Cell type-dependent effects of ellagic acid on cellular metabolism

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Non-standard Abbreviations:

2DG: 2-Deoxy-D-glucose

EA: Ellagic Acid

OCR: Oxygen consumption rate

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, likely limiting the chemotherapeutic utility of this compound.

INTRODUCTION

Ellagic acid (EA) is a polyphenolic compound enriched in nuts and berries. Like all polyphenols EA is an antioxidant (Priyadarsini *et al.*, 2002; Di Meo *et al.*, 2013) and has many cellular effects including inhibiting cancer cell proliferation (Zhang *et al.*, 2014; Garcia-Nino and Zazueta, 2015). Ellagic acid is bioavailable when taken orally and can briefly reach serum concentrations up to several hundred nanomolar in human subjects (Seeram *et al.*, 2004; González-Sarrías *et al.*, 2015; Kang *et al.*, 2016). 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 (Kang *et al.*, 2016). Like EA, bioactive urolithin metabolites are antioxidants and have anti-cancer activity (Espin *et al.*, 2013; Kang *et al.*, 2016) but may have unique cellular targets (Vicinanza *et al.*, 2013).

The number of cellular processes purported to be modulated by EA is impressive (Garcia-Nino and Zazueta, 2015). In non-transformed cells, EA is protective against oxidative insults (Sepand *et al.*, 2016; Dhingra *et al.*, 2017; Firdaus *et al.*, 2018). 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 (Edderkaoui *et al.*, 2008; Alfredsson *et al.*, 2014; Ho *et al.*, 2014; Salimi *et al.*, 2015), 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 (Salimi *et al.*, 2015). There is also evidence that EA can affect cellular ATP production by targeting glycolysis. Ellagic acid downregulates the expression of the

sodium/hydrogen exchanger NHE1 leading to cellular acidification and inhibition of glycolytic flux in endometrial cancer cells (Abdelazeem *et al.*, 2017). In T cell lymphoma EA can inhibit the activity of lactate dehydrogenase to decrease glycolysis (Mishra and Vinayak, 2015). 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 500nM (Espin *et al.*, 2013; Zhang *et al.*, 2014; Zeb, 2018)).

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 was 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.

RESULTS AND DISCUSSION

Ellagic acid inhibits ATP production

We first investigated the effects of EA on total ATP levels in four commonly used cell lines: human embryonic kidney HEK293 cells, cervical carcinoma HeLa cells, metastatic breast cancer MCF7 cells, and hepatocellular carcinoma HepG2 cells. Each cell line was incubated with either vehicle (DMSO) or 0.1, 1.0, or 10 µM EA for 24 hours. The cells were then lysed and total ATP levels were quantified using a luciferase-based assay (Turman and Mathews, 1996). At all doses EA had no effect on ATP levels in HEK293 cells (Figure 1A). HeLa cells had a complex response to EA, with increases at 0.1 and 1.0 µM, and a slight reduction at 10 µM compared to control (Figure 1B). Both MCF7 and HepG2 cells had no differences in ATP concentration when treated with 0.1 µM and 1.0 µM EA, and a decrease at 10 µM EA. To investigate whether EA was affecting glycolysis or respiration, we measured ATP levels in the

presence of the glycolysis inhibitor 2-deoxy-D-glucose (2DG; red plots in Figure 1A-D). As expected, 2DG reduced cellular ATP levels in all cell types. In HEK293, HeLa and HepG2 cells, EA further reduced ATP levels in the presence of 2DG (Figure 1A, C-D). In contrast to the other three cell types, in MCF7 cells there was no reduction in ATP levels in the presence of 2DG (Figure 1C). To determine the glycolytic capacity at each EA concentration, we subtracted total ATP levels from the levels measured in the presence of 2DG and normalized the data to control (vehicle). As shown in Figure 1E, EA only reduced glycolysis in MCF7 cells. Normalized respiratory capacity was reduced in all cells types except MCF7 cells using this assay consistent with the large body of literature indicating EA can affect mitochondrial function. One important caveat to these findings is that 2DG uptake and retention is dependent upon glucose transporter and hexokinase activity respectively. It is well known that cancer cells upregulate both of these enzymatic activities to different extents (Macheda *et al.*, 2005; Mathupala *et al.*, 2006), so therefore some of these cell type-specific differences may relate to alterations in glycolytic pathways. It is also not ideal to use an endpoint assay to measure ATP production. We therefore tested more directly the effects of EA on cellular respiration.

