ELSEVIER

Flavonoid transport by mammalian endothelial cells

Derek D. Schramm,* Heather E. Collins,† and J. Bruce German*

**Departments of Food Science and Technology and †Genetics, Section of Molecular and Cellular Biology, University of California, Davis, CA USA*

Despite the ever-growing body of literature reporting the effects of flavonoids on animals at both the cellular and systemic levels, one of the most basic questions—"Are the effects of flavonoids on animal cells initiated through their interaction with extracellular targets or intracellular targets?"—has yet to be addressed. Because many effects of flavonoids on cells can be detected within minutes of flavonoid application and because flavonoids diffuse across lipid membranes slowly or not at all, intracellular mechanisms would necessitate a flavonoid transport system for rapid flavonoid uptake. The specific aims of this investigation were (1) to determine if endothelial cells contain a mechanism that mediates rapid flavonoid uptake and (2) to provide evidence for or against the hypothesis that rapid flavonoid effects on endothelial cell synthesis of prostacyclin and endothelin are initiated through the interaction of flavonoids with intracellular targets. Data show that bovine and human aortic endothelial cells possess a transport system that mediates rapid uptake of the flavonoid morin and suggest that the flavonoid uptake system utilizes a variety of oxygenated phenolic compounds as substrates. Further investigation into flavonoid transport should expedite future investigation into the mechanisms of flavonoid actions, because it may allow research to focus on the cellular locations where flavonoids are concentrated. Although endothelial cells contain a mechanism for the rapid uptake of morin, data reported herein suggest that morin initiates its rapid effects on endothelial cell synthesis of prostacyclin and endothelin through an interaction with extracellular targets. (J. Nutr. Biochem. 10:193–197, 1999)

Keywords: flavonoid; transport; endothelial cell; endothelin; prostacyclin; morin

Introduction

Flavonoids are secondary metabolites synthesized by plants for use in defense and reproduction. Traditionally, human consumption of flavonoids and other phytochemicals has occurred through the ingestion of fruits, vegetables, legumes, and plant-derived products such as tea, wine, cocoa, coffee, and fermented foods.[1] Today, the pattern of flavonoid consumption is changing. For example, crop engineers are pursuing increased plant flavonoid content with the intent of improving disease resistance in plants and plant nodulation efficiency.[2,3] In addition, due to marketing, flavonoids are consumed as oral supplements and are being considered as potential food additives because they can reduce food spoilage.[4–6] As a result, a complete understanding of both systemic and cellular flavonoid effects is necessary.

Dozens of flavonoid-induced endothelial cell (EC) effects have been described in the scientific literature.[1,7–11] For example, flavonoids alter EC synthesis of eicosanoids,[9] nitric oxide,[10] and endothelin (ET)[11] within minutes following flavonoid application. Due to its proximity to blood-borne flavonoids, the endothelium is likely to be one of the primary sites of flavonoid action. Because flavonoids diffuse across lipid membranes slowly or not at all,[12,13] a transport system for rapid flavonoid uptake would be necessary for flavonoids to initiate their rapid effects on ECs through intracellular mechanisms. This investigation was conducted (1) to determine if mammalian ECs contain a system for rapid flavonoid uptake and (2) to provide evidence for or against the hypothesis that the rapid effects of flavonoids on EC synthesis of prostacyclin and ET are

Address correspondence to Dr. Derek D. Schramm, Department of Food Science and Technology, Section of Molecular and Cellular Biology, University of California at Davis, One Shields Avenue, Davis, CA 95616 USA.
Funding provided in part by USDA/BARD grant IS2260-93RC and NIH grant 1 U24 AI37627 from the Office of Alternative Medicine, NIH.
Received May 12, 1998; accepted December 16, 1998.

655 Avenue of the Americas, New York, NY 10010
0955-2863/99/$–see front matter
PII S0955-2863(98)00104-1

initiated through flavonoid interaction with intracellular targets.

