Blackberry, raspberry and black raspberry polyphenol extracts attenuate angiotensin II-induced senescence in vascular smooth muscle cells
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Activation of angiotensin II (Ang II) signaling during aging increases reactive oxygen species (ROS) leading to vascular senescence, a process linked to the onset and progression of cardiovascular diseases (CVD). Consumption of fruits and vegetables, particularly berries, is associated with decreased incidence of CVD, which has mainly been attributed to the polyphenol content of these foods. Thus, the objective of this study was to investigate the role of blackberry (BL), raspberry (RB), and black raspberry (BRB) polyphenol extracts in attenuating Ang II-induced senescence in vascular smooth muscle cells (VSMCs) and to determine the molecular mechanisms involved. BL, RB and BRB polyphenol extracts (200 µg ml⁻¹) attenuated Ang II-induced senescence, denoted by decreased number of cells positive for senescence associated β-galactosidase (SA-β-gal) and down-regulation of p21 and p53 expression, which were associated with decreased ROS levels and Ang II signaling. BL polyphenol extract increased superoxide dismutase (SOD) 1 expression, attenuated the up-regulation of Nox1 expression and the phosphorylation of Akt, p38MAPK and ERK1/2 induced by Ang II, and reduced senescence in response to Nox1 overexpression. In contrast, RB and BRB polyphenol extracts up-regulated the expression of SOD1, SOD2, and glutathione peroxidase 1 (GPx1), but exerted no effect on Nox1 expression nor on senescence induced by Nox1 overexpression. BRB reduced signaling similar to BL, while RB was unable to reduce Akt phosphorylation. Furthermore, we demonstrated that inhibition of Akt, p38MAPK and ERK1/2 as well as down-regulation of Nox1 by siRNA prevented senescence induced by Ang II. Our findings indicate that Ang II-induced senescence is attenuated by BL polyphenols through a Nox1-dependent mechanism and by RB and BRB polyphenols in a Nox1-independent manner, likely by increasing the cellular antioxidant capacity.

1. Introduction

Aging is considered as the main risk factor for the development of chronic diseases, including cardiovascular diseases (CVD). The prevalence and incidence of CVD, the number one cause of death worldwide, continues to rise as life expectancy increases. Cellular senescence, one of the common intrinsic aging processes, is linked to the onset and progression of CVD. In the presence of various stimuli, including oxidative stress, senescent cells become dysfunctional and resistant to apoptosis as they enter a state of irreversible growth arrest. During this process, senescent cells remain metabolically active, secreting abnormal amounts of a variety of molecules, including inflammatory cytokines, extracellular matrix components, and reactive oxygen species (ROS), which characterize the senescence associated secretory phenotype (SASP). Excess ROS can react with proteins, lipids, and DNA altering gene expression. Altogether, these intrinsic and extrinsic changes have detrimental effects on the tissue microenvironment, increasing the chance of diseases. For instance, in vitro and in vivo studies showed that ROS levels and senescence associated β-galactosidase (SA-β-gal), a senescent marker, were elevated in vascular smooth muscle cells (VSMCs) with aging and atherosclerosis. Furthermore, in human atheroma, SA-β-gal-positive cells exhibited increased expression of p53 and p16, which are negative regulators of the cell cycle and also serve as markers of cellular senescence.
In the vasculature, NADPH oxidases (Noxes) are major sources of ROS.\(^9\) Nox1 and Nox4 are two isoforms found in VSMCs and they generate superoxide anion (O\(_2^−\)) and hydrogen peroxide (H\(_2\)O\(_2\)), respectively.\(^{10,11}\) Found on the cell surface and in endosomes, Nox1 is an inducible enzyme, associated with p22phox, that co-localizes with caveolin and its activation requires interaction with cytosolic subunits, including p47phox, p67phox, and Rac1.\(^{12,13}\) Nox4 is also associated with p22phox; however, it is a constitutive enzyme that co-localizes with vinculin and is mainly found in focal adhesions and in the endoplasmic reticulum.\(^{14,15}\) The expression of both Nox1 and Nox4 is elevated in many CVDs, including atherosclerosis.\(^7\) Sheehan \textit{et al.}\(^{16}\) found that aortic O\(_2^−\) levels and atherosclerotic lesions decreased in ApoE\(^{−/−}\)/ Nox1\(^{−/−}\) compared to ApoE\(^{−/−}\) control mice. Additionally, Vendrov \textit{et al.}\(^{17}\) demonstrated that Nox4 expression increased in aging mice under hyperlipidemic conditions.

Angiotensin II (Ang II), a peptide hormone, is a potent stimulus that contributes to the development of vascular senescence \textit{in vivo}\(^{18}\) and \textit{in vitro} \textit{in} VSMCs.\(^5\) Circulating Ang II exerts its pathological effects through binding to the Ang II type 1 receptor (AT1R), a G protein-coupled receptor (GPCR) abundant in the vasculature.\(^{19}\) In VSMCs, this binding pro-motes the phosphorylation and activation of downstream tyrosine and serine/threonine kinases,\(^{20,21}\) such as p38MAPK, Akt, and ERK1/2, which mediate Ang II-induced growth effects in VSMCs.\(^{22,23}\) The binding of Ang II to AT1R also increases ROS production, partially due to the up-regulation of Nox1 protein expression. In fact, Ang II-induced production of ROS is inhibited by Nox1 siRNA.\(^{24}\) Another mechanism by which Ang II increases ROS levels is through the down-regulation of antioxidant enzymes. We previously demonstrated that down-regulation of catalase, an antioxidant enzyme that reduces H\(_2\)O\(_2\) to H\(_2\)O and O\(_2\), induced senescence similarly to Ang II treatment.\(^6\) Furthermore, Ang II induces senescence in VSMCs through a p53/p21-dependent mechanism.\(^5\) However, whether nutritional interventions can modulate this pathway by reducing ROS and senescence is not fully understood.

