(–)-Epicatechin alters reactive oxygen and nitrogen species production independent of mitochondrial respiration in human vascular endothelial cells

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Abstract

Introduction: Vascular endothelial dysfunction is characterised by lowered nitric oxide (NO) bioavailability, which may be explained by increased production of reactive oxygen species (ROS), mitochondrial dysfunction and altered cell signalling. (-)-Epicatechin (EPI) has proven effective in the context of vascular endothelial dysfunction, but the underlying mechanisms associated with EPI's effects remain unclear.

Objective(s): Our aim was to investigate whether EPI impacts reactive oxygen and nitrogen species (RONS) production and mitochondrial function of human vascular endothelial cells (HUVECs). We hypothesised that EPI would attenuate ROS production, increase NO bioavailability, and enhance indices of mitochondrial function.

Methods: HUVECs were treated with EPI (0-20 μM) for up to 48 h. Mitochondrial and cellular ROS were measured in the absence and presence of antimycin A (AA), an inhibitor of the mitochondrial electron transport protein complex III, favouring ROS production. Genes associated with mitochondrial remodelling and the antioxidant response were quantified by RT-qPCR. Mitochondrial bioenergetics were assessed by respirometry and signalling responses determined by western blotting.

Results: Mitochondrial superoxide production without AA was increased 32% and decreased 53% after 5 and 10 μM EPI treatment vs. CTRL ($P<0.001$). With AA, only 10 μM EPI increased mitochondrial superoxide production vs. CTRL (25%, $P<0.001$). NO bioavailability was increased by 45% with 10 μM EPI vs. CTRL ($P=0.010$). However, EPI did not impact mitochondrial respiration. NRF2 mRNA expression was increased 1.5- and 1.6-fold with 5 and 10 μM EPI over 48 h vs. CTRL ($P=0.015$ and $P=0.001$, respectively). Finally, EPI transiently enhanced ERK1/2 phosphorylation (2.9 and 3.2-fold over 15 min and 1 h vs. 0 h, respectively; $P=0.035$ and $P=0.011$).

Conclusion(s): EPI dose dependently alters RONS production of HUVECs but does not impact mitochondrial respiration. The induction of NRF2 mRNA expression with EPI might relate to enhanced ERK1/2 signalling, rather than RONS production. In humans, EPI may improve vascular endothelial dysfunction via alteration of RONS and activation of cell signalling.

63 **Introduction**

64 Globally, cardiovascular disease (CVD) is the leading cause of morbidity and mortality
65 (WHO, 2018). One major risk factor for CVD is vascular endothelial dysfunction, which is
66 typified by impaired vasodilation and diminished blood flow (Heitzer et al., 2001; Suwaidi et
67 al., 2000). Several factors contribute to vascular endothelial dysfunction, including reduced
68 nitric oxide (NO) bioavailability and elevated oxidative stress (Ungvari et al., 2018). A decline
69 in NO bioavailability has been attributed to lower endothelial nitric oxide synthase (eNOS)
70 content and activity, in part due to lower phosphorylation of eNOS at Ser1177 - at least in the
71 aged vascular endothelium (Gliemann et al., 2018; Musicki et al., 2005). Elevated oxidative
72 stress might be explained increased production of ROS (Cernadas et al., 1998; Chou et al.,
73 1998; Donato et al., 2007, 2009), that are cytosolic and mitochondrial in origin (Csiszar et al.,
74 2002, 2007; Donato et al., 2007; Hamilton et al., 2001; Jablonski et al., 2007; Sun et al., 2004;
75 Van Der Loo et al., 2000).
76
77 Vascular endothelial health is also impacted by mitochondrial function. Indeed, ageing is
78 associated with reduced mitochondrial content in endothelial cells of conduit arteries, feed
79 arteries and capillaries (Burns et al., 1979; S. H. Park et al., 2018; S.-Y. Park et al., 2018;
80 Ungvari et al., 2008), which could be due to blunted transcriptional responses (S.-Y. Park et
81 al., 2018; Ungvari et al., 2008). This potential reduction in mitochondrial biogenesis with
82 ageing may result from diminished NO bioavailability (Gouill et al., 2007; Miller et al., 2013)
83 and/or lowered AMP-activated protein kinase (AMPK) signalling (Lesniewski et al., 2012),
84 both of which are known to activate peroxisome proliferator-activated receptor gamma
85 coactivator 1-alpha (PGC-1α). A link between mitochondria and vascular endothelial
86 dysfunction has also been made from observations that human skeletal muscle feed arteries
87 exhibit impaired respiratory capacity and lower coupling efficiency in middle- (55 years) and
88 older-age (70 years) compared to young adults (S. H. Park et al., 2018; S.-Y. Park et al., 2020).
89 Furthermore, mitochondrial-targeted antioxidants like Mitoquinone (MitoQ) restore vascular
90 endothelial dysfunction in aged mice and patients with peripheral artery disease, which likely
91 results from reductions in levels of mitochondrial superoxide (Gioscia-Ryan et al., 2014; S.-Y.
92 Park et al., 2020). Together, evidence points towards mitochondria as promising targets for
93 interventions aimed at combatting vascular endothelial dysfunction.
94

95 (-)-Epicatechin (EPI) belongs to a subclass of flavonoids known as the flavanols. Not only
96 is EPI highly bioavailable – reaching up to 10 μM in circulation in humans (Hollands et al.,
97 2013) - but EPI is also associated with several health benefits (Arts et al., 2001; Hertog et al.,
98 1993), including improved vascular endothelial function (Galleano et al., 2013; Karim et al.,
99 2000; Schroeter et al., 2006) and increased NO bioavailability (Schroeter et al., 2006).
100 Additional mechanisms thought to underly the therapeutic effects of EPI include: increased
101 eNOS phosphorylation (Ramirez-Sanchez et al., 2011, 2012, 2018), enhanced content or
102 activity of enzymatic antioxidant proteins (Moreno-Ulloa, Nogueira, et al., 2015; Ramirez-
103 Sanchez et al., 2014) and augmented mitochondrial biogenesis (Lee et al., 2015; Ramirez-
104 Sanchez et al., 2018; Taub et al., 2012, 2016). Whilst the potential of EPI to enhance markers
105 of mitochondrial biogenesis and antioxidant capacity seems promising, it is unclear whether
106 this effect translates to enhanced respiratory function or altered ROS production of vascular
107 endothelial cells. In-fact, one recent study reported no impact of EPI on mitochondrial
108 respiration (Keller et al., 2020), but the authors demonstrated that EPI may lower mitochondrial
109 ROS production of HUVECs in the presence of high glucose. The challenge remains to resolve
110 whether EPI modulates ROS production and mitochondrial function of vascular endothelial
111 cells.
112
113 To this end, our aim was to investigate whether EPI modulates reactive oxygen and nitrogen
114 species (RONS) production and mitochondrial function of human vascular endothelial cells.
115 We hypothesised that EPI would attenuate ROS production, augment NO bioavailability, and
116 enhance indices of mitochondrial function.
117
118
119 **Materials and Methods**

