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# Title: The dietary flavonol quercetin ameliorates angiotensin II-induced redox signalling imbalance in a human umbilical vein endothelial cell model of endothelial dysfunction via ablation of $p47^{phox}$ expression.

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## Abstract

Scope: Quercetin is reported to reduce blood pressure in hypertensive but not normotensive humans, but the role of endothelial redox signalling in this phenomenon has not been assessed. This study investigated the effects of physiologically-obtainable quercetin concentrations in a human primary cell model of endothelial dysfunction in order to elucidate the mechanism of action of its antihypertensive effects.

Methods and results: Angiotensin II (100 nM, 8 h) induced dysfunction, characterised by suppressed nitric oxide availability (85 ± 4% $p<0.05$) and increased superoxide production (136 ± 5 %, $p<0.001$). These effects were ablated by an NADPH oxidase inhibitor. Quercetin (3 μM, 8 h) prevented angiotensin II induced changes in nitric oxide and superoxide levels, but no effect on upon nitric oxide or superoxide in control cells. The NADPH oxidase subunit $p47^{phox}$ was increased at the mRNA and protein levels in angiotensin II-treated cells (130 ± 14% of control, $p<0.05$), which was ablated by quercetin co-treatment. Protein kinase C activity was increased after angiotensin II treatment (136 ± 51%), however this was unaffected by quercetin co-treatment.

Conclusions: Physiologically-obtainable quercetin concentrations are capable of ameliorating angiotensin II-induced endothelial nitric oxide and superoxide imbalance via protein kinase C-independent restoration of $p47^{phox}$ gene and protein expression.

## 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

activity (13-15, 17). Interestingly, this suggestion is in apparent conflict with the later study of Larson *et al*. (2012), which concluded that the clinical effects of quercetin in hypertensive individuals were independent of plasma nitrite concentrations; a clinical marker of circulating $NO^{\cdot}$ availability. In addition, the barrier function of the endothelium makes this structure most likely to be exposed to plasma quercetin following oral administration.

This study therefore aimed to determine the potential effects of physiologically-obtainable quercetin concentrations on $NO^{\cdot}$ and $O_2^{\cdot -}$ signalling imbalance. For this study primary vascular endothelial cells (Human Umbilical Vein Endothelial Cells; HUVEC) have been used rather than endothelial cell lines, which have been reported to vary in characteristics quite dramatically when compared to *ex vivo* tissues. HUVEC are well characterised, relatively easily accessible, primary cells that have been reported to exhibit key endothelial cell functions, and are extensively used in the study of the effects of dietary chemicals upon cardiovascular biology and diseases (18-24). Specifically, we have assessed the effects of quercetin on $NO^{\cdot}$ availability, $O_2^{\cdot -}$ production and NADPH oxidase subunit protein levels and activity. The concentrations of quercetin added have been chosen to replicate those which may arise from dietary intake, providing meaningful information on the mechanisms which underlie clinical observations. This use of physiologically-obtainable quercetin concentrations is an important part of the novelty of this study as although the role of NADPH oxidase and $NO^{\cdot}$ production in the mechanism of action has been previously investigated in a variety of model systems, these studies have mainly assessed supra-physiological concentrations of quercetin. Thus there is a possibility that the previously reported effects of quercetin upon vascular dysfunction are actually due to off-target effects and are therefore not relevant to the mechanism of action in humans. Our study aims to address this important consideration.

## Materials and methods

*Materials*

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

Human Umbilical Vein Endothelial Cells were purchased from Promocell (Heidlberg, Germany) as cryopreserved aliquots of 5 x $10^5$ cells pooled from 4 donors. Endothelial Cell Growth Medium (ECGM) was also purchased from Promocell (Heidlberg, Germany). Medium 199 (M199) was obtained from Life Technologies (Paisley, UK). Nunc Nunclon plasma-treated black 96-well plates were bought from Fisher Scientific (Leicestershire, UK). 4, 5-Diaminofluorescein diacetate (DAF-2 DA) was purchased from Enzo Life Sciences (Exeter, UK). The DC protein assay kit, 2x Lamelli buffer, Mini-Protean 12% TGX gels, 10x TGS buffer, 0.2 μm PVDF Turboblot membrane packs, Precision Plus protein marker ladder, Clarity Western ECL solution, Aurum total RNA extraction kit and iScript cDNA synthesis kit were all obtained from Bio-Rad (Hertfordshire, UK). Marvel low-fat powdered milk was purchased from a local supermarket. Autoradiography films (Amersham ECL) were purchased from Fisher Scientific (Loughborough, UK). An Endothelin-1 ELISA assay kit was purchased from Enzo Life Sciences (Exeter, UK). Solaris PCR expression assays for NOS3 (eNOS), NCF-1, EF1α and β-actin, and the Solaris qPCR mastermix were purchased from GE Healthcare (Buckinghamshire, UK).

The following antibodies were used: $p47^{phox}$ (Abcam, Cambridge, UK, Ab63361, Lot # 803556), $gp91^{phox}$ (Abcam, Cambridge, UK, AB129068, Lot # GR83718-3), β-actin (Abcam, Cambridge, UK, ab20272, Lot # GR88824-1), Phospho-(Ser) PKC substrate antibody (Cell Signalling, 2261,Lot # 18), anti-beta tubulin (Millipore, 06-661, Lot # 239882), Goat anti-rabbit HRP-conjugate (Sigma-Aldrich, Poole, UK, A6154, Lot # 090116176), Goat anti-mouse

HRP-conjugate (Abcam, Cambridge, UK, ab97023 , Lot # GR87150-8), Goat anti-rabbit HRP-conjugate (GE Healthcare, RPN4301, Lot # 9526414).

