1 **Phenolic metabolites of anthocyanins modulate mechanisms of endothelial function**

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ABSTRACT

Anthocyanins are reported to have vascular bioactivity, however their mechanisms of action are largely unknown. Evidence suggests that anthocyanins modulate endothelial function, potentially by increasing nitric oxide (NO) synthesis, or enhancing NO bioavailability. This study compared the activity of cyanidin-3-glucoside, its degradation product protocatechuic acid and phase II metabolite, vanillic acid. Production of NO and superoxide, and expression of endothelial NO synthase (eNOS), NADPH oxidase (NOX) and haem oxygenase-1 (HO-1), was established in human vascular cell models. Nitric oxide levels were not modulated by the treatments, although eNOS was upregulated by cyanidin-3-glucoside, and superoxide production was decreased by both phenolic acids. Vanillic acid upregulated $p22^{phox}$ mRNA but did not alter NOX protein expression, although trends were observed for $p47^{phox}$ downregulation and HO-1 upregulation. Anthocyanin metabolites may therefore modulate vascular reactivity by inducing HO-1 and modulating NOX activity, resulting in reduced superoxide production and improved NO bioavailability.

INTRODUCTION

Epidemiological evidence suggests that higher consumption of anthocyanins, a sub-class of the flavonoid family of polyphenols [1], is inversely associated with risk of hypertension [2] and cardiovascular disease mortality [3, 4]. In recent randomised controlled trials, 12-week consumption of anthocyanins (320 mg/day) was associated with enhanced endothelial function in hypercholesterolaemic individuals [5], while acute consumption (724 mg) elicited a dose-dependent (biphasic) increase in endothelial-dependent vasodilation [6]. Mechanistic studies suggest that anthocyanins may act to enhance vascular function through modulating levels of nitric oxide (NO) [7, 8]. The reduced bioavailability of endothelial-derived NO is critical in the development of atherosclerosis [9]; and a loss of NO in vascular pathologies is mediated by reaction with superoxide anion (O_2^-) [10, 11] generated by vascular NADPH oxidase (NOX) enzymes [9, 12] which constitute a major source of reactive oxygen species in the vasculature. Anthocyanins have been reported to elevate the expression of the cytoprotective enzyme haem oxygenase-1 (HO-1) in human vascular endothelial cells [13] and upregulation of HO-1 with subsequent inhibition of NOX activity has been described in cell culture [14] and animal models [15]. Therefore, anthocyanins could potentially improve endothelial function, by increasing the bioavailability of endothelial-derived NO and thus improving vascular homeostasis, by decreasing endothelial NOX activity and O_2^- levels as a result of HO-1 induction.

Anthocyanins are generally reported to have a low relative bioavailability [16, 17], suggesting their bioactivity is mediated by their metabolites, which exist in the systemic circulation at much higher concentrations [17, 18] than their precursor structures. However, most previous studies have explored the activity of anthocyanins *in vitro* as unmetabolised precursor structures, whilst very few have examined the activity of their phenolic metabolites. The aim of the present study was therefore to compare the bioactivity of a parent anthocyanin with its physiologically relevant phenolic acid derivatives, to establish if anthocyanin metabolites share a common or have a differential biological

64 activity to their unmetabolised structures. Cyanidin-3-glucoside (Figure 1A) was chosen for this study

65 as Czank *et al* (2013) have recently reported the systemic concentrations of its metabolites in humans

66 using an isotope tracer study design [17, 19]. Of the 24 isotope-labelled metabolites identified, the

67 phenolic acid degradation product protocatechuic acid (Figure 1B), and its mono-*O*-methylated

68 metabolite vanillic acid (Figure 1C), were selected for comparison with the parent anthocyanin, as

69 they share structural similarities with the known vasoactive compound apocynin [2].

70

71 Bioactivity was assessed by screening physiologically relevant concentrations of the treatments (at

72 0.1, 1, 10 µM [17]) for effects on eNOS expression and activity, and angiotensin II-stimulated

73 superoxide production, in human umbilical vein endothelial cells (HUVEC). Vanillic acid was

74 ultimately selected to explore mechanisms potentially underlying the observed activity, by examining

75 the modulation of NOX isoforms (and subunits) and HO-1, using both HUVEC and human coronary

76 artery endothelial cells (HCAEC).

77

78   **MATERIALS AND METHODS**

79

80 **Standards and reagents.** Cyanidin-3-glucoside was purchased from Extrasynthese (Genay Cedex,

81 France); VAS2870 from Enzo Life Sciences (Exeter, U.K.); and all other reagents were from

82 Sigma-Aldrich (Poole, U.K.) unless otherwise noted. Stock solutions were prepared in dimethyl

83 sulphoxide (DMSO) and stored at -80°C. Foetal bovine serum (FBS, heat-inactivated) was

84 purchased from Biosera (Ringmer, UK) and tumour necrosis factor-alpha (TNF-α), TRIzol[®]

85 reagent, and SuperScript[®] II Reverse Transcriptase were obtained from Life Technologies (Paisley,

86 UK).

87

88 Precision 2x real-time PCR MasterMix with SYBR[®]Green was obtained from PrimerDesign Ltd

89 (Southampton, UK). Custom primer sets for human NOX2, NOX4, p22[phox], p47[phox] and p67[phox]

90 were supplied by PrimerDesign Ltd, and custom primers for human HO-1 (HMOX-1) by Life

91 Technologies. Primer sequences are provided as Supporting Information (Table S1).

92

93 NuPAGE sample reducing agent and LDS sample buffer were purchased from Life Technologies,

94 and Precision Plus Protein Dual Colour standards from Bio-Rad Laboratories, Inc (Hemel

95 Hempstead, UK).

96

97 **Cell culture.** Early passage, pooled HUVEC were purchased from TCS Cellworks (Buckingham,

98 UK) and used between passages 2 to 4. Cells were routinely cultured in large vessel endothelial cell

99 growth medium (TCS CellWorks) at 37°C and 5% CO_2. HUVEC were sub-cultured using 0.025%

100 trypsin and 0.01% ethylenediaminetetraacetic acid (EDTA) (TCS CellWorks).

