Jones

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2   Title: The dietary flavonol quercetin ameliorates angiotensin II-induced redox signalling

3   imbalance in a human umbilical vein endothelial cell model of endothelial dysfunction via

4   ablation of p47[phox] expression.

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22 **Abstract**

23 Scope: Quercetin is reported to reduce blood pressure in hypertensive but not normotensive

24 humans, but the role of endothelial redox signalling in this phenomenon has not been

25 assessed. This study investigated the effects of physiologically-obtainable quercetin

26 concentrations in a human primary cell model of endothelial dysfunction in order to elucidate

27 the mechanism of action of its antihypertensive effects.

28 Methods and results: Angiotensin II (100 nM, 8 h) induced dysfunction, characterised by

29 suppressed nitric oxide availability ($85 \pm 4\%$ $p<0.05$) and increased superoxide production

30 ($136 \pm 5\%$, $p<0.001$). These effects were ablated by an NADPH oxidase inhibitor. Quercetin

31 ($3\ \mu M$, 8 h) prevented angiotensin II induced changes in nitric oxide and superoxide levels,

32 but no effect on upon nitric oxide or superoxide in control cells. The NADPH oxidase

33 subunit p47^{phox} was increased at the mRNA and protein levels in angiotensin II-treated cells

34 ($130 \pm 14\%$ of control, $p<0.05$), which was ablated by quercetin co-treatment. Protein kinase

35 C activity was increased after angiotensin II treatment ($136 \pm 51\%$), however this was

36 unaffected by quercetin co-treatment.

37 Conclusions: Physiologically-obtainable quercetin concentrations are capable of ameliorating

38 angiotensin II-induced endothelial nitric oxide and superoxide imbalance via protein kinase

39 C-independent restoration of p47^{phox} gene and protein expression.

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Introduction

Evidence from a wide variety of studies suggests that particular dietary polyphenolic plant secondary metabolites are capable of modulating clinical markers of cardiovascular health in humans (1-5). Of the many thousands of these structures present in fruit and vegetable-rich diets, the flavonol quercetin is amongst the most ubiquitous, being found most commonly in berries, apples and onions, and being freely available as an isolate marketed as a nutritional supplement. Quercetin has been shown to reduce blood pressure in hypertensive but not normotensive human volunteers (6-10). These studies showed significant decreases of both systolic and diastolic blood pressure in hypertensive individuals, apparently independent of brachial artery vasodilation, angiotensin converting enzyme activity, and plasma nitrite concentrations (7, 8). However, the molecular mechanism underpinning the potential beneficial effects of quercetin have remained elusive. In models of rodent hypertension modulation of NADPH oxidase expression and reactive oxygen species (ROS) formation (11, 12) have been suggested as key targets for quercetin. However, most of these experiments have used rodent aortic ring segments which do not allow specific assessment of endothelial function (13-15).

The endothelium is an important source of vasodilatory and constrictive signalling factors involved in the downstream regulation of blood pressure and normal vascular function. Notably, the redox signalling agents nitric oxide ($NO^\cdot$) and superoxide ($O_2^{\cdot-}$) rapidly and antagonistically mediate such effects (16). Imbalance in this redox equilibrium is strongly implicated in the pathophysiology of several cardiovascular diseases, and it may therefore be hypothesised that restoration of endothelial function plays a significant role in mediating the effects of quercetin in endothelial dysfunction. Indeed, relaxation studies using stimulated aortic ring segments have suggested the involvement of endothelial $NO^\cdot$ availability in the mechanism of action of quercetin, and a cell culture model of HOCl-induced endothelial dysfunction has shown that supraphysiological concentrations of quercetin can increase NOS

69   activity (13-15, 17). Interestingly, this suggestion is in apparent conflict with the later study

70   of Larson *et al*. (2012), which concluded that the clinical effects of quercetin in hypertensive

71   individuals were independent of plasma nitrite concentrations; a clinical marker of circulating

72     $NO^{\cdot}$ availability. In addition, the barrier function of the endothelium makes this structure most

73   likely to be exposed to plasma quercetin following oral administration.

74   This study therefore aimed to determine the potential effects of physiologically-obtainable

75   quercetin concentrations on $NO^{\cdot}$ and $O_2^{\cdot-}$ signalling imbalance. For this study primary vascular

76   endothelial cells (Human Umbilical Vein Endothelial Cells; HUVEC) have been used rather

77   than endothelial cell lines, which have been reported to vary in characteristics quite

78   dramatically when compared to *ex vivo* tissues. HUVEC are well characterised, relatively

79   easily accessible, primary cells that have been reported to exhibit key endothelial cell functions,

80   and are extensively used in the study of the effects of dietary chemicals upon cardiovascular

81   biology and diseases (18-24). Specifically, we have assessed the effects of quercetin on $NO^{\cdot}$

82   availability, $O_2^{\cdot-}$ production and NADPH oxidase subunit protein levels and activity. The

83   concentrations of quercetin added have been chosen to replicate those which may arise from

84   dietary intake, providing meaningful information on the mechanisms which underlie clinical

85   observations. This use of physiologically-obtainable quercetin concentrations is an important

86   part of the novelty of this study as although the role of NADPH oxidase and $NO^{\cdot}$ production

87   in the mechanism of action has been previously investigated in a variety of model systems,

88   these studies have mainly assessed supra-physiological concentrations of quercetin. Thus there

89   is a possibility that the previously reported effects of quercetin upon vascular dysfunction are

90   actually due to off-target effects and are therefore not relevant to the mechanism of action in

91   humans. Our study aims to address this important consideration.

92

93 **Materials and methods**

94 *Materials*

95 Unless otherwise listed below, all reagents were sourced from Sigma-Aldrich (Poole, UK).

96 Human Umbilical Vein Endothelial Cells were purchased from Promocell (Heidlberg,

97 Germany) as cryopreserved aliquots of 5 x 10^5 cells pooled from 4 donors. Endothelial Cell

98 Growth Medium (ECGM) was also purchased from Promocell (Heidlberg, Germany).

99 Medium 199 (M199) was obtained from Life Technologies (Paisley, UK). Nunc Nunclon

100 plasma-treated black 96-well plates were bought from Fisher Scientific (Leicestershire, UK).

