Metal Ions in Life Sciences

Effects of Polyphenols on Metabolic Activity in Stimulated Immune Cells

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ABSTRACT

Energy metabolism is one of the main sources of reactive oxygen species leading to oxidation and inflammation in pathophysiological processes. Lipopolysaccharide (LPS)-activated mouse embryonic fibroblast (MEF) cell lines from knock-out mice for paraoxonase-1 and from transgenic mice overexpressing monocyte chemoattractant protein-1 were obtained as model of pro-oxidant and pro-inflammatory scenarios. *Theobroma cacao* and *Lippia citriodora* (worldwide consumed and common ingredient of many food products) were tested in these cell models to assess the action of polyphenols in the energy management. Our metabolomics experiments show a different behavior of polyphenols: *T. cacao* extract partially reverts the effect of LPS in a pro-oxidant scenario through the antioxidant properties of theobromine, flavonols and procyanidins, while *L. citriodora* seems to act mainly in a pro-inflammatory cell model through the action of verbascoside decreasing the production of pro-inflammatory cytokines and MCP-1. Nevertheless, the action of polyphenols cannot be attributed only to a mechanism of action but the sum of different modulations in biological pathways. The capacity of both plant extracts to decrease α-ketoglutarate levels merits special attention due to the implications in future medicine. The action of polyphenols modulating oxidative stress, cytokine production and epigenetic changes make an interesting source of bioactive compounds for nutraceutical or functional food purposes.

1. INTRODUCTION

Oxidative stress and inflammation are the basis of the most diseases and their mechanisms are inextricably linked. An increased oxidative stress starts a cascade of pro-inflammatory cytokines that triggers inflammation (Camps et al., 2014). Dysfunctions in mitochondria, as the main organelle producing reactive oxygen species (ROS) (Handy & Loscalzo, 2012), lead to non-communicable diseases (obesity, diabetes, atherosclerosis, cancer, etc.) characterized by oxidation, inflammation and metabolic disorders (Camps & García-Heredia, 2014).

Polyphenols are secondary metabolites naturally synthetized in plants and have demonstrated several biological activities, including antioxidant and anti-inflammatory properties (A. N. Li et al., 2014). *Theobroma cacao* (cocoa) is worldwide consumed and common ingredient of many food products. Cocoa is a valuable source of antioxidant compounds with health benefits mainly attributed to the flavanol content (Andujar, Recio, Giner, & Rios, 2012; M. L. Cadiz-Gurrea et al., 2017) and the proanthocyanidins with high-degree of polymerization (Belscak, Komes, Horzic, Ganic, & Karlovic, 2009; M.L. Cadiz-Gurrea et al., 2014; Martin & Ramos, 2016; Schinella et al., 2010; Yasmeen, Fukagawa, & Wang, 2017). In the past decades, the bioactive constituents of cocoa have been targeted in multiple research studies confirming its health benefits and, for these reasons, the market has developed new cocoa-based products such as cocoa liquor, cocoa powder, chocolate and other derivatives and has remained stable over the last few years (Ellam & Williamson, 2013).

Lippia citriodora (lemon verbena) is a perennial plant originally from South America which was introduced into Europe at the end of the 17th century and has been widely used in infusions for its antispasmodic, antipyretic, sedative, and digestive properties. Furthermore, this plant is used in the food industry to flavor different products. Lemon verbena infusion contains significant amounts of polyphenols, including phenylpropanoid glycosides (mainly verbascoside) and flavone diglucuronides such as luteolin 7-diglucuronide, with high anti-inflammatory and antioxidant properties (Funes et al., 2009; Quirantes-Piné, Funes, Micol, Segura-Carretero, & Fernández-Gutiérrez, 2009). In the last decade, the potential of *L. citriodora* extract supplementation as a nutraceutical is under consideration with the aim to find phytotherapies, which contribute to ameliorate diseases with less adverse effects than synthetic drugs (Cádiz-Gurrea et al., 2017).

