Cardiovascular Research (2010) **85**, 175–183
doi:10.1093/cvr/cvp290

Expression of bilitranslocase in the vascular endothelium and its function as a flavonoid transporter

Alessandra Maestro[1†], Michela Terdoslavich[1‡], Andreja Vanzo[2], Adenike Kuku[1,3], Federica Tramer[1], Vanessa Nicolin[4], Fulvio Micali[1], Giuliana Decorti[1], and Sabina Passamonti[1*]

[1] Dipartimento di Scienze della Vita, Università di Trieste, via L. Giorgieri 1, 34127 Trieste, Italy; [2] Agricultural Institute of Slovenia, Ljubljana, Slovenia; [3] Department of Biochemistry, Obafemi Awolowo University, Ile-Ife, Nigeria; and [4] Dipartimento Universitario Clinico di Biomedicina, Università di Trieste, Trieste, Italy

Received 18 March 2008; revised 3 August 2009; accepted 19 August 2009; online publish-ahead-of-print 25 August 2009

Time for primary review: 26 days

Aims Ingestion of flavonoid-rich beverages acutely affects endothelial function, causing vasodilation. This effect might be dependent on flavonoid transport into the endothelium. We investigated flavonoid uptake into vascular endothelial cells and whether this was mediated by bilitranslocase (TC 2.A.65.1.1), a bilirubin-specific membrane carrier that also transports various dietary flavonoids.

Methods and results Human and rat aortic primary endothelial cells as well as Ea.hy 926 cells were found to express bilitranslocase, as assessed by immunocytochemistry and immunoblotting analysis using anti-sequence bilitranslocase antibodies targeting two distinct extracellular epitopes of the carrier. Bilitranslocase function was tested by measuring the rate of bromosulfophthalein (a standard bilitranslocase transport substrate) uptake into endothelial cells and was inhibited not only by bilitranslocase antibodies but also by quercetin (a flavonol). Similarly, uptake of both quercetin and malvidin 3-glucoside (an anthocyanin) were also found to be antibody-inhibited. Quercetin uptake into cells was inhibited by bilirubin, suggesting flavonoid uptake via a membrane pathway shared with bilirubin.

Conclusion The uptake of some flavonoids into the vascular endothelium occurs via the bilirubin-specific membrane transporter bilitranslocase. This offers new insights into the vascular effects of both flavonoids and bilirubin.

Keywords Vascular endothelium • Flavonoids • Bilirubin • Membrane transport • Bilitranslocase

1. Introduction

Epidemiological studies suggest that diets rich in fruits and vegetables decrease the risk of cardiovascular diseases.[1] This seems to be related to the intake of polyphenols,[2] plant-specific metabolites that have a multitude of molecular targets in mammalian cells.[3] The risk-reducing action of dietary polyphenols has been examined from various perspectives, taking into account their antioxidant, anti-inflammatory, hypo- and norm-lipidaemic, and vasodilating activities,[4] all of which concur to improve vascular function and slow down the progression of atherosclerosis. Improvement of endothelial function can be observed *in vivo* following ingestion of chocolate,[5,6] a flavonoid-rich food, highlighting the relevance of previous *in vitro* and *in vivo* data on experimental animals that show vasoactivity of dietary flavonoids.[7,8]

Various molecular targets of flavonoids have been identified in endothelial cells, such as nitric oxide synthase,[9] NADPH oxidase,[10,11] hypoxia-induced factor-1,[12] adenosine deaminase,

†Present address. IRCCS Burlo Garofolo, 34137 Trieste, Italy.

‡Present address. Rottapharm Biotech Srl, Centro Ricerche Area Science Park, 34012 Trieste, Italy.

* Corresponding author. Tel: +39 0405583680, Fax: +39 0405583691, Email: spassamonti@units.it

and 5'-nucleotidase.[13,14] This would in turn postulate a role of membrane transporters in mediating rapid and specific influx of flavonoids into the endothelium, a question thus far addressed by only few studies,[15–17] in spite of its obvious, fundamental importance.

This investigation was prompted by the somehow unexpected finding that human umbilical vein endothelial cells (HUVEC) express bilitranslocase (TC 2.A.65.1.1; http://www.tcdb.org/). The latter is a liver plasma membrane bilirubin transporter[18] found to have high affinity for various dietary flavonoids[19,20] and to mediate their uptake into the human liver cell line HepG2.[21] Thus, this study was aimed, first, at confirming the expression of bilitranslocase in the rat aorta and in isolated endothelial cells of various origins and, subsequently, at verifying the hypothesis that bilitranslocase may mediate the specific uptake of flavonoids therein. The data obtained suggest that flavonoids are rapidly taken up into the endothelium via bilitranslocase and, consistent with the latter's role as a bilirubin transporter, that their uptake can be modulated by bilirubin via a competitive mechanism.

2. Methods

2.1 Chemicals

All chemicals were purchased from Sigma-Aldrich, unless otherwise specified.

2.2 Animals

Male Wistar rats (250 g) were used for this study. They were fed standard laboratory chow (Harlan Teklad 2018) and tap water *ad libitum*, and were housed in temperature-controlled rooms at 22–24°C and 50–60% humidity. All animals were maintained and handled at the Animal House of the University of Trieste according to the provisions of the European Community Council Directive (n.86/609/CEE) and to the provisions of Italy (D.L.vo. 116/92). Care was taken not to hurt animals during the procedures.[22] Rats were anaesthetized by intraperitoneal injection of 2.5 mL of a 2.5% (mass:vol) solution of 2,2,2-tribromoethanol in ethanol:0.15 M NaCl (1:9, vol:vol). The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.3 Explant, culture, and phenotype assessment of endothelial cells from rat aorta

Primary endothelial cells were explanted from rat aorta using the method of McGuire and Orkin,[23] with slight modifications. Briefly, the aorta was carefully cleaned of peri-adventitial fat and connective tissue, longitudinally opened and pieces (2 mm^2) were laid with the intima side down on 24-well plates containing 200 μL of Matrigel (BD Biosciences), a reconstituted basement membrane derived from Engelbreth-Holm-Swarm EHS sarcoma.[24] Matrigel was diluted 1:3 (vol:vol) in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 12.5% fetal bovine serum (FBS), 1% of ECGS (Endothelial Cell Growth Supplement), penicillin 200 U/mL, 200 μg/mL streptomycin, 1% amphotericin B, and 1% non-essential aminoacids. After 3–5 days, the pieces were removed and the endothelial cells, migrated from aortic segments, were allowed to grow to confluence. Cells were detached from Matrigel following incubation with dispase (Gibco) (0.02 g/mL) at 37°C for 90 min for further growing in flasks. Their endothelial phenotype was ascertained by fluorescence microscopy visualization both of endocytosed acetylated LDL[25] (data not shown) and of membrane-bound von Willebrand factor (data not shown) using specific antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA.).