120 ***Cell culture and treatment***

121 Human umbilical endothelial vein endothelial cells (HUVECs; Thermo Fisher Scientific,
122 Waltham, MA, USA) at passages 3-7 were used in this study. HUVECs were not passaged
123 more than 7 times because of changes in cell phenotype that can occur with multiple population
124 doublings that ultimately lead to senescence (Chang et al., 2005; Cheung, 2007; Grillari et al.,
125 2000). Following the plating of cells onto pre-gelatinised well-plates (0.2% gelatin) in
126 complete endothelial cell growth medium (EGM; Cell Applications Inc, San Diego, CA, USA),
127 ~80% confluent HUVECs were washed twice with Dulbecco's phosphate-buffered saline (D-

128 PBS) and switched to pre-warmed (37°C) EGM in the absence (vehicle [H_2O], 'CTRL') or
129 presence of EPI (0.5-20 µM) over 24 h and 48 h. Human umbilical endothelial vein endothelial
130 cells (HUVECs; Thermo Fisher Scientific, Waltham, MA, USA) at passages 3-7 were used in
131 this study. HUVECs were not passaged more than 8 times because of changes in cell phenotype
132 that can occur with multiple population doublings that ultimately lead to senescence (Chang et
133 al., 2005; Cheung, 2007; Grillari et al., 2000). Following the plating of cells onto pre-
134 gelatinised well-plates (0.2% gelatin) in complete endothelial cell growth medium (EGM; Cell
135 Applications Inc, San Diego, CA, USA), ~80% confluent HUVECs were washed twice with
136 Dulbecco's phosphate-buffered saline (D-PBS) and switched to pre-warmed (37°C) EGM in
137 the absence (vehicle [H_2O], 'CTRL') or presence of EPI (0-20 µM) over 24 h and 48 h.

138

139 ***Cell viability***

140 The fluorescent CyQUANT® Proliferation Assay kit was used to determine cell viability.
141 HUVECs were grown to 60-70% confluency in EGM in gelatinised 96-well plates. Cells were
142 subsequently dosed for 24 h in EGM +/- EPI at 0-20 µM. After 24 h, wells were aspirated,
143 washed twice with D-PBS, and then frozen immediately at -80°C. On the day of the assay,
144 plates were thawed at room temperature and CyQUANT® GR dye/cell-lysis buffer was added
145 to each well according to manufacturer instructions. Plates were gently mixed on an orbital
146 shaker (80 rpm) for 5 minutes protected from light. Sample fluorescence was measured using
147 a CLARIOStar plate reader (BMG Labtech, Ortenberg, Germany) with 485/520 Ex/Em.

148

149 ***Mitochondrial ROS production***

150 Mitochondrial superoxide was detected in HUVECs using MitoSOX (Thermo Fisher
151 Scientific, Waltham, USA), a hydroethidine probe which is targeted to mitochondria by a
152 conjugated triphenyl-phosphonium moiety (Zielonka et al., 2017). HUVECs were seeded at 3
153 × 10^4 cells/mL in gelatinised 12-well microplates and at ~80% confluence dosed +/- EPI (0-10
154 µM) for 24 h. Next, cells were washed in Krebs-Ringer buffer (KRH; 135 mM NaCl, 3.6 mM
155 KCl, 10 mM HEPES (pH 7.4), 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 0.5 mM NaH_2PO_4, 2 mM
156 glutamine and 5 mM D(+)-Glucose) prior to incubation at 37ºC for 30 minutes, with or without
157 15 µM antimycin A (AA) to stimulate mitochondrial superoxide production. Next, AA-
158 containing KRH was removed and MitoSOX was loaded into cells in fresh pre-warmed KRH
159 to a final concentration of 2.5 µM. Plates were immediately transferred to a plate reader

160 (ClarioStar, BMG Labtech) and fluorescence was monitored continuously at 30-sec intervals
161 over 30 min at excitation/emission of 510/580 nm. Rates of mitochondrial superoxide
162 production were determined from the slope of the resultant progress curve over the 30-minute
163 recording. Finally, plates were immediately fixed in 1% (v/v) acetic acid in methanol for the
164 determination of cell density by the Sulforhodamine B (SRB) assay, which was used to
165 normalise obtained fluorescence values. The plate reader's focal height and gain were
166 optimised and fixed between different experiments.
167
168 ***Cellular ROS***

169 Cellular ROS were detected using the CellROX® Deep Red reagent by spectrophotometry.
170 HUVECs were seeded at 3×10^4 cells/mL into gelatinised 12-well microplates and at ~80%
171 confluence dosed +/- EPI (0-10 μM) for 24 h. After treatment, HUVECs were washed in KRH
172 with or without 15 μM AA and incubated at 37ºC for 30 minutes, prior to KRH removal and
173 CellROX loading using fresh, pre-warmed KRH buffer, to a final concentration of 2.5 μM.
174 Following 30 minutes CellROX incubation protected from light, cells were washed 2 × with
175 D-PBS and immediately transferred to a plate reader (ClarioStar, BMG Labtech), where
176 fluorescent CellROX oxidation products were excited at 640 nm and light emission detected at
177 665 nm. The plate reader's focal height and gain were optimised and fixed between
178 experiments. Upon completion of the reading, plates were immediately fixed for the
179 determination of cell density by as discussed above.
180
181 ***Nitric oxide bioavailability***

182 To assess intracellular NO bioavailability, HUVECs were plated and cultured as previously
183 described. At ~80% confluency, cells were treated with 0, 5 or 10 μM EPI for 24 h. After
184 treatment, HUVECs were washed 2 × with D-PBS and loaded with DAF-FM™ diacetate (4-
185 amino-5-methylamino- 2′,7′-difluorofluorescein diacetate; Molecular Probes, Invitrogen) to a
186 final concentration of 1 μM in KRH buffer and incubated at 37°C for 45 minutes protected
187 from light. Following dye loading, cells were washed 2 × with D-PBS and immediately
188 trypsinised prior to pelleting and resuspension in D-PBS. Sample fluorescence was
189 subsequently detected at 495/515 Ex/Em by flow cytometry (BD Accuri C6, BD Biosciences,
190 Wokingham, UK). Data were recorded from 5,000 events and median values reported.
191
192 ***RT-qPCR – Gene expression quantification***

