Physiological concentrations of dietary polyphenols regulate vascular endothelial cell expression of genes important in cardiovascular health

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Previous cell culture-based studies have shown potential health beneficial effects on gene expression of dietary polyphenols, including those found in red wine and green tea. However, these studies have tended to use higher concentrations (2–100 μM) than those observed in blood (0.1–1 μM) after consuming polyphenol-rich foods or beverages. The present study investigated effects of physiological concentrations of different classes of dietary polyphenol on the expression of genes important in cardiovascular health (endothelial NO synthase (eNOS), endothelin-1 (ET-1) and vascular endothelial growth factor (VEGF)) by cultured vascular endothelial cells (human umbilical vein endothelial cells) in the absence or presence of H₂O₂. Resveratrol and quercetin (0.1–1 μM) increased eNOS and VEGF mRNA expression particularly in the absence of H₂O₂ (50 μM) and decreased H₂O₂-induced ET-1 mRNA expression (P<0.001 for polyphenol × H₂O₂ interactions). Similarly, resveratrol and quercetin decreased endothelin secretion into the media, blocking the stimulatory effect of 50 μM-H₂O₂ (P<0.001 for polyphenol × H₂O₂ interaction). Of the nine other polyphenols tested, only epigallocatechin gallate had similar effects on both the eNOS and ET-1 mRNA expression, but to a lesser extent than resveratrol at an equimolar concentration (0.1 μM). The observed effects on gene expression would be expected to result in vasodilation and thereby reduced blood pressure. Since only three of the eleven polyphenols tested had biological activity, it is unclear whether particular structures are important or whether the effects might relate to the relatively high antioxidant capacities of the three active polyphenols.

Dietary polyphenols: Endothelial cells: Endothelial NO synthase: Endothelin-1: Gene expression

Epidemiological studies(1) have shown an association between the intake of plant foods, particularly fruit and vegetables, and a decrease in CHD and other CVD. Similarly, increased consumption of green tea is associated with a reduced risk of CVD(2), while rat studies indicate that consumption of green tea reduces the increased blood pressure induced by fructose feeding(3). These beneficial effects of plant-derived foods and beverages on cardiovascular health are suggested to be due to the presence of polyphenols. Previous studies have shown that polyphenols may well be beneficial to cardiovascular health(4), particularly those present in red wines (e.g. resveratrol and quercetin), which are thought to account for the ‘French paradox’.

The mechanism of action for the beneficial effects of polyphenols on CVD may be due to their antioxidant activity. However, many polyphenols are metabolised in the body, and these metabolites have much lower antioxidant capacities than their parent compounds, suggesting that antioxidant activity may not be their only mechanism of action(5). Polyphenols thought to provide health benefits include resveratrol, quercetin, epicatechin gallate (ECG) and epigallocatechin gallate (EGCG), which belong to different classes of polyphenol and may therefore have differing effects dependent upon their chemical structures. Tea and polyphenols present in teas (particularly ECG and EGCG) have previously been reported to have anti-carcinogenic, anti-proliferative and anti-angiogenic properties(6), as well as having effects on gene expression. These anti-angiogenic properties may relate to local expression of vascular endothelial growth factor (VEGF), a potent stimulator of angiogenesis. Red wine polyphenols have been shown to reduce expression of the vasoconstrictor, endothelin-1 (ET-1) in cultured vascular cells(7–9) and to increase expression of endothelial NO synthase (eNOS), the enzyme responsible for the production of the vasodilator NO(10,11). However, cell culture-based studies investigating these effects have tended to use supraphysiological concentrations of individual polyphenols. Maximum concentrations of polyphenols in the blood after consumption of polyphenol-rich foods or beverages tend to be about 0.1–1 μM(12), whereas previous in vitro studies have tended to treat cells with much higher concentrations (2–100 μM).

Stress is known to elevate the expression of certain genes in several cell types including human umbilical vein endothelial cells (HUVEC) and smooth muscle cells(9). Different methods to induce stress or to elevate gene expression include stretching cells cultured on a flexible membrane (cyclic strain)(9) or treatment with H₂O₂(9) or angiotensin II(9). Previous studies have shown that supraphysiological concentrations

Abbreviations: ECG, epicatechin gallate; EGCG, epigallocatechin gallate; eNOS, endothelial NO synthase; ET-1, endothelin-1; HUVEC, human umbilical vein endothelial cells; VEGF, vascular endothelial growth factor.