*Human Umbilical Vein Endothelial Cell (HUVEC) culture*

HUVEC were grown to ~95% confluence with ECGM + 20% foetal bovine serum (FBS) over 3-4 d, with the culture media refreshed every 2 d. After trypsinisation, HUVEC were seeded into either 96-well or 6-well plates at a density of 14,700 cells/$cm^2$ and cultured for the durations described below and in the figure legends, with media refreshed every 2 days.

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

Passage 4 – 6 HUVEC were seeded in 96-well plates (5 x $10^3$ cells / well) and allowed to proliferate for 2 - 3 d as required. Cell cultures were serum-deprived for 24 hours in Medium-199 containing 0.5% FBS (M199) before endpoint measurement. Ang II was prepared as a 1 mM stock in dd$H_2O$ (18.2 MΩ), diluted in culture media to produce 10 nM – 10 μM final concentration. 8 hr before end-point measurement cells were treated in M199 with or without Ang II. Cells were treated with quercetin or solvent only as directed in the Figure legends for 8 hours in the presence or absence of Ang II. For cells treated with L-NAME, a stock solution of 100 mM was prepared in DMSO, and diluted 1000-fold in culture media. Cells were treated with L-NAME, or a vehicle control, for 30 minutes before end-point measurement. After incubation with the appropriate treatment(s), cells were washed once on a warmed plate with 200 μl of warmed HBSS (containing magnesium and calcium), and 70 μl of warm HBSS (containing magnesium and calcium) containing 2 μM DAF-2DA was added per well. For L-NAME treated cells, L-NAME was also included in this dye solution. Fluorescence ($\lambda_{ex}$ 485

nm, $\lambda_{em}$ 520 nm) was measured every 3 min over a 30 minute period using a Tecan infinite X200 plate reader, maintained at 37°C, with a matrix of 4x4 points per well measured (ten flashes per point), and a manual gain of 100 set. Linear rates ($r^2 \geq 0.99$) were calculated for all wells and a mean rate of fluorescence was calculated for each treatment (n=4-6 wells per treatment). Wells containing cells only, and wells without cells were also assessed

*Protein extraction from HUVEC cultures*

Cells were cultured as described and treated 8 h before extraction with either DMF (0.1% v/v), quercetin (3 μM), DMF (0.1% v/v) and Ang II (100 nM), or quercetin (3 μM) and Ang II (100 nM), in M199 containing 0.5% FBS. Monolayers were washed twice in 3 ml of PBS per well, before the addition of 200 μl of RIPA buffer (1% NP-40 substitute, 0.5% w/v sodium deoxycholate, 0.1% w/v sodium dodecyl sulphate, made up in PBS containing protease inhibitor cocktail) per well. Cells were harvested by scraping before incubation for 5 min on ice to aid lysis. The cell lysates were sonicated (Sonic Vibracell VCX130PB) for 3 x 10 s bursts on ice. Lysates were centrifuged at 16 100 x g, 5 min, and the resulting supernatant stored at -20°C.

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

Protein extracts, prepared as above, were quantified using the DC protein assay as described by the manufacturer. 20 μg of protein extract was added in a 1:1 ratio to 2x Lamelli buffer. The resulting samples were then boiled for 5 minutes at 95°C, before incubation on ice for 2 minutes, and centrifugation at 16 100 x g for 30 seconds. Proteins were separated using 12% TGX gel for 30 minutes at 250 V in 1x TGS buffer. Separated proteins were transferred PVDF membranes before washing in 1x TBST buffer (20 mM Trizma base, 137 mM sodium chloride,

1 ml of tween20, pH 7.6) and blocking in a solution of 5% w/v milk in 1x TBST for 30 minutes. Membranes were rinsed and washed 3x 10 minutes in 1x TBST buffer, before incubation for 1 hour with the appropriate primary antibody dilution ($p47^{phox}$ – 1:2000; $gp91^{phox}$ – 1:5000) in 1% w/v milk in 1x TBST buffer. Following further rinsing and 3 x 10 minute washes in 1x TBST buffer, membranes were incubated for 1 hour with the appropriate secondary antibody (Goat anti-rabbit HRP conjugate – 1:30000) diluted in 1% w/v milk in 1x TBST. The membranes were then rinsed and washed again (3x 10 minutes in 1x TBST) and processed by ECL. Membranes were stored at 4°C for use in measuring β-actin levels as a loading control. Membranes were incubated for 2x 10 minute washes in stripping buffer (200 mM glycine, 3.5 mM sodium dodecyl sulphate, 1% tween20, adjusted to pH 2.2), washed twice in TBST, and then blocked (5% w/v milk, 30 minutes) and washed a further 3 times. The membranes were incubated with the β-actin HRP conjugated antibody (1:2 000 000 dilution) in 1% w/v milk in 1xTBST buffer for 1 hour before a further 3x 10 minutes washes in 1x TBST buffer. The membranes were incubated in ECL solution and developed as previously described, with a 2.5 minute exposure to the autoradiography film.

For the assessment of PKC-phosphorylated substrate consensus sequences membranes were blocked using 10% bovine serum albumin (BSA) for 1 h followed by incubation with a PKC-phosphorylated substrate consensus sequence primary antibody diluted to 1:1000 in 1 % BSA for 1 h. An appropriate HRP-conjugated secondary antibody diluted 1:10,000 in 1% BSA was used to detect the primary antibody. Membranes were stripped and re-probed with for β-tubulin at a dilution of 1:1000 overnight at 4°C.

All films were digitised and densiometry was done using the ImageJ software. A ratio of protein of interest: β-actin was calculated for each sample with the control samples for each biological replicate set at 100%. For the PKC-phosphorylated substrate films, the entire lane was quantified by densitometry as described above, and normalised to β-tubulin.