Methods and materials

Materials

Bovine aortic ECs were a gift from M.E. O'Donnell (University of California, Davis, CA USA). Human aortic ECs were purchased from Clonetics (San Diego, CA USA). Eagle's minimal essential medium for culture (EMEM) was purchased from Mediatech (Herndon, VA USA). Catechin, flavone, caffeic acid, chlorogenic acid, coumarin, ATP, ADP, GTP, NaCl, KCL, MgCl, glucose, and the remaining cell culture materials were purchased from Sigma Scientific (Orlando, FL USA). Valinomycin and ouabain were purchased from CALBIOCHEM (La Jolla, CA USA). Morin and AMP-PCP were purchased from Fluka (Milwaukee, WI USA). 6-Keto $PGF_{1\alpha}$ and ET immunoassay kits were purchased from Cayman (Ann Arbor, MI USA). Flavanone, 3,5,7,3′,4′-pentahydroxyflavanone, and 7,3′,4′-trihydroxyisoflavone were purchased from Indofine (Somerville, NJ USA).

Cell cultures and treatments

Cell culture was conducted as previously described.[9] ECs were seeded and grown in 24-well plates, microscope cover slips, or 20- × 100-mm culture dishes with EMEM containing 2 mmol/L L-glutamine, 10% fetal bovine serum, 100 units penicillin/mL, and 0.1 mg streptomycin/mL. Cells were used at passage 10 or less. Cells were washed with sterile phosphate buffered saline (PBS) before use. EC integrity was monitored by trypan blue exclusion as described.[14]

Determination of flavonoid uptake

Cellular transport of the flavonoid morin was analyzed using fluorometry, fluorescence microscopy, and high pressure liquid chromatography (HPLC). For fluorometry and fluorescence microscopy, cells were incubated for 6 or 8 minutes in EMEM containing appropriate treatment compounds where applicable, rinsed with an excess of PBS, and analyzed by emission at 520 nm. Fluorescence was detected by a Fluorophor 2300 plate reader (Millipore) or a Zeiss (Germany) Axioplan Universal Microscope combined with a Zeiss MC-100 microscope camera. When examined using fluorescence microscopy, ECs were exposed to a mercury short arc lamp (45 V, 1.3 A, 50 W, 2,000 lm), or the mercury short arc lamp in combination with the MC-100's standard halogen bulb.

HPLC analysis was used to quantitate cellular morin following the protocol described previously.[9] As above, cells were treated for 8 minutes in EMEM containing any appropriate treatment and washed with an excess of PBS. They were then scraped from their 20- × 100-mm tissue culture dishes into water/methanol/0.1 M HCl (85:14:1), filtered through a 0.2-μ syringe filter, and centrifuged at 5,000 g for 10 minutes. HPLC was conducted on the supernatant fraction.

Measurement of ET and prostacyclin

Cells at >85% confluence were rinsed with PBS and incubated in 250 μL of phenol red-free EMEM culture medium with treatment compounds added to medium immediately prior to its application. One hundred microliters of medium was removed immediately following application and again after 10 minutes of incubation. Samples were frozen (−80°C) and stored until analyzed. Medium only was compared with medium + morin, and medium + ATP was compared with medium + ATP + morin. Immunoassay procedures were conducted as described previously.[9,11]

Statistical analysis

Experimental data were compared using one-way analysis of variance (ANOVA), testing the hypothesis that means from samples were equal, and by F-test with significance assigned at a level of $P < 0.05$.

Results

Following an 8-minute incubation of aortic ECs with EMEM containing 200 μmol/L morin and 5 mmol/L ATP, morin characteristic emission (520 nm) was observed from photon-stimulated ECs (*Figure 1A,B*). Morin appeared to be concentrated primarily in the perinuclear region of cells. Representative chromatography from cell extracts (cells incubated in EMEM, cells incubated in EMEM containing 200 μmol/L morin, cells incubated in EMEM containing 200 μmol/L morin and spiked with 100 pmol morin prior to extraction, and cells incubated with EMEM containing 200 μmol/L morin and 5 mmol/L ATP) are shown in *Figure 1C* (1 through 4, respectively). Morin eluted at approximately 57 minutes. Seventy-two pmol morin/culture dish, or approximately 1.53×10^{-16} mol morin/cell, was extracted from cells incubated for 8 minutes in EMEM containing 200 μmol/L morin (determined by morin standard curve). Cells incubated in EMEM containing 200 μmol/L morin and 5 mmol/L ATP contained 190 pmol of extractable morin, or approximately 4.0×10^{-16} mol morin/cell.