Ellagic acid has cell-type specific effects on respiration

To investigate the acute effects of EA on mitochondrial function *in situ* in living cells, we measured the oxygen consumption rate (OCR) in the presence of various inhibitors to evaluate six key parameters of mitochondrial function: basal respiration, maximal respiration, ATP production, spare respiratory capacity, proton leak, and non-mitochondrial oxygen consumption using the Seahorse extracellular flux assay (Pelletier *et al.*, 2014; Wang *et al.*, 2015). For these experiments each cell type was treated with various concentration of EA for 1 hour prior to measuring the oxygen consumption rate. Treatment of HEK293 cells with EA resulted in modest increases in basal respiration, maximal respiration, ATP production and spare respiratory capacity at a physiologically relevant dose (100 nM; Figure 2A). Higher doses did not have any effect with the exception of maximal respiratory rates. The complex dose-dependent effects on

OCR is consistent with previous studies investigating mitochondrially-targeted antioxidants (Reily *et al.*, 2013). HeLa cells also had increased basal respiration and ATP production at low EA doses, however there were also negative effects on spare respiratory capacity and proton leak (Figure 2B). In contrast to HEK293 and HeLa cells, 100 nM EA decreased basal respiration and ATP production and increased the spare respiratory capacity in MCF7 cells (Figure 2C). In all three cell types, there was no clear dose dependent effects of EA. However, the observed effects on OCR occurred at physiologically relevant concentrations of EA (100 nM). HepG2 cells only had changes in OCR at the highest dose of EA (10 µM; Figure 2D), and resulted in decreased basal respiration, maximal respiration, spare respiratory capacity, proton leak, and non-mitochondrial oxygen consumption. To facilitate visualization of the broad and dose-dependent effects of EA on mitochondrial function, the oxygen consumption rate data were combined onto a single graph for each cell type in Figure 4. Replotting in this manner was also more useful to determine the effect sizes of EA on OCR. In HEK293 cells there was clear dose-dependent effects on maximal respiration with most other parameters remaining unchanged (Figure 3A). In both HeLa and MCF7 cells, EA had very modest effects on mitochondrial OCR with no obvious patterns to its action (Figure 3B-C). In contrast to the other three cells types, EA had robust and dose-dependent effects on OCR in HepG2 cells (Figure 3D), with nearly all parameters inhibited after treatment with 10 µM EA. It can be concluded that the effects of acute treatment with EA on mitochondrial function are complex and highly cell-type specific. This may be related to differences in mitochondrial content, morphology, and ultimately function in these four cell types. To evaluate these possibilities, we next performed single cell imaging of mitochondrial polarization after acute treatment with EA.

Ellagic acid has cell-type specific effects on mitochondrial polarization

Multiple studies have shown that EA induces mitochondrial depolarization (Edderkaoui *et al.*, 2008; Alfredsson *et al.*, 2014; Ho *et al.*, 2014; Salimi *et al.*, 2015). Most studies use chronic EA treatment models which also induce cell death signaling pathways which are known to

depolarize mitochondria. Thus, it is difficult to ascertain from these studies whether the effects of EA in mitochondrial function were direct or indirect. To investigate the acute effects of EA on mitochondrial polarization, we employed real time *in situ* imaging if live cells using single-cell JC-1 imaging. The dye JC-1 aggregates and fluoresces red in polarized mitochondria, while the green monomeric form predominates in depolarized mitochondria (Reers *et al.*, 1991; Smiley *et al.*, 1991). Often a single mitochondria will have significant heterogeneity in membrane potential and will have regions where red and green fluorescence predominates (Smiley *et al.*, 1991). Staining of HEK293 cells with JC-1 reveals clear mitochondrial localization with red/green heterogeneity (Figure 4A). Treatment with 10 µM EA causes a slight reduction in mitochondrial polarization over a 40 minute time course (Figure 4B). Ellagic acid had no effects on mitochondrial polarization in HeLa or MCF7 after 40 minutes of treatment (Figure 4C-F). In contrast, EA treatment of HepG2 cells caused a dramatic and almost immediate reduction in mitochondrial polarization (Figure 4G-H). This finding likely explains the broad effects of EA on mitochondrial respiration observed in HepG2 cells determined by extracellular flux analysis (Figures 2-3). In all cell types there was no obvious changes in mitochondrial morphology after EA treatment as determined by the green JC-1 fluorescence (Figure 4 A,C,E,G). We conclude that the effects on EA on ATP production and mitochondrial respiration are remarkably cell type specific. Importantly, these findings also highlight that potential therapeutics which have an effect on mitochondrial function in one of these cell types may not be generalizable to other cell types.