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(10–100 μM) of resveratrol blocked the stimulatory effects of H2O2, cyclic strain and angiotensin II treatment on ET-1 mRNA expression.

The present study investigated the effects of physiological concentrations of different classes of dietary polyphenol on the expression of genes important in cardiovascular health (eNOS, ET-1 and VEGF) in cultured vascular endothelial cells (HUVEC) under stress-free and oxidative stress conditions (i.e. with or without H2O2).

Materials and methods

Materials and chemicals

Unless stated otherwise, all chemicals (including polyphenols) were obtained from Sigma-Aldrich (St Louis, MO, USA). The endothelin ELISA kit was obtained from Biomedica (Vienna, Austria) via Oxford Biosystems (Oxford, UK).

Preparation of individual polyphenols

A 100 μM stock solution of each polyphenol was prepared in PBS and diluted further with PBS before being added to culture media.

Cell culture

HUVEC were purchased from Clonetics (Cambrex, Inc., Walkersville, MD, USA) as a frozen stock (passage 3), defrosted and cultured in endothelial basal medium 2 growth media plus bullet kit (Cambrex, Inc.) containing 2 % fetal calf serum, antibiotics and growth factor supplements at 37°C and 5 % CO2. The cells were passaged three times to provide a pool of cells to be used for experiments. Experiments were performed in six-well plates (Corning, Inc., Corning, NY, USA) when the cells were 80–85 % confluent, at which stage the endothelial basal medium 2 growth media (2 % foetal calf serum, with growth factors) was replaced with endothelial basal medium 2 control media (0·8 % foetal calf serum, no growth factors) and incubated for 24 h (37°C, 5 % CO2). Treatments were prepared in endothelial basal medium 2 containing 0·8 % fetal calf serum and antibiotics but no other supplements. This consisted of 70 % media and 30 % polyphenol and/or H2O2 in PBS, with PBS alone used as control. Final concentrations of individual polyphenols in the culture media ranged from 0·001 to 1·0 μM, while concentrations of H2O2 ranged from 6·25 to 50 μM. After 24 h incubation with treatments, culture media were stored at −40°C for measurement of endothelin secretion using an ELISA kit and total RNA was extracted from the cells using TRIzol (Invitrogen, Carlsbad, CA, USA), both according to the manufacturer’s instructions.

Quantitative real-time PCR

Total RNA was DNase-treated before quantifying RNA yields using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and checking RNA integrity by running on 1 % agarose gels. Complementary DNA was synthesised using Moloney murine leukaemia virus reverse transcriptase and buffer (Promega, Fitchburg, WI, USA). Gene- and species-specific primers and probes for real-time PCR (Table 1) were all purchased from Applied Biosystems.
Effects of hydrogen peroxide on human umbilical vein endothelial cells gene expression

The dose-dependent effects of H$_2$O$_2$ (6.25–50 μM) on the expression of eNOS, ET-1 and VEGF mRNA were determined. Table 2 shows a dose-dependent increase in the expression of all the three genes with H$_2$O$_2$ treatment with maximum increases of about 220% (relative to controls) observed with 50 μM-H$_2$O$_2$ (P<0.001 for all).

Dose–response effects of resveratrol and quercetin on human umbilical vein endothelial cells gene expression

The dose-dependent effects of resveratrol and quercetin (0.001–1.0 μM) on the expression of eNOS, ET-1 and VEGF mRNA were investigated in the absence or presence of H$_2$O$_2$ (50 μM). Both the polyphenols had similar effects, but resveratrol was more potent than quercetin at equimolar concentrations (Tables 3 and 4).

As before, addition of 50 μM-H$_2$O$_2$ increased the eNOS, ET-1 and VEGF mRNA expression (Tables 3 and 4), but the effects of the polyphenols were gene specific. Resveratrol induced dose-dependent increases in the expression of eNOS and VEGF mRNA in the absence of H$_2$O$_2$ (Table 2), but the two treatments were not additive (P<0.001 for H$_2$O$_2$×resveratrol interactions). In contrast, resveratrol (at 0.1 and 1 μM) reduced the ET-1 mRNA expression in the absence of H$_2$O$_2$ (Table 3) and reduced or blocked the effect of 50 μM-H$_2$O$_2$ (P<0.001 for both H$_2$O$_2$×resveratrol interaction).