The properties of the flavonoid uptake system in ECs were thoroughly investigated by taking advantage of morin's fluorescence. Flavonoid transport in bovine and human aortic ECs responded to morin concentration in a similar manner (*Figure 2*), with uptake increased multifold by the addition of ATP to medium. *Table 1* details the modulation of morin uptake by a variety of substances. Although ATP, GTP, and ADP stimulated morin uptake, GTP and ADP were significantly less effective than ATP. The poorly hydrolyzable ATP analog AMP-PCP (Fluka, Milwaukee, WI USA) inhibited morin uptake in a dose-dependent manner. Neither the potassium ionophore valinomycin nor the inhibitor of Na^+- and K^+-dependent ATPases ouabain altered morin uptake in either cell type. KCl, NaCl, and MgCl also had no effect on the uptake of morin by ECs.

The addition of an equal molar amount of catechin, coumarin, 7,3′,4′-trihydroxyisoflavone, 3,5,7,3′,4′-pentahydroxyflavanone, caffeic acid, or chlorogenic acid to the incubation medium inhibited morin uptake by 58%, 23%, 60%, 68%, 49%, and 73%, respectively. In contrast, flavone and flavanone had no significant effect. No detectable photon emission that could interfere with morin measurement occurred when these compounds were excited at 495 nm. Although the uptake and localization of morin could be followed by photon emission, photobleaching occurred rapidly during prolonged excitation (>1 min). As shown in *Figure 3A*, although intracellular morin was increased several-fold by the addition of ATP, morin-induced EC prostacyclin secretion was unaffected. In contrast, increased intracellular morin drastically altered the net effect of morin on EC ET secretion (*Figure 3B*).