Ellagic acid is not a robust inducer of caspase activation

Ellagic acid has been shown to specifically induce apoptosis in cancer cells (Larrosa *et al.*, 2006; Hagiwara *et al.*, 2010; Ho *et al.*, 2014; Mishra and Vinayak, 2014; Chen *et al.*, 2015; Mishra and Vinayak, 2015; Salimi *et al.*, 2015; Wang *et al.*, 2016) while having cytoprotective activity in non-transformed cells (Atta Ur *et al.*, 2001; Priyadarsini *et al.*, 2002; Garcia-Nino and Zazueta, 2015; Sepand *et al.*, 2016; Firdaus *et al.*, 2018; Zeb, 2018). To determine whether EA

activated apoptotic pathways, we measured caspase-3 catalytic activities in cells treated with EA for 24 hours. Treatment of non-transformed HEK293 cells with EA caused a slight reduction in basal caspase-3 activity (Figure 5A). In HeLa cells we found that a supraphysiologic dose of EA (10 µM) could increase caspase-3 catalytic activity about 2 fold (Figure 5B). For comparison, a robust apoptotic inducer of apoptosis in this cell line such as staurosporine will cause a 10-50 fold increase in caspase-3 activity (Boehning *et al.*, 2003; Akimzhanov *et al.*, 2013). Ellagic acid had no statistically significant effect on caspase 3 activity in HepG2 cells (Figure 5C) despite strong effects on mitochondrial function in this cell type (Figures 2-4). MCF7 cells have a mutation in the caspase-3 gene which eliminates the expression of the pro-enzyme (Janicke *et al.*, 1998). We therefore measured caspase-6 activity in this cell line. We could not detect any caspase-6 activity in MCF7 cells, even under basal conditions. Thus, at least in these four cell lines, EA is not a robust inducer of the apoptotic program. These findings also suggest that the changes in glycolytic flux or OCR observed in Figures 1-4 cannot be explained by activation of cell death pathways.

Ellagic acid as a potential chemotherapeutic

This study demonstrates that EA has highly cell type-dependent effects on cellular metabolism. Within individual cell types, the multitude effects on mitochondrial function suggest that there is not a single target of action for this compound. Not surprisingly, some of the effects mimic the action of antioxidants (Reily *et al.*, 2013; Garcia-Nino and Zazueta, 2015). Almost all previous studies using EA have utilized doses which are not achievable in human serum. In this study we see modest effects on mitochondrial function at physiologically relevant doses (100 nM). However, our findings also highlight that the effects of EA are highly cell type specific. This may limit the usefulness of this compound as a therapeutic for cancer. Urolithin metabolites of EA achieve higher serum levels and have a longer half-life (Gonzalez-Sarrias *et al.*, 2010; Espin *et al.*, 2013). It will be useful in future studies to examine the role of these compounds on mitochondrial function.

MATERIALS AND METHODS

Cell culture

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 0.01mg/ml insulin and 10% fetal bovine serum.

Cellular ATP levels by endpoint assay

Total ATP levels using the endpoint assay in Figure 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 hours 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.

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 hour 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.

JC-1 staining and analysis

Cells were passed onto coverslips 24 hours 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 minutes to pellet any insoluble material. The supernatant was used for staining. Cells were loaded with JC-1 for 10 minutes at 37°C and the staining solution was replaced with imaging buffer until mounting on an inverted live cell imaging microscope. Alternating red and green fluorescent mages were acquired every three seconds on a Nikon TiS inverted microscope with 40X objective. Images were acquired with a Photometrics Evolve deep cooled, back thinned EMCCD using Metafluor software. Each cell type was imaged as a single field in biological triplicates, and the total number of single cells analyzed is listed directly on the traces in Figure 4. Image analysis was done using ImageJ. Mitochondrial membrane potential was inferred from

the standard deviation of the red channel fluorescence from a region of interest encompassing an entire single cell. The rationale behind this approach is that highly polarized mitochondria would have fluorescent red puncta with a high average standard deviation on average over the cell area, whereas depolarized mitochondria would cause monomerization and loss of red puncta and a subsequent decrease in standard deviation. A similar approach has been used extensively to monitor cytochrome c-GFP fusion protein release from mitochondria (Goldstein *et al.*, 2000; Boehning *et al.*, 2003). This approach also facilitated comparisons between the cell lines as those cells which had green channel fluorescence barely above background (such as HeLa) had artificially inflated red/green ratios.