Epidemiological studies conducted in the United States (US) and European populations demonstrate that high consumption of fruits and vegetables is associated with decreased mortality rates due to CVD, which is mainly attributed to the polyphenol content of these foods.\(^{25,26}\) Blackberry (BL), raspberry (RB), and black raspberry (BRB) are widely consumed in the US and have a high polyphenolic content. Polyphenols are considered bioactive compounds and are known to modulate the antioxidant response by interacting with numerous molecular targets. For instance, BL juice enhanced endothelium and vascular health by scavenging peroxynitrite (NO\(_3^−\)).\(^{27}\) In spontaneously hypertensive rats, RB decreased the blood pressure and increased superoxide dismutase (SOD) levels, while decreasing malondialdehyde (MDA) levels, a marker of oxidative stress.\(^{28}\) In mice, BRB extract increased the activity of SOD2 and decreased the levels of MDA.\(^{29}\) Lastly, anthocyanins, flavonoids found in high concentrations in BL, RB and BRB, decreased the proliferation and migration of VSMCs.\(^{30}\) Despite the abundant data available supporting the role of berries as antioxidants, to our knowledge, no study has tested their possible protective role in senescence induced by activation of Ang II signaling in VSMCs. Therefore, the goal of this study was to determine the role of BL, RB, and BRB polyphenol extracts in attenuating Ang II-induced senescence and to determine the molecular mechanisms involved. We found that BL, RB, and BRB polyphenol extracts attenuated the increase in ROS levels and senescence induced by Ang II through Nox1-dependent (BL) and -independent (RB and BRB) mechanisms.

2. Materials and methods

2.1 Reagents

Dulbecco's Modified Eagle Medium (DMEM) and Ang II (Sigma Aldrich, Saint Louis, MO); fetal bovine serum (FBS) (Seradigm, Radnor, PA); penicillin-streptomycin and \(\beta\)-glutamine (Corning Cellgro, Manassas, VA); rabbit polyclonal antibodies against: phospho-Akt (Ser 473), phospho-ERK1/2 (Thr 202/Tyr 204), ERK1/2, phospho-p38MAPK (Thr 180/Tyr 182), p38MAPK and MEK1 inhibitor PD98059 (Cell Signaling, Danvers, MA), SOD1 and p21 (Genetex, Irvine, CA), GPx1 (Abcam, Cambridge, UK), catalase and Akt inhibitor Triciribine (CABiochem, Billerica, MA), SOD2 and p38MAPK inhibitor SB203580 (Enzo Life Sciences, Farmingdale, NY), and Akt1/2/3 (Santa Cruz Biotechnology, Dallas TX); mouse monoclonal antibodies against: p53 (Genetex, Irvine, CA) and \(\beta\)-actin (Sigma-Aldrich, Saint Louis, MO); goat polyclonal antibody against Nox1 (Abcam, Cambridge, UK); adenovirus containing Nox1 (Ad-Nox1), \(4.4 \times 10^{10}\) plaque forming unit (PFU) per ml (Vector Biolabs, Philadelphia, PA) and Nox1 siRNA from Ambion (Naugatuck, CT).

2.2 Polyphenol extraction from berries

Polyphenol extracts of freeze-dried BL, RB, and BRB powders were performed using the ultrasound-assisted method as described by Kim & Lee.\(^{31}\) Briefly, 10 g of freeze-dried berry powder was combined with 80% ethanol and sonicated for 20 min at 23 °C in subdued light with nitrogen gas purging continuously. The mixture was filtered under vacuum suction and the residue rinsed with 80% ethanol and extracted using the same conditions. The two filtrates were combined with 50 ml of 80% ethanol and evaporated using a rotary evaporator (Buchi Labortechnik, New Castle, DE) at 62 °C and 50 rpm. Crude extracts were frozen overnight (−80 °C) and subsequently freeze-dried (VirTis Benchtop “K” Freeze dryer, Warminster, PA). Then, the freeze-dried crude extracts were purified according to the method described by Queires \textit{et al.}\(^{32}\) In brief, the crude extracts were mixed with two volumes of chloroform and allowed to separate. The aqueous fraction was then extracted with two volumes of ethyl acetate and incubated in a shaker at 65 °C for 30 min. The aqueous fraction was frozen overnight (−80 °C) and subsequently freeze-dried and stored at −20 °C for later analysis.
2.3 Measurement of polyphenols in berry extracts

The identification and quantification of polyphenols in the berry extracts were performed by BioProfile Testing Laboratories, LLC (Minneapolis, MN) using high-performance liquid chromatography (Phenomenex, Torrance, CA). The polyphenolic compounds were detected at the wavelength of 315 nm. Rigel C18 (150 × 4.6 mm; Stellar Phases, Inc.) was operated at 23 °C. The elution solvents were: 0.1% formic acid in water (A) and 100% acetonitrile with 0.1% formic acid (B). Samples were eluted according to the gradient time of: 0–3 min, 5% B; 3 min, 5% B; 3–30 min, 35% B; 30–31 min, 5% B; 31–35 min, 5% B, 40 min. The injection amount was 20 µl and the flow rate was 1 ml min⁻¹. Phenolic compounds were quantified using mass spectrometry over a wavelength of 200–600 nm. Peak areas were correlated with concentrations in accordance with the calibration curves, using chlorogenic acid (Sigma-Aldrich, Saint Louis, MO), rutin hydrate, ferulic acid, and quercetin dihydrate (LKT Laboratories, Saint Paul, MN) as standards.

2.4 Cell culture

Sprague-Dawley rat thoracic aortas were used to isolate VSMCs, which were prepared by enzymatic digestion as previously described.33 Experiments with animals were performed in compliance with the Public Health Service Policy and approved by the Animal Care and Use Committee at the Florida State University. VSMCs were cultured up to passage 12 in DMEM supplemented with 1 g L⁻¹ glucose, 10% FBS, 2 mM glutamine, 100 U ml⁻¹ penicillin, and 100 mg ml⁻¹ streptomycin. Cells were growth-arrested in DMEM containing 0.5% FBS and 200 µg ml⁻¹ berry polyphenol extracts 24 h prior to stimulation with 100 nM Ang II for three days. The medium was replaced every day with fresh berry polyphenol extracts and Ang II. For infection experiments, cells were incubated with 10 × 10⁷ PFU of empty or Nox1 containing virus (Ad-Nox1) in plain DMEM for 1 h and cultured for 48 h in complete media before the addition of berry polyphenol extracts. After three days, the cells were processed for SA-β-gal activity measurements.