193 HUVECs were grown to ~70% confluency in EGM in gelatinised 12-well plates and lysed
194 in 125 µL TRIzol. Total RNA was then extracted using the phenol-chloroform method. RNA
195 concentrations were determined by spectrophotometry (NanoDrop™ 2000, Thermo Fisher
196 Scientific, Waltham, USA). Specific primers used in each PCR are outlined in supplementary
197 Table 1. After preparation, reaction tubes were transferred to a Rotor-Gene Q PCR thermal
198 cycler for product amplification using a one-step protocol (QuantiFast SYBR® Green RT-PCR
199 Kit, Qiagen, UK). The amplification protocol was as follows: reverse transcription (10 minutes
200 at 50°C), transcriptase inactivation and initial denaturation (95°C for 5 min) followed by 40 ×
201 amplification cycles consisting of: 95°C for 10 s (denaturation) and 60°C for 30 s (annealing
202 and extension); followed by melt curve detection. Critical threshold (C_T) values were derived
203 from setting a threshold of 0.09 for all genes. To quantify gene expression, C_T values were used
204 to quantify relative gene expression using the comparative Delta Delta C_T ($2^{-\Delta\Delta CT}$) equation
205 (Livak & Schmittgen, 2001), whereby the expression of the gene of interest was determined
206 relative to the internal reference gene (RPL13a) in the treated sample, compared with the
207 untreated zero-hour control.
208
209 ***Mitochondrial respiration***
210 HUVECs (passages 4-6) were seeded in XFe 24 well plates (Agilent, Santa Clara, CA,
211 USA) at 30,000 cells per well in 200 µL EGM for 48 h. After 48 h, HUVECs were washed
212 twice with D-PBS and replaced with fresh EGM containing 0, 5 and 10 µM EPI for 24 h. On
213 the day of the assay, HUVECs were washed with pre-warmed XF Dulbecco's Modified Eagle
214 Medium pH 7.4 (DMEM; Agilent, Santa Clara, CA, USA), supplemented with 5.5 mM
215 glucose, 1 mM sodium pyruvate and 2 mM L-Glutamine, and brought to a final well volume
216 of 500 µL. The cells were incubated in this medium for 45 minutes at 37°C in a non-CO_2
217 incubator and then transferred to a Seahorse XFe24 extracellular flux analyser (Agilent, Santa
218 Clara, CA, USA) maintained at 37°C. After an initial 15-minute calibration, oxygen
219 consumption rate (OCR) was measured by a 3-4 loop cycle consisting of a 1-min mix, 2-min
220 wait and 3-min measure to record cellular respiration. After measuring basal respiration, 2 mM
221 oligomycin was added to selectively inhibit the mitochondrial ATP synthase. Subsequently, 3
222 µM BAM15 was added to uncouple OCR to determine maximal respiration, and finally a
223 mixture of 2 µM rotenone and 2 µM AA was added to inhibit complex I and III of the electron
224 transfer system, respectively, to determine non-mitochondrial respiration. Rates of oxygen
225 consumption were corrected for non-mitochondrial respiration and expressed relative to the

226 cell number of the appropriate well, determined by the CyQUANT® assay. The raw values of
227 extracellular acidification rate (ECAR) and OCR were divided into component rates to
228 calculate the relative contribution of glycolytic (ATP_{glyc}) and oxidative ATP-producing
229 reactions (ATP_{ox}) to total ATP production, as previously described (Mookerjee & Brand,
230 2015). Three independent experiments were performed that contained at least two technical
231 replicates.
232
233 ***SDS-PAGE and immunoblotting***

234 Total protein and phosphoprotein levels were detected by western blot. Following
235 treatment, HUVECs were lysed and scraped in ice-cold 1x precipitation assay buffer
236 containing: 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and
237 0.1% SDS, supplemented with 1x Protease Inhibitor Cocktail Set V (Merck Life Science, UK).
238 Cell lysates were centrifuged for 15 minutes at 18,000 × g (4°C) and the supernatant was stored
239 at -80°C before analyses for total protein by the Pierce BCA™ assay. Samples were
240 subsequently resuspended in 4x Laemmli buffer (Bio-Rad laboratories, Hertfordshire, UK)
241 containing reducing agent (1x working concentration: 31.5 mM Tris-HCl [pH 6.8], 10%
242 glycerol, 1% SDS, 0.005% Bromophenol Blue and 355 mM 2-mercaptoethanol) and were
243 loaded and electrophoresed on 10% SDS-stain-free polyacrylamide gels (supplementary Figure
244 1). Semi-dry transfer of proteins to a nitrocellulose membrane was performed using the Trans-
245 Blot® Turbo™ Transfer System. Following blocking for 1-hour in Tris-buffered saline Tween-
246 20 (TBS-T) containing 5% non-fat dried milk (NFDM), membranes were incubated overnight
247 with rabbit anti-phosphorylated or total antibodies: AMPKα, pThr172-AMPK, p44/42 MAPK,
248 pThr202/Tyr204-p44/42 MAPK, eNOS and pSer1177-eNOS, at a dilution of 1:500-1:4000 in
249 5% bovine serum albumin (BSA) made up in TBS-T (see Table 1). After overnight incubation,
250 the membranes were washed 3 times in TBS-T for 5 minutes and incubated for 1 hour in HRP-
251 conjugated anti-rabbit antibodies (Cell Signaling Technology, London, UK) at dilution of
252 1:5000-1:10,000, following optimisation. Proteins were visualised by enhanced
253 chemiluminescence (Thermo Fisher Scientific inc, Waltham, USA) and quantified by
254 densitometry (ChemiDoc™ MP imaging system, Bio-Rad Laboratories, Inc. CA, USA).
255 Analysed western blot images are presented in supplementary figure 2.
256
257
258

259 **Table 1.** List of antibodies and dilutions used.

Antibody	Primary Ab Dilution	Secondary Ab Dilution
AMPKα	1:1000	1:5000
pThr172-AMPK	1:1000	1:10,000
p44/42 MAPK	1:2000	1:10,000
pThr202/Tyr204-p44/42 MAPK	1:2000	1:10,000
eNOS	1:500	1:5000
pSer1177-eNOS	1:500	1:5000

260 All antibodies were purchased from Cell Signaling Technology.

261

262 **Statistical analysis**

263 One-way ANOVAs were employed to detect effects of EPI treatment. Two-way ANOVAs
264 were employed to detect main effects (e.g., dose, time or antimycin A) and potential significant
265 interactions between two main independent factors. Multiple comparisons between
266 experimental conditions were adjusted for multiple tests, using Dunnett's or Sidak's where
267 appropriate. All data are presented as mean±SEM and significance accepted when $P<0.05$.

268

269

270 **Results**

271 ***EPI does not cause vascular endothelial cell toxicity***

272 After 24 h EPI treatment (0.5-20 μM dose responses), there was a significant main effect
273 of dose on cell proliferation ($P=0.018$; Figure 1). However, multiple comparisons revealed no
274 significant difference between doses of EPI versus CTRL. Given that EPI did not cause cell
275 toxicity, and prior knowledge of physiologic EPI concentrations *in vivo* (up to 10 μM),
276 subsequent experiments were performed with doses of 5 and 10 μM.

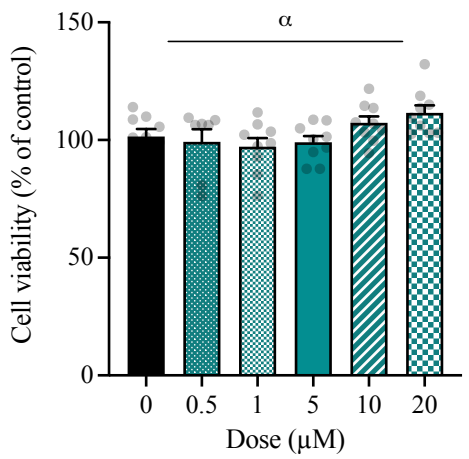


Figure 1. EPI does not cause vascular endothelial cell toxicity. HUVECs were treated with 0-20 μM EPI for 24 h. Data are means±SEM, representative of 3 independent repeats with 3 replicates of each condition. Statistical significance was tested for by one-way ANOVA and Dunnett's test for multiple comparisons. [α] Significant main effect of dose (P<0.05).