Caspase-3 activity

Caspase-3 and caspase-6 enzymatic activities were performed exactly as described previously 24 hours after treatment with ellagic acid (Boehning *et al.*, 2003). Three biological replicates averaged from three technical replicates were used for each data point. An unpaired t test was performed to compare vehicle to different concentrations of ellagic acid for each parameter quantified.

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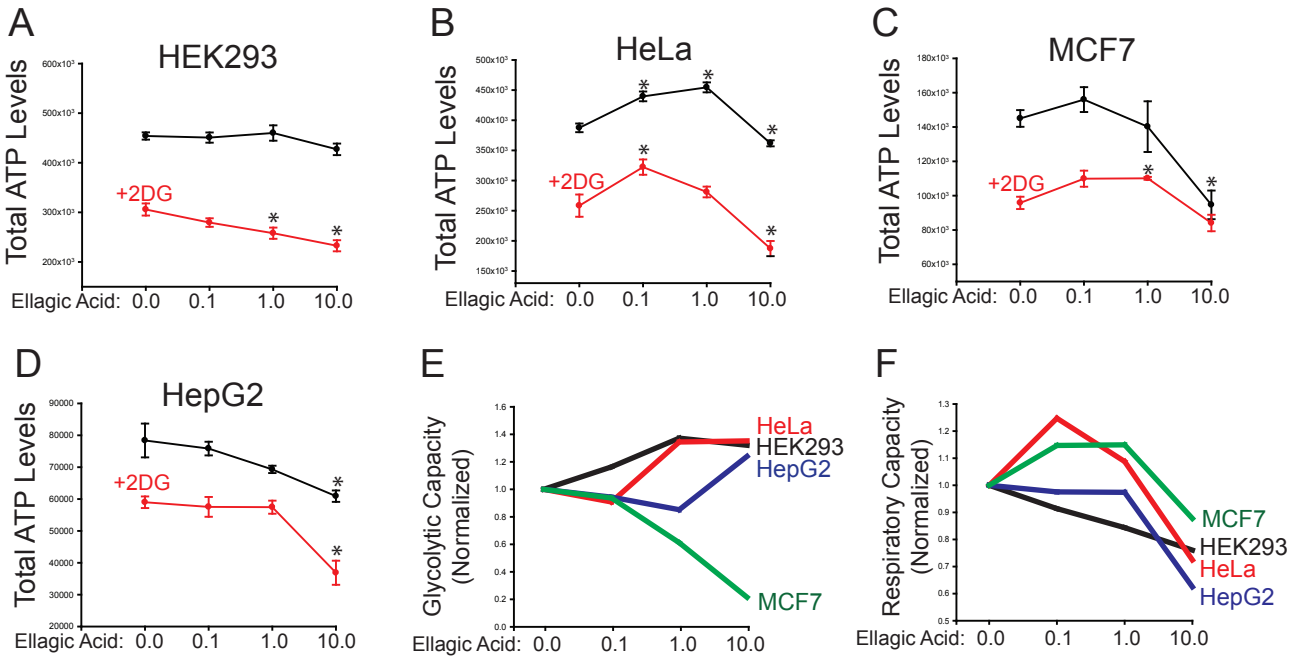

Figure 1 Ellagic acid impacts cellular ATP levels. A) Total HEK293 cellular ATP levels in the presence and a bsence of the glycolysis inhibitor 2-deoxy-D-glucose (2DG) after 24 hours treatment with the indicated concentrations of ellagic acid (in μM). The data are pooled from three separate experiments. 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.