101

102 Cryo-preserved, second passage, single donor HCAEC were purchased from PromoCell GmbH

103 (Heidelberg, Germany) and used between passages 3 to 6. Cells were routinely cultured in

104 endothelial cell medium MV (PromoCell GmbH) at 37°C and 5% CO_2. HCAEC were sub-cultured

105 using 0.04% trypsin and 0.03% EDTA (PromoCell GmbH).

106

107 **Cytotoxicity assay.** The maximal level of flavonoids and flavonoid metabolites reported in the

108 systemic circulation is generally below 10 µM [1], hence this concentration was the maximum *in vitro*

109 concentration utilised in the present study. Cell viability following exposure to 10 µM of treatment

110 compounds was determined using cell proliferation reagent WST-1 (Roche Applied Science,

111 Burgess Hill, U.K.) in accordance with the manufacturer's protocol. The assay was conducted using

112 fibronectin-coated microplates seeded with HUVECs at a density of ~10,000 cells/well, and

113 subsequently grown to confluence as determined by light microscopy. After incubation with

114 treatment compounds for 24 h, 10 µl WST-1 reagent was added to each well, and plates were

incubated for a further 4 h. Absorbance was measured at 440 - 450 nm using a microplate reader

[Fluostar/Polarstar Optima, BMG Labtech (Aylesbury, U.K.)].

Nitrite/nitrate assay and eNOS enzyme-linked immunosorbent assay (ELISA). Fibronectin-coated 24-well plates were seeded with HUVECs at a density of ~30,000 cells/well, and cells grown to confluence. Cells were then cultured in the absence or presence of treatment compounds (0.1, 1, or 10 µM) for 24 h; after which supernatants were removed and stored at -80°C. Cells were washed once with warm phosphate-buffered saline (PBS), and then harvested in trypsin/EDTA and trypsin-blocking solution. Cell suspensions were stored at -80°C until lysis. Nitric oxide production was assessed using a colourimetric microplate assay (Cayman Chemical Company Nitrate/Nitrite Colourimetric Assay Kit from Cambridge Bioscience, Cambridge, U.K.) according to the manufacturer's instructions. The average intra-assay coefficient of variation (CV) was 6.63% ± 1.10% (mean ± SD, n=3) and the inter-assay CV was 2.38% (n=3). Quantification of eNOS in HUVEC lysates was performed with the Quantikine Human eNOS Immunoassay (R&D Systems, Abingdon, U.K.) according to the manufacturer's instructions. The average intra-assay CV was 4.67% ± 1.86% (mean ± SD, n=3) and the inter-assay CV was 6.10% (n=3).

Stimulated superoxide production assay. Superoxide production was assessed using a modified cytochrome c assay [20, 21]. The modified assay utilised fibronectin-coated 24-well plates seeded with HUVECs at a density of ~50,000 cells/well, and grown to confluence; after which cells were washed once with warm Medium 199 (supplemented with 2% FBS) and incubated for 16-18 h. Cells were then washed once with warm PBS, and incubated for 6 h in supplemented phenol-red free Medium 199 (Invitrogen, Paisley, U.K.) with 0.1 µM angiotensin II, 30 µM ferricytochrome c, and 0.1, 1 or 10 µM of the treatment compounds or 5 µM VAS2870 (selective NOX inhibitor [22]); in the presence or absence of 65 units superoxide dismutase (SOD). Aliquots of cell supernatants were

140 subsequently transferred to a 96-well microplate for measurement of absorbance at 550 nm. Culture

141 plates were frozen at -80°C for protein extraction.

142

143 **Direct cytochrome c reduction.** Direct reduction of cytochrome c was assessed by co-incubation

144 of treatment compounds in cell-free extracts at concentrations of 2, 20, 200 and 2000 µM with 20

145 µM cytochrome c in PBS at 37 °C, as described previously for catechols and quinols [23]. The

146 spontaneous reduction of cytochrome c was monitored kinetically at 550 nm over 2 h. Cytochrome

147 c reduction was quantified using the millimolar extinction coefficient for reduced cytochrome c

148 (29.5 mM.cm^{-1}).

149

150 **Superoxide production.** Cell-free superoxide production by xanthine/xanthine oxidase was

151 measured using a previously described method [21]. Briefly, 200 µM of cytochrome c, 0.1 U/ml

152 xanthine oxidase and 200 µM xanthine was added to 1, 10, 100 and 1000 µM of the treatment

153 compounds in 50 mM sodium phosphate buffer (pH 7.4). The reaction kinetics of cytochrome c was

154 monitored at 550 nm at 25°C over 15 min (to reaction plateau). Superoxide generated was

155 determined by subtracting the rate of cytochrome c reduction (increase in absorbance at 550 nm) in

156 the presence of SOD versus parallel incubations in the absence of SOD.

157

158 **Stimulated NOX isoform/subunit gene expression assay.** Twenty-four well plates (SPL Life

159 Sciences) coated with fibronectin were seeded with HUVEC at a density of ~30,000 cells/well, and

160 the cells grown to confluence. Culture medium was then aspirated, and the cells were incubated for

161 16 - 18 h in M199 supplemented with 2% FBS. Thereafter, the cells were incubated for 4 h in

162 supplemented M199 alone (basal), or media with 0.1 µM angiotensin II in the presence or absence

163 of 0.1, 1 or 10 µM vanillic acid. After incubation, the plates were either frozen at -80°C or used for

164 RNA extraction.

165

Stimulated p47^phox protein expression assay. Six-well plates (SPL Life Sciences) coated with fibronectin were seeded with HUVEC or HCAEC at a density of 100,000 cells/well, and cells were grown to confluence. Culture medium was then aspirated, and the cells were incubated for 16 - 18 h in M199 supplemented with 2% FBS (HUVEC only). Cells were subsequently incubated for 5 h in supplemented M199 alone (basal), or media with 20 ng/ml TNF-α in the presence or absence of 0.1, 1, or 10 µM vanillic acid. After incubation, media was aspirated from all wells, and the plates frozen at -80°C until protein extraction.

Endothelial HO-1 expression assay. Expression of HO-1 mRNA/protein was assessed using fibronectin-coated 6-well plates (SPL Life Sciences) seeded with HUVEC or HCAEC at a density of ~100,000 cells/well, and grown to ~70% confluence. Culture medium was then aspirated, and the cells were incubated for 6 h in supplemented culture medium alone (basal), or media with 10 ng/ml phorbol 12-myristate 13-acetate (PMA, positive control), vehicle control (0.005% DMSO; HUVEC only), or 0.1, 1 or 10 µM vanillic acid. After incubation, media was aspirated from all wells and the plates either frozen at -80°C, or used for RNA or protein extraction.