Similarly, quercetin induced dose-dependent increases in the expression of eNOS and VEGF mRNA in the absence of H$_2$O$_2$ (Table 4), but there was no additive effect with H$_2$O$_2$ on eNOS mRNA expression, while higher concentrations of quercetin reduced VEGF expression induced by 50 μM-H$_2$O$_2$ (P<0.001 for both H$_2$O$_2$×quercetin interactions). Like resveratrol, quercetin (at 0.1 and 1 μM) reduced ET-1 mRNA expression compared with controls (Table 4) and reduced or blocked the effect of 50 μM-H$_2$O$_2$ (P<0.001 for both H$_2$O$_2$×quercetin interaction).

<table>
<thead>
<tr>
<th>H$_2$O$_2$ concentration (μM)</th>
<th>0</th>
<th>6.25</th>
<th>12.5</th>
<th>25</th>
<th>50</th>
<th>SED</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>3.60</td>
<td>3.60</td>
<td>3.62</td>
<td>3.60</td>
<td>3.62</td>
<td>0.02</td>
<td>0.562</td>
</tr>
<tr>
<td>eNOS*</td>
<td>0.61</td>
<td>0.77†</td>
<td>0.99†</td>
<td>1.12†</td>
<td>1.35†</td>
<td>0.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>VEGF*</td>
<td>0.62</td>
<td>0.76†</td>
<td>0.92†</td>
<td>1.08†</td>
<td>1.36†</td>
<td>0.04</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ET-1*</td>
<td>0.60</td>
<td>0.73†</td>
<td>0.96†</td>
<td>1.05†</td>
<td>1.30†</td>
<td>0.02</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; eNOS, endothelial NO synthase; VEGF, vascular endothelial growth factor; ET-1, endothelin-1.

* Gene expression normalised to GAPDH.
† Mean values were significantly different from those of the control group (P<0.05, post hoc Dunnett’s test).

Effects of resveratrol, quercetin and hydrogen peroxide on endothelin secretion

Since both quercetin and resveratrol (at 0.1 μM) reduced basal and H$_2$O$_2$ (50 μM)-induced expression of ET-1 mRNA, their effects on the secretion of endothelin by HUVEC were examined. The effects were very similar to the effects on mRNA expression: in the absence of H$_2$O$_2$, both quercetin and resveratrol (0.1 μM) reduced the secretion of endothelin relative to controls (Fig. 1). In the absence of quercetin and resveratrol, H$_2$O$_2$ (50 μM) significantly increased the secretion of endothelin, but this was blocked by the addition of either resveratrol or quercetin (0.1 μM), with resveratrol being slightly more potent (P<0.001, H$_2$O$_2$×polyphenol interaction).

Effects of other dietary polyphenols on human umbilical vein endothelial cells gene expression

In order to investigate structure–function relationships in the observed effects, a variety of polyphenols (caffeic acid, genistein, phloretin, phloridzin, gallic acid, epicatechin,
ET-1 expression compared with controls and blocked the stimulatory effect of H2O2 on ET-1 mRNA. None of the other polyphenols studied appeared to have significant effects on ET-1 expression, although there was some variability in expression.

Discussion

H2O2 alone was found to significantly increase the expression of eNOS, VEGF and ET-1 mRNA in a dose-dependent manner (6.25–50 μM) after 24 h treatment. Previous studies have shown similar effects, with 25 μM-H2O2 increasing ET-1 expression in HUVEC(9) and 100 μM-H2O2 increasing eNOS expression in bovine aortic endothelial cells(13).