101 4, 5-Diaminofluorescein diacetate (DAF-2 DA) was purchased from Enzo Life Sciences

102 (Exeter, UK). The DC protein assay kit, 2x Lamelli buffer, Mini-Protean 12% TGX gels, 10x

103 TGS buffer, 0.2 μm PVDF Turboblot membrane packs, Precision Plus protein marker ladder,

104 Clarity Western ECL solution, Aurum total RNA extraction kit and iScript cDNA synthesis kit

105 were all obtained from Bio-Rad (Hertfordshire, UK). Marvel low-fat powdered milk was

106 purchased from a local supermarket. Autoradiography films (Amersham ECL) were purchased

107 from Fisher Scientific (Loughborough, UK). An Endothelin-1 ELISA assay kit was purchased

108 from Enzo Life Sciences (Exeter, UK). Solaris PCR expression assays for NOS3 (eNOS),

109 NCF-1, EF1α and β-actin, and the Solaris qPCR mastermix were purchased from GE

110 Healthcare (Buckinghamshire, UK).

111 The following antibodies were used: p47^{phox} (Abcam, Cambridge, UK, Ab63361, Lot #

112 803556), gp91^{phox} (Abcam, Cambridge, UK, AB129068, Lot # GR83718-3), β-actin (Abcam,

113 Cambridge, UK, ab20272, Lot # GR88824-1), Phospho-(Ser) PKC substrate antibody (Cell

114 Signalling, 2261,Lot # 18), anti-beta tubulin (Millipore, 06-661, Lot # 239882), Goat anti-

115 rabbit HRP-conjugate (Sigma-Aldrich, Poole, UK, A6154, Lot # 090116176), Goat anti-mouse

116   HRP-conjugate (Abcam, Cambridge, UK, ab97023 , Lot # GR87150-8), Goat anti-rabbit HRP-

117   conjugate (GE Healthcare, RPN4301, Lot # 9526414).

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Human Umbilical Vein Endothelial Cell (HUVEC) culture

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120   HUVEC were grown to ~95% confluence with ECGM + 20% foetal bovine serum (FBS) over

121   3-4 d, with the culture media refreshed every 2 d. After trypsinisation, HUVEC were seeded

122   into either 96-well or 6-well plates at a density of 14,700 cells/cm^2 and cultured for the

123   durations described below and in the figure legends, with media refreshed every 2 days.

124

125   *Live cell measurement of* NO$^{\cdot}$ *availability in HUVEC cultures*

126   Passage 4 – 6 HUVEC were seeded in 96-well plates (5 x 10^3 cells / well) and allowed to

127   proliferate for 2 - 3 d as required. Cell cultures were serum-deprived for 24 hours in Medium-

128   199 containing 0.5% FBS (M199) before endpoint measurement. Ang II was prepared as a 1

129   mM stock in ddH$_2$O (18.2 MΩ), diluted in culture media to produce 10 nM – 10 μM final

130   concentration. 8 hr before end-point measurement cells were treated in M199 with or without

131   Ang II. Cells were treated with quercetin or solvent only as directed in the Figure legends for

132   8 hours in the presence or absence of Ang II. For cells treated with L-NAME, a stock solution

133   of 100 mM was prepared in DMSO, and diluted 1000-fold in culture media. Cells were treated

134   with L-NAME, or a vehicle control, for 30 minutes before end-point measurement. After

135   incubation with the appropriate treatment(s), cells were washed once on a warmed plate with

136   200 μl of warmed HBSS (containing magnesium and calcium), and 70 μl of warm HBSS

137   (containing magnesium and calcium) containing 2 μM DAF-2DA was added per well. For L-

138   NAME treated cells, L-NAME was also included in this dye solution. Fluorescence (λ_{ex} 485

139   nm, λ_{em} 520 nm) was measured every 3 min over a 30 minute period using a Tecan infinite

140   X200 plate reader, maintained at 37°C, with a matrix of 4x4 points per well measured (ten

141   flashes per point), and a manual gain of 100 set. Linear rates ($r^2 \geq 0.99$) were calculated for all

142   wells and a mean rate of fluorescence was calculated for each treatment (n=4-6 wells per

143   treatment). Wells containing cells only, and wells without cells were also assessed

144   *Protein extraction from HUVEC cultures*

145   Cells were cultured as described and treated 8 h before extraction with either DMF (0.1% v/v),

146   quercetin (3 μM), DMF (0.1% v/v) and Ang II (100 nM), or quercetin (3 μM) and Ang II (100

147   nM), in M199 containing 0.5% FBS. Monolayers were washed twice in 3 ml of PBS per well,

148   before the addition of 200 μl of RIPA buffer (1% NP-40 substitute, 0.5% w/v sodium

149   deoxycholate, 0.1% w/v sodium dodecyl sulphate, made up in PBS containing protease

150   inhibitor cocktail) per well. Cells were harvested by scraping before incubation for 5 min on

151   ice to aid lysis. The cell lysates were sonicated (Sonic Vibracell VCX130PB) for 3 x 10 s

152   bursts on ice. Lysates were centrifuged at 16 100 x g, 5 min, and the resulting supernatant

153   stored at -20°C.

154

155   *Western blotting for NADPH oxidase subunits p47^{phox} and gp91^{phox}*

156   Protein extracts, prepared as above, were quantified using the DC protein assay as described

157   by the manufacturer. 20 μg of protein extract was added in a 1:1 ratio to 2x Lamelli buffer.

158   The resulting samples were then boiled for 5 minutes at 95°C, before incubation on ice for 2

159   minutes, and centrifugation at 16 100 x g for 30 seconds. Proteins were separated using 12%

160   TGX gel for 30 minutes at 250 V in 1x TGS buffer. Separated proteins were transferred PVDF

161   membranes before washing in 1x TBST buffer (20 mM Trizma base, 137 mM sodium chloride,

162 1 ml of tween20, pH 7.6) and blocking in a solution of 5% w/v milk in 1x TBST for 30 minutes.

163 Membranes were rinsed and washed 3x 10 minutes in 1x TBST buffer, before incubation for 1

164 hour with the appropriate primary antibody dilution (p47^{phox} – 1:2000; gp91^{phox} – 1:5000) in

165 1% w/v milk in 1x TBST buffer. Following further rinsing and 3 x 10 minute washes in 1x

166 TBST buffer, membranes were incubated for 1 hour with the appropriate secondary antibody

167 (Goat anti-rabbit HRP conjugate – 1:30000) diluted in 1% w/v milk in 1x TBST. The

168 membranes were then rinsed and washed again (3x 10 minutes in 1x TBST) and processed by

169 ECL. Membranes were stored at 4°C for use in measuring β-actin levels as a loading control.

170 Membranes were incubated for 2x 10 minute washes in stripping buffer (200 mM glycine, 3.5

171 mM sodium dodecyl sulphate, 1% tween20, adjusted to pH 2.2), washed twice in TBST, and

172 then blocked (5% w/v milk, 30 minutes) and washed a further 3 times. The membranes were

173 incubated with the β-actin HRP conjugated antibody (1:2 000 000 dilution) in 1% w/v milk in

174 1xTBST buffer for 1 hour before a further 3x 10 minutes washes in 1x TBST buffer. The

175 membranes were incubated in ECL solution and developed as previously described, with a 2.5

176 minute exposure to the autoradiography film.