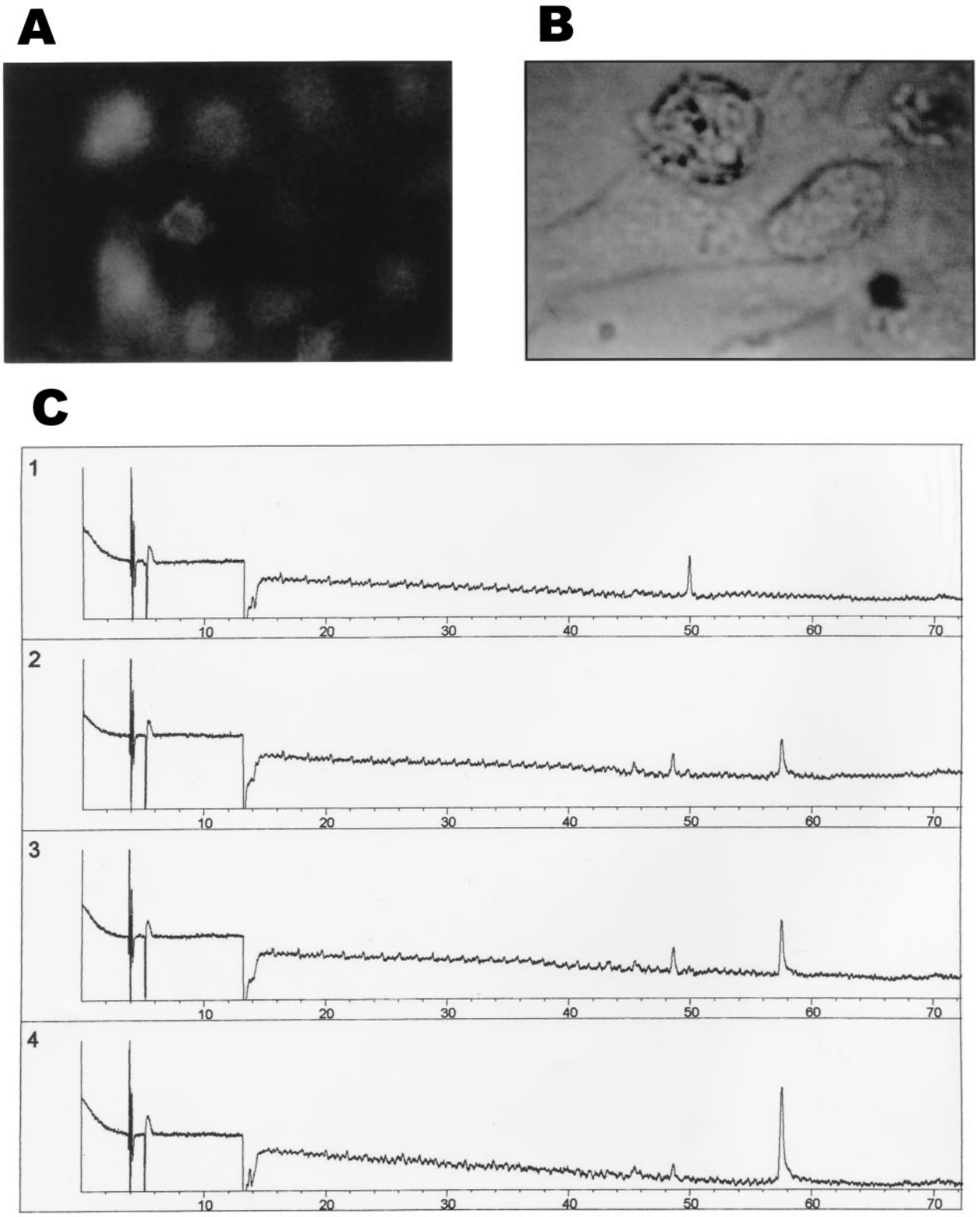

Figure 1 Illustrations of rapid morin uptake by endothelial cells from 8-minute incubations. *(A)* Photon emission (ex 495 nm, em 520 nm) was visualized by illumination with a mercury short arc lamp. *(B)* Co-illuminated with both mercury lamp and the MC-100's standard halogen bulb. Although photon emission (ex 495 nm, em 520 nm) was visible throughout cells, it was concentrated primarily in cell nuclei. *(C)* 1: Chromatography of an extract from cells incubated in EMEM. 2: Chromatography of an extract from cells incubated in EMEM containing 200 μmol/L morin. 3: Chromatography of an extract from cells incubated in EMEM containing 200 μmol/L morin and spiked prior to extraction with 100 pmol morin. 4: Chromatography of an extract from cells incubated with EMEM containing 200 μmol/L morin and 5 mmol/L ATP.

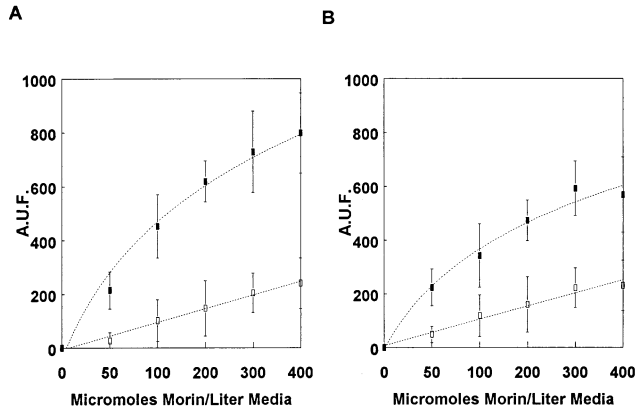

Figure 2 Morin uptake in response to dose and ATP for bovine *(A)* and human *(B)* aortic endothelial cells. Following a 6-minute cell incubation, endothelial cells were washed four times with PBS and then analyzed on a Fluorophor 2300 plate reader (Millipore; ex 495 nm, em 520 nm). A.U.F. is arbitrary units of fluorescence. □, no ATP in medium, ■, 5 mM ATP in medium.

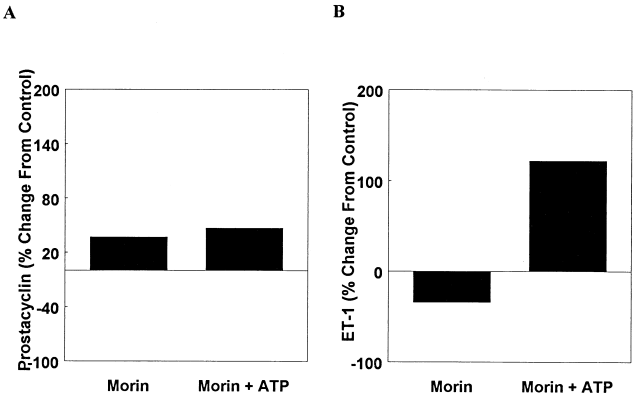

Figure 3 Effect of morin on endothelial cell synthesis of prostacyclin *(A)* and endothelin *(B)*. Data is the average of four experiments. ATP concentration was 5 mM.