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

HUVEC were cultured as described in 6-well plates and serum-deprived in M199 containing 0.5% FBS for 24 h prior to experiments. At 8 h before experiments these cultures were incubated with or without Ang II (100 nM) in the presence of vehicle (0.1% v/v DMF), 3 nM, or 3 μM quercetin, with 3 wells per treatment. For experiments using the NADPH oxidase inhibitor VAS-2870, cells were treated with or without Ang II for 8 h, with either vehicle (0.1% DMF) or 1 μM VAS-2870 for 30 minutes before assay. After 8 h incubation the cells were washed twice in excess warm PBS containing 100 μM DTPA and then incubated in darkness with 2 ml of 20 μM dihydroethidium (DHE, dissolved in DMSO at a stock concentration of 20 mM) in HBSS for 30 min . Following two further washes with excess PBS - DTPA, each well was scraped in 500 μl of methanol, and wells from the same treatment groups were pooled and stored overnight at -20 °C. The resulting lysates were centrifuged (16,100 x g, 10 minutes, 4 °C) with both the supernatant and pellet retained separately. The supernatant was evaporated to dryness using a centrifugal evaporator without heating (Genevac MiVac, Genevac, Ipswich, UK), and the resulting residue was dissolved in 1:1 water:stabilisation solution (33.3% methanol, 10 μM fluorescein internal standard, 0.1 mM DTPA, and 5 mM sodium ascorbate, made up to final volume with water) with a final volume of 120 μl for LC-MS analysis. The pellet was dissolved in 100 μl of 0.1 M sodium hydroxide and the protein content was quantified using the DC assay as directed by the manufacturer.

For LC-MS analysis of DHE oxidation products, the specific $O_2^{\cdot-}$ reaction product (2-HE+) was detected at a *m/z* ratio of 330.3, and at a retention time of 7 min. Separation was achieved using a Shimadzu prominence LC20 quaternary pump and autosampler, a Shimadzu CTO10 column oven, and an Agilent Eclipse XDB-C18 column (5 μm, 4.6 x 150 mm) at a flow rate of 0.5 ml/min. Water containing 0.5% formic acid (solvent A) and methanol containing 0.5% formic acid (solvent B) were used to achieve separation as follows; 43% B for 3.35 minutes,

increasing to 85% B by 8 minutes, maintained at 85% B until 11.5 minutes, reduced to 43% B by 11.75 minutes, held at 43% B until 18.75 minutes. Column oven temperature was set to 40 °C. The injection volume used was 20 µl. A Shimadzu LC2020 single quadrapole mass spectrometer was used as the detector with the following settings: Fluorescein was used as an internal standard, at a *m/z* ratio of 333.0 and a retention time of 11.25 minutes. Peaks corresponding to 2-HE+ and fluorescein were integrated and peak areas were calculated. The ratio of 2-HE+:fluorescein was normalised to the total protein. Control samples from each independent experiment were set to 100%, with all other treatments expressed as a percentage of control, and 3 independent experiments were analysed. A representative chromatogram is shown in Figure S1.

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

HUVEC cultures were grown as described above in 6-well plate format, with and without Ang II (8 h, 100 nM) and quercetin (3 µM, 8 h) treatments. Cells were washed and scraped in PBS, with wells from the same treatment group pooled together, centrifuged, and RNA was extracted using the Bio-Rad Aurum RNA extraction kit as directed by the manufacturer. Eluted RNA was quantified by spectrophotometry. Using the Bio-Rad iScript cDNA synthesis kit, as directed by the manufacturer, 500 ng of RNA was reverse transcribed, and the resulting cDNA was quantified by spectrophotmetry. Relative quantification of gene expression, comparing control against treated samples, was achieved using the validated Solaris PCR expression assay probes for eNOS, $p47^{phox}$, Elongation Factor 1α and β-actin, by loading 150 ng of cDNA template to the assay probes and Solaris PCR master mix as directed by the manufacturer. The following PCR conditions were used for these probe sets: 1 cycle of 95°C 15 min followed by 50 cycles of 95°C for 15 sec and 60°C for 1 min. The resulting Ct values were analysed by the

ΔΔCt method, using β-actin or Elongation Factor 1α as the reference gene. Three independent experiments were analysed, with mean fold change in eNOS or $p47^{phox}$ expression in treated samples compared to control samples calculated, ± 1 standard deviation.

*Statistical analysis*

All statistical analyses used Sigmaplot version 12.0, with comparisons between multiple groups done by ANOVA or ANOVA on ranks, as appropriate after testing for normality of distribution and homogeneity of variance. Post-hoc tests were done using Tukey post-hoc tests. Values less than $p = 0.05$ were considered statistically significant.

## Results

### *Ang II inhibits the production of* $NO^{\cdot}$ *in HUVEC*

In order to examine the effects of quercetin on endothelial dysfunction we first exposed HUVEC to a range of Ang II concentrations (10 nM – 10 μM) for up to 24 h, under optimised culture conditions (as outlined in Figure S2), and the effect of Ang II treatment upon $NO^{\cdot}$ availability was determined (Figure 1 A-D). It was observed that concentrations below 10 μM Ang II resulted in a 10 – 20% decrease in the rate of nitric oxide production, measured in live HUVEC cultures by determining the rate of fluorescence accumulation using diaminofluorescein (DAF). The greatest and most consistent reduction in $NO^{\cdot}$ was observed for an 8 h incubation at 100 nM angiotensin II (Figure 1B). We did not observe a dose-response for Ang II in these experiments over the concentration range tested, however the observation of a lack of effect at higher concentrations (1 μM and above) is consistent with previous research, as is the observed time dependency of Ang II treatment (18). Based upon these experiments future Ang II treatments were at a concentration of 100 nM for 8 h; a treatment regime that resulted in a decrease in $NO^{\cdot}$ availability of 15 ± 4% compared to untreated cells (Figure 1E, $p<0.05$). To confirm that this effect was mediated in a receptor dependent manner the experiments were repeated in the presence of the angiotensin receptor type 1 antagonist irbesartan (10 μM, 8 h). Under these conditions no change in NO production was observed (Figure 1E). It was also noted that DMSO (0.1% v/v, 8 h), a commonly-used carrier solvent, attenuated the effects of Ang II on $NO^{\cdot}$ levels in this system (Figure S2). Dimethyl-formamide (DMF) did not attenuate the effects of Ang II on $NO^{\cdot}$ and was therefore used for all future experiments.