In this work, antioxidant and anti-inflammatory properties of food polyphenols found in cocoa and lemon verbena were tested on two mouse embryonic fibroblast (MEF) cell lines: 1) knock-out mice for paraoxonaxe-1, an antioxidant endogenous enzyme, as model of oxidation (PON-1 KO cells) and 2) transgenic mice overexpressing the monocyte chemoattractant protein 1 (MCP-1), a proinflammatory cytokine, as model of inflammation (tgMCP-1 cells). To analyze the impact of polyphenols in the energy management in these conditions, a targeted metabolomics approach was performed on gas chromatography coupled to a quadrupole time-of-flight mass spectrometer and an electron impact source.

2. MATERIAL AND METHODS

2.1. Chemicals.

Methanol, dimethylsulphoxide (DMSO), methoxyamine hydrochloride, pyridine and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). N-methyl-N-trimethylsilyl-trifluoroacetamide with 1% trimethylchlorosilane (TMS) and RIPA lysis and extraction buffer were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ultrapure type-1 water was obtained from a Milli Q water system (Merck Millipore, Darmstadt, Germany). DMEM medium, fetal bovine serum (FBS), phosphate-buffered saline (PBS), L-glutamine and penicillin/streptomycin were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA).

2.2. Cell culture.

Cells (5 replicates) were grown to 80% confluence in 6-well plates using DMEM medium containing 10% FBS, 5 mM L-glutamine and penicillin/streptomycin. After depletion of FBS (0.1%), *L. citriodora* (standardized with 10% of verbascoside) and *T. cacao* extracts (Monteloeder, Elche, Spain) at a final concentration of 100 μg/mL in wells, dissolved in DMSO, were tested for 48 hours in PON-1 KO and tgMCP-1 cells. Complete polyphenol characterization of both extracts is detailed in Supplementary Information Figures S1 and S2, Tables S1 and S2. Prior to the assay, cells were incubated 24 hours with LPS from *E. coli* (100 ng/mL) to stimulate the pro-oxidant and pro-inflammatory states. Controls (with or without LPS and with or without plant extracts) were also made. Then, cells were scrapped in PBS and centrifuged at 2500 rpm 5 minutes. Supernatants were removed and pellets were stored at -80 °C until use.

The total amount of protein (in mg) was used to normalize the metabolite concentrations. For this purpose, cells (3 replicates) where scraped using 200 µL of RIPA lysis and extraction buffer, and protein concentration was calculated using the absorbance at 280 nm analyzed in a NanoDrop 2000 system (Thermo Scientific, Waltham, MA, USA).

2.3. Metabolite extraction.

Metabolites were extracted from cells following the protocol performed by (Riera-Borrull et al., 2016). Briefly, 200 µL of methanol:water (8:2) was added to cell pellets and lysed with three cycles of freezing and thawing using liquid N_2. After precipitating proteins, samples were centrifuged at 14000 rpm for 10 min, supernatants were collected, dried under N_2 and derivatized using methoxyamine in pyridine (40 mg/mL) and TMS.

2.4. Instrumentation, chromatographic conditions and mass spectrometry detection.

A 7890A gas chromatograph coupled with an electron impact source to a 7200 quadrupole time-of-flight mass spectrometer equipped with a 7693 autosampler module and a J&W Scientific HP-5MS column (30 m x 0.25 mm, 0.25 µm) (Agilent Technologies, Santa Clara, USA) were used. Optimized parameters for chromatographic separation and QTOF detection are explained in

Riera-Borrull et al., 2016. Metabolites were quantitated using standard calibration curves and values normalized using the total amount of protein.

2.5. Data analysis

Raw data were processed and compounds were detected and quantified using Qualitative and Quantitative Analysis B.07.00 software (Agilent Technologies), respectively. Statistical analysis was performed using the Kruskal-Wallis non-parametric test with SPSS 23.0 software (IBM Corporation, Armonk, NY, USA). Sparse partial least square discriminant analysis (sPLS-DA) were made using Metaboanalyst 3.0 (www.metaboanalyst.ca) (Xia, Sinelnikov, Han, & Wishart, 2015).