2.4 Explant, culture, and phenotype assessment of aortic smooth muscle cells

Rabbit arterial smooth muscle cells were isolated from rabbits as previously described.[26]

2.5 Human endothelial and hepatic cell lines

The human endothelial cell line Ea.hy 926, derived from the fusion of human umbilical vein endothelial cells (HUVEC) with the human lung carcinoma cell line A549, maintains the characteristics of differentiated endothelial cells.[27] Cells were cultured in DMEM, supplemented with 10% FBS, penicillin 100 U/mL, and streptomycin 100 μg/mL, under an atmosphere of 5% CO_2 and 95% air at 37°C, and sub-cultured twice weekly by exposure to a 0.05% trypsin and 0.02% EDTA solution. Human hepatoblastoma HepG2 cells were grown as previously described.[18]

2.6 Human primary endothelial cells

Human aortic endothelial cells derived from aorta were purchased and cultured according to the manufacturer's instructions (Promocell GmbH, Heidelberg, Germany).

2.7 Bilitranslocase antibodies

Polyclonal bilitranslocase antibodies were obtained from rabbit sera immunized with two different peptides, i.e. peptide A (EDSQGQHLSSF) and peptide B (EFTYQLTSSPTC), corresponding to segments 65–75 and 235–246 of the primary structure of bilitranslocase, yielding antibody A and antibody B. Both were purified by affinity chromatography from immune sera of rabbits, as described earlier.[28,29]

2.8 SDS–PAGE and immunoblot

Lysis buffer (10 mM Tris–HCl pH 7.4, 100 mM EDTA, 100 mM NaCl, 0.1% sodium dodecyl sulphate; 50 μL), supplemented with 5 μL Protease Inhibitor Cocktail, was added to cell monolayers (10^6 cells). Lysates were sonicated (10–15 s, in ice) in Eppendorf tubes and centrifuged at 13 000 r.p.m. for 10 min; supernatants were collected and assessed for protein concentration.[30] Human red blood cells ghosts obtained by a volunteer donor were prepared as described by Steck and Kant.[31] Aliquots thereof were separated by 12% SDS–PAGE and electro-transferred onto Immobilon-P membranes. After blocking (5%, mass:vol, powder milk in 10 mM Tris–HCl pH 7.4, 0.5 M NaCl, 0.1% Tween-20) at room temperature for 2 h, membranes were incubated overnight with bilitranslocase antibodies (Antibody A, 25 μg IgG/mL, in blocking solution) at 4°C. Horseradish peroxidase-conjugated secondary antibodies (1:6000-diluted goat anti-rabbit, Pierce) and chemiluminescent SuperSignal West Dura substrate (Thermo Scientific, Rockford, IL, USA) were used to develop the immunoblot.

2.9 Immunhistochemistry

Following anaesthesia, a rat underwent exsanguination by transcardial perfusion (at flow rate of 10 mL min^{-1}) with ~150 mL of phosphate buffered saline solution (PBS). The perfusion continued with 100 mL of fixative solution [4% (mass:vol) paraformaldehyde and 0.2% (vol:vol) glutaraldehyde in PBS]. Then, the abdominal aorta was

removed, cut in small rings and stored in the same solution for 2 h at 4°C. The rings were washed four times in PBS and twice in ultra pure water, then dehydrated in ethanol and embedded in LR White M acrylic resin. Immunolabelling of ultrathin sections (100 nm supported on 300 mesh nickel grids) was carried out by grid flotation technique at 21°C for 1 h on drops of blocking buffer [1% (mass:vol) bovine serum albumin (BSA), 20% (vol:vol) normal goat serum (NGS) in 0.1 M Tris buffered saline pH 7.4 (TBS)], and then incubated overnight at 4°C in 3 μg/mL of polyclonal anti-bilitranslocase Antibody A in incubation medium [1% (mass.vol) BSA, 1% (vol:vol) NGS, 4% (vol:vol) FBS and 0.1% (vol:vol) Tween 20 (Merck SpA, Milano, Italy) in TBS]. After several washes (six times, 5 min each) in TBS to remove the antibody excess, the sections were incubated for 2 h in the same incubation medium but at pH 8.4, containing gold-conjugated 10 nm goat anti-rabbit IgG (British BioCell, Cardiff, UK) diluted 1:100. Same grids were treated with Silver Enhancing Kit (British BioCell, Cardiff, UK) to enlarge the gold particles. Finally, the sections were counterstained with uranyl acetate (2%, mass:vol) for 15 min and then lead citrate solution (0.25%, mass:vol) for 2.5 min and observed by Philips EM 208 electron microscope at 80 kV accelerating voltages. Anti-bilitranslocase antibody A was omitted in the controls. In order to assess the endothelial phenotype of the cells lining the aortic lumen, a similar procedure was implemented with adjacent aortic rings, incubated with anti-von Willebrandt factor antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, 1:100 in incubation medium).

2.10 Immunocytochemistry

About 2.5×10^4 cells were seeded on sterile glass cover slips, para-formaldehyde-fixed, treated with 0.2% (vol:vol, in PBS) Triton X-100 for 10 min and incubated for 1 h with blocking solution (see above) at 37°C. Cells were incubated for 2 h at room temperature with bilitranslocase antibodies (antibody B, 100 μg IgG/mL). A FITC-conjugated secondary antibody (goat anti-rabbit IgG, 1: 200) was used to visualize immunocomplexes under a fluorescence microscopy.

2.11 Preparation of cell monolayers for uptake assay

Cell monolayers were obtained by seeding cells (2.5×10^5 in 7 mL of growth medium) in 25 cm^2 flasks; the growth medium was changed every second day. Uptake experiments were performed after 6 days, as cells reached confluence. In some experiments the cell monolayer was pre-incubated at 37°C in growth medium containing 0.1 mM phenylmethanesulfonyl fluoride (PMSF) for 20 min or in serum-free growth medium containing either antibody B or rabbit IgG (both 0.24 μg IgG/mL) for 30 min. Prior to uptake assays, the cell medium was removed and cells were rinsed with PBS (three times, at 37°C).