Discussion

Our results demonstrate that a rapid, energy-dependent transport system is present in aortic ECs for the uptake of morin. In addition, results from the competition experiment suggest that the transport system in mammalian aortic ECs uses hydroxylated phenolic compounds as substrates. Although little is known concerning flavonoid transport either in plant or animal cells, of the three types of flavonoid transport systems identified in plant cells,[15–18] only one—that which was identified in barley vacuoles for uptake of the flavonoid isovitexin[15]—used an unmodified flavonoid as a substrate. That system further resembled the system for flavonoid uptake identified here in its rapid transmembrane movement of flavonoids and its induction by energy molecules. Together these data suggest that the transporter in ECs could be derived from one in the plant kingdom.

Data showing that the uptake of flavonoids by ECs is increased several-fold by the presence of ATP in culture medium will allow investigators to determine if specific effects of flavonoids on ECs that are initiated within minutes of flavonoid application are initiated through the interaction of flavonoids with extracellular targets or through their interaction with intracellular targets. Because ECs were shown to contain a transport system for rapid morin uptake, this investigation established that the rapid effects of this flavonoid on ECs could be initiated through intracellular mechanisms. Despite this, because the ATP concentration of human plasma is only 1 μM,[19] only extracellular initiation is likely to occur for morin's rapid effects on EC prostacyclin and ET synthesis. We stress that this does not exclude the possibility that long-term effects of morin on EC prostacyclin and ET synthesis are initiated intracellularly.

The overall objective of this line of inquiry is to provide a better understanding of the role of food phytochemicals in promoting human health and well-being and the mechanisms through which they work. Because little information is available concerning the uptake of flavonoids by and sequestration in animal cells, proposing mechanisms of flavonoid action is currently difficult. Further investigation into flavonoid uptake and sequestration by animal cells may arm researchers with knowledge concerning preferential flavonoid uptake and sequestration into cellular organelles. This, in turn, would allow investigators to focus on the cellular or extracellular locations where specific flavonoids are preferentially concentrated, and to look for correlates between flavonoid structure, cellular location, and biological function. Alternatively, data may show that many flavonoids are concentrated along the lipid–water interface

Table 1 Modulation of morin uptake: Percent (%) of control

Group	Bovine ECs	Human ECs
ATP (5 mmol/L) (control)	100	100
ATP (5 mmol/L + Catechin (100 μmol/L)	42	52
ATP (5 mmol/L) + Flavone (100 μmol/L)	89	88
ATP (5 mmol/L) + Flavonone (100 μmol/L)	—	86
ATP (5 mmol/L) + Pentahydroxyflavonone (100 μmol/L)	—	42
ATP (5 mmol/L) + Trihydroxyisoflavone (100 μmol/L)	—	40
ATP (5 mmol/L) + Coumarin (100 μmol/L)	—	77
ATP (5 mmol/L) + Coumaric acid (100 μmol/L)	—	51
ATP (5 mmol/L) + Caffeic acid (100 μmol/L)	—	27
ATP (5 mmol/L) + AMP-PCP (1 mmol/L)	68	65
ATP (5 mmol/L) + AMP-PCP (2 mmol/L)	47	38
ATP (5 mmol/L) + Valinomycin (1 μmol/L)	109	94
ATP (5 mmol/L) + Ouabain (1 μmol/L)	116	87
GTP (5 mmol/L)	51	61
ADP (5 mmol/L)	59	64
NaCl (5 mmol/L)	15	24
KCl (5 mmol/L)	13	18
MgCl (5 mmol/L)	14	19
Glucose (5 mmol/L)	21	24
Medium only	18	22

Reactions were initiated by replacement of EMEM with fresh medium containing 2 mmol/L L-glutamine, 10% fetal bovine serum (FBS), 100 μmol/L morin, and treatment compound(s) when applicable. Incubation proceeded for 8 minutes, after which cells were washed three times with phosphate buffered saline (PBS) and analyzed at ex 495 nm, em 520 nm. Data represent the average of three experiments.
ECs–endothelial cells.

of cells and their organelles, information that would be less useful in narrowing down the potential mechanisms by which flavonoids initiate/mediate their effects. Although data reported herein do not suggest that this will be the case, the possibility cannot be overlooked.

Acknowledgments

We thank T.L. Thirkill and G.C. Douglas for use of their fluorometer, D. Rein for assistance with fluorescence microscopy, C. Dillard for editing, Dr. A. Waterhouse and J. Donovan for use of the HPLC apparatus, and Carl L. Keen for mentoring.

