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Synergism of plant-derived polyphenols in adipogenesis: Perspectives and implications

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ABSTRACT

Dietary polyphenols may exert their pharmacological effect via synergistic interactions with multiple targets. Putative effects of polyphenols in the management of obesity should be primarily evaluated in adipose tissue and consequently in well-documented cell model. We used *Hibiscus sabdariffa* (HS), a widely recognised medicinal plant, as a source of polyphenols with a number of salutary effects previously reported. We present here the full characterisation of bioactive components of HS aqueous extracts and document their effects in a model of adipogenesis from 3T3-L1 cells and in hypertrophic and insulin-resistant adipocytes. Aqueous extracts were up to 100 times more efficient in inhibiting triglyceride accumulation when devoid of fibre and polysaccharides. Significant differences were also observed in reactive oxygen species generation and adipokine secretion. We also found that, when polyphenols were fractionated and isolated, the benefits of the whole extract were greater than the sum of its parts, which indicated a previously unnoticed synergism. In conclusion, polyphenols have interactive and complementary effects, which suggest a possible application in the management of complex diseases and efforts to isolate individual components might be irrelevant for clinical medicine and/or human nutrition.

Introduction

Obesity-associated metabolic, oxidative and inflammatory disturbances are a growing epidemic and are associated with at least six of the top ten causes of death (McGeer and McGeer 2004). Adipocytes store excess energy, but when overloaded they become resistant to insulin, which compromises their ability to accumulate lipids and facilitates alterations in structure and metabolism in remote tissues, such as the pancreas, liver and muscle (Yu and Zhu 2004; Jernas et al. 2006; Rull et al. 2010). Excessive oxidation in adipose cells is common and triggers cellular stress (Furukawa et al. 2004; Yeop Han et al. 2010). The resulting sequence of events remains poorly understood in humans but tends to self-perpetuate if untreated. Initially, there is a complex process of cellular adaptation, monitored by tissue-resident macrophages. When failure and malfunction become extreme, a chronic inflammatory response is unleashed (Rull et al. 2010).

If assumptions are accurate, it is conceivable that antioxidant and/or anti-inflammatory therapies that act on adipose tissue may have potential benefits in the amelioration of obesity-related diseases. However, current available drugs have not been assayed yet. The only validated therapeutic measure consists of preventing hypertrophy in adipocytes via caloric restriction or increased caloric expenditure, but changes in lifestyle are difficult to achieve. Plant-derived polyphenols may provide a similar effect without restricting caloric intake (Lamming et al. 2004; Howitz and Sinclair 2008). Polyphenols are antioxidant and anti-inflammatory

Abbreviations: HS, *Hibiscus sabdariffa*; AHS, aqueous extract of *H. sabdariffa*; PEHS, phenolic extract of *H. sabdariffa*; FBS, foetal bovine serum; TNF-α, tumour necrosis factor-alpha; IGF1, insulin-like growth factor-1; IL-6, interleukin-6; VEGF, vascular endothelial growth factor; IL-1a, interleukin-1 alpha; IL-1b, interleukin-1 beta; MCP-1, monocyte chemoattractant protein-1; IBMX, 3-isobutyl-1-methylxanthine.

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molecules that interact in humans with molecular targets involved in stress response pathways, and increased ingestion of dietary polyphenols could be helpful. However, plant-derived polyphenols are secondary metabolites that are synthesised in response to a major stress event; consequently, the expected amount of polyphenols in our commonly consumed food is very low. We reasoned that tropical plant-derived products could be a potential source of polyphenol concentrate and could be used to design dietary supplements. Recent data indicate that aqueous extracts of *Hibiscus sabdariffa* (HS) might ameliorate metabolic disturbances (Carvajal-Zarrabal et al. 2005; Alarcon-Aguilar et al. 2007; Kim et al. 2007), but human trials have been generally unsatisfactory, due to an incomplete characterisation of the essential bioactive components (Beltrán-Debón et al. 2009; Mozaffari-Khosravi et al. 2009; Kuriyan et al. 2010). In this study, we address this issue, document the effects of polyphenols on mouse adipocytes and provide data that support multi-target action in the same signalling cascades or response networks.

Materials and methods

Materials

3T3-L1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Dexamethasone, 3-isobutyl-1-methylxanthine, insulin, crystal violet, Ascentis C18 preparative reverse phase column, formic acid, acetonitrile and ethanol were obtained from Sigma–Aldrich (Madrid, Spain). Dulbecco's modified Eagle's medium, calf serum, foetal bovine serum, and an antibiotic mixture (penicillin–streptomycin) were purchased from PAA Laboratories (Linz, Austria). Sodium pyruvate and trypsin–EDTA were obtained from Invitrogen (Carlsbad, CA). Polyvinyldifluoride (PVD) filters, 0.22 μm, were obtained from Millipore (Bedford, MA). AdipoRed™ Assay Reagent was obtained from Lonza (Walkersville, MD USA). The resin used for the preparative chromatography was Amberlite™ FPX66 (Rohm and Haas, Philadelphia, USA). The standards for the calibration curves, chlorogenic acid, quercetin-3-rutinoside, quercetin-3-glucoside, kaempferol-3-*O*-rutinoside, kaempferol 3-(*p*-coumaroylglucoside), quercetin, 4-hydroxycoumarin and delphinidin-3-sambubioside were purchased either from Fluka, Extrasynthese (Genay, France) or Polyphenols Laboratories (Hanaveien, Norway).