177 For the assessment of PKC-phosphorylated substrate consensus sequences membranes were

178 blocked using 10% bovine serum albumin (BSA) for 1 h followed by incubation with a PKC-

179 phosphorylated substrate consensus sequence primary antibody diluted to 1:1000 in 1 % BSA

180 for 1 h. An appropriate HRP-conjugated secondary antibody diluted 1:10,000 in 1% BSA was

181 used to detect the primary antibody. Membranes were stripped and re-probed with for β-tubulin

182 at a dilution of 1:1000 overnight at 4°C.

183 All films were digitised and densiometry was done using the ImageJ software. A ratio of

184 protein of interest: β-actin was calculated for each sample with the control samples for each

185 biological replicate set at 100%. For the PKC-phosphorylated substrate films, the entire lane

186 was quantified by densitometry as described above, and normalised to β-tubulin.

187 *Intracellular* $O_2{}^-$ *determination by LC-MS detection of 2HE+*

188 HUVEC were cultured as described in 6-well plates and serum-deprived in M199 containing

189 0.5% FBS for 24 h prior to experiments. At 8 h before experiments these cultures were

190 incubated with or without Ang II (100 nM) in the presence of vehicle (0.1% v/v DMF), 3 nM,

191 or 3 μM quercetin, with 3 wells per treatment. For experiments using the NADPH oxidase

192 inhibitor VAS-2870, cells were treated with or without Ang II for 8 h, with either vehicle (0.1%

193 DMF) or 1 μM VAS-2870 for 30 minutes before assay. After 8 h incubation the cells were

194 washed twice in excess warm PBS containing 100 μM DTPA and then incubated in darkness

195 with 2 ml of 20 μM dihydroethidium (DHE, dissolved in DMSO at a stock concentration of 20

196 mM) in HBSS for 30 min . Following two further washes with excess PBS - DTPA, each well

197 was scraped in 500 μl of methanol, and wells from the same treatment groups were pooled and

198 stored overnight at -20 °C. The resulting lysates were centrifuged (16,100 x g, 10 minutes, 4

199 °C) with both the supernatant and pellet retained separately. The supernatant was evaporated

200 to dryness using a centrifugal evaporator without heating (Genevac MiVac, Genevac, Ipswich,

201 UK), and the resulting residue was dissolved in 1:1 water:stabilisation solution (33.3%

202 methanol, 10 μM fluorescein internal standard, 0.1 mM DTPA, and 5 mM sodium ascorbate,

203 made up to final volume with water) with a final volume of 120 μl for LC-MS analysis. The

204 pellet was dissolved in 100 μl of 0.1 M sodium hydroxide and the protein content was

205 quantified using the DC assay as directed by the manufacturer.

206 For LC-MS analysis of DHE oxidation products, the specific $O_2{}^-$ reaction product (2-HE+)

207 was detected at a *m/z* ratio of 330.3, and at a retention time of 7 min. Separation was achieved

208 using a Shimadzu prominence LC20 quaternary pump and autosampler, a Shimadzu CTO10

209 column oven, and an Agilent Eclipse XDB-C18 column (5 μm, 4.6 x 150 mm) at a flow rate

210 of 0.5 ml/min. Water containing 0.5% formic acid (solvent A) and methanol containing 0.5%

211 formic acid (solvent B) were used to achieve separation as follows; 43% B for 3.35 minutes,

212 increasing to 85% B by 8 minutes, maintained at 85% B until 11.5 minutes, reduced to 43% B

213 by 11.75 minutes, held at 43% B until 18.75 minutes. Column oven temperature was set to 40

214 °C. The injection volume used was 20 µl. A Shimadzu LC2020 single quadrapole mass

215 spectrometer was used as the detector with the following settings: Fluorescein was used as an

216 internal standard, at a *m/z* ratio of 333.0 and a retention time of 11.25 minutes. Peaks

217 corresponding to 2-HE+ and fluorescein were integrated and peak areas were calculated. The

218 ratio of 2-HE+:fluorescein was normalised to the total protein. Control samples from each

219 independent experiment were set to 100%, with all other treatments expressed as a percentage

220 of control, and 3 independent experiments were analysed. A representative chromatogram is

221 shown in Figure S1.

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223 *RNA extraction, cDNA synthesis and qPCR of eNOS and β-actin*

224 HUVEC cultures were grown as described above in 6-well plate format, with and without Ang

225 II (8 h, 100 nM) and quercetin (3 µM, 8 h) treatments. Cells were washed and scraped in PBS,

226 with wells from the same treatment group pooled together, centrifuged, and RNA was extracted

227 using the Bio-Rad Aurum RNA extraction kit as directed by the manufacturer. Eluted RNA

228 was quantified by spectrophotometry. Using the Bio-Rad iScript cDNA synthesis kit, as

229 directed by the manufacturer, 500 ng of RNA was reverse transcribed, and the resulting cDNA

230 was quantified by spectrophotmetry. Relative quantification of gene expression, comparing

231 control against treated samples, was achieved using the validated Solaris PCR expression assay

232 probes for eNOS, p47[phox], Elongation Factor 1α and β-actin, by loading 150 ng of cDNA

233 template to the assay probes and Solaris PCR master mix as directed by the manufacturer. The

234 following PCR conditions were used for these probe sets: 1 cycle of 95°C 15 min followed by

235 50 cycles of 95°C for 15 sec and 60°C for 1 min. The resulting Ct values were analysed by the

236 ΔΔCt method, using β-actin or Elongation Factor 1α as the reference gene. Three independent

237 experiments were analysed, with mean fold change in eNOS or p47^{phox} expression in treated

238 samples compared to control samples calculated, ± 1 standard deviation.

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241 *Statistical analysis*

242 All statistical analyses used Sigmaplot version 12.0, with comparisons between multiple

243 groups done by ANOVA or ANOVA on ranks, as appropriate after testing for normality of

244 distribution and homogeneity of variance. Post-hoc tests were done using Tukey post-hoc tests.