References

1 Schramm, D.D. and German, J.B. (1998). Potential effects of flavonoids on the etiology of vascular disease. *J. Nutr. Biochem.* **9,** 560–566

2 Dixon R.A., Lamb, C.J., Masoud, S., Sewalt, V.J., and Paiva, N.L. (1996). Metabolic engineering: Prospects for crop improvements through the genetic manipulation of phenylpropanoid biosynthesis and defense responses—a review. *Gene* **179,** 61–71

3 Henkel, J. (1995). Genetic engineering: Fast forwarding to future foods. *FDA Consumer* **4,** 6–11

4 Grange, J.M. and Davey, R.W. (1990). Antibacterial properties of propolis. *Journal of the Royal Society of Medicine* **83,** 159–160

5 Nelson, D.B. (1982). Letter (April 25) to F.R. Senti; reported in Life Sciences Research Office evaluation of the health aspects of hesperidin, naringin, and citrus bioflavonoid extracts as food ingredients. *Federation of the American Society for Experimental Biology* **9,** 19

6 Ramanathan, L. and Das, N.P. (1992). Studies on the control of lipid oxidation in ground fish by some polyphenolic natural products. *Journal of Agricultural and Food Chemistry* **40,** 17–21

7 Karger, B. (1976). The flavonoids. A class of semi-essential food components: Their role in human nutrition. *World Rev. Nutr. Diet.* **24,** 117–191

8 Rice-Evans, C.A. and Packer, L. (eds.) (1998). *Flavonoids in Health and Disease.* Marcel Dekker Inc., New York, NY, USA

9 Schramm, D.D., Donovan, J.L., Kelly, P.A., Waterhouse, A.L., and German, J.B. (1998). Differential effects of small and large molecular weight wine phytochemicals on endothelial cell eicosanoid secretion. *J. Agric. and Food Chem.* **46,** 1900–1905

10 Fitzpatrick, D.F., Hirschfield, S.L., and Coffey, R.G. (1993). Endothelium-dependent vasorelaxing activity of wine and other grape products. *Am. J. Physiol.* **265,** H774–H778

11 Schramm, D.D. and German, J.B. (1998). Decreased endothelial cell-derived endothelin in media containing catechin, wine, or wine components of low or high mass (abstract). Keystone ENDOTHELIUM symposia, Lake Tahoe, NV, USA

12 Ratty, A.K., Sunamoto, J., and Das, N.P. (1988). Interaction of flavonoids with 1,1-diphenyl 2-picrylhydrazyl free radical, liposomal membranes and soybean lipoxygenase-1. *Biochem. Pharmacol.* **37,** 989–995

13 Terao, J., Piskula, M., and Yao, Q. (1994). Protective effect of epicatechin, epicatechin gallate, and quercetin on lipid peroxidation in phospholipid bilayers. *Arch. Biochem. Biophys.* **388,** 278–284

14 Boiadjieras, S., Hallberg, C., Hogstrom, M., and Busch, C. (1984). Exclusion of trypan blue from microcarriers by endothelial cells. *Methods in Lab. Invest.* **50,** 239–246

15 Klein, M., Weissenbock, G., Dufaud, A., Gaillard, C., Kreuz, K., and Martinoia, E. (1996). Different energization mechanisms derive the vacuolar uptake of a flavonoid glucoside and a herbicide glucoside. *J. Biol. Chem.* **271,** 29666–29671

16 Lamuela-Raventos, R.M. and Waterhouse, A.L. (1994). A direct HPLC separation of wine phenolics. *Am. J. Enol. Vitic.* **41,** 1–5

17 Matern, U., Reichenbach, C., and Heller, W. (1986). Efficient uptake of flavonoids into parsley (*Petroselinum hortense*) vacuoles requires acylated glycosides. *Planta* **167,** 183–189

18 Hopp, W. and Ulrich-Seitz, H. (1987). The uptake of acylated anthocyanin into isolated vacuoles from a cell suspension culture of *Daucus carota*. *Planta* **170,** 74–85

19 Jabs, M., Ferrell, W., and Robb, H. (1977). Microdetermination of plasma ATP and creatine phosphate concentrations with a luminescence biometer. *Clin. Chem.* **23,** 2254–2257