2.12 BSP and quercetin uptake

The uptake assays were similar to those previously applied in the human hepatocarcinoma cell line HepG2.[18,21] The tests started by adding the transport solution (7 mL PBS, containing either 24 μM BSP or 10 μM quercetin) to the cell monolayer, previously washed with PBS. The flasks were kept in a water bath at 37°C and gently shaken. At time intervals, samples (BSP: 300 μL; quercetin: 800 μL) of the transport medium were transferred to polycarbonate cuvettes for spectrophotometric analysis (Ultrospec 2100 pro, Amersham Biosciences, AB, Uppsala, Sweden). Quercetin was analysed at $\lambda = 375$ nm ($\varepsilon = 18.1\ M^{-1}\ cm^{-1}$); BSP was analysed in alkali (0.1 M NaOH, 2.7 mL) at $\lambda = 580$ nm ($\varepsilon = 64\,000\ M^{-1}\ cm^{-1}$). The same kind of experiment was repeated 3–5 times, yielding qualitatively similar results. This experiment was performed three times. The figures display representative experiments, carried out in triplicate on the same day.

2.13 Assay of malvidin 3-glucoside uptake

The assays started by adding the transport solution (4 mL PBS, containing 18 μM malvidin 3-glucoside) to the cell monolayer, previously washed with PBS. The flasks were kept in a water bath a 37°C and gently shaken. At time intervals, samples (300 μL) of the transport medium were added to 120 μL of 0.8% perchloric acid in methanol for further HPLC-DAD analysis, as previously described.[21] This experiment was performed three times. The figure displays a representative one, carried out in triplicate on the same day.

2.14 Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 (La Jolla, CA, USA). Differences among uptake curves were evaluated by two-way ANOVA with Bonferroni posttests. *P*-values < 0.05 (two-tailed) were regarded as statistically significant.

3. Results

3.1 Immunochemical studies with bilitranslocase antibodies

3.1.1 SDS–PAGE and immunoblot

Bilitranslocase polyclonal antibodies used in this work target two selected extracellular domains of the transporter,[18] causing inhibition of its transport activity in rat liver plasma membrane vesicles.[28,29] Their effects can be prevented by nM bilirubin,[28,29] showing their specific molecular interaction. Consistently, both antibody A and antibody B cause inhibition of BSP uptake into the human hepatocarcinoma cell line HepG2.[18,21] These antibodies were used to detect bilitranslocase in the endothelial cells. *Figure 1A* shows immunoblots of lysates of human endothelial cells (Ea.hy 926 cell line), primary rabbit aortic smooth muscle cells, human hepatocytes (HepG2 cell line, positive control), and human erythrocyte ghosts (negative control), using antibody A. As already seen in liver,[18,28,29] the antibody reacts with a single protein of apparent molecular mass of ~38 kDa. Similar data could be obtained with antibody B (data not shown). Given their specific reaction, these antibodies seemed suitable to further investigate the expression and function of bilitranslocase in the vascular endothelium.

3.1.2 Immunohistochemistry

Figure 1B shows the tunica intima and the tunica media of a section of rat aorta; endothelial cells were characterized for the expression of the von Willebrandt factor (data not shown). The immunogold electron microscopy detection of bilitranslocase in the rat aortic endothelium is shown in *Figure 1C*, where several immunogold particles were found both at the plasma membrane level and in intracellular vesicles.

3.1.3 Immunocytochemistry

Immunocytochemistry analysis with the bilitranslocase antibodies was carried out on primary aortic endothelial cells explanted from rat (*Figure 2A*), on human aortic endothelial cells (*Figure 2B*), and on the permanent cell line Ea.hy 926 (*Figure 2C*). In all instances,

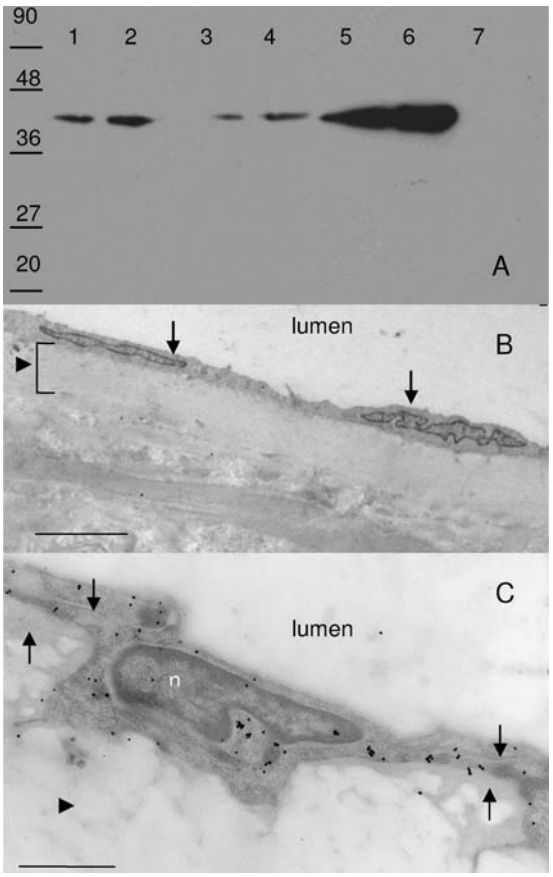

Figure 1 (*A*) Immunoblotting of lysates of Ea.hy 926 cells (lanes 1–2, 30, and 60 μg proteins, respectively), primary rabbit aortic smooth muscle cells (lanes 3–4, 30, and 60 μg), HepG2 (lanes 5–6, 30, and 60 μg), and human erythrocyte ghosts (lane 7, 50 μg) using bilitranslocase antibody A. (*B* and *C*) Immunogold electron-microscopy localization of bilitranslocase in rat aortic endothelium. (*B*) shows a transverse section of the rat thoracic aorta, with two endothelial cells (arrows) resting on the *lamina elastica interna* (black arrowhead) (scale bar 5 μm). (*C*) shows a section of the *tunica intima*, with an endothelial cell (*n* = nucleus) contacting (downward arrows) neighbouring cells; the endothelium rests on the sub-endothelial layer (upward arrows), interconnected with the *lamina elastica interna* (arrowhead). Bilitranslocase localization in the endothelium is demonstrated by immunogold particles (black dots), using antibody A (scale bar 1 μm).

cells express bilitranslocase. The antigen appears not only at the level of the cell membrane, but also in the cytoplasm; in particular, the large human primary endothelial cells offer the opportunity to visualize a network of intra-cellular tubules, thought to arise by invagination of the plasma membrane.[32]

3.2 Assays of bilitranslocase transport activity in Ea.hy 926 cells

The Ea.hy 926 cell line is an endothelial cell model, suitable for maintenance in culture for up to 30 passages, thus appropriate for characterizing the transport properties of bilitranslocase under a variety of experimental conditions. These cells were used in transport assays using three different bilitranslocase substrates, such as bromosulfophthalein, quercetin, and malvidin 3-glucoside.