EPI dose-dependently modulates mitochondrial RONS production

Next, we assessed whether EPI, in the absence or presence of the complex III inhibitor, AA, impacted mitochondrial superoxide emission. There was a significant main effect of dose and AA on rates of MitoSOX oxidation (P<0.001), and a significant dose × AA interaction (P<0.001; Figure 2A). Post-hoc comparisons revealed that, in the absence of AA (-AA), 5 μM EPI significantly increased and 10 μM EPI decreased rates of MitoSOX oxidation compared to CTRL, respectively (CTRL: $8.1\times10^{-5}\pm0.2\times10^{-5}$; 5 μM EPI: $10.7\times10^{-5}\pm0.2\times10^{-5}$; 10 μM EPI: $3.8\times10^{-5}\pm0.2\times10^{-5}$ RFU/sec^{-1}/cell^{-1}; P<0.001). The associated raw traces of MitoSOX oxidation are displayed in Figure 2B. In the presence of AA (+AA), 5 μM EPI did not affect MitoSOX oxidation versus CTRL (5 μM EPI: $35.4\times10^{-5}\pm0.4\times10^{-5}$ vs. CTRL: $35.4\times10^{-5}\pm0.5\times10^{-5}$ RFU/sec^{-1}/cell^{-1}; Figure 2A). Whereas 10 μM EPI significantly increased rates of MitoSOX oxidation compared to CTRL (10 μM EPI: $44.4\times10^{-5}\pm0.6\times10^{-5}$ vs. CTRL: $35.4\times10^{-5}\pm0.5\times10^{-5}$ RFU/sec^{-1}/cell^{-1}; P<0.001). In contrast to mitochondrial ROS, cellular ROS production (not mitochondrial-specific) was not altered by EPI (Figure 2C).

After revealing that rates of mitochondrial ROS production were dose-dependently altered by EPI, we assessed whether EPI modified intracellular NO levels. There was a significant main effect of EPI dose on NO levels (P=0.003; Figure 2D). Whilst 5 μM EPI did not impact NO levels, 10 μM EPI significantly increased NO levels compared to CTRL conditions (10 μM EPI: $4.38\times10^{5}\pm0.43\times10^{5}$ vs. CTRL: $3.02\times10^{5}\pm0.18\times10^{5}$ AU; P=0.010).

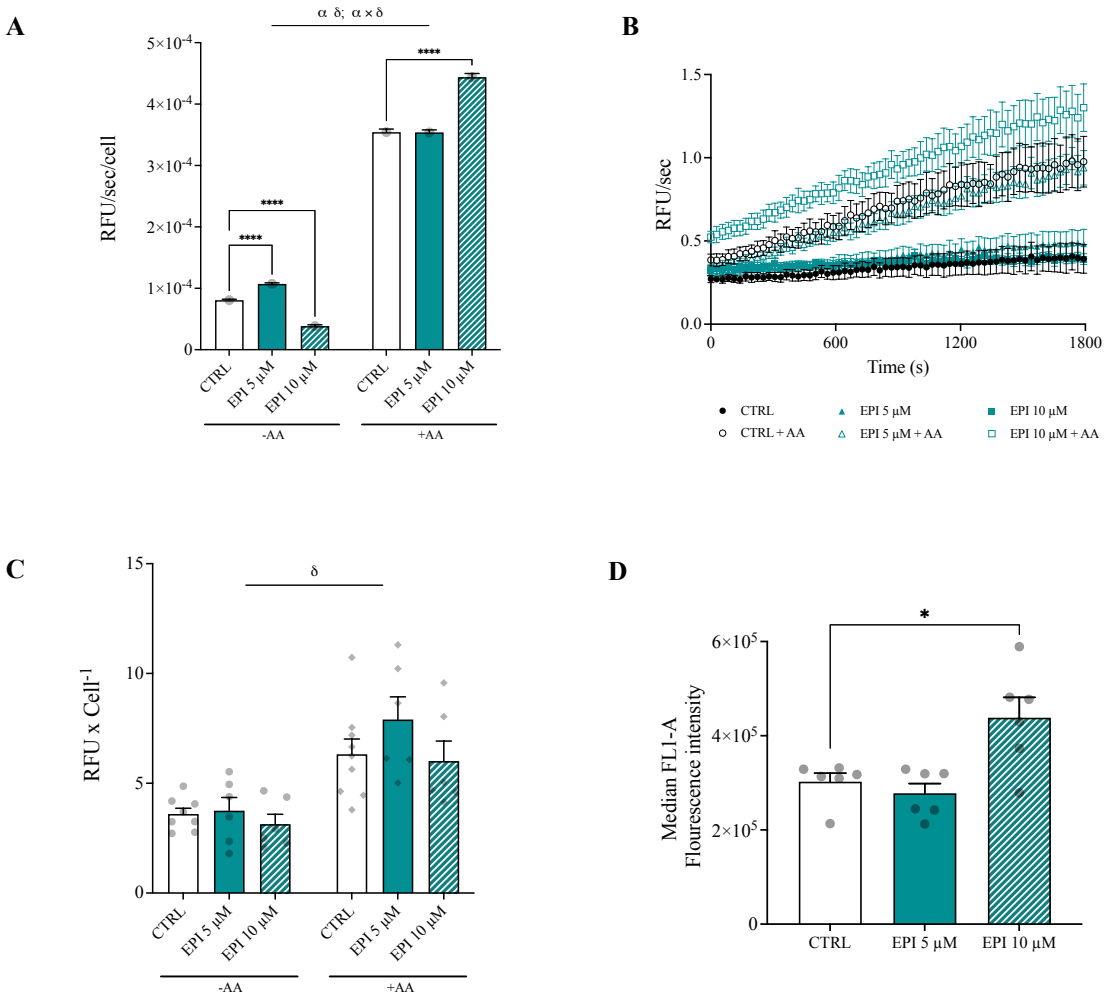


Figure 2. EPI dose-dependently impacts RONS production in vascular endothelial cells. A) MitoSOX oxidation rates in HUVECs after 24 h EPI treatment and 30 minutes incubation with or without antimycin A. B) Mean unnormalized MitoSOX oxidation rates measured in 30 second intervals over 30 minutes. C) CellROX oxidation in HUVECs after 24 h EPI treatment. D) DAF-FM oxidation in HUVECs after 24 h EPI treatment measured by flow cytometry. Data are means±SEM of three independent repeats with two replicates per treatment. Statistical significance was tested for by a two-way ANOVA, with dose and antimycin A as factors: $^{\alpha}$ Significant main effect of dose; $^{\delta}$ Significant main effect of AA (P<0.05). *P<0.05 and **** P<0.0001.

