Supporting Information

Human quercetin conjugated metabolites attenuate TNF-α-induced changes in vasomodulatory molecules in a HUASMCs/HUVECs co-culture model

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Material and Methods

Cell viability determination
When HUASMCs in culture reached 95-100% confluence, cells were incubated with quercetin or quercetin metabolites (2 μM or 10 μM) for 24 hours. Following the incubations, cell viability was assessed by trypan blue exclusion as previously described elsewhere (Tribolo et al., 2008). Cells were counted using a dual-chamber haemocytometer under a light microscope. Cell morphology was also examined.

Cell culture
HUVECs and HUASMCs were obtained from Lonza and grown in EGM-2 bullet kit and SmGM-2 bullet kit, respectively (Lonza). HUASMCs were seeded in a co-cultured system using a 12-well transwell plate, in which HUVECs were seeded onto the outer lower side of a polyester membrane (seeding density of 2 x 10^4 cells/cm^2), and when they reached confluence HUASMCs were seeded at the same seeding density onto the inner upper side of the membrane: both cell types were maintained in their respective media. HUASMCs were starved in SmBM basal medium (without any supplement or fetal calf serum added) over night before each experiment. The experiments were performed with cells in both an unstimulated state and under inflammatory conditions induced by TNF-α (10 ng/mL). All the experiments were carried out between passage number 2-4 (HUVECs) and 5-7 (HUASMCs), respectively.

mRNA expression
The probes and primers used are listed in Table 1S.
Table 1S Sequences of probes and primers for real-time RT-PCR (TaqMan).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Probe (5’→3’)</th>
<th>Sense primer  (5’→3’)</th>
<th>Antisense primer  (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>h-ET-1</td>
<td>AACACTCCCGAGCACTGGTCCG</td>
<td>TGCCACCTGGACATCATTG</td>
<td>GACCTAGGGGCTTCCAAGTCCAT</td>
</tr>
<tr>
<td>h-</td>
<td>CATGACCACAGTCCATGCCTCACT</td>
<td>TTAGCACCCCTGGCCAACT</td>
<td>GCCATCCACAGTCTTCTGGG</td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Data analysis

Statistical analyses were performed using the R data analysis software (R Development Core Team (2006). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org). Standard ANOVA models were employed to analyse these data. For all models, diagnostics were checked to determine if data transformations, outlier omissions, or alternative non-parametric models were required. All results from the models were considered significant if p<0.05.

Additional remarks on the results

An independent experiment to examine the dose-response relationship over the range of 1-15 μM with one of the conjugates (Q3GlcA) indicated that the increase in cGMP was maximal at 1 and 2 μM, although a net increase was still observed up to 10 μM (data not shown). Tribolo et al. (2008) also found some relevant vascular effects to be stronger in response to 2 μM of flavonoid compared to 10 μM. Quercetin exerts anti-inflammatory mechanisms at lower concentrations but at higher concentrations may exert pro-inflammatory mechanisms which overcome the anti-inflammatory effects.
seen at low concentrations. Moreover, it has been shown that after ingesting flavonoid-rich fruits/vegetables, humans plasma total quercetin metabolite concentrations peak at 0.3-2.2 µM (Scalbert & Williamson, 2000).