*Physiologically-obtainable quercetin concentrations prevents Ang II-induced decreases in nitric oxide production*

Using the live-cell nitric oxide assay described above, we next assessed the effect of quercetin upon the rate of nitric oxide production in HUVEC cultures with and without Ang II treatment (8 h, 100 nM). An initial test concentration of 3 μM quercetin was selected for these experiments in order to test a physiologically-obtainable concentration (7, 25, 26). Quercetin (3 μM, 8 h) was shown to prevent Ang II-induced decreases in the rate of nitric oxide production but had no effect on control cells (Figure 2A). A dose response of quercetin (3 nM – 3 μM) in Ang II treated cells showed that quercetin prevented Ang II-induced dysfunction at all concentrations tested (Figure 2B). We next assessed the effect of quercetin treatment (3 μM, 8 h) on the expression of endothelial Nitric Oxide Synthase (eNOS) using qPCR, with no changes in gene expression observed for any treatment compared to untreated cells (Figure 2C).

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

As NADPH oxidase expression and reactive oxygen species (ROS) formation have been suggested as key targets for quercetin (11, 12) we next sought to determine the effects of both Ang II and quercetin treatments on superoxide production in HUVEC cultures. Superoxide was increased by 36 ± 5% compared to control cells, and this was prevented by quercetin co-treatment at both 3 μM and 3 nM (Figure 3A). The prevention of Ang II-induced superoxide production by the NADPH oxidase inhibitor VAS-2870 (Figure 3B, 1 μM, 8 h, (27)) suggests that this ROS-producing complex was a major source of Ang II-induced superoxide in these 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 $NO^{\cdot}$, increased intracellular $O_2^{\cdot -}$, 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$^{phox}$, $O_2^{\cdot -}$ (SOD-inhibitable lucigenin detection),
325 and of $NO^{\cdot}$ production (nitrate and nitrite levels) *in vitro* (28, 29). However, these previous

studies used supraphysiological concentrations of quercetin under non-dysfunctional conditions and their findings are in conflict with clinical research. Consequently we have sought to address the discrepancy between the *in vitro* and clinical data by using physiologically-attainable quercetin concentrations and assessed effects under both non-dysfunctional and dysfunctional conditions.

Our study has shown that physiologically-obtainable concentrations of quercetin restore endothelial function (availability of $NO^{\cdot}$, intracellular $O_2^{\cdot -}$ and $p47^{phox}$ expression) to control levels in dysfunctional cells (induced by Ang II) whereas there are no effects in non-dysfunctional cells. This is in concordance with the current clinical data for the effects of quercetin in healthy and diseased populations (7, 8). The mechanism of action of quercetin that underlies these effects is currently unknown, with mechanistic insights from clinical studies and rodent studies being conflicting. Although these rodent studies have implicated changes in NADPH oxidase activity and increased availability of $NO^{\cdot}$ as part of the mechanism of action, due to the inherent multicellular and multi-tissue complexity of the aortic ring segments, they have mainly focussed upon the effects of quercetin on vascular smooth muscle rather than the endothelium in *ex vivo* aortic ring segments. The findings presented from our study demonstrate that quercetin restores endothelial function in Ang II-treated cells and suggests that the observed restoration of endothelial $NO^{\cdot}$ and $O_2^{\cdot -}$ signalling via the restoration of NADPH oxidase expression (possibly independently of PKC activity) is part of the mechanism of action of quercetin (Figure 6). Interestingly, over the concentration range tested (3 nM – 3 μM), there was no observed dose response and a similar ablation of endothelial dysfunction by quercetin was seen at all tested concentrations. There are several possible explanations for this observation including that the concentrations assessed in this study were not in the linear phase of a classical s-shaped dose response, or that quercetin potentially has multiple different mechanisms of action which can result in a variety of different shaped dose

response curves (30). Ultimately, future research should focus upon investigating the dose-response characteristics of quercetin in the context of endothelial dysfuction, as well as investigating the role of other potential mechanisms of action, such as other kinase signalling cascades (e.g. MAPK) and the phosphorylation control of eNOS and $p47^{phox}$.

Quercetin has been reported in human plasma in the nanomolar to low micromolar range, after dietary intake and supplementation (7, 8, 25, 26). Human pharmacokinetic studies also have shown that quercetin undergoes substantial conjugative metabolism, with the formation of sulfate and glucuronide conjugates, whereas the aglycone form is not detected in human plasma (31). It should be noted that these circulating conjugated forms are not currently widely available, and that as a result we have used the aglycone form of quercetin in this study. This is not necessarily a major limitation as it has recently been suggested that the anti-hypertensive effects of quercetin glucuronides are dependent on deconjugation activity and the liberation of quercetin (32). HUVEC have also been reported to express β-glucuronidase at the mRNA level (33), suggesting that they also possess at least some deconjugative machinery. Taken together, these data suggest that the use of the aglycone form of quercetin in the endothelial cell model reported is a valid approach for assessing its effects on endothelial dysfunction.

In summary, physiologically-obtainable concentrations of the dietary flavonoid quercetin restored $NO^{\cdot}$ availability, $O_2^{\cdot -}$ production and $p47^{phox}$ expression to control levels, possibly in a PKC-independent manner, in a human primary cell model of endothelial dysfunction. There were no further improvements in $NO^{\cdot}$ availability under control conditions. These results are consistent with clinical observations that quercetin reduces blood pressure in hypertensive, but not normotensive, individuals, and suggest a potential mechanism by which quercetin mediates this effect.