EPI dose-dependently impacts the expression of genes associated with energy metabolism in vascular endothelial cells

Next, experiments were performed to resolve whether EPI alters the expression of genes linked with mitochondrial function and the antioxidant response. Firstly, the expression of genes associated with mitochondrial function and remodelling were quantified. In the presence of EPI, there was a significant main effect of dose (P=0.018) and time (P=0.002) on dynamin-

316 related protein 1 (DRP1) expression (Figure 3A), but no significant dose × time interaction. At
317 48 h, 10 µM EPI increased DRP1 expression 2.2-fold compared to CTRL (*P*=0.010). There
318 was a significant main effect of dose on mitofusin-2 (MFN2) mRNA expression in cells treated
319 with EPI (*P*=0.035; Figure 3B). With 10 µM EPI, MFN2 expression increased 1.6-fold versus
320 CTRL (*P*=0.024). Parkin, PGC-1α, and transcription Factor A (TFAM) expression were not
321 changed by EPI treatment (supplementary Figure 3).
322
323 Genes associated with the antioxidant response were also quantified. There was no
324 effect of dose or time on catalase or eNOS expression. There was a significant main effect of
325 dose and time on NADPH oxidase 4 (NOX4) expression in EPI treated cells, respectively
326 (*P*=0.015 and *P*=0.006). A significant main effect of dose was found on nuclear factor-
327 erythroid factor 2-related factor 2 (NRF2) expression in the presence of EPI (*P*<0.001; Figure
328 3C). NRF2 mRNA abundance was increased 1.5-fold and 1.6-fold with 5 and 10 µM EPI over
329 48 h when compared to CTRL (*P*=0.015 and *P*=0.001, respectively). There was a significant
330 effect of time on superoxide dismutase 2 (SOD2) expression in EPI treated cells only
331 (*P*=0.024). Multiple comparisons revealed that SOD2 expression was increased 2.1-fold in the
332 presence of 10 µM EPI versus CTRL conditions (*P*=0.040; Figure 3D).
333
334

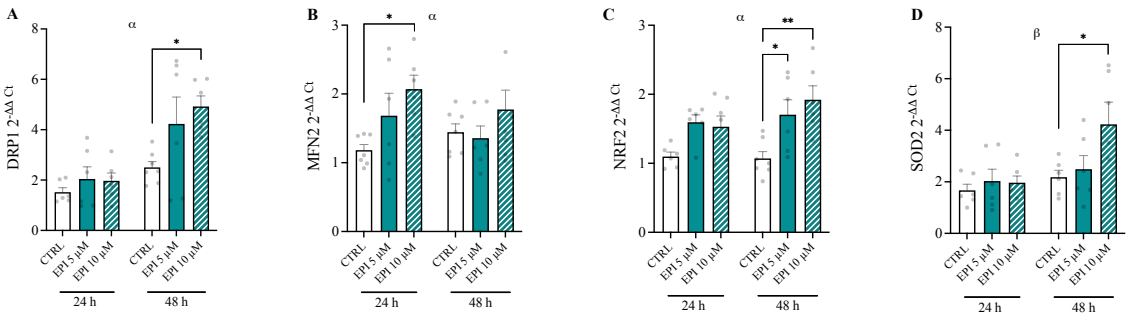


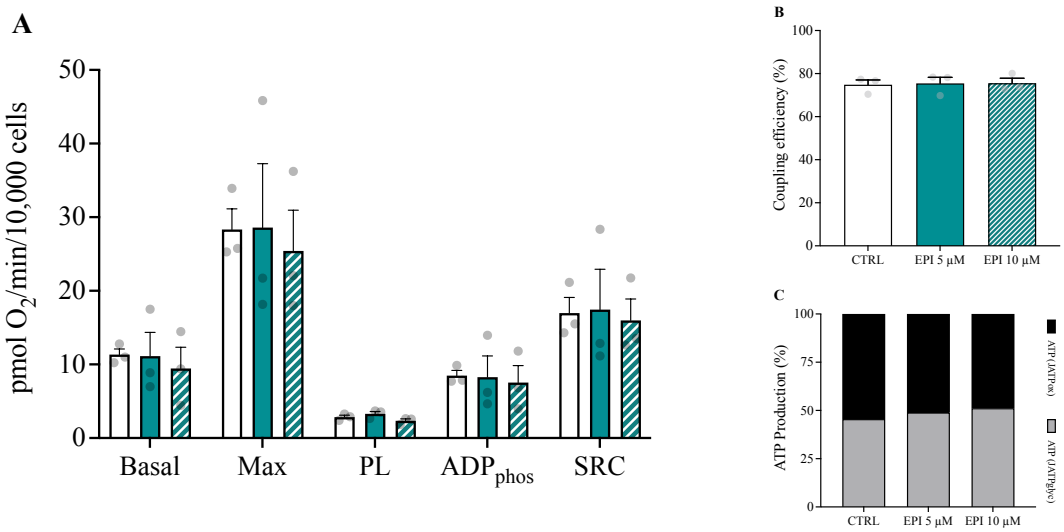
Figure 3. Gene expression responses following acute EPI treatment. HUVECs were treated with 0, 5 and 10 µM EPI over 48 h and lysed for analysis of gene expression. A) DRP1, B) MFN2, C) NRF2 and D) SOD2. Data are means±SEM from 3 independent experiments. Statistical significance was determined by a two-way ANOVA, with dose and time as factors. Multiple comparisons were performed by Dunnett's test to determine differences in gene expression between conditions. $^{\alpha}$ Main effect of dose; $^{\beta}$ main effect of time (*P*<0.05); **P*<0.05, ***P*<0.01.

335
336

337 ***EPI does not alter mitochondrial bioenergetics of vascular endothelial cells***

338 Having described that EPI influences RONS production and alters the expression of
339 genes linked with mitochondrial function, we tested whether EPI impacted vascular endothelial
340 cell bioenergetics. There was no significant main effect of EPI on rates of basal respiration,
341 maximal respiration, ADP phosphorylation, proton leak, spare respiratory capacity (%) or
342 coupling efficiency, regardless of dose (Figure 4A & 4B & 4D). There was no effect of EPI on
343 the relative contribution of ATP_{glyc} or ATP_{ox} to total ATP production (Figure 4C).

344



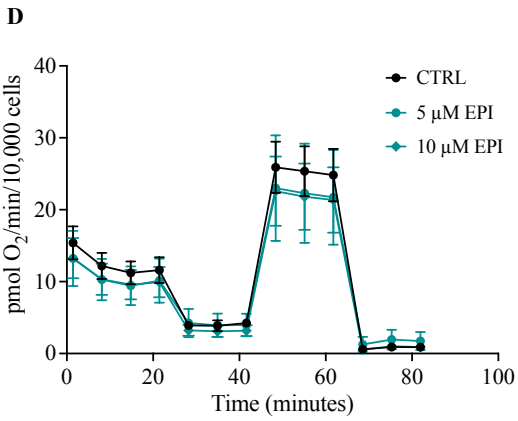


Figure 4. EPI does not directly affect mitochondrial bioenergetics. A) Mitochondrial bioenergetics of HUVECs following 24 h EPI treatment (0, 5 and 10 µM). B) Coupling efficiency of oxidative phosphorylation. C) Relative contribution of glycolytic (grey) and oxidative (black) ATP production to total ATP production rates. D) Representative trace of oxygen consumption rates during the mitochondrial stress test. Data from 3 independent experiments are normalised to cell number ($1x10^3$) and presented as mean±SEM. Max, maximal respiratory capacity; PL, proton leak; ADP_{phos}, ADP phosphorylation; SRC, spare respiratory capacity.