245 Values less than $p = 0.05$ were considered statistically significant.

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256 **Results**

257 *Ang II inhibits the production of NO· in HUVEC*

258 In order to examine the effects of quercetin on endothelial dysfunction we first exposed

259 HUVEC to a range of Ang II concentrations (10 nM – 10 μM) for up to 24 h, under optimised

260 culture conditions (as outlined in Figure S2), and the effect of Ang II treatment upon NO·

261 availability was determined (Figure 1 A-D). It was observed that concentrations below 10 μM

262 Ang II resulted in a 10 – 20% decrease in the rate of nitric oxide production, measured in live

263 HUVEC cultures by determining the rate of fluorescence accumulation using

264 diaminofluorescein (DAF). The greatest and most consistent reduction in NO· was observed

265 for an 8 h incubation at 100 nM angiotensin II (Figure 1B). We did not observe a dose-response

266 for Ang II in these experiments over the concentration range tested, however the observation

267 of a lack of effect at higher concentrations (1 μM and above) is consistent with previous

268 research, as is the observed time dependency of Ang II treatment (18). Based upon these

269 experiments future Ang II treatments were at a concentration of 100 nM for 8 h; a treatment

270 regime that resulted in a decrease in NO· availability of $15 \pm 4\%$ compared to untreated cells

271 (Figure 1E, $p<0.05$). To confirm that this effect was mediated in a receptor dependent manner

272 the experiments were repeated in the presence of the angiotensin receptor type 1 antagonist

273 irbesartan (10 μM, 8 h). Under these conditions no change in NO production was observed

274 (Figure 1E). It was also noted that DMSO (0.1% v/v, 8 h), a commonly-used carrier solvent,

275 attenuated the effects of Ang II on NO· levels in this system (Figure S2). Dimethyl-formamide

276 (DMF) did not attenuate the effects of Ang II on NO· and was therefore used for all future

277 experiments.

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279

280 *Physiologically-obtainable quercetin concentrations prevents Ang II-induced decreases in*

281 *nitric oxide production*

282 Using the live-cell nitric oxide assay described above, we next assessed the effect of quercetin

283 upon the rate of nitric oxide production in HUVEC cultures with and without Ang II treatment

284 (8 h, 100 nM). An initial test concentration of 3 μM quercetin was selected for these

285 experiments in order to test a physiologically-obtainable concentration (7, 25, 26). Quercetin

286 (3 μM, 8 h) was shown to prevent Ang II-induced decreases in the rate of nitric oxide

287 production but had no effect on control cells (Figure 2A). A dose response of quercetin (3 nM

288 − 3 μM) in Ang II treated cells showed that quercetin prevented Ang II-induced dysfunction at

289 all concentrations tested (Figure 2B). We next assessed the effect of quercetin treatment (3 μM,

290 8 h) on the expression of endothelial Nitric Oxide Synthase (eNOS) using qPCR, with no

291 changes in gene expression observed for any treatment compared to untreated cells (Figure

292 2C).

293

294 *Quercetin prevents Ang II-induced superoxide production by NADPH oxidases*

295 As NADPH oxidase expression and reactive oxygen species (ROS) formation have been

296 suggested as key targets for quercetin (11, 12) we next sought to determine the effects of both

297 Ang II and quercetin treatments on superoxide production in HUVEC cultures. Superoxide

298 was increased by $36 \pm 5\%$ compared to control cells, and this was prevented by quercetin co-

299 treatment at both 3 μM and 3 nM (Figure 3A). The prevention of Ang II-induced superoxide

300 production by the NADPH oxidase inhibitor VAS-2870 (Figure 3B, 1 μM, 8 h, (27)) suggests

301 that this ROS-producing complex was a major source of Ang II-induced superoxide in these

302 HUVEC cultures.

303 *Quercetin prevents Ang II-induced p47^{phox} expression at the protein and mRNA levels*

304 In order to explore the potential mechanism by which quercetin prevents Ang II-induced

305 dysfunction we next assessed the effect of quercetin treatment on the expression of the NADPH

306 oxidase subunits p47^{phox} and gp91^{phox}. Quercetin (3 μM, 8 h) prevented Ang II-induced p47^{phox}

307 protein levels (130 ± 14% of untreated cells), but had no effect upon p47^{phox} protein levels in

308 control cells (Figure 4A). This trend was also reflected at the mRNA level (Figure 4B). The

309 expression of gp91^{phox} protein was unaffected by either Ang II or quercetin treatments (Figure

310 4C).

311 *Ang II-induced PKC activity is not modulated by quercetin co-treatment*

312 Protein Kinase C activity is a well-characterised component of the Ang II signalling cascade

313 and known activator of NADPH oxidase activity. Treatment of HUVEC with Ang II caused a

314 trend of increase in PKC activity (116 ± 32%, p<0.05), which was unaffected by co-incubation

315 of cell with quercetin (Figure 5). Quercetin alone (3 μM, 8 h) appeared to have no PKC activity

316 (Figure 5). These data suggest the possibility that the observed effects of quercetin are

317 independent of PKC activity.

318

319 **Discussion**

320 In this study we have utilised angiotensin II to induce endothelial dysfunction *in vitro*, as

321 characterised by decreased availability of NO˙, increased intracellular O$_2$˙$^-$, increased NADPH

322 oxidase subunit expression and increased PKC activity. Using this model we have probed the

323 impacts of the flavonoid quercetin, which has previously been reported to modulate the

324 expression of the NADPH oxidase subunit p47^{phox}, O$_2$˙$^-$ (SOD-inhibitable lucigenin detection),

325 and of NO˙ production (nitrate and nitrite levels) *in vitro* (28, 29). However, these previous

326   studies used supraphysiological concentrations of quercetin under non-dysfunctional

327   conditions and their findings are in conflict with clinical research. Consequently we have

328   sought to address the discrepancy between the *in vitro* and clinical data by using

329   physiologically-attainable quercetin concentrations and assessed effects under both non-

330   dysfunctional and dysfunctional conditions.

331   Our study has shown that physiologically-obtainable concentrations of quercetin restore

332   endothelial function (availability of $NO^{\cdot}$, intracellular $O_2^{\cdot-}$ and $p47^{phox}$ expression) to control

333   levels in dysfunctional cells (induced by Ang II) whereas there are no effects in non-

334   dysfunctional cells. This is in concordance with the current clinical data for the effects of

335   quercetin in healthy and diseased populations (7, 8). The mechanism of action of quercetin

336   that underlies these effects is currently unknown, with mechanistic insights from clinical

337   studies and rodent studies being conflicting. Although these rodent studies have implicated

338   changes in NADPH oxidase activity and increased availability of $NO^{\cdot}$ as part of the mechanism

339   of action, due to the inherent multicellular and multi-tissue complexity of the aortic ring

340   segments, they have mainly focussed upon the effects of quercetin on vascular smooth muscle

341   rather than the endothelium in *ex vivo* aortic ring segments. The findings presented from our

342   study demonstrate that quercetin restores endothelial function in Ang II-treated cells and

343   suggests that the observed restoration of endothelial $NO^{\cdot}$ and $O_2^{\cdot-}$ signalling via the restoration

344   of NADPH oxidase expression (possibly independently of PKC activity) is part of the

345   mechanism of action of quercetin (Figure 6). Interestingly, over the concentration range tested

346   (3 nM – 3 µM), there was no observed dose response and a similar ablation of endothelial

347   dysfunction by quercetin was seen at all tested concentrations. There are several possible

348   explanations for this observation including that the concentrations assessed in this study were

349   not in the linear phase of a classical s-shaped dose response, or that quercetin potentially has

350   multiple different mechanisms of action which can result in a variety of different shaped dose

351   response curves (30). Ultimately, future research should focus upon investigating the dose-

352   response characteristics of quercetin in the context of endothelial dysfuction, as well as

353   investigating the role of other potential mechanisms of action, such as other kinase signalling

354   cascades (e.g. MAPK) and the phosphorylation control of eNOS and p47^{phox}.