Reverse transcription – quantitative polymerase chain reaction. RNA was extracted from cells using TRIzol® reagent, according to the manufacturer's instructions; and 1 µg of each sample utilised in a reverse transcription reaction with SuperScript® II. Analysis of gene expression was performed using the Applied Biosystems 7500 Real time PCR System (Life Technologies; 7500 software version 2.0) with SYBR®Green detection. Typically, 25 ng of cDNA was amplified with 300 nM of the appropriate primer set. Following enzyme activation at 95°C for 10 minutes, 50 cycles of denaturation (15 seconds at 95°C) and data collection (60 seconds at 60°C) were performed. Relative changes in gene expression were quantified using the comparative C_t method [24]. Optimal stably expressed human reference genes for normalisation of C_t data were identified

191 using a geNormPLUS kit with primer sets for six genes (PPIA, PRDM4, UBE2D2, UBE4A,

192 TWY1, VIPAS39) supplied by PrimerDesign Ltd.

193

194 **Immunoblot analysis of endothelial NOX isoform/subunit and HO-1 expression.** Cells were

195 harvested and lysed 1% IGEPAL® (octylphenoxy polyethoxyethanol, CA-630), 150 mM NaCl, 20

196 mM Tris and 10% glycerol (pH 8.0), supplemented with protease inhibitors (Roche Complete

197 Protease Inhibitor Cocktail). Plates were incubated with lysis buffer for 0.5 h at 4°C, and recovered

198 solutions subject to cell disruption by oscillation (50 Hz for 5 minutes with Qiagen TissueLyser

199 LT). After centrifugation at 13,000 rpm (15 minutes at 4°C) the protein content of the supernatants

200 was assayed using the Pierce BCA Protein Assay Kit (Fisher Scientific U.K. Ltd, Loughborough,

201 U.K.) according to manufacturer's instructions.

202

203 For SDS-PAGE, cell lysates were reduced using 50 mM dithiothreitol. Briefly, 15 - 25 µg of protein

204 was loaded onto a 4% polyacrylamide stacking gel, and separated across a 10% resolving gel (at 25

205 mA for 1 h) prior to semi-dry transfer to Immobilon-FL PVDF membrane (Millipore, Watford, UK)

206 at 200 mA for 1.5 h. Membranes were blocked for 1 h at room temperature and incubated overnight

207 (at 4°C) with chicken polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH,

208 Millipore) or goat polyclonal anti-actin (Santa Cruz Biotechnology, Inc., California, U.S.); and

209 either rabbit polyclonal anti-gp91phox (Millipore), rabbit polyclonal anti-NOX4 (Abcam,

210 Cambridge, U.K.), rabbit polyclonal anti-p47^{phox} (Santa Cruz Biotechnology, Inc. or Abcam), or

211 rabbit polyclonal anti-HO-1 (Abcam). Membranes were then washed prior to incubation with either

212 donkey anti-chicken IgG (IR dye 680 LT) or donkey anti-goat IgG (IR dye 680) and goat anti-rabbit

213 IgG (IR dye 800 CW) (Li-Cor, Cambridge, U.K.) for 1 h at room temperature. Membranes were

214 washed and subsequently imaged and quantified using an Odyssey Infrared Imaging System (Li-

215 Cor; Application Software version 3.0.21).

216

Statistical analysis. Analysis of variance (ANOVA) with LSD post-hoc test was performed using SPSS software (IBM, New York, USA) version 18 for Windows. Significance was determined at the 5% level relative to basal or the assay control. Three biological replicates for each control or treatment were utilised for analysis unless otherwise noted. Means of biological replicates were represented graphically, with error bars denoting standard deviation from the mean. For each control or treatment in the stimulated superoxide production assay, mean absorbance ratio (550 nm/620 nm) in the presence of SOD (n=3) was subtracted from individual absorbance ratio values in the absence of SOD (n=3), to correct for cytochrome c reduction owing to generated superoxide. Mean SOD-corrected absorbance ratio for each treatment was then represented graphically as percentage of that for the angiotensin II control.

RESULTS

Endothelial cytotoxicity. All treatments were screened for cytotoxicity at a concentration of 10 µM; representing the highest concentration used in subsequent bioactivity investigations. No significant effects ($p > 0.05$) on endothelial cell viability were observed following a 24 h incubation of HUVECs with 10 µM of any treatment compound (data not shown).

Endothelial NO production. Cyanidin-3-glucoside, and the phenolic derivatives protocatechuic acid and vanillic acid, did not significantly alter endothelial NO production as measured by levels of NO decomposition products (nitrite & nitrate) in HUVEC supernatants (Supporting Information, Figure S1). Resveratrol (100 µM) and PMA (10 nM) were used as positive controls to confirm the sensitivity of the assay, and elicited significant increases in total nitrite and nitrate (> 270% above basal levels).

eNOS expression in cultured HUVEC. Significant upregulation of eNOS ($p < 0.001$) was observed following 24 h incubation of HUVECs with 0.1, 1 & 10 µM cyanidin-3-glucoside (~4 - 7 fold increase; Figure 2A). No significant alteration in eNOS levels was observed at any concentration of protocatechuic acid (Figure 2B) or vanillic acid (Figure 2C).

Stimulated endothelial superoxide production. The generation of superoxide anion was confirmed using a previously reported NOX inhibitor VAS2870 [22], in the presence and absence of angiotensin II, where co-incubation with VAS2870 significantly reduced superoxide production ($p < 0.01$) (Supporting Information, Figure S2).

Significantly elevated superoxide levels were detected following incubation of cells with 0.1 µM and 1 µM cyanidin-3-glucoside (~3 fold increase; Figure 3A), relative to the angiotensin II-stimulated control. A significant decrease in superoxide ($p < 0.001$) was induced by protocatechuic acid, at 10 µM (~5 fold decrease; Figure 3B), whilst vanillic acid elicited statistically significant reductions ($p < 0.05$) in superoxide at all concentrations examined (~2 fold decrease at 1 µM; Figure 3C). A linear dose-response relationship was not evident for any treatment. Control experiments were conducted to exclude possible confounding through direct reduction of cytochrome c, or scavenging of NOX-derived superoxide radicals, by the treatments. There was no significant impact on cytochrome c reduction (≤ 0.02 nmole.h^{-1}) or superoxide levels (75-106% of control) during incubations with treatment compounds at concentrations up to 10 µM in cell-free systems.