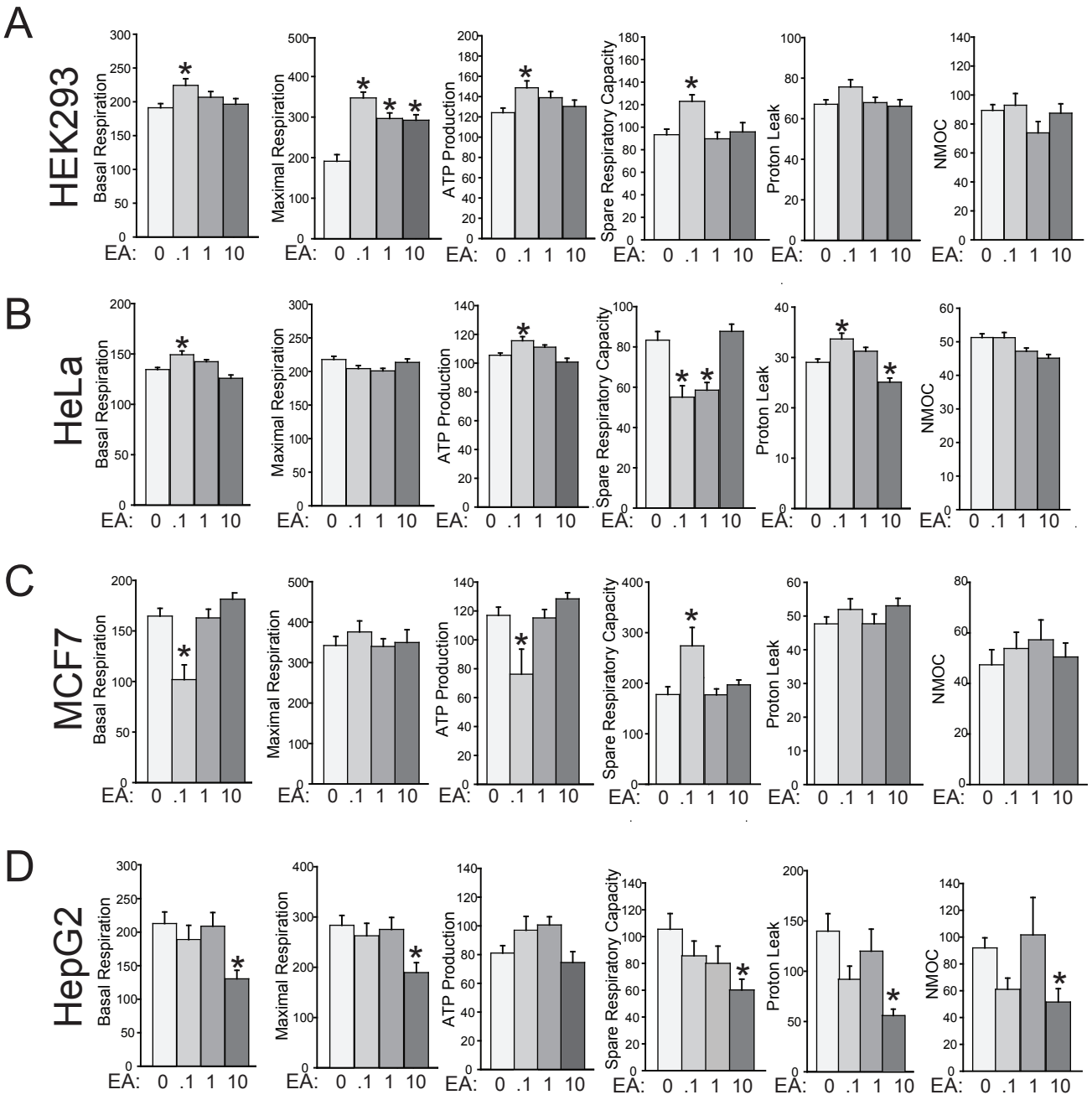

Figure 2 Dose-dependent effects of ellagic acid on oxygen consumption rate. A) Basal respiration, maximal respiration, ATP production, spare respiratory capacity, proton leak, and non-mitochondrial oxygen consumption (NMOC) in HEK293 cells determined by extracellular flux measurements at various ellagic acid (EA) concentrations (in μM). Ellagic acid was added 1 hour prior to measurement. The data are averages of the oxygen consumption rates (in pmol/min) and 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.