3. RESULTS AND DISCUSSION

Understanding oxidation and inflammation as the two fronts of a same coin is crucial to assess the mechanisms involved in appearance, progression and resolution (or chronicity) of these pathophysiological conditions and how they are interconnected to metabolism (Griffiths, Gao, & Pararasa, 2017). Inflammation and oxidation are related to several “metabolic diseases”, including obesity, atherosclerosis and cancer (Camps, García-Heredia, Hernández-Aguilera, & Joven, 2016), and mitochondrial impairments play a key role in these pathophysiological processes (Hernández-Aguilera et al., 2013).

Dietary polyphenols are one of the most valuable sources of these compounds, and, for this reason, numerous efforts have been realized to demonstrate how polyphenols can help to prevent or ameliorate these pathologies characterized by oxidation and inflammation (Hussain et al., 2016; Joven et al., 2014; Santini, Tenore, & Novellino, 2017).

To test the antioxidant and anti-inflammatory properties of food polyphenols, MEF PON-1 KO and tgMCP-1 cell lines previously incubated with LPS were used as oxidant and inflammatory scenarios, respectively. WT cells also were used as control cells. Although the concentration of plan extracts (100 μg/mL) used in this study is in a supra-physiological dose, these results demonstrate the effect of dietary polyphenols contained in cocoa and lemon verbena to act as antioxidant and/or anti-inflammatory compounds.

Our results showed changes in the concentrations of metabolites in both PON-1 KO and tgMCP-1 cells in comparison with WT cells. These changes include intermediates of glycolysis, tricarboxylic acid cycle (TCA), amino acids catabolism, glutamine catabolism and pentose phosphate pathway (Supplementary Information, Table S3). LPS-activated WT MEF cells experience, specially, a decrease in amino acid concentrations (Supporting information, Table S3) compared to the control WT cells, mostly branched-chain amino acids (BCAAs) (valine, leucine, isoleucine), serine, alanine and glutamate.

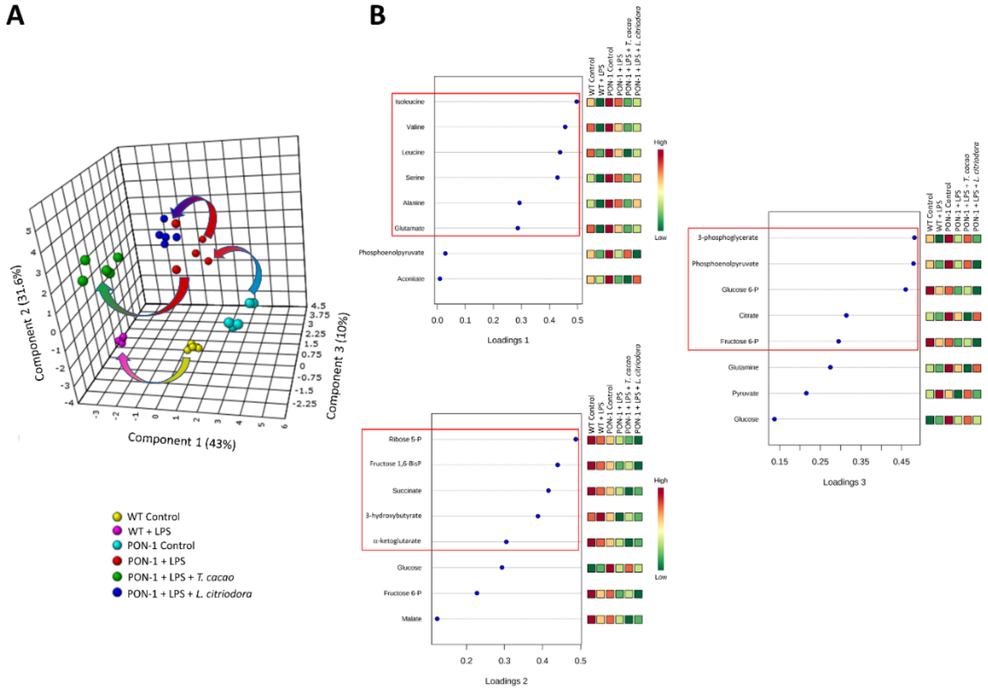

Figure 1. Effect of the treatment with cocoa and lemon verbena extracts in LPS-activated PON-1 KO MEF cells. A) Sparse partial least square discriminant analysis (sPLS-DA). B) Importance in loading metabolites in each sPLS-DA component.