3.2.1 Bromosulfophthalein uptake

The transport substrate was bromosulfophthalein (BSP), an organic anion dye (*Figure 3A*), whose quinoidal tautomer is transported by liver bilitranslocase (Km = 5 μM).[33,34] As shown in *Figure 3B*, BSP (24 μM) is rapidly taken up into cells, reaching equilibrium by 1 min. Both antibody A and B (0.24 μg IgG/mL added to the cell growth medium for 1 h prior to the uptake test) markedly inhibited BSP uptake into cells. The partial effect might be ascribed to: (i) the transport activity of other, unidentified BSP membrane carriers contributing to the uptake; (ii) the residual activity of bilitranslocase molecules not involved in the formation of immuno-complexes; (iii) partial functional inhibition of the carrier, in spite of full complexation with antibodies. Notwithstanding, a substantial share of BSP uptake into cells is bilitranslocase-mediated. *Figure 3C* shows that BSP uptake is inhibited by the flavonoid quercetin (5 μM, its structure is insetted in *Figure 4B*), a competitive inhibitor of bilitranslocase transport activity displaying some structural analogies with phthaleins.[19] It should be observed that under such conditions quercetin interacts with bilitranslocase below its saturation (quercetin Ki = 21.1 μM,[19]), thus stronger inhibition might be expected at higher quercetin concentrations. These tests were, however, not done, due to the limited quercetin solubility in the transport assay medium.

3.2.2 Quercetin uptake

Figure 4A shows that quercetin (10 μM) is also rapidly taken up into Ea.hy 926 cells. Its uptake could be inhibited either by BSP (24 μM) or by bilirubin (as low as 50 nM), each added to the quercetin solution (*cis*-inhibition). In a *trans*-inhibition test, cells were pre-loaded with 1 μM bilirubin added to the cell culture medium for 1 h and then removed prior to the uptake test. Again in this condition, quercetin uptake was inhibited (*trans*-inhibition) (*Figure 4B*). The latter findings suggest that quercetin entered into cells via a high-affinity bilirubin carrier, presumably bilitranslocase (bilirubin Kd = 2 nM,[18,20,28]). Consistently, both bilitranslocase antibodies (0.24 μg IgG/mL added to the cell growth medium for 1 h prior to the uptake test) inhibited quercetin uptake (*Figure 4C*). *Figure 4C* shows that an even stronger inhibition could be obtained by pre-treating the cell monolayer with the serine-specific reagent phenylmethanesulfonyl fluoride. This serine-specific reagent is an inhibitor of bilitranslocase transport activity in rat liver plasma membrane vesicles[35,36] and a blocker of bilirubin uptake into the liver cell line HepG2.[18]

3.2.3 Malvidin 3-glucoside uptake

Quercetin, though extensively used *in vitro* studies, is found in human plasma, mainly as glucurono- and sulfo-conjugated derivatives[37] that are not substrates of bilitranslocase.[19] Quercetin derivatives could be, however, deconjugated in sites of inflammation[38] and converted to the aglycone, that is a substrate of

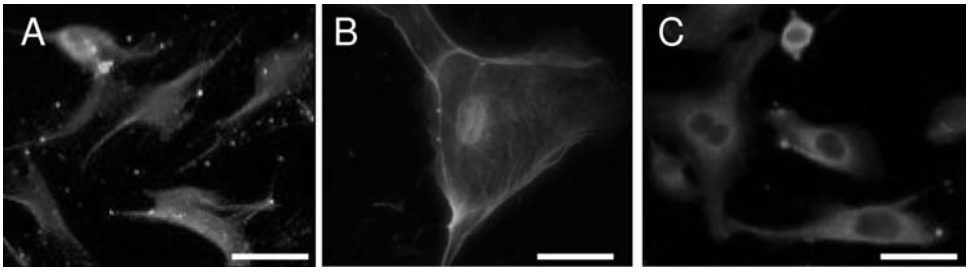

Figure 2 Immunocytochemistry of vascular endothelial cells. (*A*) Primary endothelial cells explanted from rat aorta. (*B*) Primary endothelial cells from human aorta. (*C*) Ea.hy 926 cells. Primary immunocomplexes formed with bilitranslocase antibodies (antibody A) were detected by a FITC-conjugated anti-rabbit antibody and visualized under epifluorescence microscopy (scale bar 50 μm).