Quercetin and resveratrol were found to significantly increase the expression of eNOS in a dose-dependent manner after 24 h. This agrees with previous studies using supraphysiological concentrations of resveratrol, where 33 μM and 10–100 μM up-regulated eNOS expression in HUVEC and HUVEC-derived EA.hy 926 cells, respectively(10). In the present study, the combination of 50 μM-H2O2 and quercetin or resveratrol (0.001–1.0 μM) increased eNOS expression, but the two effects were not completely additive, suggesting that the effects are either via similar mechanisms or have simply reached a maximum level. Although eNOS protein or activity levels were not measured, a recent study(14) showed that both resveratrol and quercetin

Table 3. Dose-dependent effects of resveratrol (R) in the absence or presence of hydrogen peroxide (50 μM) on human umbilical vein endothelial cells gene expression

<table>
<thead>
<tr>
<th>R concentration (μM)</th>
<th>0</th>
<th>0.001</th>
<th>0.005</th>
<th>0.01</th>
<th>0.05</th>
<th>0.1</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>3.68</td>
<td>3.62</td>
<td>3.70</td>
<td>3.69</td>
<td>3.67</td>
<td>3.63</td>
<td>3.65</td>
</tr>
<tr>
<td>eNOS*</td>
<td>0.64</td>
<td>0.73</td>
<td>0.82</td>
<td>0.93</td>
<td>1.08</td>
<td>1.19</td>
<td>1.22</td>
</tr>
<tr>
<td>VEGF*</td>
<td>0.63</td>
<td>0.68</td>
<td>0.73</td>
<td>0.80</td>
<td>0.88</td>
<td>0.91</td>
<td>0.97</td>
</tr>
<tr>
<td>ET-1*</td>
<td>0.66</td>
<td>0.84</td>
<td>0.76</td>
<td>0.69</td>
<td>0.59</td>
<td>0.45</td>
<td>0.43</td>
</tr>
</tbody>
</table>

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; eNOS, endothelial NO synthase; VEGF, vascular endothelial growth factor; ET-1, endothelin-1.

* Gene expression normalised to GAPDH.

Table 4. Dose-dependent effects of quercetin (Q) in the absence or presence of hydrogen peroxide (50 μM) on human umbilical vein endothelial cells gene expression

<table>
<thead>
<tr>
<th>Q concentration (μM)</th>
<th>0</th>
<th>0.001</th>
<th>0.005</th>
<th>0.01</th>
<th>0.05</th>
<th>0.1</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>3.68</td>
<td>3.64</td>
<td>3.63</td>
<td>3.64</td>
<td>3.66</td>
<td>3.64</td>
<td>3.68</td>
</tr>
<tr>
<td>eNOS*</td>
<td>0.67</td>
<td>0.74</td>
<td>0.78</td>
<td>0.81</td>
<td>0.87</td>
<td>0.90</td>
<td>0.93</td>
</tr>
<tr>
<td>VEGF*</td>
<td>0.62</td>
<td>0.64</td>
<td>0.76</td>
<td>0.81</td>
<td>0.87</td>
<td>0.90</td>
<td>0.93</td>
</tr>
<tr>
<td>ET-1*</td>
<td>0.62</td>
<td>0.87</td>
<td>0.72</td>
<td>0.64</td>
<td>0.62</td>
<td>0.57</td>
<td>0.52</td>
</tr>
</tbody>
</table>

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; eNOS, endothelial NO synthase; VEGF, vascular endothelial growth factor; ET-1, endothelin-1.

* Gene expression normalised to GAPDH.
(at 100 μM) increased NO synthesis by HUVEC-derived EA.hy 926 cells, indicating that the effects on mRNA expression described here are likely to be matched at the protein level.

In contrast, treatment of HUVEC with either quercetin or resveratrol (at 0.1 or 1.0 μM) resulted in decreased expression of ET-1 mRNA, particularly in the presence of 50 μM H₂O₂. These findings are consistent with previous studies in HUVEC showing that resveratrol (100 μM) inhibited the expression of ET-1 mRNA induced by H₂O₂ (25 μM) or cyclic strain(9). Similarly, resveratrol (at 1–100 μM) reduced ET-1 mRNA expression and gene promoter activity induced by angiotensin II in rat aorta smooth muscle cells(8). Therefore, resveratrol’s ability to block stress-induced ET-1 mRNA expression is not specific to treatment with H₂O₂, but importantly the present studies demonstrate the effects at physiologically relevant concentrations.

As well as increasing ET-1 mRNA expression, H₂O₂ (50 μM) also significantly increased secretion of endothelin protein into the media. A similar effect on endothelin protein levels was observed in human aortic vascular smooth muscle cells treated with 200 μM-H₂O₂ for 8 h(15). Quercetin and resveratrol (0.1 μM) significantly reduced the secretion of endothelin and blocked the stimulatory effects of H₂O₂ (50 μM); again in agreement with Ruef et al.(15), who showed that supraphysiological concentrations of resveratrol and quercetin (100 and 50 μM, respectively) blocked the effect of H₂O₂ (200 μM) on ET-1 protein content in human aortic vascular smooth muscle cells.

