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Cell type-dependent effects of ellagic acid on cellular metabolism

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ABSTRACT

Ellagic acid is a botanical polyphenol which has been shown to have numerous effects on cellular function. Ellagic acid can induce apoptosis and inhibit the proliferation of various cancer cell types *in vitro* and *in vivo*. As such, ellagic acid has attracted significant interest as a potential chemotherapeutic compound. One mechanism by which ellagic acid has been proposed to affect cellular physiology is by regulating metabolic pathways. Here we show the dose-dependent effects of ellagic acid on cellular energy production and downstream induction of the apoptotic program in HEK293, HeLa, MCF7, and HepG2 cells. At physiologically relevant doses, ellagic acid has pleiotropic and cell-type specific effects on mitochondrial function. At high doses ellagic acid can also influence glycolytic pathways and induce cell death. Our results demonstrate that ellagic acid can influence mitochondrial function at therapeutically relevant concentrations. The observed effects of ellagic acid on cellular respiration are complex and cell type-specific, which may limit the chemotherapeutic utility of this compound.

1. Introduction

Ellagic acid (EA)[2]is a polyphenolic compound enriched in nuts and berries. Like all polyphenols EA is an antioxidant [1,2] and has many cellular effects including inhibiting cancer cell proliferation [3,4]. Ellagic acid is bioavailable when taken orally and can briefly reach serum concentrations up to several hundred nanomolar in human subjects [5–7]. Metabolism of EA by microbial action in the gut leads to the production of urolithins which are also bioactive and achieve much higher serum concentrations with a longer half-life compared to EA [5]. Like EA, bioactive urolithin metabolites are antioxidants and have anti-cancer activity [5,8] but may have unique cellular targets [9].

The number of cellular processes purported to be modulated by EA is impressive [4]. In non-transformed cells, EA is protective against oxidative insults [10–12]. In contrast, EA has anti-proliferative and apoptotic effects in cancer cells. These seemingly contradictory activities of EA may be due to the unique energetic demands of the tumor microenvironment. Transformed cells require high levels of ATP, NADPH, and cellular building blocks such amino acids to support cell division and migration, often in the context of a hypoxic environment. In cancer cells EA appears to target these unique metabolic demands by regulating mitochondrial function. Ellagic acid causes mitochondrial depolarization in pancreatic, B cell, neuroblastoma, and bladder cancers [13–16], in most cases indirectly by activating apoptotic pathways. In B cell chronic lymphocytic leukemia EA has direct effects on mitochondria resulting in ROS production and release of pro-apoptotic factors from mitochondria [13]. There is also evidence that EA can affect cellular ATP production by targeting glycolysis. Ellagic acid downregulates the expression of the sodium/hydrogen exchanger 1 (NHE1) leading to cellular acidification and inhibition of glycolytic flux in endometrial cancer cells [17]. In T cell lymphoma EA can inhibit the activity of lactate dehydrogenase to decrease glycolysis [18]. One important caveat regarding many *in vitro* studies investigating EA is that the experimental effects on cellular physiology are not apparent at EA concentrations which are attainable in human serum (less than 500 nM [3,8,19]).

In this study, we investigated the dose-dependent effects of EA on cellular ATP production in one non-transformed cell line (HEK293) and three commonly used cancer cell lines derived from different primary tumors (HeLa, MCF7, and HEPG2). Total ATP levels and *in situ* mitochondrial function in living cells were determined to evaluate the generality of the effects of EA on cellular metabolism at physiologically relevant concentrations. Finally the ability of EA to induce apoptotic cell death was investigated.

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[2] Non-standard abbreviations: 2DG: 2-Deoxy-D-glucose; EA: Ellagic Acid; OCR: Oxygen consumption rate.

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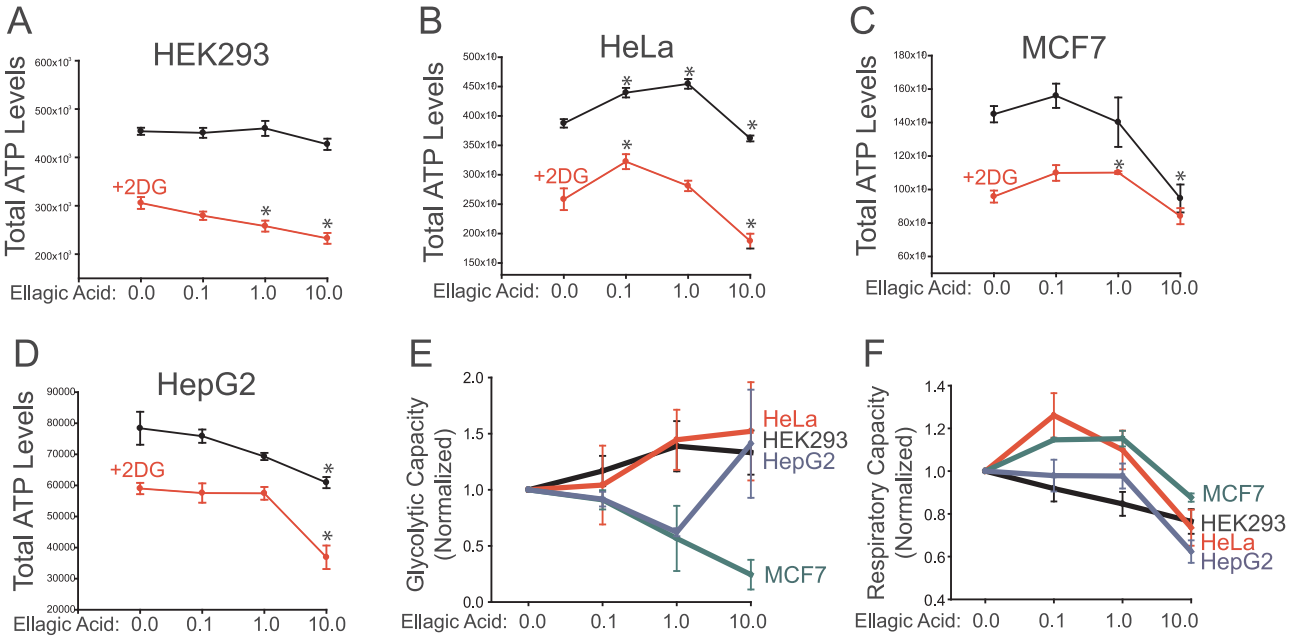