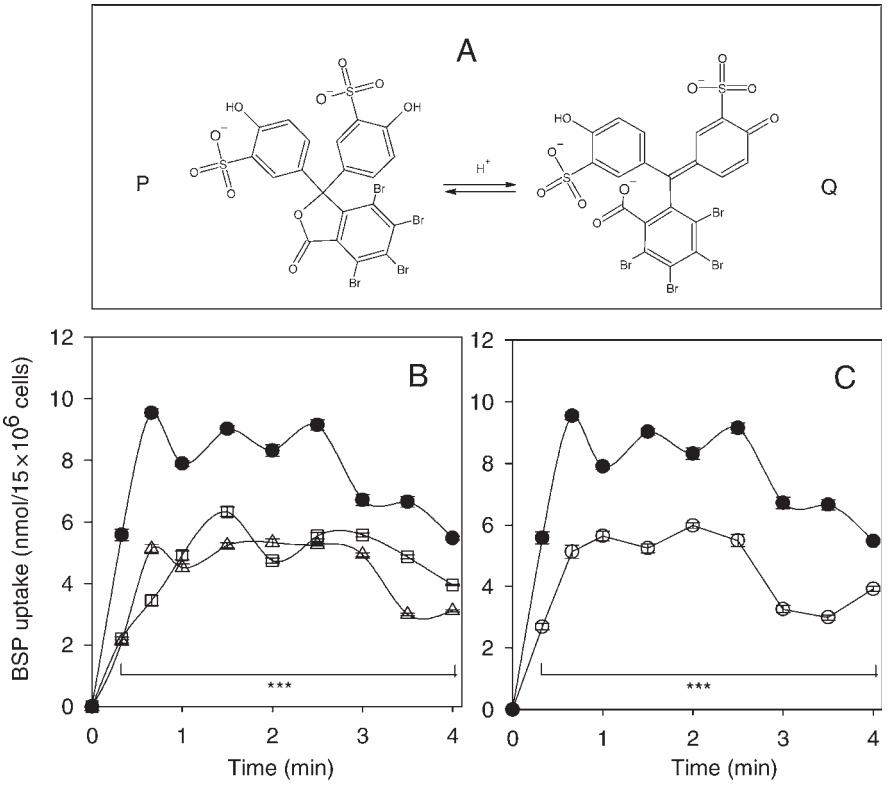

Figure 3 (*A*) Chemical structures of BSP tautomers. The colourless phenolic species (P) converts to the coloured (A_{max} = 580 nm) quinoidal one (Q) upon release of an H^+ into the medium. (*B*) BSP uptake into Ea.hy 926 monolayers: the effect of bilitranslocase antibodies. Uptake tests were carried out as described in Sections 2.11 and 2.12, using cell mono-layers pre-incubated either without (filled circles) or with anti-bilitranslocase antibodies (antibody A, open triangles; antibody B, open squares). (*C*) *Cis*-inhibition of BSP uptake into Ea.hy 926 monolayers by extracellular quercetin. Uptake tests were carried out using 24 μM BSP either in the absence (filled circles) or in the presence of 10 μM quercetin (open circles). Data are means ± SEM ($n = 3$). All data obtained with the inhibitors are significantly different from BSP alone at ***$P < 0.001$.

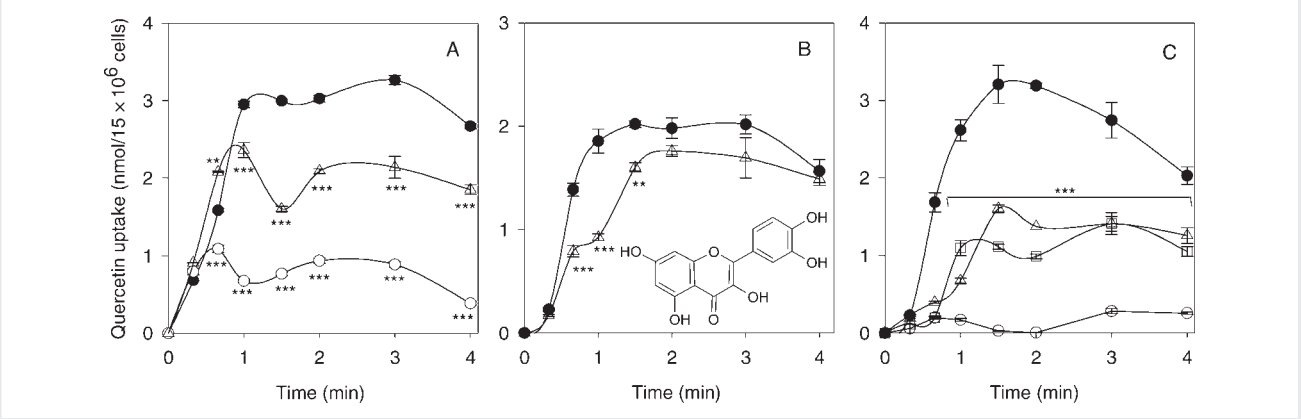

Figure 4 Quercetin uptake into Ea.hy 926 monolayers. (*A*) *Cis*-inhibitions by either extracellular BSP or bilirubin. Uptake tests were carried out as described in Sections 2.11 and 2.12, using 10 μM quercetin either in the absence (filled circles) or in the presence of either 24 μM BSP (open circles) or 50 nM bilirubin (open triangles). (*B*) *Trans*-inhibition by intracellular bilirubin. Uptake of 10 μM quercetin was carried out as described in Sections 2.10 and 2.11; cell monolayers were pre-incubated for 1 h either in the absence (filled circles) or in the presence of 1 μM bilirubin (open triangles) added to culture medium. Then, cells monolayers were washed and incubated with transport assay solution containing 10 μM quercetin. The inset displays quercetin chemical structure. (*C*) The effect of bilitranslocase antibodies and of the irreversible serine-specific reagent PMSF. Cell monolayers were pre-incubated either without (filled circles) or with bilitranslocase antibodies (antibody A, open triangles; antibody B, open squares) or with 0.1 mM PMSF (open circles). Then, cells monolayers were washed and incubated with transport assay solution containing quercetin. Data are means ± SEM ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$.

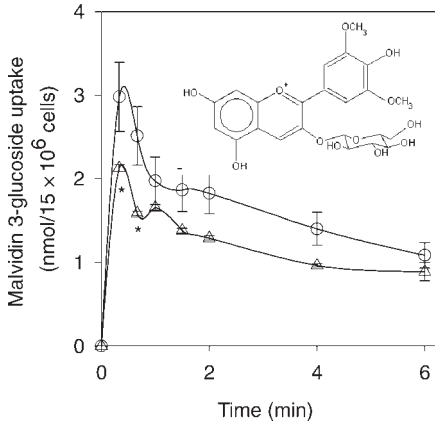

Figure 5 Malvidin 3-glucoside uptake into Ea.hy 926 monolayers: the effect of bilitranslocase antibodies. Monolayers of Ea.hy 926 cells were pre-incubated either without (filled circles) or with 0.24 μg/mL antibody B (open squares). Then, cells were washed and incubated with 18 μM malvidin 3-glucoside. Data are means ± SEM ($n = 3$). Significantly different from malvidin 3-glucoside (chemical structure in the inset) alone at $^{*}P < 0.05$.

bilitranslocase. In contrast, malvidin 3-glucoside (*Figure 5A*), a flavonoid pigment of red fruits and berries and a strong inhibitor of bilitranslocase activity (Ki = 1.42 μM),[34] is found intact in plasma following ingestion.[39] Its uptake into Ea.hy 926 cells might be of physiological interest. *Figure 5B* shows that the uptake of this anthocyanin into Ea.hy 926 cells was inhibited by antibody B, similar to what seen in liver cells (HepG2).[21]

3.3 Quercetin uptake in primary endothelial cells

In order to test bilitranslocase function also in normal cells, either primary endothelial cells explanted from rat aorta or human aortic endothelial cells were used in quercetin uptake tests. *Figure 6A* shows that quercetin uptake into primary rat endothelial cells was similar both in rate and in extent to that seen in Ea.hy 926 cells and much higher than in smooth muscle cells explanted from rabbit aorta and tested to provide a reference, since they poorly express bilitranslocase (data not shown).