345

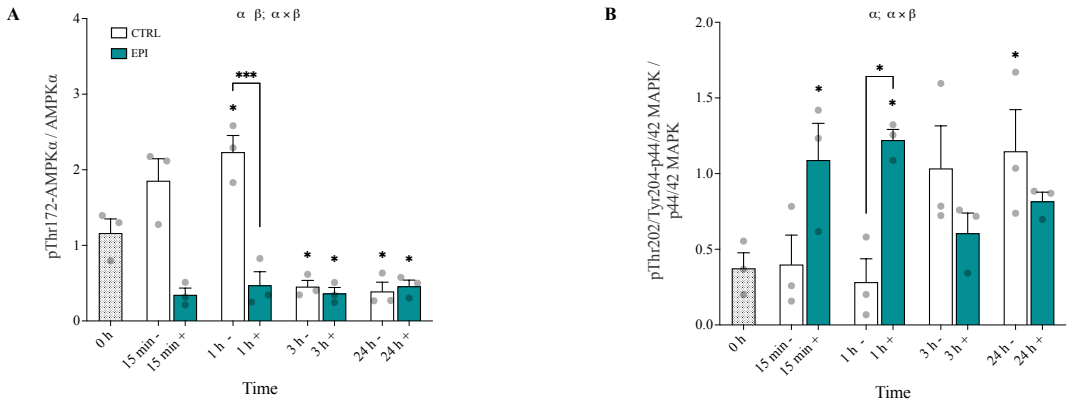
346

347 ***EPI rapidly and transiently promotes ERK1/2 phosphorylation, independent of AMPK***

348 To further probe how EPI might alter vascular endothelial transcription, we assessed
349 cell signalling responses. There was a significant main effect of treatment and time on AMPKα
350 phosphorylation, and a significant interaction was observed (P<0.001; Figure 5A). Multiple

comparisons revealed a significant increase in phosphorylation of AMPKα at Thr172 at 1 h versus 0 h (1 h: 2.24±0.22 vs. 0 h: 1.16±0.19 AU) under CTRL conditions (*P*=0.006), whereas there was no significant change at 1 h versus 0 h with EPI (1 h: 0.47±0.18 vs. 0 h: 1.16±0.19 AU; *P*=0.157). However, there was a significant reduction in AMPKα phosphorylation in the presence of EPI vs. CTRL at 15 min (EPI: 0.35±0.09 vs. CTRL: 1.86±0.29 AU; *P*<0.001) and 1 h (EPI: 0.47±0.18 vs. CTRL: 2.24±0.22 AU; *P*<0.001). From 3 hours, AMPKα phosphorylation was suppressed under both CTRL (3 h: 0.54±0.08 and 24 h: 0.39±0.12 AU vs. 0 h: 1.16±0.19 AU; *P*=0.046 and *P*=0.026, respectively) and EPI conditions compared to 0 h CTRL (3 h: 0.37±0.08 and 24 h: 0.46±0.08 AU vs. 0 h: 1.16±0.19 AU; *P*=0.021 and *P*=0.049, respectively).

Whilst ERK1/2 is not involved in the AMPK/eNOS pathway, ERK1/2 signalling may mediate the effects of EPI upon vascular endothelial cell adaptation. There was no main effect of treatment (*P*=0.141), but a significant main effect of time on ERK1/2 phosphorylation (*P*=0.039; Figure 5B). There was also a significant treatment × time interaction (*P*=0.003). Under CTRL conditions, despite a 2.7-fold increase in ERK1/2 phosphorylation at 3 h, ERK1/2 phosphorylation did not reach significance vs. 0 h until 24 hours post treatment (0 h: 0.38±0.10 vs. 24 h: 1.15±0.28 AU; *P*=0.022). By contrast, EPI significantly increased ERK1/2 phosphorylation at 15 minutes vs. 0 h (15 min: 1.09±0.24 vs. 0 h: 0.38±0.10 AU; *P*=0.035), which was retained at 1 hour (1 h: 1.22±0.07 vs. 0 h: 0.38±0.10 AU; *P*=0.011), before returning to baseline levels, suggesting a change in the temporal pattern of ERK1/2 activation because of EPI treatment. Indeed at 1-hour, EPI treatment resulted in a 4.3-fold increase in ERK1/2 phosphorylation vs. the 1-hour untreated CTRL (*P*=0.007).



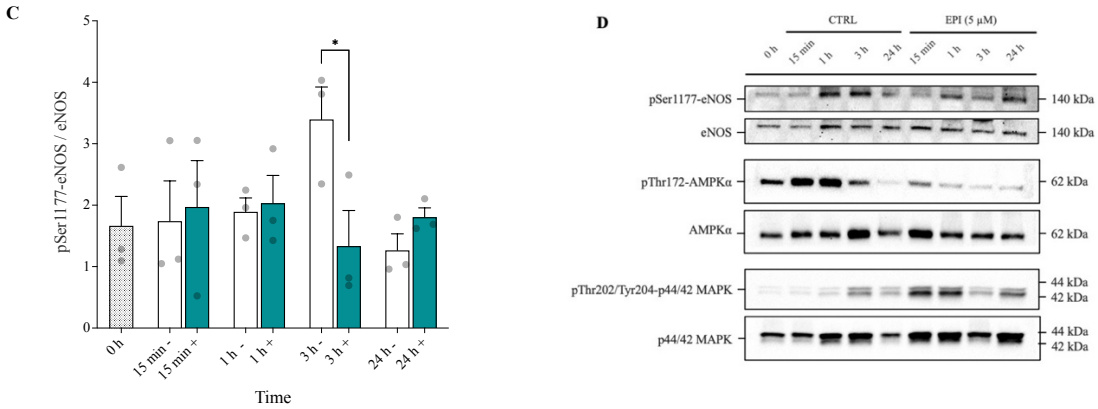


Figure 5. EPI rapidly and transiently activates ERK1/2 signalling whilst blunting AMPK phosphorylation. A) AMPKα phosphorylation at Thr172 in HUVECs in the absence (-; clear bars) or presence (+; green bars) of EPI. B) ERK1/2 phosphorylation at Thr202/Tyr204. C) eNOS phosphorylation at Ser1177. D) Representative images of n=3 independent experiments. Cell lysates were analysed by SDS-PAGE and western blotting with indicated antibodies. Data are expressed as means±SEM; **P*<0.05 and ****P*<0.001. $^{\alpha}$ Significant main effect of treatment; $^{\beta}$ significant main effect of time (*P*<0.05).

To help establish whether the increase in NO brought about by EPI was associated with eNOS signalling, phosphorylation of eNOS at Ser1177 was assessed. There was no main effect of treatment or time on eNOS phosphorylation (Figure 5C), and no treatment × time interaction (*P*=0.100). At 3 h, eNOS phosphorylation was ~60% higher under CTRL versus EPI conditions (CTRL: 0.38±0.10 vs EPI: 1.15±0.28 AU, *P*=0.038).