355   Quercetin has been reported in human plasma in the nanomolar to low micromolar range, after

356   dietary intake and supplementation (7, 8, 25, 26). Human pharmacokinetic studies also have

357   shown that quercetin undergoes substantial conjugative metabolism, with the formation of

358   sulfate and glucuronide conjugates, whereas the aglycone form is not detected in human plasma

359   (31). It should be noted that these circulating conjugated forms are not currently widely

360   available, and that as a result we have used the aglycone form of quercetin in this study. This

361   is not necessarily a major limitation as it has recently been suggested that the anti-hypertensive

362   effects of quercetin glucuronides are dependent on deconjugation activity and the liberation of

363   quercetin (32). HUVEC have also been reported to express β-glucuronidase at the mRNA level

364   (33), suggesting that they also possess at least some deconjugative machinery. Taken together,

365   these data suggest that the use of the aglycone form of quercetin in the endothelial cell model

366   reported is a valid approach for assessing its effects on endothelial dysfunction.

367   In summary, physiologically-obtainable concentrations of the dietary flavonoid quercetin

368   restored NO˙ availability, O_2˙⁻ production and p47^{phox} expression to control levels, possibly in

369   a PKC-independent manner, in a human primary cell model of endothelial dysfunction. There

370   were no further improvements in NO˙ availability under control conditions. These results are

371   consistent with clinical observations that quercetin reduces blood pressure in hypertensive, but

372   not normotensive, individuals, and suggest a potential mechanism by which quercetin mediates

373   this effect.

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375 **Author contributions**

376 Experiments were designed by HSJ and FLC. Experiments were done by HSJ, FLC, AG and

377 SM. KN provided reagents and resources for the PKC experiments. Funding was provided

378 by SLA. All authors reviewed and approved the final manuscript.

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386 **Author disclosure statement**

387 For all authors: No competing financial interests exist.

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395 **List of Abbreviations**

396 Ang II – Angiotensin II

397 cDNA – Copy deoxyribose nucleic acid

398 DAF-2 DA – Diaminofluorescein diacetate

399 ddH_2O – distilled water

400 DHE - Dihydroethidium

401 DMF – Dimethyl-formamide

402 DMSO – Dimethyl-sulfoxide

403 DTPA – Diethylene triamine penta-acetic acid

404 ECGM – Endothelial cell growth media

405 ECL – Enhanced luminol chemiluminescence

406 eNOS – Endothelial nitric oxide synthase

407 FBS – Fetal bovine serum

408 HBSS – Hank's balanced salt solution

409 HRP – Horseradish peroxidase

410 HUVEC – Human umbilical vein endothelial cells

411 kDa – Kilo Dalton

412 LC-MS – Liquid chromatography mass spectrometry

413 L-NAME – NG-Nitro-L-arginine methyl ester

414   M199 – Medium 199

415   NADPH – Nicotinamide adenine dinucleotide 2'-phosphate (reduced form)

416   NO$^{\cdot}$ - Nitric oxide

417   O$_2^{\cdot-}$ - Superoxide

418   PBS – Phosphate buffered saline

419   PKC – Protein kinase C

420   PVDF – Polyvinylidiene difluoride

421   qPCR – Quantitative polymerase chain reaction

422   RNA – Ribose nucleic acid

423   ROS – Reactive oxygen species

424   SOD – Superoxide dismutase

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531

Figure legends

Figure 1: Angiotensin II reduces NO˙ availability in HUVEC cultures.

HUVEC cultures were incubated with Ang II for 1 – 24 h at A) 10 nM, B) 100 nM, C) 1000 nM and D) 10 000 nM, with NO˙ availability assessed post incubation in live cells using diaminofluorescein as described in the methods. E) HUVEC cultures were incubated with Ang II (100 nM, 8 h) ± the Angiotensin receptor type 1 antagonist Irbesartan (10 μM, 8 h). Filled bars indicate Ang II treatment (100 nM, 8 h). Graphs show mean values ± 1 S.D., $n \geq 3$ independent experiments, * $p<0.05$.

Figure 2: Physiologically-obtainable quercetin concentrations prevent Ang II-induced decreases in nitric oxide production.

A) Ang II (100 nM, 8 h) induced decreases in NO˙ availability were restored to control levels by quercetin treatment (3 μM, 8 h), whereas quercetin treatment alone had no effect on NO˙ availability. This trend was observed for a range of physiologically-obtainable quercetin concentrations (3 nM – 3000 nM, B). C) endothelial nitric oxide synthase gene expression was not altered by either Ang II (100 nM, 8 h) or quercetin (3 μM, 8 h) treatments. Filled bars indicate Ang II treatment (100 nM, 8 h). Graphs show mean values ± 1 S.D., $n \geq 3$ independent experiments, * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

554 **Figure 3: Quercetin prevents Ang II-induced superoxide production by NADPH oxidases.**

555 A) Ang II-induced increases in intracellular superoxide are restored to control levels by

556 quercetin co-treatment (8 h) at both 3 nM and 3 μM concentrations, with no effect in quercetin

557 only treated cells. B) Ang II-induced increases in intracellular superoxide are ameliorated to

558 control levels by the NADPH oxidase inhibitor VAS-2870 (1 μM, 8 h). Filled bars indicate

559 Ang II treatment (100 nM, 8 h). Graphs show mean values ± 1 S.D., n ≥ 3 independent

560 experiments, * p<0.05.

561

562 **Figure 4: Quercetin prevents Ang II-induced p47^{phox} expression at the protein and mRNA**

563 **levels.**

564 Quercetin co-treatment (3 μM, 8 h) restored Ang II-induced p47^{phox} protein expression (100

565 nM, 8 h) to control levels in HUVEC cultures as assessed by Western blotting (A). This trend

566 was reflected at the mRNA level, assessed using qPCR (B). The NADPH oxidase subunit

567 gp91^{phox} was unaffected by either Ang II (100 nM, 8 h) or quercetin (3μM, 8 h) treatments (C).