Stimulated endothelial gene expression of NOX isoforms and subunits. Vanillic acid was examined for effects on stimulated endothelial gene expression of the NOX2 and NOX4 isoforms, and the associated subunits p22^{phox}, p47^{phox}, and p67^{phox}. Real time PCR melt curve data indicated non-specific amplification for NOX2, p47^{phox}, and p67^{phox} primer sets (data not shown); therefore

268 relative quantification of gene expression was performed for NOX4 and p22^{phox} only. Co-incubation

269 of HUVEC with angiotensin II and vanillic acid (0.1, 1 and 10 μM) for four hours elicited no

270 significant differences in NOX4 mRNA compared to the angiotensin II control ($p > 0.05$; Figure

271 4A). A statistically significant ($p < 0.05$) upregulation of p22^{phox} mRNA levels was observed with

272 vanillic acid at 0.1 μM and 1 μM (Figure 4B).

273

274 **Stimulated endothelial protein expression of NOX isoforms and subunits.** The modulation of

275 NOX2 and NOX4 isoforms by vanillic acid was further characterised at the protein level. Following

276 angiotensin II stimulation, weak immunoreactive bands corresponding to NOX2 were visualised by

277 immunoblotting of endothelial lysates, such that quantification of bands by densitometry was not

278 possible (data not shown). By contrast, angiotensin II-induced upregulation of NOX4 expression

279 was observed, although this was not significantly modulated ($p > 0.05$) by co-incubation of cells

280 with angiotensin II and vanillic acid at any concentration examined (0.1-10 μM) (Figure 5).

281

282 The effect of vanillic acid on stimulated protein expression of the key NOX2 subunit p47^{phox} was

283 also investigated as a possible mechanism of NOX inhibition. Following co-incubation of HUVEC

284 with 20 ng/ml TNF-α and increasing concentrations of vanillic acid (0.1, 1, or 10 μM) for five

285 hours, a trend towards decreased p47^{phox} expression was observed relative to TNF-α alone (Figure

286 6A), although these changes were not statistically significant ($p = 0.06$ at 1 μM and 10 μM vanillic

287 acid). A confirmatory experiment was conducted using the HCAEC model, but here no effect was

288 detected on TNF-α stimulated p47^{phox} expression ($p > 0.05$; Figure 6B).

289

290 **Endothelial HO-1 expression.** Modulation of HO-1 expression by vanillic acid was examined as a

291 putative indirect mechanism of NOX inhibition. Incubation of HUVEC with the protein kinase C

292 activator PMA (at 10 ng/ml) for six hours induced a significant increase in basal HO-1 mRNA

293 levels ($p < 0.001$ versus vehicle control; Figure 7). Vanillic acid, at concentrations of 0.1, 1 and 10

μM, elicited a concentration-dependent elevation in endothelial HO-1 mRNA (~1.6-fold versus unstimulated cells with 10 μM vanillic acid), with a trend towards significance compared to vehicle control (p = 0.1) at 10 μM (Figure 7). Trends towards elevated expression of HO-1 protein in HUVEC were also observed following a six hour incubation with PMA (~1.9-fold increase versus unstimulated cells) or vanillic acid (~1.8-fold increase at 1 μM vanillic acid) relative to the vehicle control (p = 0.07 and p = 0.1 respectively; Figure 8A). Since the vehicle control (0.005% DMSO) also appeared to upregulate HO-1 protein, vanillic acid was prepared directly in aqueous solution for use in the HCAEC model, to exclude any vehicle-related effects. Expression of HO-1 protein was slightly elevated following a 6 hour incubation with PMA in the HCAEC model (~1.2-fold increase versus unstimulated cells; Figure 8B). An apparent upregulation of HO-1 protein was observed at all concentrations of vanillic acid tested (Figure 8B), with a trend towards significance at 1 μM (~1.8-fold increase; p = 0.07) relative to basal.

DISCUSSION

The low bioavailability of parent anthocyanins suggests their bioactivity *in vivo* is mediated by phenolic metabolites, which have recently been reported as the main circulating species following anthocyanin consumption [17-19]. Here, we report that phenolic metabolites appear to modulate vascular endothelial cell function through alternative mechanisms to those previously described for parent anthocyanins. In the present study, the parent anthocyanin increased eNOS expression, whereas phenolic derivatives had no effect. However, these metabolites elicited reductions in superoxide production, which could subsequently decrease scavenging of NO. Recent studies by Czank *et al* (2013) and de Ferrars *et al* (2014) have confirmed a physiologically appropriate range for anthocyanin metabolites in humans of 0.1 - 10 μM [17-19] and the present study assessed potential mechanisms of activity at these concentrations. Indeed, following consumption of 500 mg ^{13}C-labelled cyanidin-3-glucoside, a serum C_{max} of 1.85 μM has been reported for vanillic acid, with 1

µM concentrations persisting for up to 24 hours [19]; suggesting phenolic derivatives of anthocyanins may be present in the systemic circulation at low micromolar levels for at least 18-24 hours after ingestion of parent anthocyanins. Based on our findings, physiologically relevant levels of anthocyanin metabolites are likely to act indirectly to maintain vascular homeostasis, through induction of HO-1 and decreased endothelial superoxide generation, as opposed to directly stimulating eNOS activity and NO production.

Endothelium-derived nitric oxide is a key component of vascular homeostasis [11] and prior *in vitro* studies have described both upregulation and activation of the eNOS enzyme by anthocyanins (including cyanidin-3-glucoside) in cultured endothelial cells at concentrations ranging from 0.001 µM up to 250 µM [7, 8, 13]. Interestingly, in the present study we observed differential bioactivity for cyanidin-3-glucoside relative to its phenolic acid derivatives. Specifically, no treatment compounds significantly modulated endothelial NO production, however, cyanidin-3-glucoside significantly upregulated eNOS protein levels, while the phenolic derivatives remained inactive (Figure 2).