2.5 Cell viability

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay as described by the manufacturer (Cayman Chemical, Ann Arbor, MI). In brief, cells were plated at 5 × 10⁵ per well and cultured with BL, RB, and BRB polyphenol extracts for three days. The cells were then incubated with the MTT reagent for 4 h, followed by the addition of a crystal dissolving solution, which produced a purple solution. Absorbance was measured at 570 nm.

2.6 Senescence associated β-galactosidase staining

Staining for SA-β-gal activity was determined as previously described.6 Cells were washed twice with PBS, fixed with 0.2% glutaraldehyde in PBS for 10 min and incubated in 40 mM phosphate buffer containing 1 mg ml⁻¹ X-Gal, 150 mM NaCl, 2 mM MgCl₂, 5 mM K₃Fe(CN)₆, and 5 mM K₄Fe(CN)₆ overnight at 37 °C. The cells were then washed twice with PBS and kept in 70% glycerol. Images were acquired using an Axio Observer. A1 microscope (Carl Zeiss, Cambridge, UK) using a 10× objective. The cells positive for SA-β-gal were counted in six different fields per well (three to six individual wells per condition) and expressed as a percentage of the total cell number. Additionally, SA-β-gal was detected using fluorescein-di-β-D-galactopyranoside (FDG) (Ex/Em: 488/515), a fluorescent analogue of X-gal, as previously described.34

2.7 Small RNA interference

Nox1 was down-regulated by small interfering RNA (siRNA) using a basic Nucleofector® Kit for primary VSMCs and the following oligonucleotides: Nox1 sense GGAUUUAAUUUGAG-CUCUUTT, and antisense AAGAGCUCAAAUAAUUCCTG, as previously reported.35

2.8 ROS measurements

Levels of ROS were assessed, as previously described,6 using 2′,7′-dichlorodihydrofluorescein diacetate (H₂DCFDA) and dihydroethidium (DHE) (Invitrogen, Carlsbad, CA). Briefly, VSMCs were washed with Dulbecco’s PBS and incubated with 10 µM H₂DCFDA or DHE for 30 min. The fluorescence intensity for H₂DCFDA was determined at 485/520 nm (Ex/Em) and for DHE at 405/570 nm (Ex/Em) in a BioTek Synergy H1 microplate reader (Winooski, VT, USA).

2.9 Western Blot

Cells were lysed in buffer A (50 mM HEPES, pH 7.4, 50 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 mM Na₃O₇P₂, 10 mM NaF, and 2 mM Na₃VO₄) plus protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO). Total homogenates were sonicated and separated on 10% SDS-PAGE gels. Enhanced chemiluminescence was used to determine the expression of target proteins.

2.10 NADP/NADPH ratio measurements

The levels of NADP and NADPH were measured using an Enzymochrom NADP/NADPH assay kit (Bioassay Systems, Hayward, CA) as described by the manufacturer. Briefly, cells were re-suspended in 100 µl of extraction buffer, homogenized, and sonicated for 20 seconds before heating at 65 °C for 5 min. Glucose dehydrogenase was used for NADP and NADPH quantification. Absorbance was measured at 565 nm in a BioTek Synergy H1 microplate reader. The levels of NADP and NADPH were expressed as the NADP/NADPH ratio.

2.11 Statistical analyses

Analysis of variance (ANOVA) was used to assess the differences in treatments and the Tukey–Kramer post-hoc test was used for pairwise comparisons. Significant differences were determined at p ≤ 0.05. Values are presented as mean ± standard deviation of mean (SD). Data analyses were performed using SSPS version 20.0 for Windows (SPSS Inc., Chicago, IL).
3. Results

3.1 Polyphenol extracts from berries prevent Ang II signaling in VSMCs

Analyses of the polyphenol profile of ethanol-based BL, RB, and BRB polyphenol extracts (Table 1) show that the BL polyphenol extract had the highest amount of phenolic acids, such as gallic and 3-O-caffeoylquinic (3-CQA) acids and was high in flavonols, such as epicatechin (EC) and epigallocatechin (EGC). The highest amount of EC was present in the RB polyphenol extract, which was also the only polyphenol extract containing the anthocyanin delphinidin-3-O-glucoside. On the other hand, the BRB polyphenol extract had the highest concentration of the flavonol quercetin, the anthocyanin cyanidin-3-glucoside, and the flavanol EGC. In addition, BRB was the only polyphenol extract tested that contained ferulic acid.

To examine whether BL, RB, and BRB polyphenol extracts attenuate Ang II-induced senescence in VSMCs, we first focused on Ang II signaling, since Ang II-induced Akt phosphorylation promotes cellular senescence in VSMCs. We incubated VSMCs with Ang II (100 nM) and monitored the phosphorylation of Akt and ERK1/2 for 60 min. We observed that phosphorylation of these two kinases peaked at 5 min, suggesting that phosphorylation promotes cellular senescence in VSMCs. We focused on Ang II signaling, since Ang II-induced Akt phosphorylation promotes cellular senescence in VSMCs.

For senescence experiments, VSMCs were pre-incubated with 200 µg ml⁻¹ of BL, RB, or BRB polyphenol extracts for 24 h followed by treatment with Ang II for 5 min (Fig. 1B). Our results indicate that Ang II-induced Akt phosphorylation was efficiently attenuated by BL, RB, and BRB polyphenol extracts at concentrations of 100 µg ml⁻¹ (0.39 ± 0.09, 0.55 ± 0.12, and 0.28 ± 0.15-fold, respectively, n = 3, p < 0.05) and 200 µg ml⁻¹ (0.25 ± 0.09, 0.31 ± 0.14, and 0.16 ± 0.06-fold, respectively, n = 3, p < 0.01) when compared to Ang II treated cells (Fig. 1B and C).