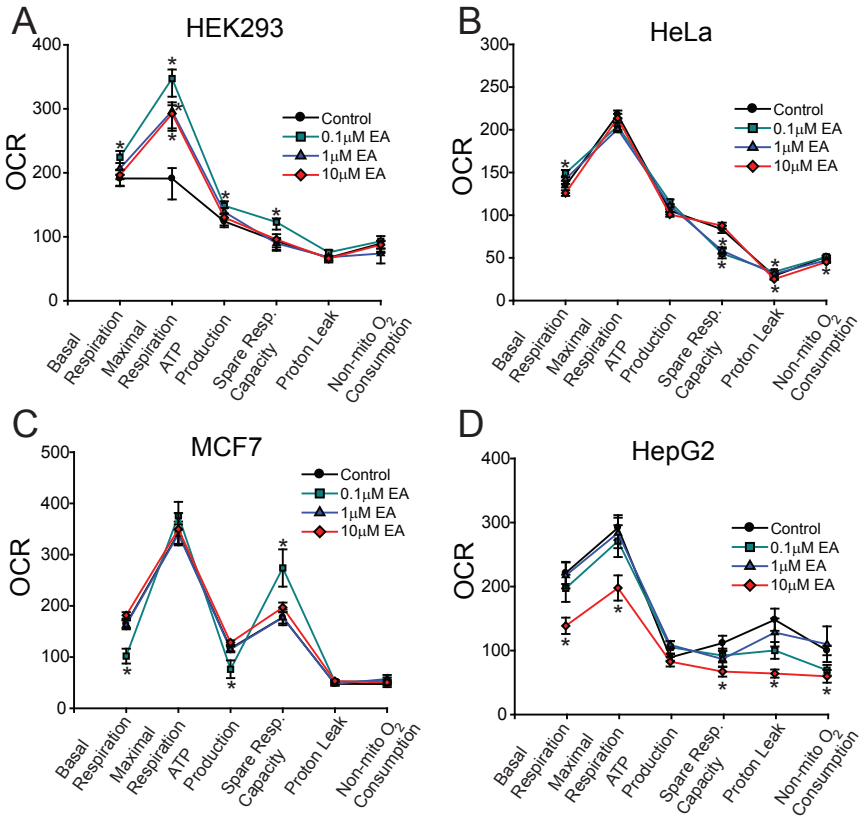

Figure 3 Ellagic acid has cell-type specific effects on mitochondrial function. A) Pooled respiratory function data for HEK293 cells at various ellagic acid (EA) concentrations. Data points represent the mean +/- s.e.m. of the oxygen consumption rate (OCR) of three separate determinations. *$p < 0.05$ versus control (vehicle) using an unpaired t test. The same experiment was repeated in HeLa (B), MCF7 (C), and HepG2 (D) cells.

