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Original Contribution

INCORPORATION OF THE ELDERBERRY ANTHOCYANINS BY ENDOTHELIAL CELLS INCREASES PROTECTION AGAINST OXIDATIVE STRESS

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Abstract—The objective of this study was to investigate the ability of endothelial cells (EC) to incorporate anthocyanins and to examine their potential benefits against various oxidative stressors. Endothelial dysfunction has been proposed to play an important role in the initiation and development of vascular disease, with studies having shown that administration of antioxidants improves endothelial function. Elderberry extract contains 4 anthocyanins, which where incorporated into the plasma membrane and cytosol of EC following 4 h incubation at 1 $mg.ml^{-1}$. However, incorporation within the cytosol was considerably less than that in the membrane. Uptake within both regions appeared to be structure dependent, with monoglycoside concentrations higher than that of the diglucosides in both compartments. The enrichment of EC with elderberry anthocyanins conferred significant protective effects in EC against the following oxidative stressors: hydrogen peroxide (H_2O_2); 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH); and $FeSO_4$/ascorbic acid (AA). These results show for the first time that vascular EC can incorporate anthocyanins into the membrane and cytosol, conferring significant protective effects against oxidative insult. These findings may have important implications on preserving EC function and preventing the initiation of EC changes associated with vascular diseases. © 2000 Elsevier Science Inc.

Keywords—Flavonoids, Anthocyanins, Endothelial cells, Oxidative stress, Free radicals

INTRODUCTION

During the past two decades, an increasing number of studies have investigated the diverse protective effects elicited by polyphenolics (flavonoids) present in various fruits and vegetables. Examples of their biological potency include: protection against the incidence and mortality rates of cancer [1], protection against ischemic heart disease mortality [2], as well as demonstrating antitumorigenic [3], antimicrobial [4], anti-inflammatory-allergic [5], and antimutagenic [6] properties.

A large proportion of the flavonoids found in fruits and vegetables have sugar residues bound to their structures. Initially it was thought that only flavonoid aglycones (without bound sugars) were able to pass through the gut wall, and that enzymes capable of cleaving the β-glycosidic bonds were not secreted into the gut or the intestinal wall. However, numerous studies have clearly shown that flavonoid glycosides can indeed enter into the circulation intact [7–9], including anthocyanins [10–14]. Some positive therapeutic effects thought to be elicited by anthocyanins include: maintenance of normal vascular permeability [15], vasoprotective and anti-inflammatory properties [16], and anticancer activity [17]. Reports from our lab have also shown dietary supplementation with a blueberry (*Vaccinium* spp.) extract primarily composed of anthocyanins retarded age-related declines in aspects of neurological function [18].

Although these studies provide useful information

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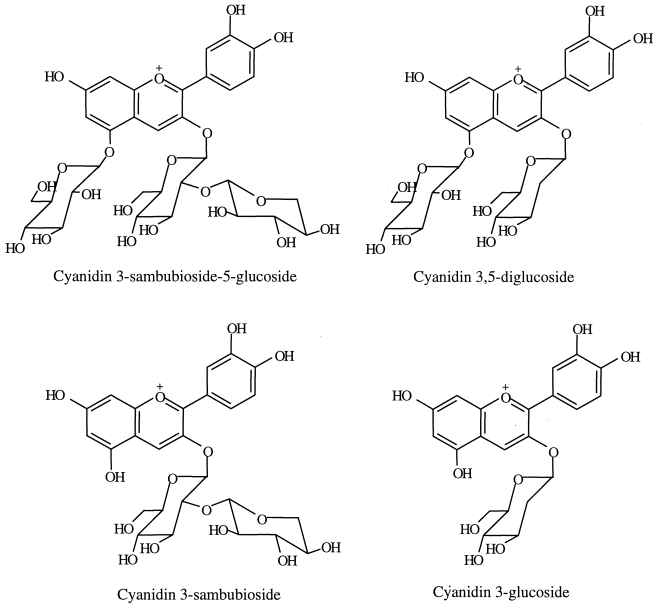

