of differentiation. In differentiated CM-treated 3T3-L1, extracts increased GSH levels and GSH/GSSG ratio, CA and MA prevented caspase-3 induction, and MA decreased MCP-1, while CA increased IL-6 gene expressions. Finally, MA reverted CM specific IRS-1 phosphorylation, and CA improved insulin-stimulated glucose uptake. Thus, treatments with extracts of Chilean native fruits were able to block the development of oxidative stress, inflammation and insulin-resistance *in vitro*.

1. Introduction

Obesity is a pathology with major world prevalence. This disease is defined by an abnormal fat accumulation in adipose tissue depots, resulting in increased body mass (Hartroft, 1960), mainly that of white adipose tissue (WAT) by means of hypertrophy or hyperplasia (adipogenesis) of adipocytes due to a higher influx of triacylglycerols (Spiegelman & Flier, 2001). During adipogenesis, a dynamic interaction between adipocytes and

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Abbreviations: ADIPOQ, adiponectin; WAT, white adipose tissue; ECM, extracellular matrix; MMPs, metalloproteinases; ROS, reactive oxygen species; TNF-α, tumoral necrosis factor alpha; IL-6, interleukin 6; MCP-1, monocyte chemoattractant protein-1; NO, nitric oxide; CM, conditioned media; MA, maqui; CA, calafate; BLU, blueberry; INS, insulin; LPS, lipopolysaccharides; SOD, superoxide dismutase; CAT, catalase; GSH, reduced glutathione; GSSG, oxidized glutathione; γ-GCL, gamma glutathione cysteine ligase

http://dx.doi.org/10.1016/j.jff.2016.08.052
1756-4646/

extracellular matrix (ECM) exists, which is regulated by matrix metalloproteinases (MMPs). In fact, ECM remodelling is a key feature that promotes adipogenesis itself (Catalan, Gomez-Ambrosi, Rodriguez, & Fruhbeck, 2012). On the other hand, the WAT presents a recognized endocrine function, and it is known that a pathological increase in the mass of this tissue produces a deregulation of the production of its endogenous products, termed adipokines (Bray, 2004), in part related to the establishment of an oxidative stress status by overproduction of reactive oxygen species (ROS) (Furukawa et al., 2004). Furthermore, it has been demonstrated that WAT expansion leads to an increase in macrophage population within this tissue, mainly from a pro-inflammatory phenotype (Weisberg et al., 2003). This macrophage infiltration is also known to be induced by programmed cell death (apoptosis) of resident adipocytes (Alkhouri et al., 2010). Consequent macrophage–adipocyte interaction stimulates further production of TNF-α, IL-6, NO and MCP-1 by the tissue, jointly with a decrease in the secretion of anti-inflammatory molecules, such as adiponectin (Klionsky et al., 2012). This phenomenon, which is defined as a chronic inflammatory state (Ros Perez & Medina-Gomez, 2011), is responsible for the proliferation of obesity-related insulin-resistance (Dandona, Aljada, & Bandyopadhyay, 2004). Therefore, the use of anti-inflammatory agents could be considered as a promising strategy in order to prevent adverse obesity-associated consequences, such as insulin-resistance. In this sense, anthocyanins have been reported to possess antioxidant, anti-inflammatory, anti-obesity and anti-diabetic features (He & Giusti, 2010; Jennings, Welch, Spector, Macgregor, & Cassidy, 2014; Seeram, Zhang, & Nair, 2003). It has been described that the Chilean native fruits, maqui (MA, *Aristotelia chilensis*) and calafate (CA, *Berberis microphylla*) contain high amounts of anthocyanins. Previously, we reported anti-inflammatory features (mRNA expression and secretion of key molecules) of extracts of these fruits *in vitro* in mouse macrophages, induced adipocytes, and in a co-culture involving both types of cells (Reyes-Farias et al., 2015). In order to study the benefits of these fruits in an *in vitro* scenario, the present study evaluated the effects of MA and CA extract treatment on ECM remodeling, oxidative stress, apoptosis, inflammation, and insulin-induced glucose uptake, specifically in adipocytes that were treated with conditioned media from activated macrophages.