NOX enzymes represent a major source of reactive oxygen species in the vasculature [12, 25] and anthocyanin metabolites could act as endothelial NOX inhibitors, based on previously reported structure-activity studies [21]. In the current study, differential bioactivity was again observed for cyanidin-3-glucoside relative to its phenolic acid derivatives. A significant elevation in superoxide levels was elicited by cyanidin-3-glucoside; and in contrast, both protocatechuic acid and vanillic acid significantly reduced superoxide levels (Figure 3). Whilst previous reports have described superoxide scavenging [26] and direct cytochrome c reduction [27] elicited by flavonoids, such activity is reported at much higher concentrations (~40 - 100 µM) than those used in the current investigation ($\leq$ 10µM), and control experiments in cell-free incubations confirmed negligible superoxide scavenging or direct cytochrome c reduction by the treatments. High concentrations of phenolic compounds in culture medium have also been reported to result in the generation of

346   hydrogen peroxide [28], which could potentially interfere with assay methodologies; however, only

347   minimal hydrogen peroxide formation has previously been reported for delphinidin (at 10 µM) [28]

348   which is one of the most reactive anthocyanins [29]. Therefore, ==data from cell-free experiments in the==

349   ==current study indicate that the treatment compounds do not directly scavenge superoxide radicals, as==

350   ==negligible activity was observed, and the present findings likely reflect reduced endothelial==

351   ==superoxide generation as opposed to radical scavenging.==

352

353   The phenolic derivative vanillic acid (the methylated metabolite of protocatechuic acid) was

354   subsequently selected to explore mechanisms potentially underlying the observed reductions in

355   superoxide production. In the present study, vanillic acid at a concentration of 1 µM significantly

356   reduced endothelial superoxide levels, which is comparable to the C_{max} value reported by Czank *et*

357   *al* (2013) for ^{13}C-labelled phase II conjugates of protocatechuic acid (2.35 µM) following ingestion

358   of ^{13}C-labelled cyanidin-3-glucoside [17]. Steffen *et al* (2008) have previously described NOX

359   inhibitory activity of vanillic acid in disintegrated HUVEC (IC_{50} = 8.1 µM), with minimal direct

360   superoxide scavenging activity in a cell-free system (IC_{50} > 100 µM) [21]; which was confirmed by

361   the absence of direct superoxide scavenging by vanillic acid in the present study.

362

363   NOX4 is reported to be the major vascular endothelial isoform [30, 31], producing mainly hydrogen

364   peroxide [32]; and whilst NOX4-derived hydrogen peroxide would not act to limit NO bioavailability

365   [33], it might induce vasodilation through hyperpolarisation independently of NO activity [34]. In

366   contrast NOX2 generates superoxide [35] and may have a key role in regulating vascular function [32,

367   36]. Both NOX2 and NOX4 associate with the integral membrane protein p22^{phox} [37], but activation of

368   NOX2 follows the recruitment of additional cytosolic proteins including p47^{phox} ('organiser'

369   subunit) and p67^{phox} ('activator' subunit) [37, 38]. Modulation of the expression of NOX2 and NOX4

370   isoforms, and associated subunits (p22^{phox}, p47^{phox}, and p67^{phox}) by vanillic acid was therefore

371   explored using RT-qPCR. Co-incubation of angiotensin II with vanillic acid elicited no significant

372   changes in levels of endothelial NOX4 mRNA relative to angiotensin II alone (Figure 4A).

373   However, a significant upregulation of p22^{phox} mRNA levels was observed with 0.1 and 1 μM

374   vanillic acid (Figure 4B), whilst we were unable to assess transcriptional modulation of the

375   superoxide-generating NOX2 isoform, or p47^{phox} and p67^{phox}. Therefore the effects of vanillic acid

376   on the stimulated expression of NOX2 and p47^{phox}, and also NOX4, were explored at the protein

377   level.

378

379   There was no significant modulation of stimulated NOX4 protein expression by vanillic acid

380   (Figure 5), indicating that this particular metabolite does not affect endothelial NOX4 protein.

381   NOX2 expression was more difficult to discern by immunoblotting of cell lysates, which may

382   reflect low mRNA/protein expression in vascular cells, and/or poor antibody specificity; although

383   upregulation of NOX2 in HUVECs following angiotensin II stimulation has been previously

384   reported [20]. Interestingly, the observed elevation in p22^{phox} mRNA induced by vanillic acid did not

385   appear to be associated with increased NOX4 or NOX2 protein levels. Modulation of p47^{phox}

386   protein expression was examined as a possible mechanism of NOX2 inhibition, and here a trend

387   was observed towards downregulation of p47^{phox} protein following co-incubation with TNF-α and

388   vanillic acid (Figure 6A). Interestingly, in the HCAEC model, vanillic acid did not modulate

389   stimulated p47^{phox} expression (Figure 6B), and thus vanillic acid does not appear to significantly

390   alter p47^{phox} levels (under the present conditions) and therefore affect NOX2 activity by this

391   mechanism. However, the anthocyanidin delphinidin has recently been reported to inhibit p47^{phox}

392   translocation and NOX activity in human dermal fibroblasts [39], suggesting another potential

393   mechanism by which anthocyanins and/or their degradation products could modulate NOX

394   function.

395

396   Upregulation of HO-1 has been reported to inhibit NOX function [14, 15]; and previous studies have

397   described induction of HO-1 expression by anthocyanins and/or their metabolites in human

398   endothelial cells [13, 40]. For example, Nrf2 nuclear translocation and HO-1 expression in cultured

399   HUVEC was elicited by serum samples obtained from healthy subjects following ingestion of 160

400   mg purified anthocyanins, which may reflect activity of anthocyanin metabolites as opposed to

401   parent compounds [40]. Moreover, delphindin-3-glucoside has previously been reported to enhance

402   survival of murine hepatocytes exposed to a cytotoxic concentration of epigallocatechin-3-gallate

403   through upregulation of HO-1 mRNA levels [41]. Endothelial homeostasis is maintained through the

404   action of vasodilators such as NO, however NO bioactivity is diminished by radicals such as O_2^-

405   which are produced through activation of NOX [9, 12]. Anthocyanin metabolites could potentially

406   indirectly maintain vascular homeostasis by inducing HO-1, with subsequent inhibition of NOX

407   activity, thus reducing the scavenging of NO by NOX-derived superoxide [14, 15, 40]. Therefore the