Quercetin uptake was strongly inhibited by antibody B both in rat and human cells (*Figure 6B* and *C*, respectively), allowing to conclude that results obtained with Ea.hy 926 cells can be extrapolated to normal endothelial cells.

4. Discussion

The results of this study show that the uptake of quercetin into endothelial cells has the features of a carrier-mediated process, for the following reasons: (i) it is inhibited by the serine-specific reagent phenylmethanesulfonyl fluoride to an extent indicating that other phenomena possibly concurring to quercetin disappearance from the medium are irrelevant (e.g. plain adsorption onto membrane phospholipids or extracellular break-down) (*Figure 4C*). The sensitivity of quercetin uptake to serine-blockers implies that also organophosphorous compounds, used as insecticides and pesticides in the food production chain, might interfere with uptake of flavonoids into the endothelium; (ii) it is also inhibited by pure anti-peptide antibodies targeting two distinct extracellular domains of liver bilitranslocase[18] (*Figure 4C*); (iii) it is *cis*-inhibited by BSP (*Figure 4A*), whose uptake into endothelial

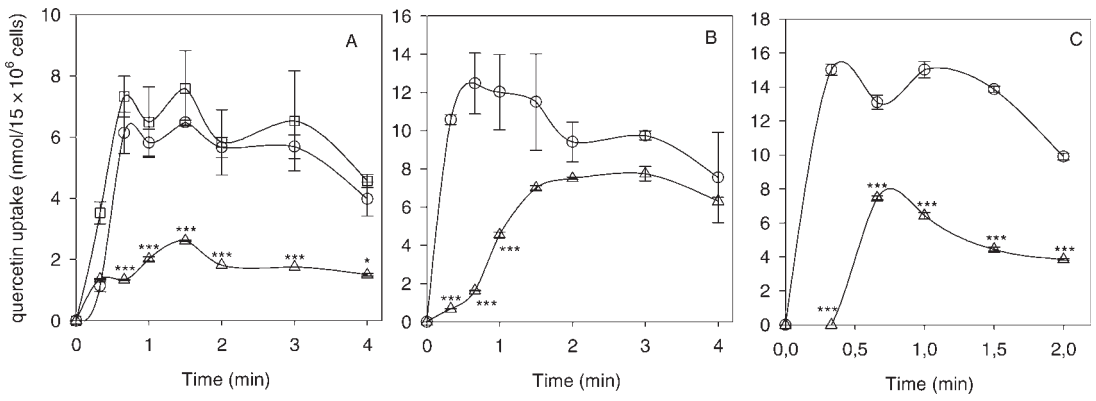

Figure 6 Quercetin uptake into endothelial cells from either rat or human aorta. (*A*) Quercetin uptake was carried in primary endothelial cells explanted from rat aorta (squares) as described in *Figure 4A*. For comparison, the same assay was also carried out with Ea.hy 926 cells (circles) and aortic smooth muscle cells (triangles). (*B*) Quercetin uptake into primary endothelial cells explanted from rat aorta: the effect of bilitranslocase antibodies. Monolayers were pre-incubated either without (circles) or with 0.24 μg/mL antibody B (triangles); quercetin uptake assay was carried out as described in *Figure 4*. (*C*) Quercetin uptake into primary endothelial cells explanted from human aorta: the effect of bilitranslocase antibodies. Monolayers were pre-incubated either without (circles) or with 0.24 μg/mL antibody B (triangles) and assayed for quercetin uptake as described earlier. Data are means ± SEM ($n = 3$). Significantly different from quercetin alone at $^{*}P < 0.05$, $^{***}P < 0.001$.

cells is not only inhibited by the same bilitranslocase antibodies (*Figure 3B*) but also *cis*-inhibited by quercetin itself (*Figure 3C*); (iv) it is both *cis*- and *trans*-inhibited by physiological concentrations of unbound (i.e. albumin-free) bilirubin[40] (*Figure 4A* and *B*, respectively), a high-affinity transport substrate of liver bilitranslocase.[18] The possible contribution of glucose transporters (in particular the ubiquitous GLUT1, shown to transport quercetin into erythrocytes[41]) to endothelial quercetin uptake cannot be ruled out and is, indeed, quite likely to take place both *in vivo* and in the above experimental models. For all that, the sensitivity of quercetin uptake to both bilirubin and anti-bilitranslocase antibodies is not less, but rather more meaningful, since it is clearly observed in systems where multiple flavonoid transporters occur.

Altogether, it can be concluded that a high-affinity bilirubin membrane transporter, similar if not identical to liver bilitranslocase, promotes the uptake of some flavonoids, such as quercetin and malvidin 3-glucoside, into the vascular endothelium. From the above uptake data and taking into account the volume of the 25 cm^2-cell monolayer (about 20 μL), it can be calculated that flavonoids may attain an intracellular concentration higher by at least one order of magnitude than the initial extracellular one, therefore potentially making the vascular endothelium, extending for up to about 240 m^2,[42] one of the largest tissutal targets of dietary flavonoids. Noticeable is, however, the observation that the apparent cellular uptake of flavonoids is transient, the likely consequence of their active efflux from cells.[43] The concentrations of flavonoids in these assays, chosen to be reliably quantified, are well above the reported circulating levels following ingestion.[44] However, preliminary tests done in our laboratories indicate that much lower extracellular concentrations ($<10^{-6}$ M) of flavonoids can be used to demonstrate both their bilitranslocase-dependent uptake and their intracellular bioactivity.

Quite meaningful is the finding that bilirubin inhibits quercetin uptake, an indirect though still compelling demonstration that bilirubin itself permeates the endothelial plasma membrane through a carrier protein, rather than by passive diffusion. Lipophilic bilirubin is taken up into the liver, the exclusive site of its disposition, by a carrier-mediated mechanism, as demonstrated first in rats in 1975[45] and as expected of a di-anion at the plasma pH.[46] The advantage for endothelial cells to express one or more types of bilirubin membrane carrier(s) would be to establish yet another point of control on intracellular bilirubin concentration, downstream from the first one at the level of the heme oxygenase reaction. The latter enzyme (HO-1, EC-Number 1.14.99.3) is rapidly and markedly induced by transcriptional activation triggered by the most various stressors,[47] thus leading to large intracellular fluctuations of its products, i.e. carbon monoxide and biliverdin, the latter subsequently reduced to bilirubin by biliverdin reductase (EC-Number 1.3.1.24). If bilirubin generated intracellularly by heme oxygenase improves the endothelial function, by decreasing superoxide anion generation by NADPH oxidase (EC-Number 1.6.3.1)[48] and suppressing pro-inflammatory genes,[49] also serum bilirubin confers vascular protection[50,51] and reduced risk of cardiovascular disease.[52] It is therefore, reasonable to speculate that one, so far unappreciated, mechanism whereby flavonoids are beneficial for the cardiovascular system might also be the inhibition of bilirubin efflux from endothelial cells, so that bilirubin would attain the effective intracellular concentration to act like both an antioxidant[53] and an inhibitor of reactive oxygen species' generation by NADPH oxidase.