Discussion

Resolving EPI's mode of action using vascular endothelial cells as a model will help to establish its potential efficacy in mitigating vascular endothelial dysfunction. We tested the hypothesis that EPI would attenuate ROS production, augment NO bioavailability and enhance mitochondrial function of human vascular endothelial cells in culture. We demonstrated that physiologic EPI concentrations, dose-dependently, modulated RONS emission but did not directly impact mitochondrial respiration. Moreover, the influence of EPI on RONS emission was associated with the induction of increased NRF2 mRNA, which appears downstream of rapid and transient activation of ERK1/2 signalling. Taken together our findings expand our knowledge of EPI's mechanisms of action *in vitro* and support further research on EPI as a potential instigator of cell signalling and NRF2 activation *in vivo*.

393 ***EPI dose-dependently modulates RONS production***

394 We have demonstrated that EPI dose-dependently altered mitochondrial ROS production
395 in the absence of additional cell stress. Increased rates of mitochondrial ROS production,
396 reported in the presence of 5 μM EPI supports previous observations that 10 days EPI
397 supplementation increased superoxide production in mitochondria isolated from mouse heart
398 tissue (Panneerselvam et al., 2013). However, EPI has been demonstrated to increase the
399 abundance and/or activity of key antioxidant proteins like SOD2 and catalase (Bettaieb et al.,
400 2014; Calabró et al., 2016), and even to blunt hydrogen peroxide production from isolated brain
401 and heart mitochondria (Lagoa et al., 2011). In a similar way, we found that higher (10 μM)
402 EPI concentrations attenuated the rate of mitochondrial superoxide production, which may
403 have been facilitated by increased SOD2 mRNA expression. Clearly and importantly, the
404 divergent effects of EPI on mitochondrial ROS production suggest EPI's biological effects are
405 highly dose dependent. Future studies should investigate whether EPI alters the vascular
406 endothelial cell redox state and its potency relative to known mitochondrial antioxidants.
407 Another noteworthy observation of this study was that EPI did not rescue AA-induced
408 increases in mitochondrial ROS. Likewise, one recent study reported that 1 μM EPI did not
409 lower the production of mitochondrial superoxide in HUVECs after acute AA treatment (Keller
410 et al., 2020), suggesting limited efficacy for EPI in overcoming conditions associated with
411 elevated mitochondrial ROS production in the vascular endothelium of humans.

412

413 The potent stimulation of NO production by EPI is well documented in studies using cell,
414 human and rodent models (Loke et al., 2008; Moreno-Ulloa, Mendez-Luna, et al., 2015;
415 Ramirez-Sanchez et al., 2011, 2018; Schroeter et al., 2006), although not all studies have
416 reported such effects (Dower et al., 2015). Here, we demonstrated that EPI increased NO levels
417 of vascular endothelial cells, suggesting that EPI may be a promising strategy to combat
418 vascular endothelial dysfunction, although further *in vivo* studies are required. Previous studies
419 have attributed elevated NO bioavailability in the presence of EPI to increased phosphorylation
420 of eNOS at Ser177 (Carnevale et al., 2014; Ramirez-Sanchez et al., 2010, 2012, 2018; Ramírez-
421 Sánchez et al., 2016). Given that we and others demonstrated unaltered eNOS phosphorylation
422 in the presence of EPI (Keller et al., 2020), it is plausible that arginase inhibition is the potential
423 mechanism by which EPI increases NO production in HUVECs (Schnorr et al., 2008).
424 However, this may not be the case in arterial endothelial cells, where EPI has repeatedly been

425 shown to enhance eNOS Ser1177 phosphorylation (Ramirez-Sanchez et al., 2012, 2018;
426 Ramírez-Sánchez et al., 2016).

427

428 ***EPI augments the expression of genes linked with the antioxidant response and***
429 ***mitochondrial dynamics***

430 To better understand the mechanisms underlying EPI's effects, we measured the
431 transcription of genes associated with energy metabolism. Interestingly, EPI increased the
432 expression of genes DRP1 and MFN2, respectively involved in mitochondrial fission and
433 fusion. Despite these effects, EPI did not impact mitochondrial respiration, at least over 24
434 hours. Although mitochondrial dynamics can influence respiratory function (Chen et al., 2010;
435 Glancy et al., 2020), the lack of functional change in mitochondrial respiration could reflect a
436 discord between cellular mRNA responses and changes in protein abundance and/or function.
437 Regardless of dose, EPI significantly enhanced NRF2 mRNA expression. This observation
438 implies that the induction of NRF2 mRNA by EPI is independent of RONS production and
439 might be explained by alternate factors that control NRF2 activity, like phosphorylation status
440 (Robledinos-Antón et al., 2019). Although post-translational modifications of NRF2 were not
441 assessed in this study, flavonoids have been shown to promote NRF2 phosphorylation and
442 nuclear translocation (Lan et al., 2017; Shi et al., 2018), which would likely increase the
443 transcription of genes related to the antioxidant response, including NRF2 itself.

444

445 ***EPI does not impact mitochondrial respiration***

446 One important finding was that EPI does not impact mitochondrial respiration of HUVECs
447 in culture. Although mitochondria have been proposed as potential molecular targets of EPI
448 (Duluc et al., 2012), previous investigations into EPI's effects on mitochondrial respiration
449 have produced equivocal results. Some studies have demonstrated increased state 3 respiration
450 in rat beta cells following EPI (0.1-2.5 μM) supplementation (Kener et al., 2018; Rowley et al.,
451 2017), whilst others have reported inhibited or similar state 3 respiration rates with EPI,
452 depending on the substrates provided (Kopustinskiene et al., 2015). One recent study using
453 HUVECs as a model reported that 0.1 and 1 μM EPI treatment over 2 hours had negligible
454 impact on mitochondrial respiration (Keller et al., 2020). Taken together, our data suggest that
455 the therapeutic potential of EPI is not related to changes in mitochondrial respiration in vascular

456 endothelial cells, pointing to alternate potential mechanisms of action like cell signalling
457 activation (Fraga et al., 2018).

458

459 ***EPI rapidly and transiently activates ERK1/2 signalling***

460 The serine/threonine protein kinase AMPK is an important regulator of mitochondrial
461 adaptation (Herzig & Shaw, 2018). However, in our studies AMPK phosphorylation was
462 suppressed in the presence of EPI for up to 1 h and was without further impact for up to 24 h,
463 compared with controls. Supporting these findings, 2 h EPI treatment (1 μM) did not affect the
464 phosphorylation of AMPK in HUVECs (Keller et al., 2020). Although EPI is capable of
465 augmenting AMPK activity in liver and muscle tissue, and several cell types (Murase et al.,
466 2009; Papadimitriou et al., 2014; Si et al., 2011), it seems that AMPK activation is not
467 responsible for the therapeutic actions of EPI in HUVECs in culture. Together with the
468 negligible impact of EPI on mitochondrial respiration, the data suggest that EPI does not
469 directly alter vascular endothelial cell energy metabolism, *in vitro*.