## Author contributions

Experiments were designed by HSJ and FLC. Experiments were done by HSJ, FLC, AG and SM. KN provided reagents and resources for the PKC experiments. Funding was provided by SLA. All authors reviewed and approved the final manuscript.

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## Author disclosure statement

For all authors: No competing financial interests exist.

## List of Abbreviations

Ang II – Angiotensin II

cDNA – Copy deoxyribose nucleic acid

DAF-2 DA – Diaminofluorescein diacetate

dd$H_2O$ – 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

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## 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, * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

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

A) Ang II-induced increases in intracellular superoxide are restored to control levels by quercetin co-treatment (8 h) at both 3 nM and 3 μM concentrations, with no effect in quercetin only treated cells. B) Ang II-induced increases in intracellular superoxide are ameliorated to control levels by the NADPH oxidase inhibitor VAS-2870 (1 μM, 8 h). Filled bars indicate Ang II treatment (100 nM, 8 h). Graphs show mean values ± 1 S.D., n ≥ 3 independent experiments, * $p<0.05$.

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

Quercetin co-treatment (3 μM, 8 h) restored Ang II-induced $p47^{phox}$ protein expression (100 nM, 8 h) to control levels in HUVEC cultures as assessed by Western blotting (A). This trend was reflected at the mRNA level, assessed using qPCR (B). The NADPH oxidase subunit $gp91^{phox}$ was unaffected by either Ang II (100 nM, 8 h) or quercetin (3μM, 8 h) treatments (C). Filled bars indicate Ang II treatment (100 nM, 8 h). For the Western blotting data shown, the densitometry of the protein band of interest has been normalised to that of beta-actin, and it is this ratio that is presented in the bar graphs. Graphs show mean values ± 1 S.D., n ≥ 3 independent experiments, * $p<0.05$; ** $p<0.01$.

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

There was a trend of increase in PKC activity induced by Ang II exposure (100 nM, 8 h) but this effect was not ablated by quercetin co-treatment (3 μM, 8 h). Filled bars indicate Ang II treatment (100 nM, 8 h). For this Western blotting data the densitometry values for each lane

of the PKC substrates blot has been normalised to that of tubulin. It is this ratio that is presented in the bar graphs. Graphs show mean values ± 1 S.D., n ≥ 3 independent experiments

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

Quercetin restores angiotensin II-induced redox imbalance, centred around nitric oxide and superoxide. Changes in $p47^{phox}$ protein expression are also restored to control levels, via modulation of gene expression, by a mechanism that seems independent of PKC activity. Arrows indicate the effects of angiotensin II treatment, which were ameliorated by quercetin co-exposure.

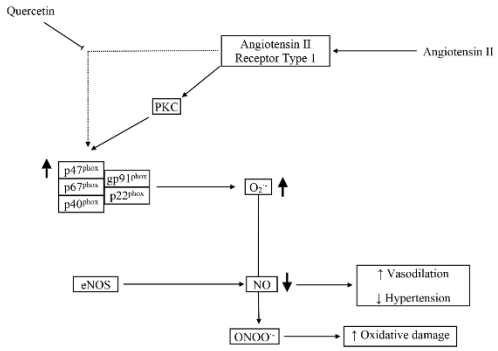
**Supplementary Figure 1**

A representative chromatogram of dihydroethidium (HE, precursor), the superoxide-specific reaction product of HE (2HE+), and fluorescein (internal standard). Dihydroethidium was detected at $m\backslash z = 316.3$, 2HE+ at $m\backslash z = 330$, and the internal standard fluorescein at $m\backslash z = 333$.

**Supplementary Figure 2**

A) Cell media composition affects HUVEC response to Ang II (100 nM, 8 h). 24 h serum deprivation in M199 containing 0.5% (v/v) FBS resulted in a consistent decrease in $NO^{\cdot}$ availability (15 ± 4% of control) when HUVEC were treated with Ang II (100 nM, 8 h). Filled bars indicate Ang II (100 nM, 8 h) treatment. B) The effect of cell passage number upon Ang II (100 nM, 8 h) induced decreases in $NO^{\cdot}$ availability. Passages 4-6 are demonstrated to be

599 responsive to Ang II, measured by $NO^{\cdot}$ availability. C) The effect of confluency upon Ang II
600 induced decreases in $NO^{\cdot}$ availability. Cells grown for 2-3 days post seeding are demonstrated
601 to be responsive to Ang II, measured by $NO^{\cdot}$ availability, with decreasing responsiveness by 4
602 days post seeding. D) The effects of DMSO and DMF upon Ang II induced decreases in $NO^{\cdot}$
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 $NO^{\cdot}$ 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