Table 1 Polyphenol profile and quantification of blackberry, raspberry and black raspberry and black raspberry extracts

<table>
<thead>
<tr>
<th>Analytes (ppm)</th>
<th>Blackberry Rubus L.</th>
<th>Raspberry Rubus idaeus L.</th>
<th>Black raspberry Rubus occidentalis L.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolic acids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gallic acid</td>
<td>243.5</td>
<td>74.6</td>
<td>63.5</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>32.9</td>
<td>27.9</td>
<td>30.9</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>—</td>
<td>—</td>
<td>104.5</td>
</tr>
<tr>
<td>Chlorogenic acids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-O-Caffeoylquinic acid</td>
<td>235.3</td>
<td>114.4</td>
<td>9.4</td>
</tr>
<tr>
<td>4-O-Caffeoylquinic acid</td>
<td>13.0</td>
<td>10.4</td>
<td>59.0</td>
</tr>
<tr>
<td>5-O-Caffeoylquinic acid</td>
<td>14.1</td>
<td>—</td>
<td>18.8</td>
</tr>
<tr>
<td>Flavonoids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>95.0</td>
<td>26.7</td>
<td>102.8</td>
</tr>
<tr>
<td>Cyanidin-3-glucoside</td>
<td>116.6</td>
<td>33.8</td>
<td>733.7</td>
</tr>
<tr>
<td>Cyanidin-3-galactoside</td>
<td>7.1</td>
<td>3.6</td>
<td>—</td>
</tr>
<tr>
<td>Delphinidin-3-O-glucoside</td>
<td>—</td>
<td>45.0</td>
<td>—</td>
</tr>
<tr>
<td>Flavan-3-ols</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(–)-Epicatechin</td>
<td>323.4</td>
<td>477.8</td>
<td>—</td>
</tr>
<tr>
<td>(–)-Epigallocatechin</td>
<td>319.8</td>
<td>198.8</td>
<td>434.7</td>
</tr>
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</table>

The same was true for Ang II-induced ERK1/2 phosphorylation, which was best reduced by BL, RB, and BRB polyphenol extracts at concentrations of 100 µg ml⁻¹ (0.55 ± 0.09, 0.65 ± 0.12, and 0.71 ± 0.16-fold, respectively, n = 3, p < 0.05) and 200 µg ml⁻¹ (0.5 ± 0.09, 0.56 ± 0.13 and 0.37 ± 0.18-fold, respectively, n = 3, p < 0.01) when compared to Ang II treated cells (Fig. 1B and D).

Senescence is measured after at least three days of treatment with Ang II, a time in which senescence markers such as SA-β-gal, p21, and p53 are significantly up-regulated leading to the development of the SASP. Thus, we determined cell viability using 200 µg ml⁻¹ of berry polyphenol extracts after three days of treatment and found that this dose did not produce any cytotoxicity (Fig. 1E). Low levels of toxicity were observed at a concentration of 400 µg ml⁻¹ for the three extracts tested (data not shown). Thus, for the three berry polyphenol extracts used in this study, a dose of 200 µg ml⁻¹ elicited the most robust response without any toxic effect; thus, it was used to perform the subsequent experiments.

For senescence experiments, VSMCs were pre-incubated with 200 µg ml⁻¹ of BL, RB, or BRB polyphenol extracts for 24 h followed by treatment with Ang II (100 nM) for three days. We first measured the levels of phosphorylated ERK1/2, p38MAPK, and Akt to confirm the inhibitory role of the berry polyphenol extracts in Ang II signaling in long-term incubation periods. As expected, the phosphorylation levels of ERK1/2, p38MAPK, and Akt increased (3.43 ± 0.6, 4.2 ± 0.5, and 3.87 ± 0.29-fold, respectively, n = 4, p < 0.05) in response to Ang II (Fig. 2A–D). Pre-incubation with BL, RB, and BRB polyphenol extracts significantly decreased Ang II-induced ERK1/2 phosphorylation (0.53 ± 0.17, 1.06 ± 0.29, and 0.59 ± 0.3-fold, respectively, n = 5, p < 0.05) (Fig. 2A and B). Similarly, phosphorylation of p38MAPK by Ang II was significantly reduced when cells were pre-incubated with BL (2.14 ± 0.77-fold, n = 4, p < 0.05), RB (2.72 ± 0.65-fold, n = 4, p < 0.05) or BRB (1.03 ± 0.58-fold, n = 4, p < 0.001) extracts (Fig. 2A and C). Nonetheless, phosphorylation of Akt by Ang II was significantly decreased by BL (0.68 ± 0.71-fold, n = 3, p < 0.05) and BRB (0.72 ± 0.24-fold, n = 3, p < 0.001), but not by RB (2.8 ± 1.3-fold, n = 3, p > 0.05) polyphenol extracts (Fig. 2A and D). Thus, these data indicate that BL, RB, and BRB have distinct effects on Ang II signaling and that the BL and BRB extracts are more effective than the RB polyphenol extract in decreasing the phosphorylation of Ang II-induced kinases.