Methods of extraction and fractionation of polyphenols

Primary aqueous extract (AHS) was obtained from sun-dried calyces from plants harvested by investigators in Senegal with an approximate plant-to-extract ratio of 5:1 as previously described (Beltrán-Debón et al. 2009). The purified extract (PEHS) was prepared by removing fibre and polysaccharides by precipitation in 85% ethanol (v/v). Extracts were reconstituted in water at 170 mg/ml and loaded onto a 1.5 cm × 25 cm chromatography column containing Amberlite™ FPX66. The retained phenolic fraction was finally eluted with 95% ethanol and 0.01% trifluoroacetic acid, rotary evaporated and freeze-dried. Total phenolic content in AHS and PEHS was measured with the Folin–Ciocalteu method (Huang et al. 2005). To further characterise the bioactive components, PEHS was dissolved in distilled water to a concentration of 230 mg/ml, filtered through a 0.45 μm PVD filter and fractionated using a WellChrom preparative HPLC system (Merck-Knauer, Berlin, Germany). We used an Ascentis C18 preparative reverse phase column (10 μm, 25 cm × 21.2 mm), and elution was performed using acetonitrile as a mobile phase in a multistep linear gradient at room temperature with a flow rate of 19 ml/min. The preparative version of EuroChrom® software, version 3.01, was used for data acquisition and analysis. We obtained 35 fractions representing distinct combinations of components, which were identified and quantified. We then lyophilised the resulting fractions for assays described below.

Characterisation and quantification of polyphenols

Analysis was performed in a Rapid Resolution Liquid Chromatography 1200 (Agilent Technologies, Palo Alto, CA) using a Zorbax Eclipse Plus C_{18}, 4.6 mm × 150 mm, 1.8 μm column at room temperature with a flow rate of 0.5 ml/min and an injection volume of 10 μl (Rodríguez-Medina et al. 2009). The chromatographic system was coupled to a time-of-flight (TOF) mass spectrometer (MS) (Bruker Daltonic Bremen, Germany) that was equipped with an orthogonal electrospray interface (ESI; model G1607A from Agilent Technologies, Palo Alto, CA, USA) that operated in negative and positive modes of ionisation. Compound identification was made by comparing the retention times and mass spectra obtained by TOF-MS with those of authentic standards or interpreted according to previously obtained mass spectra. Quantification of the major compounds in AHS, PEHS and the isolated fractions was carried out using commercially available standards when available or previously reported structurally similar compounds (Fernandez-Arroyo et al., 2011).

In vitro experimental models

The 3T3-L1 preadipocytes were propagated and differentiated according to described procedures (Green and Kehinde 1975) (see also supporting information). Differential effects on adipogenesis were assayed by adding extracts and fractions in pre-designed concentrations to the media at the beginning of the induction period; these conditions were maintained until cells were harvested. The absence of cytotoxicity was ascertained by the crystal violet method. In all experiments, more than 90% of the cells were mature adipocytes after 8–10 days of incubation. For other experiments, we used hypertrophied, insulin-resistant adipocytes obtained by increasing the time of incubation (22 days) in 25 mM glucose (Yeop Han et al. 2010). In these cases, extracts and fractions were added at day 18 and allowed to incubate for 4 days before harvesting. We assessed triglyceride accumulation with AdipoRed™; extracts and fractions were added either at day 8 (mature adipocytes) or at day 18 (hypertrophied adipocytes) after induction and were incubated for 2 or 4 additional days, respectively. Fat droplets were analysed with a Nikon Eclipse TE 2000U fluorescence microscope controlled by NIS-Elements software.

Measurement of intracellular reactive oxygen species (ROS) and secreted adipokines

Measurements were performed on hypertrophied adipocytes in 25 mM glucose to assess the effect of proposed extracts. These extracts were added to adipocytes at day 18 after inducing differentiation, and incubation proceeded for four additional days under the same conditions. ROS generation was assessed with 2′,7′-dichlorodihydrofluorescein diacetate as described (Yeop Han et al. 2010), and fluorescence was measured in a multiwell plate reader (POLARstar Omega microplate) with excitation at 485 nm and emission at 520 nm. In separate experiments, several cytokines (leptin, tumour necrosis factor-alpha (TNF-α), insulin-like growth factor-1 (IGF-1), interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), interleukin-1 alpha (IL-1a), interleukin-1 beta (IL-1b) and monocyte chemoattractant protein-1 (MCP-1)) were measured by ELISA (Signosis, Inc., Sunnyvale, CA, USA) in resulting supernatants following the manufacturer's instructions.