Fig. 1. Chemical structures of elderberry anthocyanins.

of flavonoid bioavailability/bioactivity, the identification of cell distribution and functional properties remain unknown. As such the use of simpler, more adaptable cell model systems has become more applicable. In this regard, the absorption of flavonoid glycosides has been investigated using the human intestinal Caco-2 cell model [19,20]. In these studies, particular emphasis has been directed towards quercetin glycosides, where it has been suggested that absorption is mediated via a sodium-dependant intestinal glucose/galactose transporter [19]. Surprisingly, there is little data with respect to other flavonoid classes such as anthocyanins, a common component of fruits, in particular berries [21]. In addition, though numerous studies have focused on flavonoid bioactivities using cell model systems [22–27], an extensive review of the literature revealed a sparse number of reports having investigated the biological fate of flavonoids following incubation with cells [28,29], that may help delineate whether the effects reported are mediated through an association with the cell membrane or through direct intracellular mechanisms.

The studies reported here were performed to expand on previous studies to determine the biological fate of anthocyanins following incubation with endothelial cells, and their effect on cell viability against oxidative stress. Rubini, an elderberry (*Sambucus nigra*) extract that contains only 4 anthocyanins (Fig. 1), was used for this particular investigation, to simplify the experimental determination of its localization and distribution in the cell.

MATERIALS AND METHODS

Chemicals

All reagents were of the highest purity. Sodium acetate, trichloroacetic acid, hydrochloric acid, hexane, and methanol were from Fisher Scientific, (Pittsburgh, PA, USA). Phosphate-buffered saline (PBS) tablets were from Sigma Chemical Co. Ltd (St. Louis, MO, USA). Medium 199 (M-199) with Earle's salts, L-glutamine, and 2.2 $mg.l^{-1}$ sodium bicarbonate; Dulbecco's Modified Eagle Medium (DMEM) with high glucose, L-glutamine, pyridoxine hydrochloride and sodium pyruvate; penicillin-streptomycin with 10,000 U/ml penicillin G, 10,000 U/ml, streptomycin sulfate in 0.85% saline; amphotericin B and fetal bovine serum (FBS) were from Gibco Ltd (Grand Island, NY, USA). Cyanidin, cyanidin 3-glucoside, and cyanidin 3,5-diglucoside were from Indofine Chemical Company Inc., (Somerville, NJ, USA). Rubini (elderberry extract) was a kind gift from Artemis International (Fort Wayne, IN, USA).

Bovine aortic endothelial cell culture

Bovine aortic endothelial cells were obtained from segments of bovine aorta from a local meat-processing plant. Aortas were immediately immersed in cold sterile Hank's balanced salt solution (HBSS) containing 100 U/ml penicillin, 100 U/ml streptomycin, and 1.25 $\mu g.ml^{-1}$ amphotericin B in a sterile stainless steel container. After rinsing in cold HBSS, one end of the vessel was clamped, and then filled with 0.1% collagenase in

HBSS and then clamped at the other end. Vessels were then incubated in HBSS at 37°C for 2 h, after which contents were decanted into a 50 ml centrifuge tube and then centrifuged at 800 rpm at 4°C for 5 min. The resulting pellet was resuspended and maintained in plated cultures at 37°C in M-199 and DMEM (1:1v/v) containing 5% heat inactivated FBS, 100 U/ml penicillin, 100 U/ml streptomycin, and 1.25 μg/ml amphotericin B. The cells were grown to confluence in a humidified atmosphere of 95% air, 5% CO_2. The culture medium was replaced every 2 d until the cells attained confluence and were subcultured every 4 d using calcium and magnesium free PBS, 0.25% trypsin, and 0.2% EDTA.