470

471 Importantly and of novelty, we reported that ERK1/2 phosphorylation at Thr202/Tyr204
472 was rapidly and transiently increased by EPI in HUVECs (Figure 5). This finding supports
473 recent observations of increased ERK1/2 signalling after 0.1 μM EPI treatment in bovine
474 coronary artery endothelial cells, that may be associated with phosphorylation of CaMKII
475 (Moreno-Ulloa, Mendez-Luna, et al., 2015). How EPI promotes ERK1/2 phosphorylation in
476 vascular endothelial cells remains to be described, but current evidence suggests EPI activates
477 ERK1/2 by binding to the G-protein coupled estrogen receptor (GPER) on the cell membrane
478 (Moreno-Ulloa, Mendez-Luna, et al., 2015; Yang & Chan, 2018). Given the induction of NRF2
479 mRNA expression found in this study, it would be useful to determine if acute activation of
480 ERK1/2 signalling by EPI is a prerequisite for the induction of NRF2 activity.

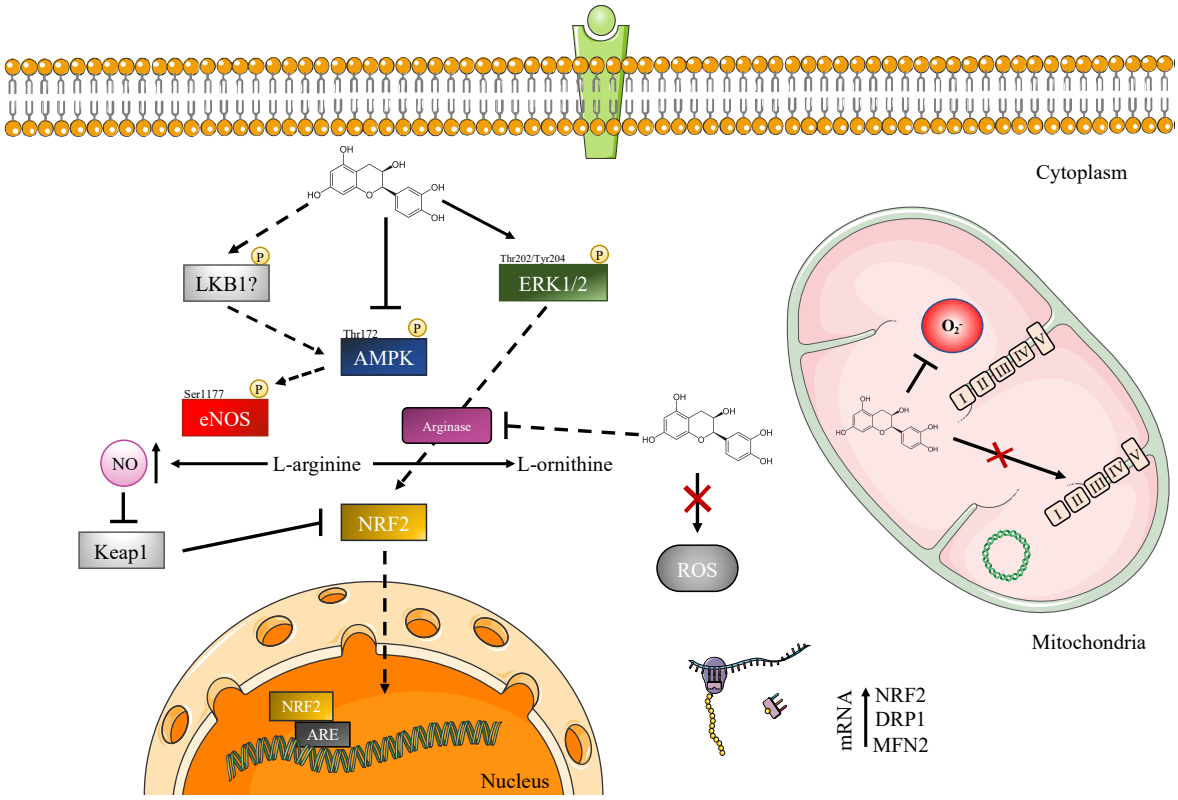
481

482 **Limitations**

483 We did not use metabolites of EPI that appear in circulation after ingestion of EPI-
484 containing foods or supplements *in vivo* (Ottaviani et al., 2016). Therefore, the present findings
485 may have limited translational potential. In our study we harnessed HUVECs to model the
486 vascular endothelial cell, and given that these cells are venous in nature, their physiology may
487 not well reflect the arterial vasculature or microcirculation where EPI potentially exerts its
488 beneficial effects. Further, not all assays were performed in the presence of additional cell

489 stress. Thus, caution should be taken when translating our findings to human populations with
490 disease. Finally, post-translational modifications of NRF2, which are critical for regulating
491 NRF2's activity, were not measured in this study.
492
493 **Conclusion**

494 We demonstrate that physiologic EPI concentrations do not impact mitochondrial
495 respiration but do modulate reactive oxygen and nitrogen species production and the signalling
496 and transcriptional activities of vascular endothelial cells *in vitro*. EPI's dose-dependent
497 alteration of reactive oxygen and nitrogen species production occurred in parallel with
498 enhanced and transient ERK1/2 signalling, and the induction of NRF2 mRNA (Figure 6). The
499 fact that EPI enhanced NRF2 mRNA expression regardless of dose, implies that alterations in
500 reactive oxygen and nitrogen species production alone were not solely responsible. Further
501 research will help clarify the precise way in which EPI promotes ERK1/2 signalling and NRF2
502 activity, and its relevance to vascular endothelial health *in vivo*.
503



504 **Figure 6.** Schematic of the potential mechanisms by which EPI exerts its biological effects in vascular
505 endothelial cells. Solid arrows/lines signify EPI's mode of action demonstrated in this study. Dashed
506 arrows represent potential activity of EPI not examined in this study but reported previously.
507

Availability of data and material

The datasets generated and analysed during this study are available from the corresponding author upon request.

Conflict of interest

Daniel G. Sadler, Jonathan Barlow, Helen Jones, Dick H. J. Thijssen and Claire E. Stewart had no conflict of interest associated with this manuscript. Richard Draijer is employed by Unilever.

Funding

The study was supported by funding received from the Biotechnology and Biological Sciences Research Council (BBSRC) and Unilever.

Authors' contributions

DGS and CES conceived the study and designed experiments. DGS and JB designed the respiration experiments using the Seahorse XFe96 Analyzer. DGS collected and analysed the data, DGS, JB, RD, HJ, DHJT and CES interpreted the data. DGS and CES wrote the manuscript and all authors revised it critically. All authors provided final approval of the version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All people designated as authors qualify for authorship, and all those who qualify for authorship are listed. CES is the guarantor for the work and/or conduct of the study, had full access to all the data in the study and takes responsibility for the integrity of data and the accuracy of the data analysis, and controlled the decision to publish.

Acknowledgements

The authors acknowledge use of the Mitochondrial Profiling Centre, a core resource supported by the University of Birmingham. This study was supported by the BBSRC funded Industrial CASE (iCASE) award (BB/P504385/1).

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