3.2 Polyphenol extracts from berries prevent Ang II-induced senescence in VSMCs

Activation of Akt, p38MAPK and ERK1/2, has been associated with increased senescence. Therefore, the decrease in Ang II-induced signaling by the berry polyphenol extracts tested in this study, particularly from BL and BRB, suggest that these berries should decrease Ang II-induced senescence. We determined senescence by measuring biomarkers of cellular senescence including SA-β-gal activity, p21, and p53 expression, after three days of treatment with BL, RB, or BRB polyphenol extracts and Ang II. As anticipated, Ang II increased the...
number of SA-β-gal positive cells compared to control cells (3.00 ± 1.30 vs 1.01 ± 0.18-fold, n = 9, p < 0.001) (Fig. 3A and B). Pre-incubation with BL, RB, and BRB polyphenol extracts attenuated Ang II-induced senescence as indicated by the decreased number of SA-β-gal positive cells (1.67 ± 0.32, 1.60 ± 0.53 and 1.23 ± 0.25-fold, respectively, n = 9, p < 0.001) compared to control cells (Fig. 3A and B). Similar to SA-β-gal, both p21 and p53 levels were significantly increased in Ang II-treated cells (2.45 ± 0.26 and 2.08 ± 0.43-fold, respectively, n = 3, p < 0.05) compared to control cells (Fig. 3C, D and E). As hypothesized, BL, RB, and BRB polyphenol extracts significantly decreased p21 levels (1.06 ± 0.30, 1.13 ± 0.33 and 0.83 ± 0.30-fold, respectively, n = 3, p < 0.05) when compared to control cells (Fig. 3C and D). Regarding p53, while all the extracts tested decreased its levels compared to Ang II-treated cells, the decrease elicited by BL was of a greater magnitude (0.55 ± 0.30-fold) compared with RB and BRB (1.66 ± 0.25 and 1.23 ± 0.60-fold, respectively, n = 3, p < 0.05) (Fig. 3C and E). These data demonstrate that BL, RB, and BRB polyphenol extracts strongly attenuated Ang II-induced senescence. Yet, RB was less effective than BL and BRB polyphenol extracts in reducing Ang II signaling, suggesting that the polyphenols in these berries act through different molecular mechanisms.

To further test whether Akt, p38MAPK and ERK1/2 are in fact involved in senescence mediated by Ang II in VSMCs, we inhibited these kinases using the Akt inhibitor V Triciribine (TCN), the p38MAPK inhibitor SB203580 (SB) and the MEK (up-stream regulator of ERK1/2) inhibitor PD98059 (PD), as we previously described6,36 and measured senescence using FDG (Fig. 3F). Using this fluorescent analogue, we found that senescence was up-regulated 1.25 ± 0.08-fold compared with control cells (0.98 ± 0.1-fold, n = 12, p < 0.01). Inhibition of p38MAPK produced the strongest inhibition of senescence (0.51 ± 0.11, n = 13, p < 0.01) compared with both Ang II and basal conditions. Inhibition of Akt and MEK significantly reduced Ang II-induced senescence (0.94 ± 0.08-fold, n = 14 and 0.85 ± 0.19-fold, n = 12, respectively, p < 0.01), but not basal senescence. Altogether, these data support the idea that down-regulation of Ang II-induced signaling by BL, RB and BRB polyphenol extracts contributes to the protective effects of these berries.
and peroxyl radicals, and DHE, which detects $O_2^{•−}$. Ang II significantly increased $H_2O_2$ levels compared to control cells (1.29 ± 0.24 vs. 0.99 ± 0.07-fold, n = 13, p < 0.001) (Fig. 4A). Pre-incubation with BL, RB, or BRB polyphenol extracts significantly decreased the basal levels of $H_2O_2$ (0.60 ± 0.10, 0.66 ± 0.15 and 0.64 ± 0.16-fold, respectively, n = 13, p < 0.001) as well as Ang II-induced increases in $H_2O_2$ (0.77 ± 0.22, 0.82 ± 0.25 and 0.77 ± 0.19-fold, respectively, n = 13, p < 0.001) (Fig. 4A). Furthermore, Ang II significantly increased the levels of $O_2^{•−}$ compared to control cells (1.21 ± 0.07 vs. 0.94 ± 0.1-fold, n = 12, p < 0.001) (Fig. 4B). Only pre-incubation with BL (0.84 ± 0.09-fold, p < 0.05), but not RB (0.88 ± 0.13-fold, p > 0.05) or BRB (0.94 ± 0.1-fold, p > 0.05) polyphenol extracts significantly reduced the basal levels of $O_2^{•−}$ (Fig. 4B). However, all three berry polyphenol extracts significantly decreased Ang II-induced $O_2^{•−}$ levels (BL: 0.99 ± 0.07, RB: 1.02 ± 0.13 and BRB: 1.03 ± 0.09-fold, n = 14, p < 0.05) (Fig. 4B).

One of the main sources of ROS in VSMCs are Noxes, in particular Nox1, whose activity and protein levels are known to increase in response to Ang II in vivo and in vitro in VSMC.39

Since Nox1 produces $O_2^{•−}$, we hypothesized that BL, RB, and BRB polyphenol extracts should attenuate the increase in Nox1 activity and/or Nox1 protein expression in response to Ang II treatment. Noxes use NADPH, which is converted to NADP, to produce $O_2^{•−}$. Up-regulation of Nox activity leads to increased levels of NADP and decreased levels of NADPH. Thus, an increase in the NADP/NADPH ratio suggests that Nox activity should be up-regulated. Ang II increased the NADP/NADPH ratio compared with control cells (1.32 ± 0.11-fold, n = 4, p < 0.05) (Fig. 4C). Consistent with the observation that BL is more effective than RB and BRB polyphenol extracts in reducing basal and Ang II-induced increases in ROS levels, BL showed a more robust down-regulation in the NADP/NADPH ratio (0.43 ± 0.24-fold, n = 3, p < 0.01) compared with RB (0.99 ± 0.08-fold, n = 3, p < 0.05) and BRB (0.79 ± 0.07-fold, n = 3, p < 0.05) polyphenol extracts (Fig. 4C). Next, we determined whether the decrease in the NADP/NADPH ratio correlated with a decrease in Nox1 protein levels (Fig. 4D and E). Ang II significantly increased Nox1 expression compared to control cells (6.25 ± 0.83-fold, n = 5, p < 0.05) (Fig. 4D and E). Ang II significantly increased Nox1 expression compared to control cells (6.25 ± 0.83-fold, n = 5, p < 0.05), which was significantly reduced by BL (3.00 ± 1.09-fold, n = 5, p < 0.001), but not by RB or BRB (8.9 ± 2.8 and 5.03 ± 0.25-fold, respectively, n = 3, p > 0.05) polyphenol extracts. No changes were observed in the protein levels of Nox4, another Nox expressed in VSMCs (data not shown).