Studies on the effects of equimolar concentrations of resveratrol and nine other dietary polyphenols showed that resveratrol, gallic acid and EGCG all increased eNOS mRNA expression in the presence and absence of H₂O₂ (50 μM), but only resveratrol and EGCG decreased ET-1 expression in the absence and presence of H₂O₂ (50 μM). In contrast, genistein, phloridzin and phloretin reduced the expression of eNOS, particularly in the absence of H₂O₂ (50 μM) but had little or no effect on ET-1 mRNA expression. The stimulatory effects of gallic acid on eNOS mRNA expression agree with a previous study(11) showing that 1 μM-gallic acid increased eNOS expression in HUVEC-derived EA.hy 926 cells, although another study(14) showed no effect of gallic acid on NO synthesis by EA.hy 926 cells. Interestingly, Appeldoorn et al.(14) also demonstrated the stimulatory effects of resveratrol, quercetin, EGCG and ECG (all at 100 μM) on NO synthesis, with no effects of epicatechin, epigallocatechin, phloretin, caffeic acid and a number of other polyphenols. The stimulatory effects of resveratrol, quercetin and EGCG were matched by increases in eNOS mRNA expression(14), whereas the effect of EGCG on eNOS mRNA expression was not statistically significant, agreeing with the data described here using lower concentrations of individual polyphenols. Although the observed inhibitory effects of phloretin on eNOS mRNA expression contrast with its lack of effect on NO synthesis(14), inhibitory effects on NO synthesis would be difficult to measure in the culture system used, since the basal level of NO synthesis was already low(15). The inhibitory effects of genistein on eNOS mRNA expression in HUVEC described here are in contrast to

Table 5. Effects of polyphenols (Poly) with differing structures (all 0-1 μM) in the absence or presence of hydrogen peroxide (50 μM) on human umbilical vein endothelial cells gene expression

<table>
<thead>
<tr>
<th>Poly (all 0-1 μM)</th>
<th>0</th>
<th>50</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cont</td>
<td>R</td>
<td>C</td>
</tr>
<tr>
<td>GAPDH</td>
<td>3.47</td>
<td>3.52</td>
<td>3.45</td>
</tr>
<tr>
<td>eNOS*</td>
<td>0.64</td>
<td>1.23</td>
<td>0.75</td>
</tr>
<tr>
<td>ET-1*</td>
<td>0.59</td>
<td>0.47</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Cont, control; R, resveratrol; C, caffeic acid; G, genistein; Pt, phloretin; Pd, phloridzin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; eNOS, endothelial NO synthase; ET-1, endothelin-1.

* Gene expression normalised to GAPDH.

Table 6. Effects of individual polyphenols (Poly; all 0-1 μM) in the absence or presence of hydrogen peroxide (50 μM) on human umbilical vein endothelial cells gene expression

<table>
<thead>
<tr>
<th>Poly (all 0-1 μM)</th>
<th>0</th>
<th>50</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cont</td>
<td>Ga</td>
<td>EC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>3.49</td>
<td>3.49</td>
<td>3.47</td>
</tr>
<tr>
<td>eNOS*</td>
<td>0.67</td>
<td>0.97</td>
<td>0.55</td>
</tr>
<tr>
<td>ET-1*</td>
<td>0.62</td>
<td>0.60</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Cont, control; Ga, gallic acid; EC, epicatechin; ECG, epicatechin gallate; EGCG, epigallocatechin gallate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; eNOS, endothelial NO synthase; ET-1, endothelin-1.

* Gene expression normalised to GAPDH.
Dietary polyphenols alter gene expression

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References


Fig. 1. Effects of resveratrol and quercetin (0-1 μM) in the presence or absence of hydrogen peroxide (50 μM) on secretion of endothelin by cultured human umbilical vein endothelial cells. Means and standard deviations (n=3) are shown. ○, Minus hydrogen peroxide (50 μM); ●, plus hydrogen peroxide (50 μM).

diagram showing endothelin secretion (fmol/ml) with control, resveratrol (0-1 μM), and quercetin (0-1 μM).