568 Filled bars indicate Ang II treatment (100 nM, 8 h). For the Western blotting data shown, the

569 densitometry of the protein band of interest has been normalised to that of beta-actin, and it is

570 this ratio that is presented in the bar graphs. Graphs show mean values ± 1 S.D., n ≥ 3

571 independent experiments, * p<0.05; ** p<0.01.

572

573 **Ang II-induced PKC activity is not modulated by quercetin co-treatment**

574 There was a trend of increase in PKC activity induced by Ang II exposure (100 nM, 8 h) but

575 this effect was not ablated by quercetin co-treatment (3 μM, 8 h). Filled bars indicate Ang II

576 treatment (100 nM, 8 h). For this Western blotting data the densitometry values for each lane

577 of the PKC substrates blot has been normalised to that of tubulin. It is this ratio that is presented

578 in the bar graphs. Graphs show mean values ± 1 S.D., n ≥ 3 independent experiments

579

580 **Figure 6: Mechanistic insights into the effects of quercetin upon endothelial cell**

581 **hypertension.**

582 Quercetin restores angiotensin II-induced redox imbalance, centred around nitric oxide and

583 superoxide. Changes in p47^{phox} protein expression are also restored to control levels, via

584 modulation of gene expression, by a mechanism that seems independent of PKC activity.

585 Arrows indicate the effects of angiotensin II treatment, which were ameliorated by quercetin

586 co-exposure.

587

588 **Supplementary Figure 1**

589 A representative chromatogram of dihydroethidium (HE, precursor), the superoxide-specific

590 reaction product of HE (2HE+), and fluorescein (internal standard). Dihydroethidium was

591 detected at $m\backslash z = 316.3$, 2HE+ at $m\backslash z = 330$, and the internal standard fluorescein at $m\backslash z = 333$.

592

593 **Supplementary Figure 2**

594 A) Cell media composition affects HUVEC response to Ang II (100 nM, 8 h). 24 h serum

595 deprivation in M199 containing 0.5% (v/v) FBS resulted in a consistent decrease in NO˙

596 availability (15 ± 4% of control) when HUVEC were treated with Ang II (100 nM, 8 h). Filled

597 bars indicate Ang II (100 nM, 8 h) treatment. B) The effect of cell passage number upon Ang

598 II (100 nM, 8 h) induced decreases in NO˙ availability. Passages 4-6 are demonstrated to be

599 responsive to Ang II, measured by NO˙ availability. C) The effect of confluency upon Ang II

600 induced decreases in NO˙ availability. Cells grown for 2-3 days post seeding are demonstrated

601 to be responsive to Ang II, measured by NO˙ availability, with decreasing responsiveness by 4

602 days post seeding. D) The effects of DMSO and DMF upon Ang II induced decreases in NO˙

603 availability. It was shown that DMSO (0.1% v/v), but not DMF (0.1% v/v), prevented Ang II

604 induced decreases in NO˙ availability. Thus DMF was used for all experiments. All

605 experiments minimum n=3 independent experiments with mean ± SD plotted. Filled bars

606 indicates Ang II treatment. ** $p<0.01$.
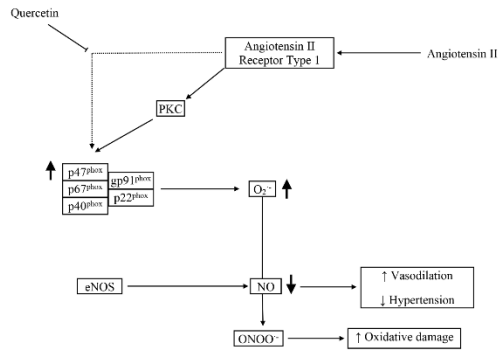
Quercetin restores key cellular signalling molecules (nitric oxide and superoxide) to normal levels in a model of vascular endothelial cell (the cells that line blood vessels) dyfunction. This is achieved by restoring the levels of p47^{phox} (a key component in the superoxide generating machinery NADPH oxidase) to normal levels. The assessment of the modulation of these pathways by quercetin concentrations similar to those measured in human blood is a major part of the novelty of this work.
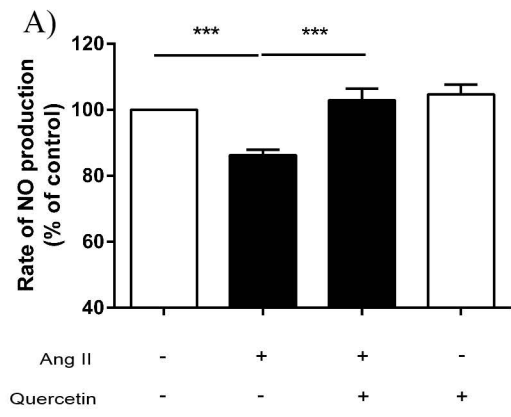
List of Abbreviations

Ang II – Angiotensin II

cDNA – Copy deoxyribose nucleic acid

DAF-2 DA – Diaminofluorescein diacetate

ddH_2O – distilled water

DHE - Dihydroethidium

DMF – Dimethyl-formamide

DMSO – Dimethyl-sulfoxide

DTPA – Diethylene triamine penta-acetic acid

ECGM – Endothelial cell growth media

ECL – Enhanced luminol chemiluminescence

eNOS – Endothelial nitric oxide synthase

FBS – Fetal bovine serum

HBSS – Hank's balanced salt solution

HRP – Horseradish peroxidase

HUVEC – Human umbilical vein endothelial cells

kDa – Kilo Dalton

LC-MS – Liquid chromatography mass spectrometry

L-NAME – NG-Nitro-L-arginine methyl ester

M199 – Medium 199

NADPH – Nicotinamide adenine dinucleotide 2'-phosphate (reduced form)

$NO^{\cdot}$ - Nitric oxide

$O_2^{\cdot-}$ - Superoxide

PBS – Phosphate buffered saline

PKC – Protein kinase C

PVDF – Polyvinylidiene difluoride
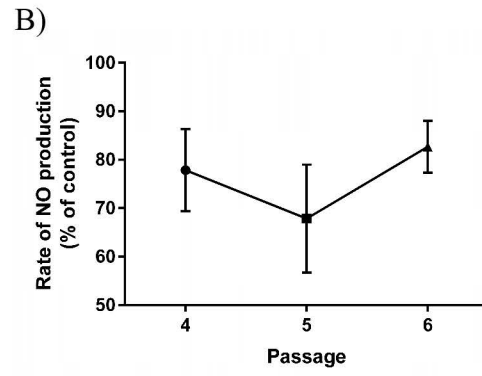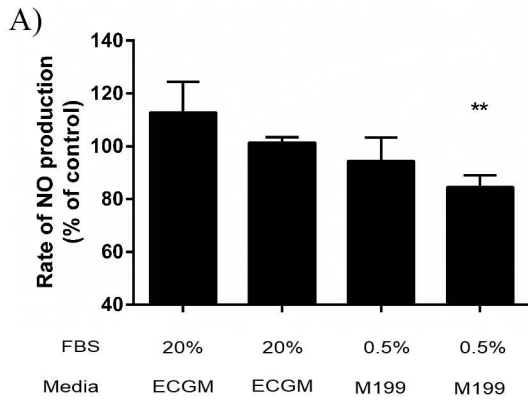
qPCR – Quantitative polymerase chain reaction