To test whether Nox1 in fact mediates senescence induced by Ang II, we down-regulated Nox1 by siRNA (siNox1), as previously described35 and measured Nox1 protein expression (Fig. 4F) and senescence using FDG (Fig. 4G). Ang II increased senescence 1.22 ± 0.01-fold, compared with control (0.99 ± 0.03-fold, n = 3, p < 0.01) in siControl-treated cells.
cells, which was significantly reduced in siNox1-treated cells (1.04 ± 0.07-fold, n = 3, p < 0.05).

In accordance with these findings, which indicate that BL polyphenol extract attenuates Ang II-induced Nox1 expression and that Nox1 mediates Ang II-induced senescence, we hypothesized that BL should protect VSMCs from senescence induced by Nox1 overexpression. To this end, we infected cells with Ad-Nox1 or empty virus (Fig. 4H) and treated cells with BL, RB, and BRB polyphenol extracts for three days to measure senescence (Fig. 4I). As anticipated, the overexpression of Nox1 increased senescence compared with control (1.48 ± 0.12 vs. 1.03 ± 0.05-fold, n = 13, p < 0.001), which was down-regulated by BL (1.13 ± 0.13-fold, n = 13, p < 0.001), but not by RB (1.35 ± 0.19-fold, n = 13, p > 0.05) or BRB (1.49 ± 0.17-fold, n = 15, p > 0.05) polyphenol extracts (Fig. 4I). Altogether, these data demonstrate that BL polyphenol extract is more effective in decreasing Ang II signaling and attenuating senescence by a mechanism that depends on the down-regulation of Nox1.

3.4 Polyphenol extracts from berries increase the expression of antioxidant enzymes

The fact that RB and BRB polyphenol extracts decrease H$_2$O$_2$ and O$_2$$^•$− levels as well as senescence in the presence of Ang II by a Nox1-independent mechanism suggests that these polyphenol extracts may reduce ROS levels by increasing the expression of antioxidant enzymes. To test this idea, we measured the expression of the peroxidases catalase and GPx1, as well as the dismutases SOD1 and SOD2. As previously reported, Ang II induced the down-regulation of catalase compared to control cells (0.32 ± 0.10-fold, n = 6, p < 0.001) (Fig. 5A and B). Pre-incubation with BL (0.27 ± 0.14-fold, n = 7, p > 0.05), RB (0.32 ± 0.18-fold, n = 7, p > 0.05), and BRB (0.39 ± 0.20-fold, n = 6, p > 0.05) polyphenol extracts did not attenuate this down-regulation (Fig. 5A and B). Ang II did not affect the expression of SOD1 (1.10 ± 0.22-fold, n = 7, p > 0.05), SOD2 (0.99 ± 0.21-fold, n = 8, p > 0.05), or GPx1 (0.88 ± 0.21-fold, n = 8, p > 0.05).
Fig. 4  Differential effects of berry polyphenol extracts on ROS levels, Nox1 expression and senescence induced by Nox1. Cells were incubated in media containing 0.5% FBS with and without 200 µg ml⁻¹ of BL, RB, or BRB polyphenol extracts for 24 h prior to stimulation with Ang II for 48 h. ROS levels were determined after 30 min incubation with (A) H₂DCFDA or (B) DHE. (C) The NADP/NADPH ratio was determined after cells were pre-incubated with 200 µg ml⁻¹ of berry polyphenol extracts for 24 h and treated with Ang II for 6 h. (D and E) Nox1 protein expression was determined by western blot in cells pre-incubated with 200 µg ml⁻¹ of berry polyphenol extracts for 24 h and treated with Ang II for 72 h. (F and G) Cells treated with siRNA to down-regulate Nox1 expression or siRNA control (siCon) were tested for (F) Nox1 protein expression or for (G) SA-β-gal measurements using FDG. (H and I) Cells were infected with an empty or with an adenovirus containing Nox1 (Ad-Nox1). After three days, cells were lysed for Nox1 protein expression analysis (H) or processed for SA-β-gal measurements using FDG (I). Quantification of Nox1 was performed using Image J. Values are expressed as mean ± SD from three independent experiments. ‡Significant difference (p < 0.05) compared to untreated control (con) and *significant difference (p < 0.05) compared to Ang II treatment alone.

Fig. 5  Effects of berry polyphenol extracts on antioxidant enzymes in VSMCs. (A) Cells were incubated in media containing 0.5% FBS with and without 200 µg ml⁻¹ of BL, RB or BRB polyphenol extracts for 24 h prior to stimulation with Ang II. After 72 h, cells were lysed and protein expression was determined by western blot. Quantification of (B) catalase, (C) SOD1, (D) SOD2, and (E) GPx1 was performed using Image J. Values are expressed as mean ± SD from three independent experiments. ‡Significant difference (p < 0.05) compared to untreated control (con) and *significant difference (p < 0.05) compared to Ang II treatment alone.
n = 4, p > 0.05). However, treatment with BL, RB, and BRB polyphenol extracts increased the expression of SOD1 at basal levels (2.46 ± 0.30, 2.33 ± 0.51 and 2.17 ± 0.10-fold, respectively, n = 3, p < 0.001), as well as after Ang II treatment (2.10 ± 0.48, 2.27 ± 0.30 and 1.82 ± 0.38-fold, respectively, n = 3, p < 0.001) compared to control cells (Fig. 5A and C).