dd$H_2O$ – 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

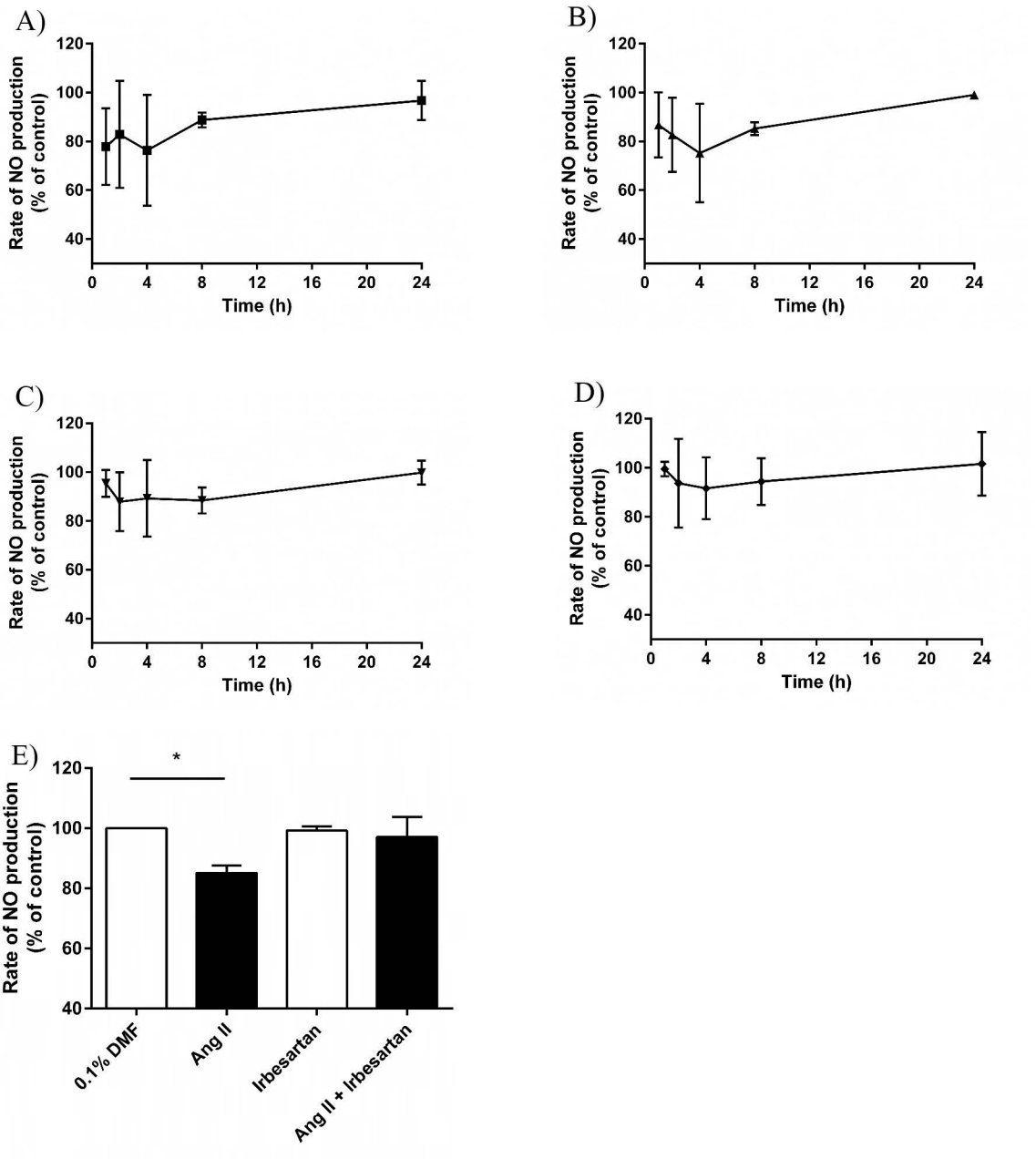
PVDF – Polyvinylidiene difluoride

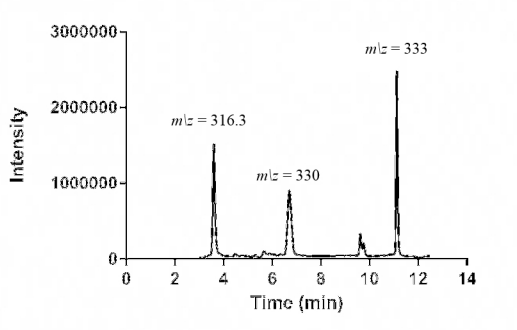
qPCR – Quantitative polymerase chain reaction

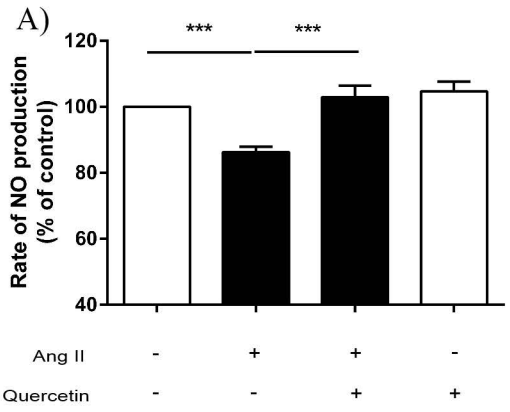
RNA – Ribose nucleic acid

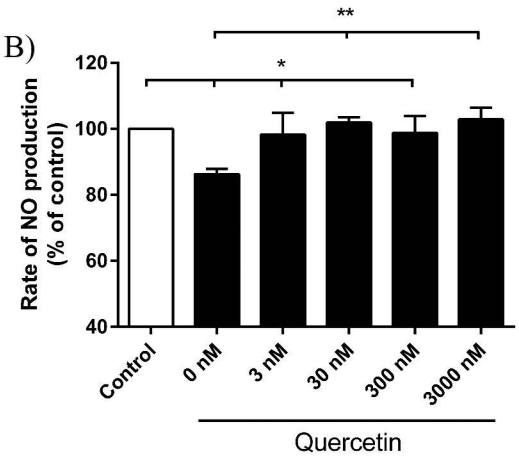
ROS – Reactive oxygen species

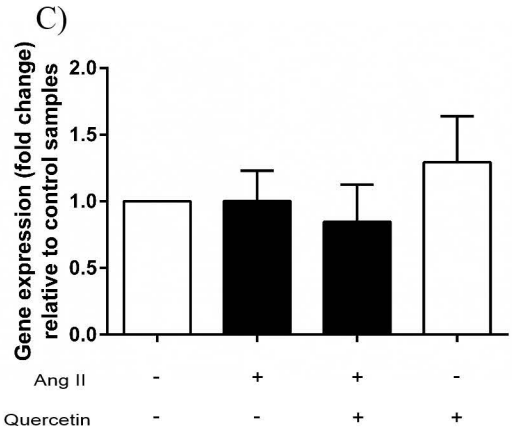
SOD – Superoxide dismutase


A)
B)
C)
D)
E)
Rate of NO production (% of control)
Time (h)
0
4
8
12
16
20
24
40
60
80
100
120
*
0.1% DMF
Ang II
Irbesartan
Ang II + Irbesartan