Human aortic endothelial cell culture

Human aortic endothelial cell (HAEC) were purchased from Clonetics Laboratories (San Diego, California, USA) and cultured in M-199 medium containing 5% fetal bovine serum. Cultured media also contained 5 μg.ml^{-1} endothelial cell-derived growth factor (EDGF), 100 μg/ml heparin, 100 U/ml penicillin, 100 U/ml streptomycin, and 1.25 μg/ml amphotericin B. Cells were seeded into T-25 flasks (Corning, Corning, NY, USA) coated with 5% gelatin; grown to confluence in a humidified atmosphere of 5% CO_2 at 37°C; and passaged using calcium and magnesium-free Hanks balanced saline solution (HBSS), 0.05% trypsin, and 0.02% EDTA. The culture medium was replaced every 2 d until the cells attained confluence. Human aortic endothelial cells were characterized by the presence of von Willebrand factor antigen using immunofluorescent microscopy [30]. The HAEC had been tested by the vendor for sterility, mycoplasma, HIV and HBV infection, normal morphology, and rate of proliferation.

Incubation measurements

Elderberry extract (1 mg.ml^{-1}) was prepared under sterile conditions in M-199:DMEM (1:1 v/v), for time and dose analysis of anthocyanin incorporation into cells. Confluent aortic endothelial cells were washed with PBS (5 ml) and incubated with 10 ml of elderberry stock solution at 37°C in a humidified atmosphere. Incubations were performed in triplicate for 1, 2, 4, 6, 8, 16, and 24 h. Dose response incubations were performed at 0.1, 0.2, 0.4, 0.6, 0.8, and 1 mg.ml^{-1} for 4 h under the various conditions previously described. Appropriate incubations in the absence of cells were also performed as controls.

Cell collection

Following incubation, culture plates were placed onto ice, the media removed, and the cells washed 3 times with copious amounts of cold PBS. Cells were subsequently collected in 1 ml PBS and centrifuged for 10 min at 10 000 rpm, after which the supernatant was removed and the cells washed with PBS. This was repeated a further 2 times and the pellet finally reconstituted in 300 μl of 1% Triton X-100. Cells were then homogenized and allowed to stand at 4°C for 30 min. Cell membranes and cytosol were subsequently separated by centrifugation at 14,000 rpm for 15 min. A small amount of cytosol was removed for protein determination. An aliquot (20 μl) of 1N HCl was added to 100 μl of cytosol prior to HPLC analysis.

Anthocyanin extraction procedure

In order to validate the extraction procedure a number of preliminary stability studies were performed. Chemical stability of elderberry anthocyanins during incubations with cells was examined by incubating the extract in media at 37°C. No significant decreases in peak areas were observed over 8 h ($n = 5$, paired two-tailed Student's *t*-test). Our preliminary studies also showed that an acidic medium facilitated extraction of the anthocyanins from the cell membrane. When elderberry extract was incubated in an acidic aqueous solution (1N HCl), anthocyanins components were chemically stable for at least 24 h at 4°C, as there were no significant decreases in peak areas over this time ($n = 5$, paired two-tailed Student's *t*-test) (data not shown). Consequently 100 μl 1N HCl was added to each pellet precipitate, vortexed vigorously and allowed to stand at 4°C for 20 min. Samples were subsequently vortexed, centrifuged at 10,000 rpm for 10 min and the supernatant collected. The extraction procedure was repeated 2 times and the supernatants combined for HPLC analysis.

HPLC analysis of anthocyanins

Chromatographic analysis was performed on a HP1100 series HPLC (Hewlett Packard, Palo Alto, CA, USA) equipped with a quaternary HPLC pump, column heater, diode array detector, and HP Chemstation for data collection and manipulation. Chromatographic separation was performed at 30°C using a HP Zorbax SB-C18 (stable bond) rapid resolution column (150 mm $\times$ 4.6 mm I.D.; particle size 3.5 μm, 80 Å) fitted with a Zorbax SB-C18 analytical guard column (12.5 mm $\times$ 4.6 mm; particle size 5 μm, 300 Å). Aliquots (200 μl) of each extraction were injected for analysis using a Bioanalytical Systems Sample Sentinel autosampler (Model MF-9069)(West Lafayette, IN, USA) maintained at 4°C. Normal phase separation of anthocyanins was achieved using binary gradient program. Mobile phase A was 25