In contrast, SOD2 expression was up-regulated by RB and BRB polyphenol extracts at basal levels (1.55 ± 0.22-fold, n = 6, p < 0.001 and 1.35 ± 0.20-fold, n = 5, p < 0.05, respectively) as well as in Ang II-treated cells (1.37 ± 0.19-fold, n = 6, p < 0.001 and 1.46 ± 0.24-fold, n = 5, p < 0.001, respectively) compared to control cells (Fig. 5A and D). However, BL polyphenol extract had no effect on SOD2 expression at basal levels (1.06 ± 0.17-fold, n = 8, p > 0.05) or in the presence of Ang II (1.03 ± 0.11-fold, n = 8, p > 0.05) (Fig. 5A and D). Similar to SOD2, GPx1 expression was up-regulated by RB (2.11 ± 0.54-fold, n = 5, p < 0.001) and BRB (2.16 ± 0.74-fold, n = 3, p < 0.001) polyphenol extracts under basal conditions compared to control cells. Furthermore, RB (1.65 ± 0.41-fold, n = 4, p < 0.001) and BRB (1.41 ± 0.18-fold, n = 3, p < 0.001) polyphenol extracts increased GPx1 expression in the presence of Ang II compared to control cells (Fig. 5A and E). Again, BL polyphenol extract did not affect GPx1 expression at basal levels (0.99 ± 0.18-fold, n = 4, p > 0.05) nor in the presence of Ang II (1.13 ± 0.36-fold, n = 4, p > 0.05) (Fig. 5A and E). Collectively, these data suggest that RB and BRB polyphenol extracts attenuate Ang II-induced increases in ROS levels and senescence likely by increasing the expression of SOD1, SOD2, and GPx1.

4. Discussion

The overall objective of the present study was to elucidate the effects of BL, RB, and BRB polyphenol extracts on Ang II-induced senescence in VSMCs. Our findings, summarized in Fig. 6, indicate that BL, RB, and BRB polyphenol extracts reduced ROS levels and attenuated senescence induced by Ang II in VSMCs through different molecular mechanisms. We demonstrate that BL polyphenol extract down-regulated Nox1 expression, the NADP/NADPH ratio, decreased the phosphorylation of kinases induced by Ang II, and reduced senescence induced by Nox1 overexpression in VSMCs. In contrast, RB and BRB polyphenol extracts reduced the NADP/NADPH ratio but were not able to decrease Nox1 expression or senescence in response to Nox1 overexpression. Thus, they likely reduced ROS levels and senescence by increasing GPx1, SOD1, and SOD2 expression. We show that these berries are dissimilarly disrupting Ang II signaling and its effects in VSMCs, which can be due to their distinct phenolic composition (Table 1). This fact is of significance as these berries could be an effective alternative and/or complementary therapy to alleviate age- and oxidative stress-related diseases, such as atherosclerosis.

Nox1-derived ROS, promotes the phosphorylation of redox-sensitive kinases including p38MAPK and Akt leading to contraction, hypertrophy, inflammation, and senescence of VSMCs. In fact, inhibition of Nox1 decreased the phosphorylation of p38MAPK and Akt induced by Ang II in VSMCs. Additionally, p38MAPK and ERK1/2 can be also activated by Ang II-induced transactivation of the epidermal growth factor receptor. Thus, Ang II signaling is mediated by ROS-sensitive and -insensitive pathways. Our findings demonstrate that BL, RB, and BRB polyphenol extracts blunted the phosphorylation of p38MAPK and ERK1/2 by Ang II, suggesting that they act on the ROS-insensitive pathway. In contrast, only BL and BRB polyphenol extracts attenuated Akt phosphorylation by Ang II, suggesting that these berries regulate the ROS-sensitive pathway. In agreement with published data, we show that the inhibition of Akt, p38MAPK and MEK or down-regulation of Nox1 by siRNA reduced Ang II-induced senescence. It is unknown, however, whether inhibition of Akt, p38MAPK or ERK1/2 by BL mediates the down-regulation of Nox1 expression or whether decreased expression of Nox1 by BL reduces the activity of these kinases.

The distinct effects of BL, RB and BRB on Ang II signaling may be due to their unique phenolic composition. The three berry polyphenol extracts have high levels of EGC, which may
contribute to the strong down-regulation of ERK1/2 and p38MAPK in response to Ang II. In fact, EGC reduced Ang II-induced phosphorylation of these two kinases in VSMCs leading to reduced proliferation.43 On the other hand, the high levels of EC observed in the RB polyphenol extract are likely to be involved in its effect on p38MAPK and ERK1/2, but not on Akt phosphorylation. We showed that the RB polyphenol extract had no significant effects on Ang II-induced Akt phosphorylation after three days of incubation. This suggests that other polyphenols in BL and BRB extracts may be responsible for the decrease in Akt phosphorylation induced by Ang II. For instance, quercetin levels were less abundant in RB than in BL and BRB polyphenol extracts. Interestingly, quercetin was shown to inhibit the activation of the PI3K/Akt pathway, but not the activation of p38MAPK and ERK1/2 by Ang II in VSMCs.44 It is also possible that the reduced effect of RB on Akt phosphorylation and Nox1 expression is due to its anthocyanin profile and/or content compared to the other two berries. For example, cyanidin-3-glucoside was less abundant in RB compared to BL and BRB polyphenol extracts. In fact, cyanidin-3-glucoside was shown to exert a synergistic effect with atorvastatin, a cholesterol-lowering drug, in reducing Ang II-induced inflammation in VSMCs. This anthocyanin was also reported to reduce Ang II-induced Nox1 expression in VSMCs.45

Although both Ang II signaling axes were partially inhibited, none of the extracts were able to attenuate the down-regulation of catalase induced by Ang II. Catalase down-regulation is dependent on Akt6 and ERK1/2 signaling.6 Thus, it is possible that a combination of BL polyphenol extract, which had the strongest effect on Akt phosphorylation and BRB polyphenol extract, which strongly inhibited ERK1/2 and p38MAPK phosphorylation, may be more effective in attenuating catalase down-regulation. It is also possible that catalase expression could be regulated by other mechanisms, not investigated here, such as protein stability. The fact that catalase down-regulation was not attenuated by BL, RB, nor BRB polyphenol extracts supports the idea that the polyphenols in these extracts do not affect the AT1R function, as suggested for other polyphenols. For example, sirtuin 1 (SIRT1), a longevity gene activated by resveratrol, down-regulated AT1R expression and inhibited ERK1/2 phosphorylation in VSMCs.46 In fact, we found that AT1R levels were not down-regulated by BL, RB, or BRB polyphenol extracts (data not shown). Hence, the effects of BL, RB, and BRB polyphenol extracts described here are different from the effects of resveratrol.