While this work addressed the issue, whether some selected flavonoids can be taken up into the vascular endothelium, these data open an even more general question, i.e. which other molecules can be targeted to the endothelium by exploiting the transport

properties of bilitranslocase? Further work, currently in progress in our laboratories, is needed to understand the transporting potential of this membrane carrier protein.

Acknowledgements

Mr Gianluca Petris (graduate student of Medical Biotechnology, University of Trieste) contributed to part of this work as a trainee of Prof. G.D. Thanks are due to Mr Claudio Gamboz for electron microscopy, to Prof. Albin Hermetter (Institute of Biochemistry, Graz University of Technology, Austria) for inspiring discussions, and to Prof. Gorazd Drevenšek and Lovro Ziberna (University of Ljubljana, Slovenia) for testing bilitranslocase antibodies in experimental models of flavonoid-induced cardioprotection.

Conflict of interest: none declared.

Funding

Financial support was provided by Università di Trieste; Ministero degli Affari Esteri (Cooperazione scientifica e tecnologica Italia-Slovenia 2006–2009); Slovenian Research Agency (projects J4-7146, BI-IT/05-08-031, V4-0321); International Centre for Theoretical Physics, Trieste, Italy (TRIL fellowship to A.K.); Soc. Az. Agr. Tenuta Villanova, 34072 Farra d'Isonzo, Italy; Regione Autonoma Friuli-Venezia Giulia (L.R. 26/2005 art. 17).

References

1. Sofi F, Cesari F, Abbate R, Gensini GF, Casini A. Adherence to Mediterranean diet and health status: meta-analysis. *BMJ* 2008;**337**:a1344.
2. Arts IC, Hollman PC. Polyphenols and disease risk in epidemiologic studies. *Am J Clin Nutr* 2005;**81**:317S–325S.
3. Middleton E Jr, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol Rev* 2000;**52**:673–751.
4. Manach C, Mazur A, Scalbert A. Polyphenols and prevention of cardiovascular diseases. *Curr Opin Lipidol* 2005;**16**:77–84.
5. Vlachopoulos C, Aznaouridis K, Alexopoulos N, Economou E, Andreadou I, Stefanadis C. Effect of dark chocolate on arterial function in healthy individuals. *Am J Hypertens* 2005;**18**:785–791.
6. Flammer AJ, Hermann F, Sudano I, Spieker L, Hermann M, Cooper KA *et al.* Dark chocolate improves coronary vasomotion and reduces platelet reactivity. *Circulation* 2007;**116**:2376–2382.
7. Stoclet JC, Chataigneau T, Ndiaye M, Oak MH, El Bedoui J, Chataigneau M *et al.* Vascular protection by dietary polyphenols. *Eur J Pharmacol* 2004;**500**:299–313.
8. Perez-Vizcaino F, Duarte J, Andriantsitohaina R. Endothelial function and cardiovascular disease: effects of quercetin and wine polyphenols. *Free Radic Res* 2006;**40**:1054–1065.
9. Schmitt CA, Dirsch VM. Modulation of endothelial nitric oxide by plant-derived products. *Nitric Oxide* 2009;**21**:77–91.
10. Sanchez M, Galisteo M, Vera R, Villar IC, Zarzuelo A, Tamargo J *et al.* Quercetin downregulates NADPH oxidase, increases eNOS activity and prevents endothelial dysfunction in spontaneously hypertensive rats. *J Hypertens* 2006;**24**:75–84.
11. Steffen Y, Gruber C, Schewe T, Sies H. Mono-O-methylated flavanols and other flavonoids as inhibitors of endothelial NADPH oxidase. *Arch Biochem Biophys* 2008;**469**:209–219.
12. Wilson WJ, Poellinger L. The dietary flavonoid quercetin modulates HIF-1 alpha activity in endothelial cells. *Biochem Biophys Res Commun* 2002;**293**:446–450.
13. Kavutcu M, Melzig MF. In vitro effects of selected flavonoids on the 5'-nucleotidase activity. *Pharmazie* 1999;**54**:457–459.
14. Melzig MF. Inhibition of adenosine deaminase activity of aortic endothelial cells by selected flavonoids. *Planta Med* 1996;**62**:20–21.
15. Schramm DD, Collins HE, German JB. Flavonoid transport by mammalian endothelial cells. *J Nutr Biochem* 1999;**10**:193–197.
16. Youdim KA, Dobbie MS, Kuhnle G, Proteggente AR, Abbott NJ, Rice-Evans C. Interaction between flavonoids and the blood-brain barrier: in vitro studies. *J Neurochem* 2003;**85**:180–192.
17. Youdim KA, Martin A, Joseph JA. Incorporation of the elderberry anthocyanins by endothelial cells increases protection against oxidative stress. *Free Radic Biol Med* 2000;**29**:51–60.
18. Passamonti S, Terdoslavich M, Margon A, Cocolo A, Medic N, Micali F *et al.* Uptake of bilirubin into HepG2 cells assayed by thermal lens spectroscopy. *FEBS J* 2005;**272**:5522–5535.
19. Karawajczyk A, Drgan V, Medic N, Oboh G, Passamonti S, Novic M. Properties of flavonoids influencing the binding to bilitranslocase investigated by neural network modelling. *Biochem Pharmacol* 2007;**73**:308–320.
20. Passamonti S, Terdoslavich M, Franca R, Vanzo A, Tramer F, Braidot E *et al.* Bioavailability of flavonoids: a review of their membrane transport and the function of bilitranslocase in animal and plant organisms. *Curr Drug Metab* 2009;**10**:369–394.
21. Passamonti S, Vanzo A, Vrhovsek U, Terdoslavich M, Cocolo A, Decorti G *et al.* Hepatic uptake of grape anthocyanins and the role of bilitranslocase. *Food Res Int* 2005;**38**:953–960.
22. Demers G, Griffin G, De Vroey G, Haywood JR, Zurlo J, Bedard M. Animal research. Harmonization of animal care and use guidance. *Science* 2006;**312**:700–701.
23. McGuire PG, Orkin RW. Isolation of rat aortic endothelial cells by primary explant techniques and their phenotypic modulation by defined substrata. *Lab Invest* 1987;**57**:94–105.
24. Kleinman HK, McGarvey ML, Hassell JR, Star VL, Cannon FB, Laurie GW *et al.* Basement membrane complexes with biological activity. *Biochemistry* 1986;**25**:312–318.
25. Voyta JC, Via DP, Butterfield CE, Zetter BR. Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. *J Cell Biol* 1984;**99**:2034–2040.
26. Decorti G, Klugmann FB, Crivellato E, Malusa N, Furlan G, Candussio L *et al.* Biochemical and microscopic evidence for the internalization of drug-containing mast cell granules by macrophages and smooth muscle cells. *Toxicol Appl Pharmacol* 2000;**169**:269–275.
27. Edgell CJ, McDonald CC, Graham JB. Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc Natl Acad Sci USA* 1983;**80**:3734–3737.
28. Battiston L, Passamonti S, Macagno A, Sottocasa GL. The bilirubin-binding motif of bilitranslocase and its relation to conserved motifs in ancient biliproteins. *Biochem Biophys Res Commun* 1998;**247**:687–692.
29. Passamonti S, Cocolo A, Braidot E, Petrussa E, Peresson C, Medic N *et al.* Characterization of electrogenic bromosulfophthalein transport in carnation petal microsomes and its inhibition by antibodies against bilitranslocase. *FEBS J* 2005;**272**:3282–3296.
30. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;**193**:265–275.
31. Steck TL, Kant JA. Preparation of impermeable ghosts and inside-out vesicles from human erythrocyte membranes. *Methods Enzymol* 1974;**31**:172–180.
32. Rippe B, Rosengren BI, Carlsson O, Venturoli D. Transendothelial transport: the vesicle controversy. *J Vasc Res* 2002;**39**:375–390.
33. Passamonti S, Battiston L, Sottocasa GL. Bilitranslocase can exist in two metastable forms with different affinities for the substrates–evidence from cysteine and arginine modification. *Eur J Biochem* 1998;**253**:84–90.
34. Passamonti S, Vrhovsek U, Mattivi F. The interaction of anthocyanins with bilitranslocase. *Biochem Biophys Res Commun* 2002;**296**:631–636.
35. Passamonti S, Battiston L, Sottocasa GL. Arylsulfonylation of bilitranslocase in plasma membranes from rat liver enables to discriminate between natural and artificial substrates. *Biochim Biophys Acta* 1997;**1323**:130–136.
36. Passamonti S, Battiston L, Sottocasa GL. On the mechanism of bilitranslocase transport inactivation by phenylmethylsulphonyl fluoride. *Mol Membr Biol* 1999;**16**:167–172.
37. Mullen W, Boitier A, Stewart AJ, Crozier A. Flavonoid metabolites in human plasma and urine after the consumption of red onions: analysis by liquid chromatography with photodiode array and full scan tandem mass spectrometric detection. *J Chromatogr A* 2004;**1058**:163–168.
38. Shimoi K, Nakayama T. Glucuronidase deconjugation in inflammation. *Methods Enzymol* 2005;**400**:263–272.
39. Prior RL, Wu X. Anthocyanins: structural characteristics that result in unique metabolic patterns and biological activities. *Free Radic Res* 2006;**40**:1014–1028.
40. Ahlfors CE. Bilirubin-albumin binding and free bilirubin. *J Perinatol* 2001;**21**(Suppl. 1):S40–S42. discussion S59–S62.
41. Cunningham P, Afzal-Ahmed I, Naftalin RJ. Docking studies show that D-glucose and quercetin slide through the transporter GLUT1. *J Biol Chem* 2006;**281**:5797–5803.
42. Muro S, Koval M, Muzykantov V. Endothelial endocytic pathways: gates for vascular drug delivery. *Curr Vasc Pharmacol* 2004;**2**:281–299.