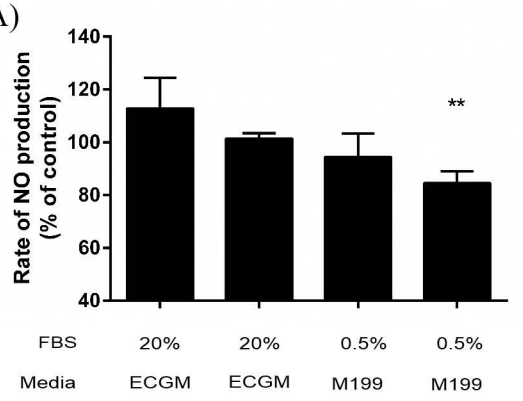

3000000
2000000
1000000
0
Intensity
m/z = 316.3
m/z = 330
m/z = 333
0
2
4
6
8
10
12
14
Time (min)


A)
***
***
Rate of NO production (% of control)
120
100
80
60
40
Ang II - + + -
Quercetin - - + +

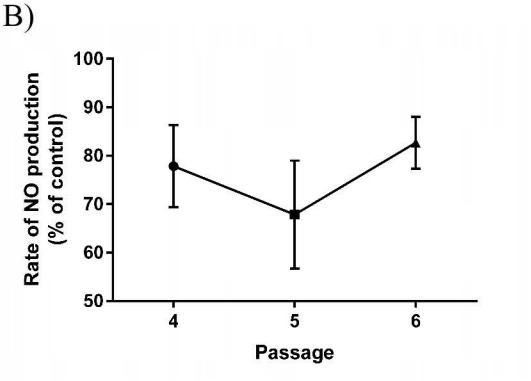

B)
**
*
Rate of NO production (% of control)
120
100
80
60
40
Control
0 nM
3 nM
30 nM
300 nM
3000 nM
Quercetin


C)
Gene expression (fold change) relative to control samples
2.0
1.5
1.0
0.5
0.0
Ang II - + + -
Quercetin - - + +

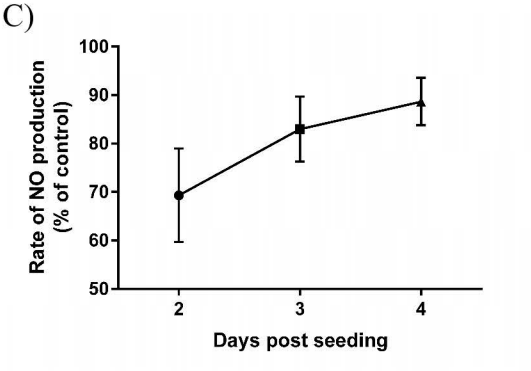
A)


Rate of NO production (% of control)
140
120
100
80
60
40
**
FBS 20% 20% 0.5% 0.5%
Media ECGM ECGM M199 M199

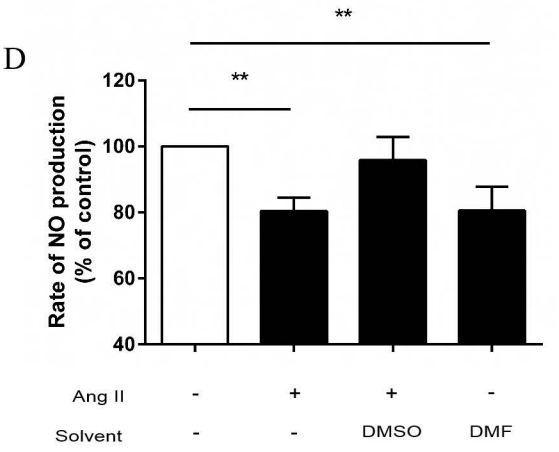
B)

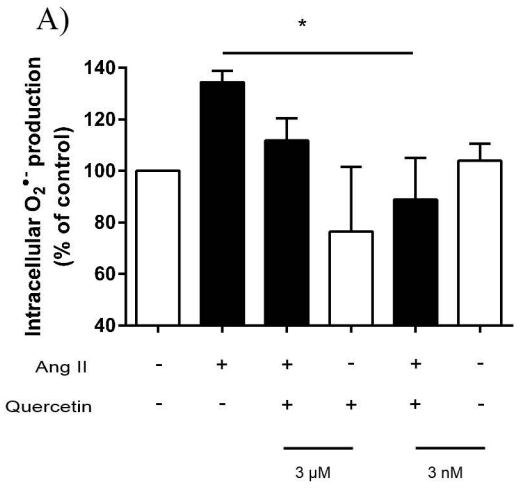

Rate of NO production (% of control)
100
90
80
70
60
50
4
5
6
Passage

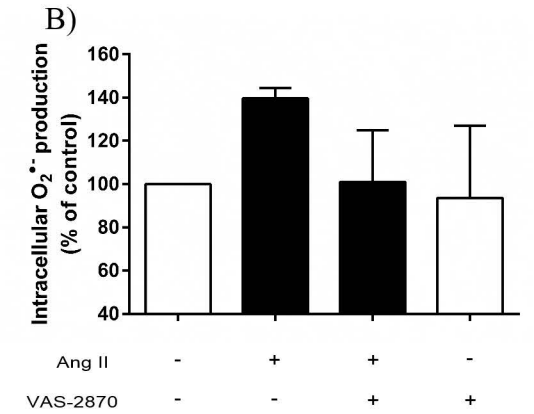
C)