mM sodium acetate in water, mobile phase B was 25 mM sodium acetate in methanol, each adjusted to pH 1.5 with trichloroacetic acid. Initial starting conditions were 30% B, between 0–15 min % B increased from 30–35%, 15–20 min % B increased from 35–40%, 20–25 min % B increased from 40–50%, at 25 min mobile phases were switched to original starting conditions (30% B) and held at this condition for 10 min prior to the next injection. The various compounds were characterized by chromatographic comparison with available authentic standards; cyanidin, cyanidin 3-glucoside and cyanidin 3,5-diglucoside. All compounds were quantified as cyanidin 3,5-diglucoside or cyanidin 3-glucoside equivalents (mg^{-1} protein), as no standards were available for sambubioside derivatives.

Effects of different oxidative stressors on cellular proliferation: Mitochondrial dehydrogenase function (MTT method)

Bovine aortic endothelial cells were seeded onto 96-well plates at a density of 10^3 cells/well. Elderberry extract was incubated at 0.05, 0.1, and 0.5 $mg.ml^{-1}$ with BAEC for 4 h. The extract solutions were then removed, and cells washed twice with Hank's solution. The effects of different oxidative stressors: hydrogen peroxide (H_2O_2) at 75 μM, 150 μM, and 300 μM; 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) at 8 μM, 16 μM, 32 μM; and $FeSO_4$/ascorbic acid (AA) at 2.5 μM/0.15 μM, 25 μM/1.5 μM, 250 μM/1.5 μM, were investigated using the MTT (3,(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) method as previously described [31]. Following 2 h incubation with the various stressors, cells were washed with PBS and 10 μl of a 5 $mg.ml^{-1}$ stock-solution of MTT in PBS was added to each well, then incubated for 4 h at 37°C, after which 100 μl of extraction buffer (comprising 20% SDS, 50% DMF) was added and the cells left overnight in a humidified atmosphere. The optical densities at 570 nm were subsequently measured using a Bio-Tek Instruments EL-340 micro plate reader (Winooski, VT, USA). Anthocyanins have an Abs_{max} around 530 nm; this, however, was not found to interfere with measurements at 570 nm. The cytotoxicity was calculated based on the difference in the optical density between the treatment (with inducer) and the control group (without inducer), according to the equation shown. The values recorded for control cultures in the absence of inducer were recorded as 0% cytotoxicity. Results are expressed as percentage of those in corresponding control cells in the presence of inducer. No differences in % cytotoxicity of control and elderberry supplemented cells (0.1–1 $mg.ml^{-1}$), were observed in the absence of inducer (data not shown).

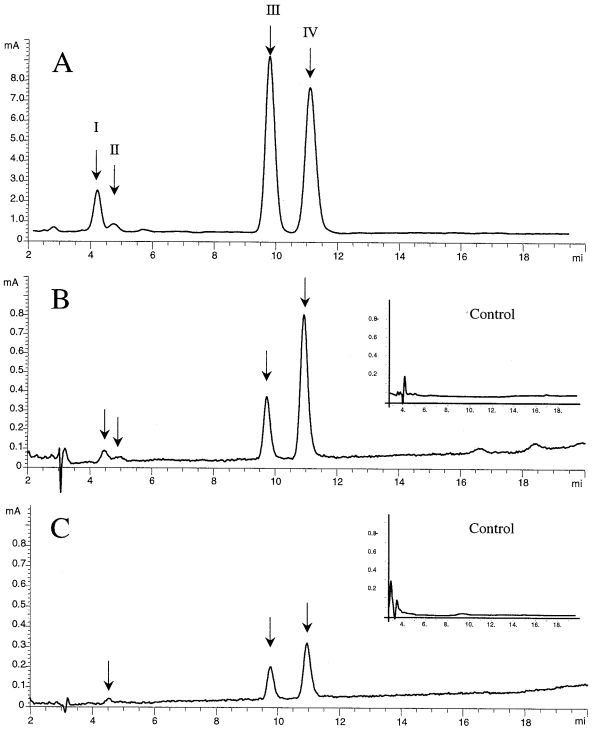

Fig. 2. (A) UV-Vis HPLC chromatograms showing anthocyanin composition of elderberry extract (50 $\mu g.ml^{-1}$), (I) cyanidin 3-sambubioside-5-glucoside, (II) cyanidin 3,5-diglucoside, (III) cyanidin 3-sambubioside, (IV) cyanidin 3-glucoside. (B) Anthocyanins located in cell membranes (insert ~ control cell membrane); (C) anthocyanins located in cytosol (insert ~ control cell cytosol), following incubation with elderberry extract (1 $mg.ml^{-1}$) for 4 h at 37°C.