408   potential indirect effect of vanillic acid on NOX activity was explored by assessing modulation of

409   endothelial HO-1 expression. Following exposure of HUVEC to vanillic acid, elevated HO-1

410   mRNA levels were observed in what appeared to be a concentration-dependent response (Figure 7),

411   with a trend towards significance relative to the vehicle control (at 10 µM vanillic acid). Likewise,

412   vanillic acid increased HUVEC HO-1 protein expression (Figure 8A), and densitometric analysis

413   indicated a trend towards significance at 1 µM. The vehicle control also appeared to moderately

414   upregulate HO-1, reflecting previously reported studies in HUVEC with low concentrations of

415   DMSO (0 - 0.8%) [42]. As such, vanillic acid was prepared directly in aqueous solution for

416   investigation in the HCAEC model; where a similar trend for upregulation of HO-1 protein was

417   evident (at 1 µM; Figure 8B), suggesting an inverted U-shaped dose-response.

418

419   A possible limitation of the current investigation was the use of HUVEC as an *in vitro* model.

420   Whilst HUVEC are widely used for research concerning general properties of endothelial cells [43],

421   an arterial endothelial cell type may be more appropriate for studies investigating the potential

422   modulation of NOX activity in relation to endothelial dysfunction. Thus as part of the current

423   investigation confirmatory studies were conducted for potential modulation of p47^{phox}/HO-1

expression, using HCAEC as a more physiologically representative endothelial model. Furthermore, in the present study there was no effect of cyanidin-3-glucoside on endothelial NO production (Supporting Information, Figure S1), despite a previous report of activation of eNOS by cyanidin-3-glucoside at concentrations of ≤5 µM in HUVECs [7]. It is possible that assessing eNOS activity by investigating enzyme phosphorylation, as described previously for blackcurrant anthocyanins in a HUVEC model [44], could have provided further information as to the effects of treatment compounds upon eNOS. NO production was clearly increased by PMA and resveratrol in control experiments, indicating adequate assay sensitivity for the detection of NO-derived nitrite/nitrate in aqueous solution. It is however possible that the assay was not sensitive enough to detect subtle changes in nitrite/nitrate. Interestingly, and with the possible exception of the upregulation of eNOS by cyanidin-3-glucoside, linear dose-response relationships were not observed for any treatment in the current investigation, suggesting differential bioactivity of anthocyanins and their metabolites across dose ranges, as described previously for cyanidin-3-glucoside [8]. However, as only three concentrations of the treatment compounds were assessed in the current study, it is not possible to draw definitive conclusions regarding dose-response relationships, and a wider dosage range is required to confirm our observations.

Another limitation of the present study was the poor qPCR specificity observed for NOX2, p47^{phox} and p67^{phox}, which precluded determination of relative changes in expression for these transcripts. Previously reported studies have suggested low or no endothelial expression of NOX2 mRNA [30, 45], and low expression of p47^{phox} and p67^{phox} mRNA in HUVEC [30]. Moreover, minimal changes were detected in NOX4 gene expression, and p47^{phox} protein expression, following stimulation with angiotensin II or TNF-α respectively; although limited upregulation of p47^{phox} was consistent in both HUVEC and HCAEC models.

449 In the present study, cyanidin-3-glucoside significantly upregulated endothelial expression of eNOS

450 as previously reported for unmetabolised anthocyanins [8, 13], however the phenolic acid derivatives

451 were not active. By contrast, both the degradation product protocatechuic acid, and its phase II

452 metabolite vanillic acid, significantly reduced endothelial superoxide levels; whereas the parent

453 anthocyanin did not. These data suggest a differential bioactivity of anthocyanins relative to their

454 phenolic derivatives. Our findings therefore indicate that anthocyanins may directly stimulate

455 eNOS, eliciting improved endothelial function; however, when anthocyanins are metabolized, this

456 direct effect upon eNOS is lost. Nevertheless, their metabolites could maintain vascular homeostasis

457 through indirectly preserving NO bioactivity, by mechanisms involving NOX or HO-1. It must be

458 noted that Czank *et al* (2013) identified 16 phenolic metabolites of cyanidin-3-glucoside in human

459 serum [17], which should all be investigated for potential vascular bioactivity in future studies.

460 Indeed, preliminary data from ongoing studies in our laboratory suggest PCA, and PCA in

461 combination with VA, upregulate HO-1 protein expression in rat aortic smooth muscle cells *in vitro*

462 (data not shown).

463

464 In conclusion, there was no direct effect of vanillic acid on endothelial protein expression of eNOS

465 or NOX isoforms in the present investigation, whereas HO-1 protein was modestly increased;

466 indicating different mechanisms of bioactivity for phenolic derivatives relative to parent

467 anthocyanins. This also suggests a potential indirect activity of anthocyanin metabolites in

468 maintaining vascular homeostasis *in vivo*.

469

ABBREVIATIONS USED

Ang II, angiotensin II; ANOVA, analysis of variance; CV, coefficient of variation; DMSO, dimethyl sulphoxide; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; eNOS, endothelial nitric oxide synthase; FBS; foetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCAEC, human coronary artery endothelial cell; HO-1, haem oxygenase-1; HUVEC, human umbilical vein endothelial cell; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NOX, NADPH oxidase; $O_2^{\cdot-}$, superoxide; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; SOD, superoxide dismutase; TNF-α, tumour necrosis factor-alpha; VA, vanillic acid.

ACKNOWLEDGEMENTS

SUPPORTING INFORMATION DESCRIPTION

Primer sequences for custom primer sets (human HMOX-1, NOX2, NOX4, p22^{phox}, p47^{phox} and p67^{phox}) are provided as Supporting Information.

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615

616

Figure 1. Chemical structures of the anthocyanin cyanidin-3-glucoside (A), its B-ring degradation product protocatechuic acid (B), and the mono-*O*-methylated metabolite of protocatechuic acid, vanillic acid (C). COMT, catechol-O-methyltransferase.

Figure 2. Modulation of endothelial eNOS expression (as quantified by ELISA of cell lysates) following 24 h incubation of HUVECs with vehicle control [0.05% DMSO in supplemented culture medium] (VC), or 0.1-10 µM of cyanidin-3-glucoside (A), protocatechuic acid (B), and vanillic acid (C). Data are graphed as mean eNOS protein as percentage of vehicle control (designated as 100% and marked by dashed line); mean ± SD (n=3). *Significant difference versus vehicle control ($p < 0.05$).