In this study, we also measured ROS levels using H2DCFDA, which detects mainly H2O2 in addition to hydroxyl and peroxyl radicals, and DHE, which detects O2•−. Our findings indicate that BL, RB, and BRB polyphenol extracts not only decreased ROS levels but also reduced the NADP/NADPH ratio in the presence of Ang II. Nox1 and Nox4 are the two isoforms activated by Ang II in VSMCs and have been implicated in the development and progression of atherosclerosis. Deficiency of Nox1 decreases atherosclerotic plaque deposition in ApoE−/− mice fed a high fat diet,16 while Nox4 expression increased with atherosclerotic lesion progression and increased plaque instability in ApoE−/−/LDLR−/− mice.47 Interestingly, in the present study, the expression of Nox4 was not affected by Ang II or by BL, RB, and BRB polyphenol extracts (data not shown). Supporting the role of Nox1 in this pathway, we found that down-regulation of Nox1 by siRNA reduced Ang II-induced senescence. Only the BL polyphenol extract mitigated the up-regulation of Nox1 by Ang II and senescence induced by Nox1 over-expression. Although RB and BRB polyphenol extracts significantly reduced the NADP/NADPH ratio, they did not attenuate the up-regulation of Nox1 expression induced by Ang II or senescence induced by Nox1 overexpression. The decrease in the NADP/NADPH ratio by the BL extract, which correlated with decreased Nox1 expression, suggests that Nox activity is reduced. However, other sources of NADPH, like the pentose phosphate pathway (PPP) cannot be ruled out. For instance, activation of the PPP generates NADPH that regenerates glutathione, increasing the antioxidant capacity of the cell. Glucose-6-phosphate dehydrogenase, a key enzyme in this pathway, was shown to be activated by the longevity gene SIRT2 leading to the up-regulation of the PPP and the antioxidant capacity.48 Thus, it is possible that BL, RB and BRB may activate the PPP leading to increased levels of NADPH and decreased NADP/NADPH ratio.

Nox1 expression is up-regulated by the redox sensitive transcription factors NF-xB49 and activator protein 1 (AP-1).50 It is possible that BL polyphenol extract attenuates the up-regulation of Nox1 transcriptionally by inhibiting NF-xB and/or AP-1 activity, which requires further investigation. The inhibitory effect of the BL polyphenol extract on Nox1 activity and expression could be mediated by the phenolic acid, 3-CQA (Table 1), which is more abundant in BL (235.3 ppm) than in RB (114.4 ppm) or BRB (9.4 ppm) polyphenol extracts. 3-CQA decreases ROS production by inhibiting c-Src, a molecule involved in the activation of Rac1.51 In VSMCs, the binding of Ang II to AT1R promotes phosphorylation and translocation of p47phox, Nox activator 1 and Rac1 from the cytosol to the membrane.12 Activation of Rac1 results in Nox1 activation with subsequent production of O2•−.52 Whether 3-CQA reduces ROS levels and senescence induced by Nox1 over-expression remains to be elucidated.

The inhibitory effect of RB and BRB polyphenol extracts on ROS levels and senescence are likely mediated by up-regulation of the cellular antioxidant capacity. Both RB and BRB polyphenol extracts increased SOD1, SOD2, and GPx1 protein expression at basal levels and in the presence of Ang II, thus, reducing both H2O2 and O2•− levels. GPx1 localizes in the cytosol and mitochondria53 and reduced activity of this enzyme has been associated with Ang II-induced vascular dysfunction.54 Furthermore, GPx1−/− fibroblasts exhibit decreased growth, reduced DNA synthesis, increased expression of senescence markers, and a morphology similar to senescent-like cells.55 GPx1 reduces H2O2 to H2O and O2 and has a lower Km for H2O2 than catalase,56 suggesting that up-regulation of GPx1 may compensate for the down-regulation of catalase. This observation may explain the decrease in ROS levels.
elicited by RB and BRB polyphenol extracts when catalase and Nox1 expression were not altered under basal conditions and in the presence of Ang II. SOD1, highly expressed in the cytosol and inter-mitochondrial membrane, and SOD2, found in the mitochondria, catalyse the conversion of \( \text{O}_2^{\cdot-} \) into \( \text{H}_2\text{O}_2 \).\textsuperscript{57} As previously reported,\textsuperscript{58} our results show that Ang II did not alter the expression of SODs in VSMCs. Even though SOD and GPx activity were not measured in this study, Cao et al.\textsuperscript{59} reported that ferulic acid, a major phenolic acid found in BRB polyphenol extract, increased SOD and GPx activity in \( \text{H}_2\text{O}_2 \) stimulated VSMCs. Since ferulic acid was not present in BL nor RB polyphenol extracts (Table 1), the up-regulation of SOD1 by BL polyphenol extract and the concomitant up-regulation of SOD1, SOD2, and GPx1 by RB polyphenol extract could be a result of a synergistic effect among the many polyphenolic compounds found in the extracts rather than a specific compound, which remains to be investigated.

5. Conclusion

Altogether our data clearly indicate that BL, RB and BRB polyphenol extracts attenuate Ang II-induced senescence in VSMCs as indicated by the decrease in the number of cells positive for SA-\( \beta \)-gal. Furthermore, the expressions of p21 and p53 were down-regulated by pre-incubation with all three berries tested in this study. We have shown that BL polyphenol extract inhibits the effects of Ang II on VSMCs by reducing the up-regulation of Nox1, increasing SOD1 levels, decreasing ROS levels, and inhibiting the activation of the redox-sensitive Akt and p38MAPK pathways that lead to senescence. On the other hand, RB and BRB polyphenol extracts reduce ROS levels and inhibit senescence in a Nox1-independent manner by increasing the levels of SOD1, SOD2, and GPx1. These different effects may be explained by the distinct polyphenolic profile of these berries. Thus, studies to examine the effects of isolated polyphenolic compounds from these berries are warranted in order to identify whether one particular polyphenol or a combination of polyphenols found in these berries are responsible for the vascular protective effects observed in this study. In addition, in vivo studies to confirm our findings are needed and are underway.

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