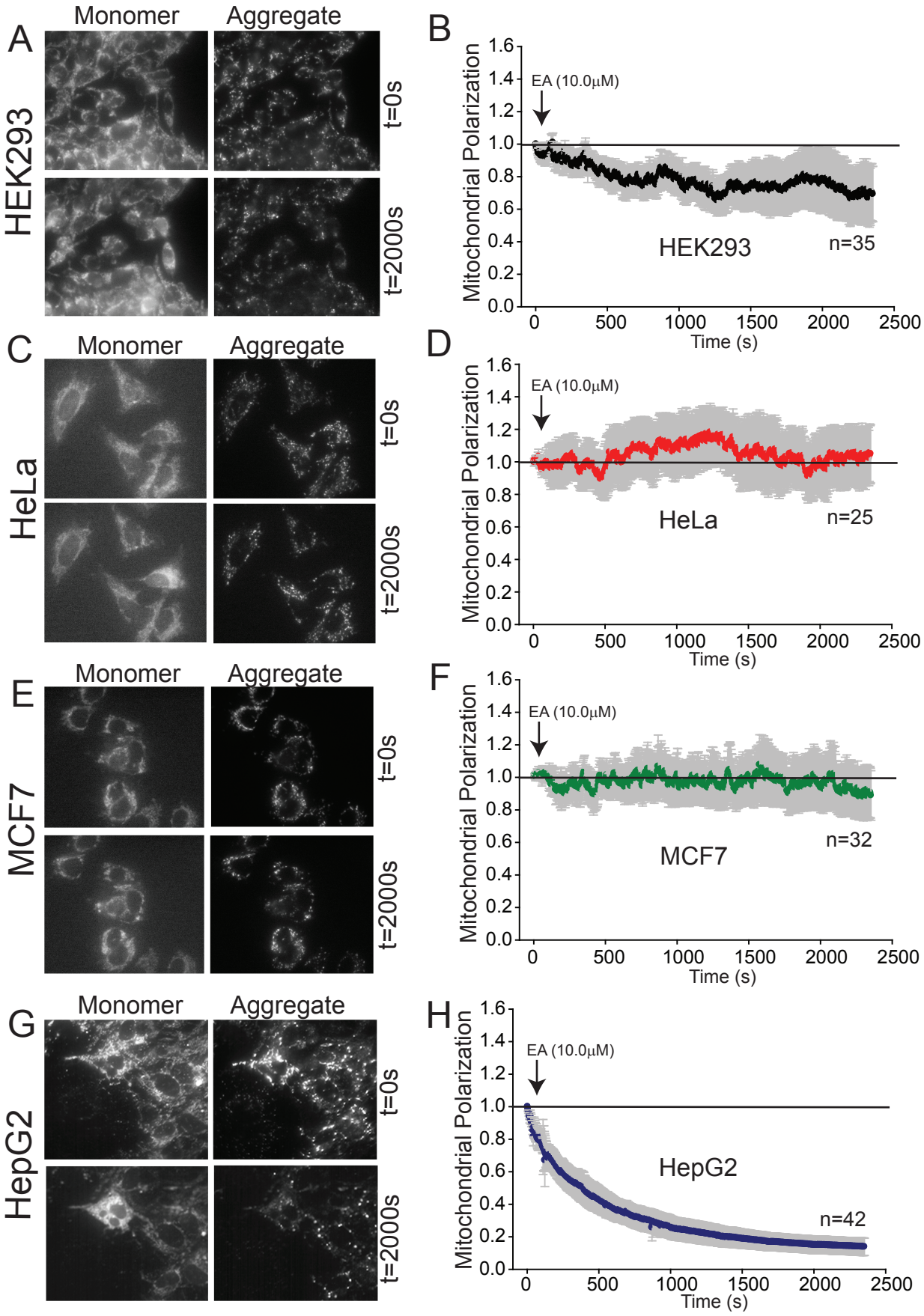

Figure 4. Effects of acute administration of ellagic acid on mitochondrial membrane potential. A) Representative JC-1 images of the green fluorescent monomer and red fluorescent aggregate at time zero and 2000 seconds after 10 μM ellagic acid (EA) addition in HEK293 cells. B) Normalized mitochondrial polarization (as determined by red aggregate fluorescence) before and after EA addition in HEK293 cells. Total number of single cells analyzed is indicated. Data points represent the mean +/- s.e.m. of three separate determinations. C,D) Same experiment in HeLa cells. Resting green fluorescence (monomer) is very low in this cell line. E,F) Same experiment in MCF7 cells. G,H) Same experiment in HepG2 cells. In all graphs a solid line represents no change from time zero.

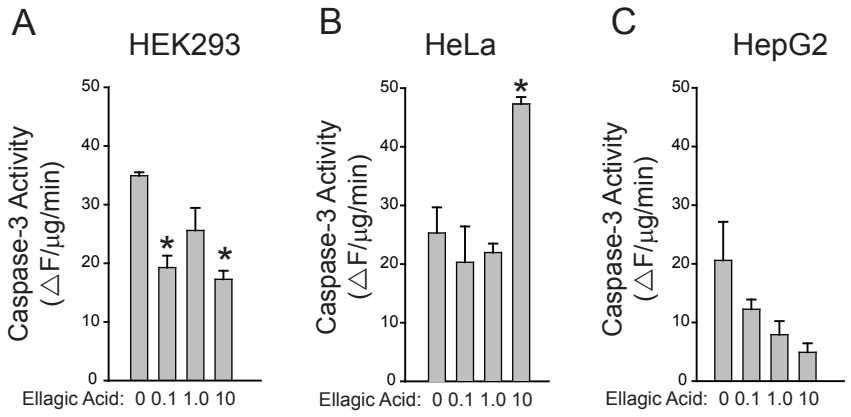

Figure 5. Ellagic acid is not a potent regulator of caspase activity. A) Caspase-3 enzymatic activity in HEK293 cells after treatment with the indicated concentrations of ellagic acid for 24 hours. Caspase-3 enzymatic activity was measured using a fluorogenic caspase-3 substrate on cell lysates ( ). Data points represent the mean +/- s.e.m. of three separate determinations. The units are change in fluorescent intensity per µg per minute. *$p < 0.05$ versus control (vehicle) using an unpaired t test. The same experiment was repeated in HeLa (B), and HepG2 (C) cells. As noted in the text MCF-7 cells have a mutation at the *CASP3* locus eliminating expression of this enzyme. Using an identical enzyme assay with a fluorogenic caspase-6 substrate resulted in no detectable caspase-6 activity in control or EA treated cells and as such is not graphed.