Figure 3. Modulation of angiotensin II (Ang II)-stimulated HUVEC ~~endothelial~~ superoxide production by 0.1-10 µM cyanidin-3-glucoside (A), protocatechuic acid (B), or vanillic acid (C). Superoxide production was quantified by reduction of extracellular ferricytochrome c. Data are graphed as SOD-corrected mean absorbance (OD) ratio (550 nm/620 nm) as percentage of Ang II control (designated as 100% and marked by dashed line); mean ± SD (n=3). *Significant difference versus Ang II-stimulated control ($p < 0.05$).

Figure 4. Modulation of HUVEC NOX4 (A) or $p22^{phox}$ (B) mRNA levels following 4 h incubation with 0.1 µM angiotensin II control (Ang II), or Ang II with 0.1 µM, 1 µM or 10 µM vanillic acid, presented as fold change versus unstimulated cells (basal; designated as 1 and marked by dashed line). Relative quantification was performed by RT-qPCR using the comparative C_t method, incorporating the geometric mean of reference genes UBE4A and VIPAS39 as the normalisation

642　factor. Data are graphed as mean ± SD (n=3). *Significant difference versus Ang II-stimulated

643　control (p < 0.05).

644

645　**Figure 5**. Expression of NOX4 protein in cell lysates from unstimulated HUVECs (basal) and

646　following 6 h incubation with 0.1 µM angiotensin II (Ang II), or Ang II with 0.1 µM, 1 µM or 10

647　µM vanillic acid (VA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was utilised as a

648　loading control for immunoblotting of cell lysates. Graphs show fold increase in NOX4 expression

649　relative to basal (designated as 1 and marked by dashed line), after quantification by densitometry

650　and normalisation to loading control (mean ± SD, n=5).

651

652　**Figure 6.** Expression of p47^{phox} protein in HUVEC (A) or HCAEC (B) lysates from unstimulated

653　cells (basal) and following 5 h incubation with 20ng/ml TNF-α, or TNF-α with 0.1 µM, 1 µM or 10

654　µM vanillic acid. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was utilised as a loading

655　control for immunoblotting of cell lysates. Graphs show fold increase in p47^{phox} expression relative

656　to basal (designated as 1 and marked by dashed line), after quantification by densitometry and

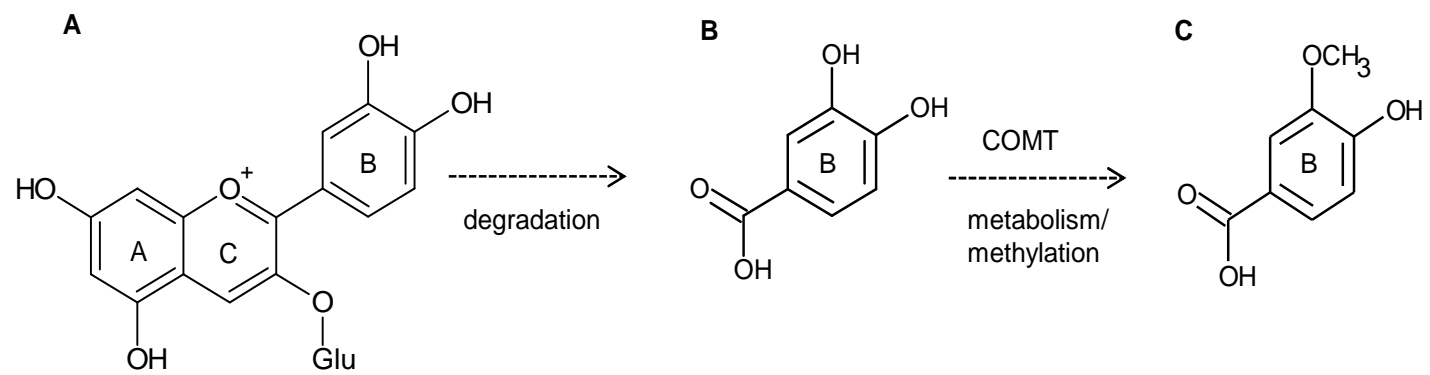657　normalisation to loading control. Data are graphed as mean ± SD (n=5 for HUVEC and n=4 for

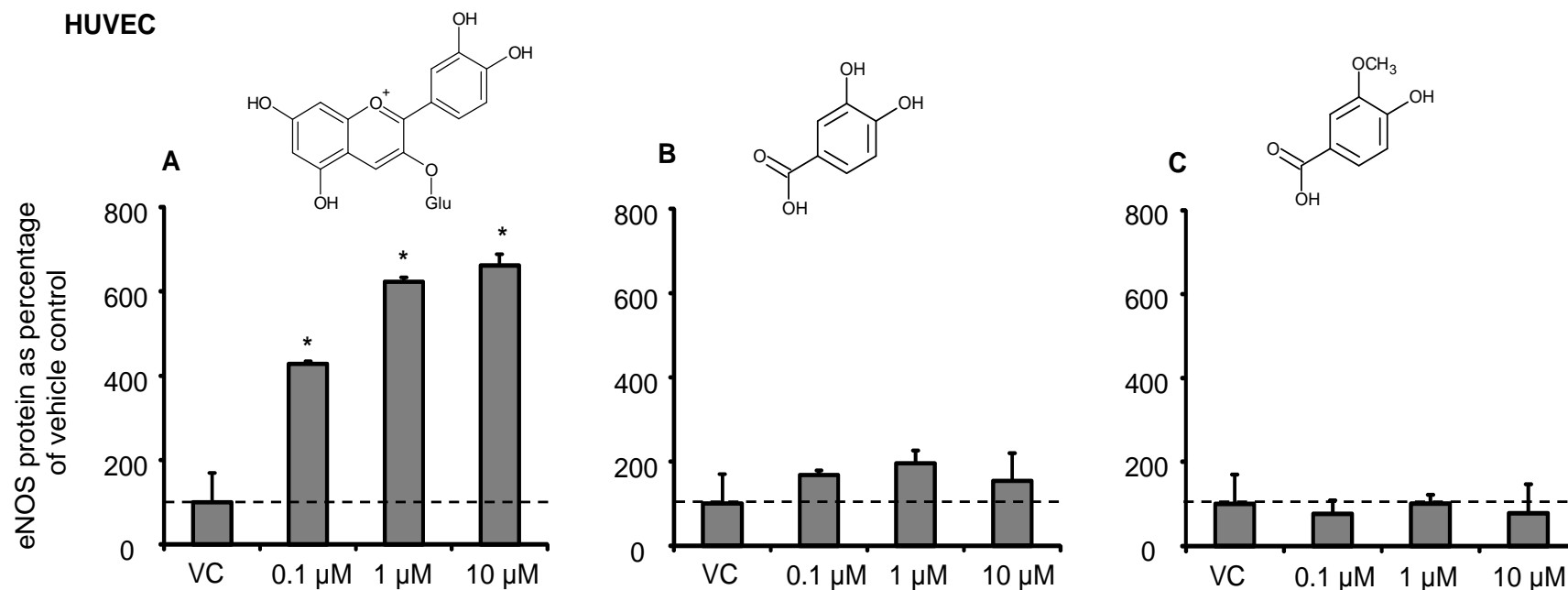658　HCAEC).