In LPS-activated PON-1 KO cells, as a pro-oxidant scenario, a shift in the sPLS-DA in the 3 components (Figure 1A) was also observed as result of a decrease in the concentrations of, mainly, amino acids and glycolysis intermediates (Figure 1B). *T. cacao* extract had an effect on energy metabolism in LPS-activated MEF PON-1 KO cells (Figure 1A), partially recovering the glycolysis intermediates and amino acids to similar values to these found in MEF WT (Figure 1B). Although, this effect is not so obvious when cells are incubated with *L. citriodora*. In this case, significant shifts in the component of the sPLS-DA (Figure 1A) were not detected, but only a partial recovery in the concentration of some amino acids (alanine, aspartate, glutamate and serine) and intermediates of TCA (aconitate, fumarate and malate) (Supporting information, Table S3).

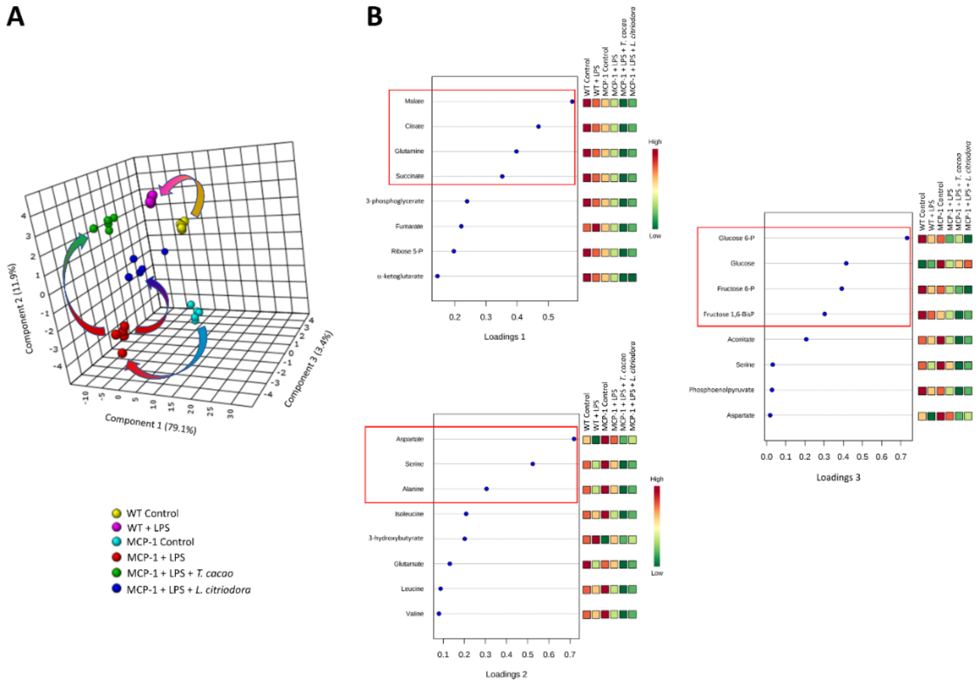

Figure 2. Effect of the treatment with cocoa and lemon verbena extract in LPS-activated tgMCP-1 MEF cells. A) Sparse partial least square discriminant analysis (sPLS-DA). B) Importance in loading metabolites in each sPLS-DA component.

In a pro-inflammatory scenario, LPS-activated tgMCP-1 cells as well as PON-1 KO cells experienced a decrease in all of the intermediates in the energy production. Incubation with *T. cacao* extract did not recover cells from the effects of LPS when regarding the concentrations of the analyzed metabolites (Supplementary Information Table S3), even though in the sPLS-DA we can observe this group in a position near the WT cells. Contrarily, *L. citriodora* had a slight action protecting tgMCP-1 MEF cells from LPS (Figure 2A).