2. Materials and methods

2.1. Fruit extracts preparation and characterization

Ripe fruits of MA (*Aristotelia chilensis*), CA (*Berberis microphylla*) and blueberry (BLU, *Vaccinium corymbosum*; as non-native control) were obtained from SAAUTCHILE (Valdivia, Chile). Fruit extracts were prepared and characterized (in terms of anthocyanin content) as previously described by Reyes-Farias et al. (2015). Briefly, polyphenolic content was determined by the Folin–Ciocalteu method (Singleton & Rossi, 1965). Relative anthocyanin content was assayed by liquid chromatography coupled to mass spectrometry. Samples were filtered and analysed using an HPLC Agilent 1100 (Agilent Technologies, Inc., Santa Clara, CA, USA) connected to an Esquire 4000 ion trap LC/MS system (Bruker Daltoniks, Bremen, Germany). At the exit of a C18 column, a splitter divided the eluant into a UV detector and to a mass spectrometer. A volume of 20 μL was injected. The mobile phases were water/acetonitrile/formic acid (87:3:10, v/v/v) and water/acetonitrile/formic acid (40:50:10, v/v/v) at a 0.8 mL/min flow rate. Phenolic compounds were detected at 520 nm. The mass spectral data were acquired in a positive mode. Ionization (nebulization) was performed with nitrogen as drying gas. Collision-induced dissociation was performed by collisions with the helium background gas in the trap.

Absolute anthocyanin content of calafate and blueberry extract was determined by liquid chromatography (Jasco PU-2089 equipment) coupled to UV/VIS detector and interface LC-NetII/ADC, with a C18 Kromasil 150 mm column, incubated in oven at constant 30 °C. Anthocyanins were detected at 520 nm. The mobile phase used was CAN/methanol/TFA (8:8:0.2%, v/v/v) in water, maintaining isocratic condition, at 1 mL/min flow rate, according to a previously reported protocol, with modifications (Giusti, Rodriguez-Saona, & Wrolstad, 1999). The stock standards were prepared in acidified methanol with 0.01% TFA, and diluted in water with 10% acetic acid and 0.2% TFA. Samples were concentrated in a Savant SpeedVac Thermo Scientific model RVT5105, resuspended in water with 10% acetic acid and 0.2% TFA, and filtered with a 0.22 μm pore filter.

2.2. Cell culture and experimental design

3T3-L1 mouse preadipocytes and RAW264.7 mouse monocytes were obtained from the Laboratory of Cellular and Molecular Biology (INTA, University of Chile, Santiago, Chile) and the Laboratory of Biochemistry, Metabolism and Drug Resistance (ICBM, University of Chile, Santiago, Chile), respectively. Both cell lines were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. RAW264.7 cells were cultured in DMEM containing 4.5 g/L glucose and 10% fetal bovine serum. Cells were activated with 5 μg/mL lipopolysaccharides (LPS, Sigma-Aldrich, St. Louis, MO, USA) to obtain conditioned media (CM + LPS). 3T3-L1 cells were cultured in DMEM containing 4.5 g/L glucose and 10% calf serum. Two days after full confluence, cells were differentiated by incubation with 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone, 10 μg/mL insulin (all reagents from Sigma-Aldrich), in 4.5 g/L glucose DMEM supplemented with 10% fetal bovine serum for 2 days, and for the next 2 days with 10 μg/mL insulin, in 4.5 g/L glucose DMEM supplemented with 10% fetal bovine serum. Thereafter, cells were maintained and refed every 2 or 3 days with media without any hormones until it reached a fully differentiated phenotype (10–12 days). Experiments were set as follows:

1 Preadipocytes were treated with 100 μM [total polyphenols] of each extract from the start of differentiation (day 0), and at each time the culture medium was changed (every 48 hours) until the end of the differentiation period. MMP-2 and MMP-9 extracellular activities were analyzed at days 3, 7 and 10. At each time, culture media was changed to DMEM supplemented with 0.1% bovine serum albumin and 1% antibiotics for 24 h (restrictive media), and then collected and stored at –80 °C for further analysis.
2 Fully differentiated adipocytes were treated with CM from activated macrophages (CM + LPS), in the presence or absence