43. Liu Z, Hu M. Natural polyphenol disposition via coupled metabolic pathways. *Expert Opin Drug Metab Toxicol* 2007;**3**:389–406.
44. Manach C, Williamson G, Morand C, Scalbert A, Remesy C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am J Clin Nutr* 2005;**81**:230S–242S.
45. Scharschmidt BF, Waggoner JG, Berk PD. Hepatic organic anion uptake in the rat. *J Clin Invest* 1975;**56**:1280–1292.
46. Boiadjiev SE, Watters K, Wolf S, Lai BN, Welch WH, McDonagh AF *et al.* pKa and aggregation of bilirubin: titrimetric and ultracentrifugation studies on water-soluble pegylated conjugates of bilirubin and fatty acids. *Biochemistry* 2004;**43**: 15617–15632.
47. Ryter SW, Alam J, Choi AM. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiol Rev* 2006;**86**:583–650.
48. Datla SR, Dusting GJ, Mori TA, Taylor CJ, Croft KD, Jiang F. Induction of heme oxygenase-1 in vivo suppresses NADPH oxidase derived oxidative stress. *Hypertension* 2007;**50**:636–642.
49. Kawamura K, Ishikawa K, Wada Y, Kimura S, Matsumoto H, Kohro T *et al.* Bilirubin from heme oxygenase-1 attenuates vascular endothelial activation and dysfunction. *Arterioscler Thromb Vasc Biol* 2005;**25**:155–160.
50. Erdogan D, Gullu H, Yildirim E, Tok D, Kirbas I, Ciftci O *et al.* Low serum bilirubin levels are independently and inversely related to impaired flow-mediated vasodilation and increased carotid intima-media thickness in both men and women. *Atherosclerosis* 2006;**184**:431–437.
51. Perlstein TS, Pande RL, Beckman JA, Creager MA. Serum total bilirubin level and prevalent lower-extremity peripheral arterial disease: National Health and Nutrition Examination Survey (NHANES) 1999 to 2004. *Arterioscler Thromb Vasc Biol* 2008;**28**:166–172.
52. Djousse L, Levy D, Cupples LA, Evans JC, D'Agostino RB, Ellison RC. Total serum bilirubin and risk of cardiovascular disease in the Framingham offspring study. *Am J Cardiol* 2001;**87**:1196–1200. A1194, 1197.
53. Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. *Science* 1987;**235**:1043–1046.