Although *T. cacao* has previously demonstrated anti-inflammatory activity (Selmi, Mao, Keen, Schmitz, & Eric Gershwin, 2006), our results are highly related with its antioxidant properties. The antioxidant capacity of cocoa extract can be attributed to its main compounds theobromine (a methylxanthine), flavonols (specially catechin and derivatives) and procyanidins, among other compounds that are present in a minor proportion. These compounds have been strongly reported for their antioxidant capacity (Hu, Kim, & Baik, 2016; S. Li et al., 2015; Schinella et al., 2010). Moreover, high quantities of theobromine and catechin derivatives, and, in a minor quantity, procyanidins, were found in human plasma after ingestion of these compounds at a dietary dose (Baggott et al., 2013; Holt et al., 2002; Lee et al., 2002). These data, together with the ability of catechin and derivatives to interact with, and cross, the cell membrane (Sirk, Brown, Sum, & Friedman, 2008), result in an antioxidant activity of cocoa polyphenols not only at plasma level but also intracellularly.

L. citriodora showed the opposite effect, acting as anti-inflammatory more than as antioxidant agent. The phenylethanoid glycoside verbascoside, also called acteoside, is the main compound of this extract (10% w/w) and anti-inflammatory properties have been already conferred on it (Alipieva, Korkina, Orhan, & Georgiev, 2014). Its mechanism of action is through inhibiting the production of cytokines such as TNF-α, IL-1β and IL-6, among others. Verbascoside also contributes to the inhibition of the nuclear factor kappa B (NF-κB) (Jing, Chunhua, & Shumin, 2015). Because MCP-1 expression depends, partially, on the action of NF-κB, the inhibition of this nuclear factor may decrease the production of MCP-1 and, thus, the inflammatory process.

Although the anti-inflammatory properties of polyphenols can be attributed to an interaction with cytoplasmic and nuclear pathways rather than their direct antioxidant properties (Pastore et al., 2012), the action of a complex mixture of polyphenols, such as cocoa or lemon verbena extracts, where synergic effects occur, cannot be assigned only to one mechanism of action. As reported previously, energy generation impairment and inflammatory conditions are closely related to disease. A correlation between pathophysiological processes that present oxidative stress and/or inflammation produce mitochondrial imbalance that affects the energy management. The properties of polyphenols as exogenous antioxidant/anti-inflammatory molecules modulates the energy expenditure by the action on the AMP-activated protein kinase (AMPK), the cytokine production or acting as epigenetic regulators (Charles et al., 2013; Joven et al., 2013; Most, Goossens, Jocken, & Blaak, 2014). Thus, the action of polyphenols improving the endogenous antioxidant systems, modulating several biological pathways related to inflammation, cancer and other pathophysiological processes make these molecules an attractive source of bioactive compounds for pharmaceutical, nutraceutical or functional food formulations.

Special attention merits α-ketoglutarate (α-KG). This key and pivotal intermediate of the TCA cycle is formed, mainly, from isocitrate or glutamate. α-KG is involved in multiple diseases such as non-alcoholic fatty liver disease (Rodríguez-Gallego et al., 2015) or cancer (Dang, Yen, & Attar, 2016). It is also related to the inhibition of histone and DNA demethylases (Lempradl, Pospisilik, & Penninger, 2015). Interestingly, both plant extracts decrease strongly the levels of α-KG. The mechanism of action of polyphenols decreasing α-KG is still unclear and deserves more efforts due to the implications in future treatments for pathophysiological processes with an hypermethylated phenotype.

4. CONCLUSIONS

In the present study, we highlight the different behavior of polyphenols as antioxidant and anti-inflammatory molecules. *T. cacao* extract is responsible to partially return the impairment in the energy management during a pro-oxidant status in PON-1 KO cells probably through the antioxidant properties of theobromine, flavan-3-ol monomers and procyanidins. On the contrary, *L. citriodora* extract possess a partial effect on the energy metabolism in a pro-inflammatory scenario probably due to the action of verbascoside which may inhibit NF-κB and, thus, decrease the expression of MCP-1. However, action of polyphenols, especially in complex mixtures, cannot be attributed to only one mechanism of action but to the sum of different pathways and mechanisms that can be modulated.

More investigations are needed to assess the implications and impact of polyphenols to decrease the levels of α-KG.

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