RNA – Ribose nucleic acid
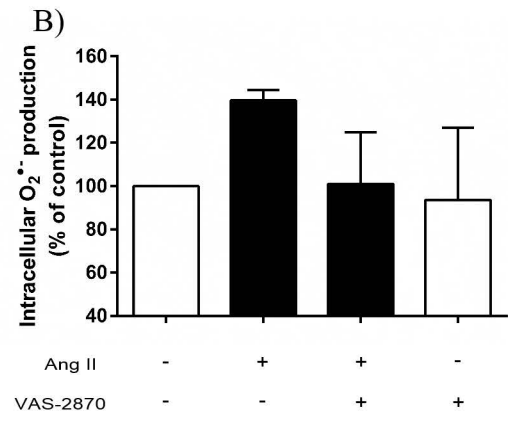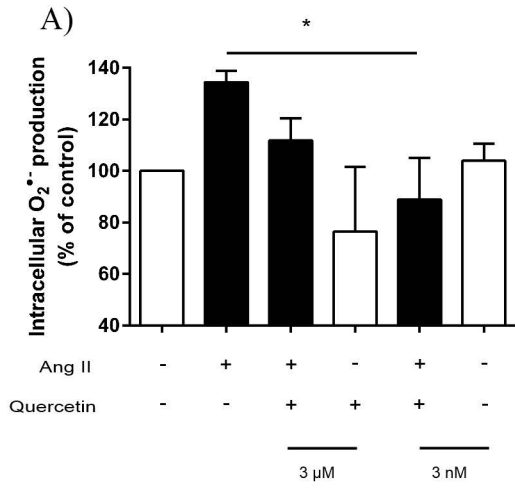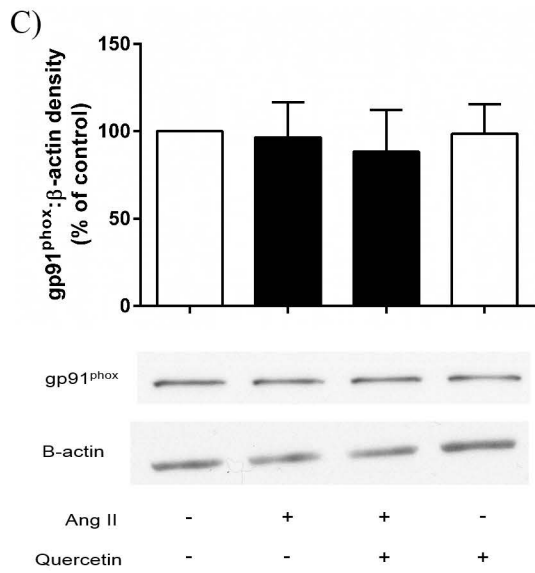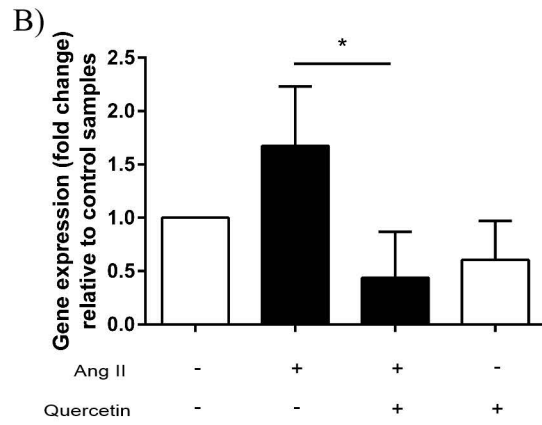
ROS – Reactive oxygen species

SOD – Superoxide dismutase

A)

p47phox:β-actin density (% of control)

200
150
100
50
0

**

p47phox

B-actin

| Ang II | - | + | + | - |
| Quercetin | - | - | + | + |

B)

Gene expression (fold change) relative to control samples

2.5
2.0
1.5
1.0
0.5
0.0

*

| Ang II | - | + | + | - |
| Quercetin | - | - | + | + |

C)

gp91phox:β-actin density (% of control)