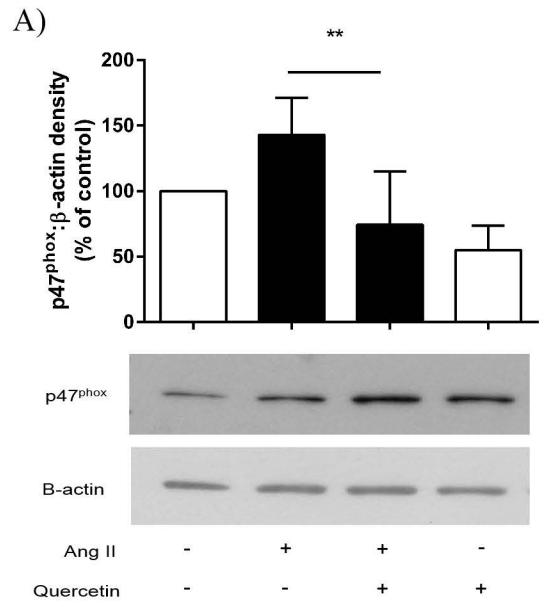

Rate of NO production (% of control)
100
90
80
70
60
50
2
3
4
Days post seeding

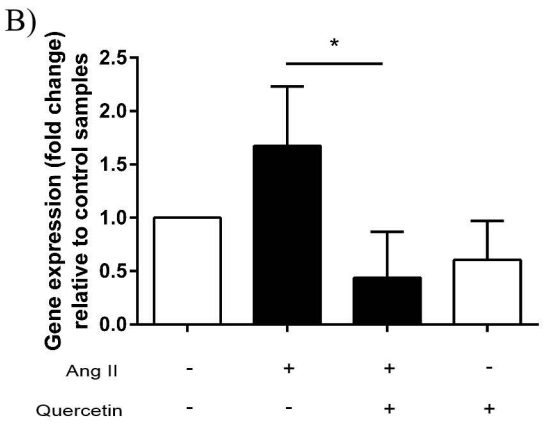
D

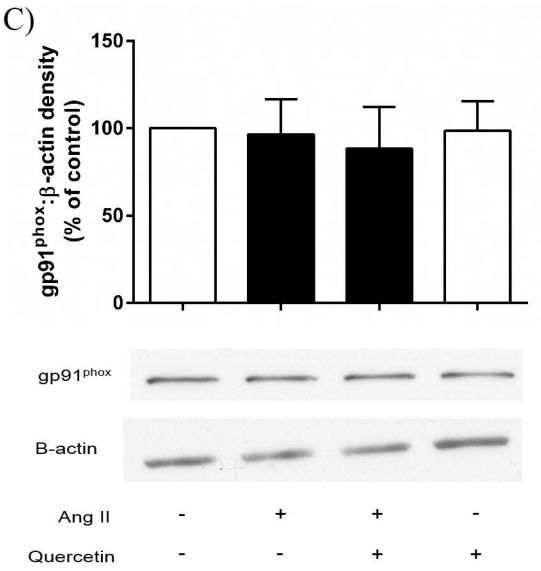

**
**
Rate of NO production (% of control)
120
100
80
60
40
Ang II - + + -
Solvent - - DMSO DMF

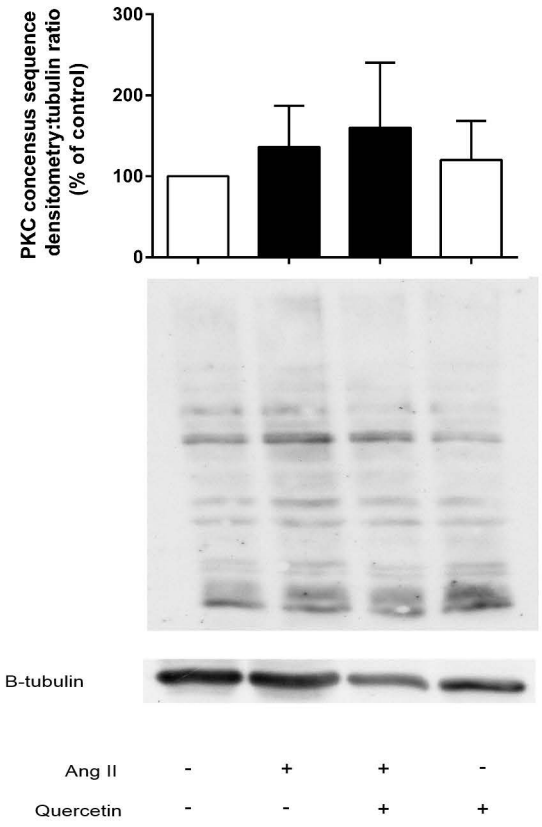

A)
*
Intracellular $O_2^{\bullet-}$ production (% of control)
140
120
100
80
60
40
Ang II - + + - + -
Quercetin - - + + + -
3 µM
3 nM


B)
Intracellular $O_2^{\bullet-}$ production (% of control)
160
140
120
100
80
60
40
Ang II - + + -
VAS-2870 - - + +


A)
**
200
150
100
50
0
p47phox:β-actin density
(% of control)
p47phox
B-actin
Ang II - + + -
Quercetin - - + +


B)
*
2.5
2.0
1.5
1.0
0.5
0.0
Gene expression (fold change)
relative to control samples
Ang II - + + -
Quercetin - - + +


C)
150
100
50
0
gp91phox:β-actin density
(% of control)
gp91phox
B-actin
Ang II - + + -
Quercetin - - + +


PKC concensus sequence densitometry:tubulin ratio (% of control)
300
200
100
0
B-tubulin
Ang II - + + -
Quercetin - - + +