Fig. 1. Ellagic acid impacts cellular ATP levels. A) Total HEK293 cellular ATP levels in the presence and absence of the glycolysis inhibitor 2-deoxy-D-glucose (2DG) after 24 h treatment with the indicated concentrations of ellagic acid (in μM). The data are pooled from three separate experiments. The same experiment was repeated in HeLa (B), MCF7 (C), and HepG2 (D) cells. Data points represent the mean +/- s.e.m. of three separate determinations. *$p < 0.05$ *versus* control (vehicle) using an unpaired *t*-test. E) Normalized glycolytic capacity of each cell type. F) Normalized respiratory capacity of each cell type.

2. Materials and methods

2.1. Cell culture and materials

HepG2 human hepatocellular carcinoma, MCF7 human breast adenocarcinoma, HeLa human cervical adenocarcinoma, and HEK293 human embryonic kidney epithelial cells were purchased from ATCC. HepG2, HeLa, and HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. MCF7 cells were cultured in minimal essential medium (MEM) supplemented with 0.01 mg/ml insulin and 10% fetal bovine serum. Cell lines were authenticated by ATCC by STR analysis and were certified mycoplasma free. Culture media and fetal bovine serum were purchased from Gibco/ThermoFischer Scientific. Extracellular flux reagents were purchased from Agilent Technologies. JC-1 was purchased from Molecular Probes/ThermoFischer Scientific. All other materials were purchased at the highest purity available from Sigma-Aldrich.

2.2. Cellular ATP levels by endpoint assay

Total ATP levels using the endpoint assay in Fig. 1 were quantified using the CellTiter-Glo luminescent kit (Promega) in 96 well format. Three biological replicates averaged from eight technical replicates were used for each data point. Where noted, addition of 5 mM 2-deoxy-D-glucose (2DG) was concurrent with ellagic acid (or vehicle), and the cells were lysed for the endpoint assay 24 h later. Luciferase activity was measured using a microplate reader essentially as described by the manufacturer. Glycolytic capacity was calculated as the difference between ATP in the presence and absence of 2DG normalized to vehicle. Respiratory capacity was simply ATP levels in the presence of 2DG normalized to vehicle. An unpaired *t*-test was performed to compare vehicle to different concentrations of ellagic acid for each parameter quantified. For all experiments in this paper a statistical comparison was considered significant compared to vehicle control at $p < 0.05$ and is indicated on the figures with an asterisk. A two-tailed Student's *t*-test was used for all comparisons between two groups. Data in all figures are presented as the mean +/- standard error of the mean. Investigators were not blinded to the treatment conditions.

2.3. Extracellular flux measurements

Cellular oxygen consumption rate was calculated in 96 well format using the Seahorse XF Cell Mito Stress Kit (Agilent Technologies). Assays were set up essentially as described by the manufacturer. The night before the assay, each cell type was plated at a density of 20,000 cells per well. All cells were treated the next day with ellagic acid in octuplet 1 h prior to measurement of OCR rate. The concentration of FCCP uncoupler was varied based upon the cell type: 0.5μM final in HeLa, HepG2 and HEK293 assays; 0.25μM final in the MCF7 assay. Oxygen consumption rates were measured in a Seahorse XF Analyzer. The following parameters were quantified post-acquisition using formulas provided by the manufacturer: basal respiration, non-mitochondrial consumption, maximal respiration, proton leak, ATP production, and spare respiratory capacity from data pooled from three separate biological replicates. All values are reported as oxygen consumption rate (OCR), with units of pmol/min. Values on the outer edges of the plate were omitted. Also eliminated were any values reporting a negative OCR. An unpaired *t*-test was performed to compare vehicle to different concentrations of ellagic acid for each parameter quantified.

2.4. JC-1 staining and analysis

Cells were passed onto coverslips 24 h prior to imaging. Cell loading and imaging was performed in imaging buffer composed of 107 mM NaCl, 20 mM HEPES, 2.5 mM $MgCl_2$, 7.25 mM KCl, 11.5 mM glucose, 1 mM $CaCl_2$, and 1% bovine serum albumin. The JC-1 dye was purchased from Thermofisher. The JC-1 dye was reconstituted in DMSO to a final concentration of 20 mg/ml and stored at -80 until use. Loading solution was prepared by diluting this stock to 50 μg/ml in imaging buffer. This solution was then vortexed extensively to facilitate dispersion. Finally, the solution was briefly sonicated with a tip sonicator and centrifuged at 5000 xg for 15 min to pellet any insoluble material. The supernatant was used for staining. Cells were loaded with JC-1 for 10 min at 37 °C and the staining solution was replaced with imaging