150
100
50
0

gp91phox

B-actin
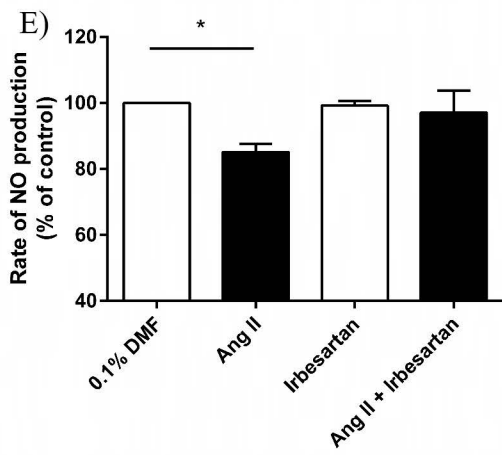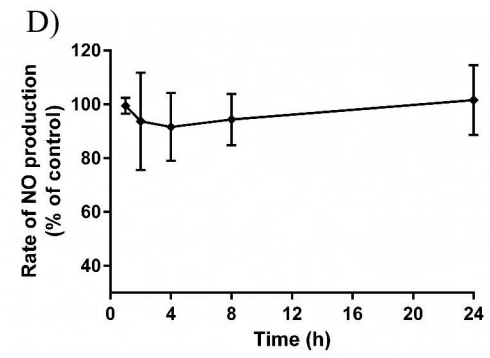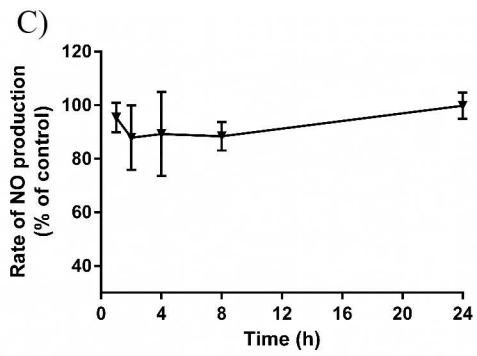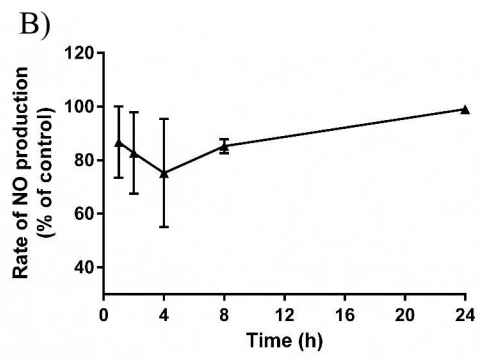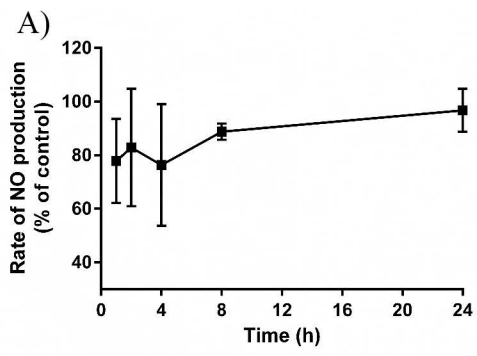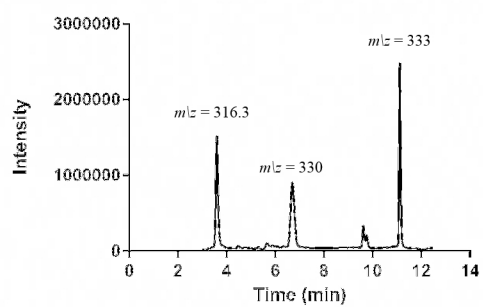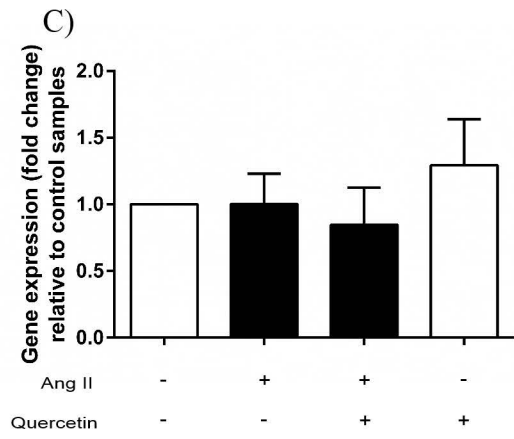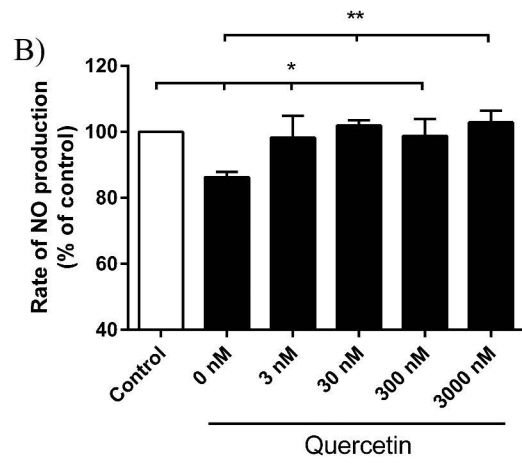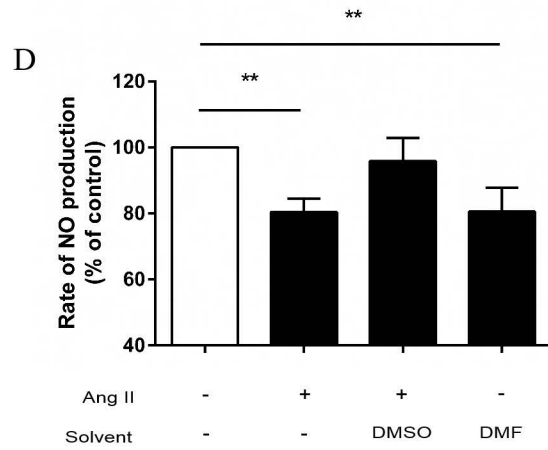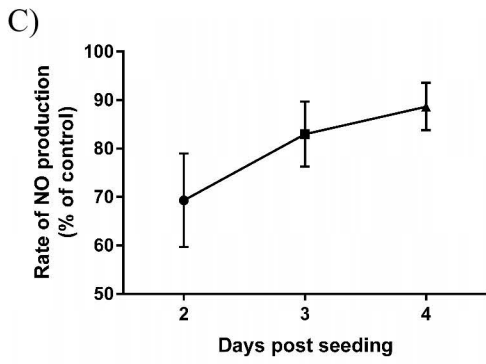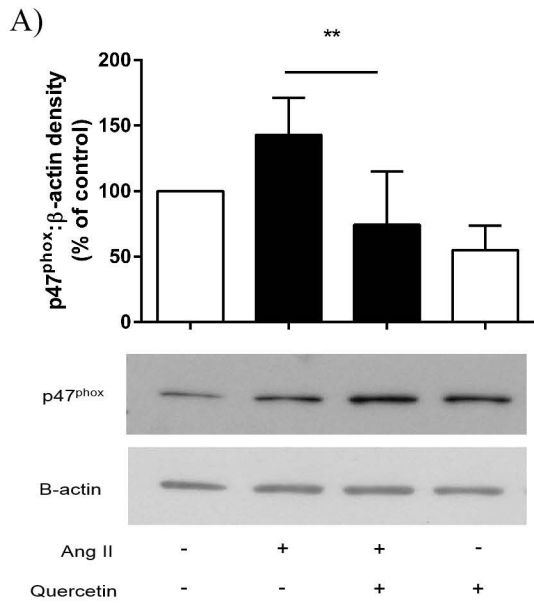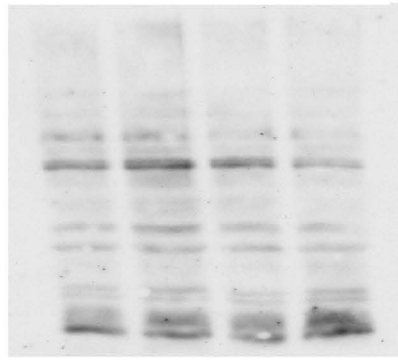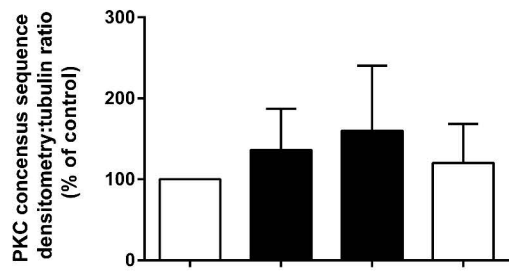
| Ang II | - | + | + | - |
| Quercetin | - | - | + | + |

B-tubulin

Ang II	-	+	+	-
Quercetin	-	-	+	+