659

660　**Figure 7.** Modulation of HUVEC HO-1 mRNA levels following 6 h incubation with vehicle control

661　(VC, 0.005% DMSO), 10 ng/ml phorbol 12-myristate 13-acetate (PMA), or 0.1 µM, 1 µM or 10

662　µM vanillic acid, presented as fold change versus untreated (basal) cells (designated as 1 and

663　marked by dashed line). Relative quantification was performed by RT-qPCR using the comparative

664　C_t method, incorporating the geometric mean of human reference genes TYW1 and PPIA as the

665　normalisation factor. Data are graphed as mean ± SD (n=3). *Significant difference versus vehicle

666　control (*p < 0.01).

667

668 **Figure 8.** Expression of HO-1 protein in HUVEC (A) or HCAEC (B) lysates from untreated cells

669 (basal) and following 6 h incubation with 10 ng/ml phorbol 12-myristate 13-acetate (PMA), vehicle

670 control (VC, 0.005% DMSO; HUVEC only) or 0.1 μM, 1 μM or 10 μM vanillic acid.

671 Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was utilised as a loading control for

672 immunoblotting of cell lysates. Graphs show fold increase in HO-1 expression relative to basal

673 (designated as 1 and marked by dashed line), after quantification by densitometry and normalisation

674 to loading control. Data are graphed as mean ± SD (n=4).

675
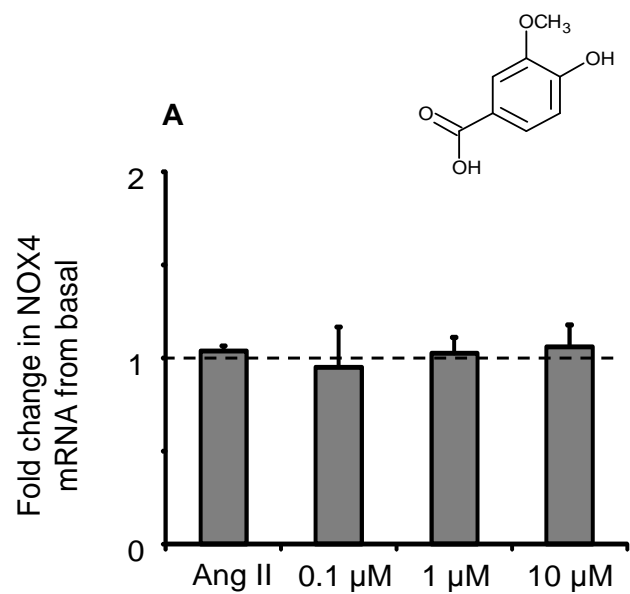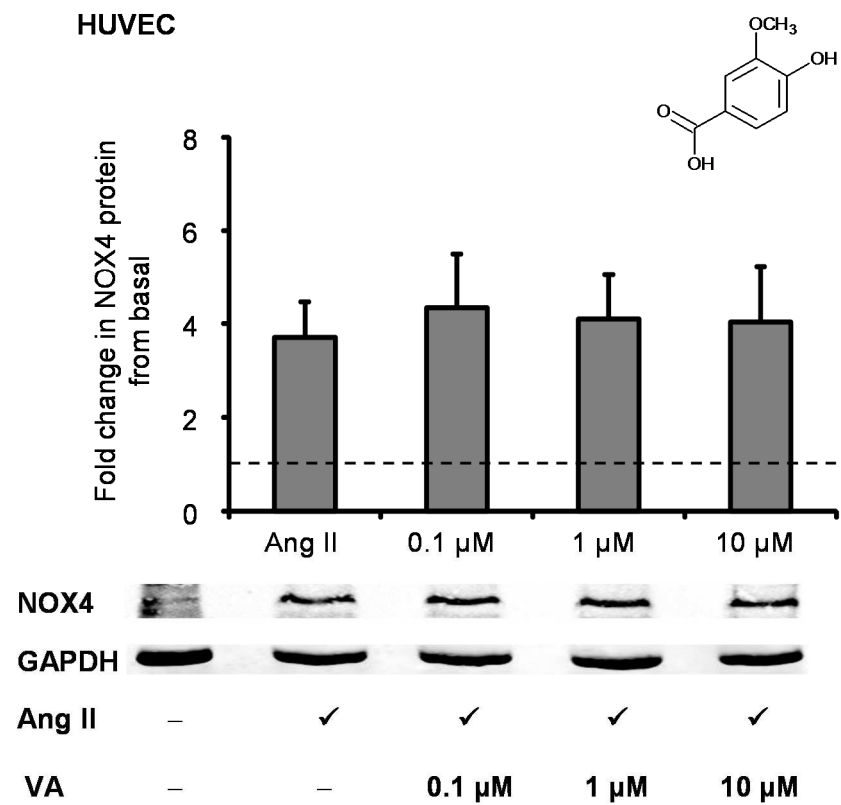
Figure 1

A

B

degradation

COMT

metabolism/
methylation

C

Figure 2

Figure 3

Figure 4

HUVEC

Figure 5

Figure 6

Figure 7

HUVEC

Figure 8

Graphic for Table of Contents only