$$\text{Cytotoxicity (\%)} = (1 - \text{OD of treated cells/OD of control cells}) \times 100$$

Statistical analysis

Results are expressed as mean ± SEM. Statistical analyses were performed using unpaired two-tailed Student's *t*-test using Systat (SPSS, Inc., Chicago, IL, USA).

RESULTS

Elderberry anthocyanin composition

UV-Vis HPLC was used to resolve elderberry anthocyanins. These anthocyanins showed good separation, the order of dilution being cyanidin 3-sambubioside-5-diglucoside (peak I), cyandin-3,5-diglucoside (peak II), cyanidin 3-sambubioside (peak III), and finally cyanidin 3-glucoside (peak IV) (Fig 2A). Anthocyanin concentrations were calculated using authentic standards; cyanidin 3,5-diglucoside (C3,5diG) and cyanidin 3-glucoside

(C3G). Using C3,5diG to determine concentrations of diglucosides, and C3G to determine glucoside concentrations, total anthocyanins present equaled 100 mg.g^{-1} extract (peak I~ 19.48 mg.g^{-1}; peak II~ 11.85 mg.g^{-1}; peak III~ 32.39 mg.g^{-1}; peak IV~ 36.31 mg.g^{-1}). However, if all peaks were based upon C3G equivalents the calculated concentration was much lower, only 84 mg.g^{-1} extract (peak I~ 10.93 mg.g^{-1}; peak II~ 8.07 mg.g^{-1}; peak III~ 38.19 mg.g^{-1}; peak IV~ 42.81 mg.g^{-1}).

Incorporation of anthocyanins by aortic endothelial cells

The incorporation of elderberry anthocyanins into EC was initially investigated using extract at 1 mg.ml^{-1}. Results showed that maximum incorporation was reached within 4 h (Fig. 3). There were no significant differences between total concentrations incorporated expressed as μg.mg^{-1} protein after 4 h as compared with 8 h. A dose-response effect after 4 h incubation was also investigated. Anthocyanin concentration (μg.mg^{-1} protein) present in EC increased significantly ($p < .01$) when incubating EC with extract at concentrations between 0.2–0.8 mg.ml^{-1} (Fig. 3). No significant differences were observed between supplementation at 0.8 mg.ml^{-1}as compared with 1 mg.ml^{-1}.

Separation of the cell membranes and cytosol showed that the anthocyanins were not only incorporated into the plasma membrane but also found to penetrate into the cell cytosol. It appeared that all four components were able to localize in the membrane (Fig 2B), whereas in the cytosol only cyanidin-3-sambubioside (C3S) and C3G were positively identified by retention time and peak purity comparisons. A small peak was also observed at 4.5 min similar to the retention time of C3S-5-G (Peak I) in the standard extract (Fig 2A). Concentrations of anthocyanins (μg.mg^{-1} protein) were found to be higher in the membrane fraction (peak I~ 0.29; peak II~ 0.16; peak III~ 1.67; peak IV~ 5.04) as compared with those in the cytosol (peak I~ 0.02; peak II~ ND; peak III~ 0.27; peak IV~ 0.44). Interestingly the concentration of C3S was significantly lower than that of C3G in both the membrane and cytosolic fractions, by 66% and 45% respectively, despite supplementation at approximately the same concentration. Under similar experimental conditions, total anthocyanin incorporation by HAEC was found to be slightly higher than in BAEC, 7.89 μg.mg^{-1} protein as compared with 7.49 μg.mg^{-1} protein, though this was not found to be statistically significant.

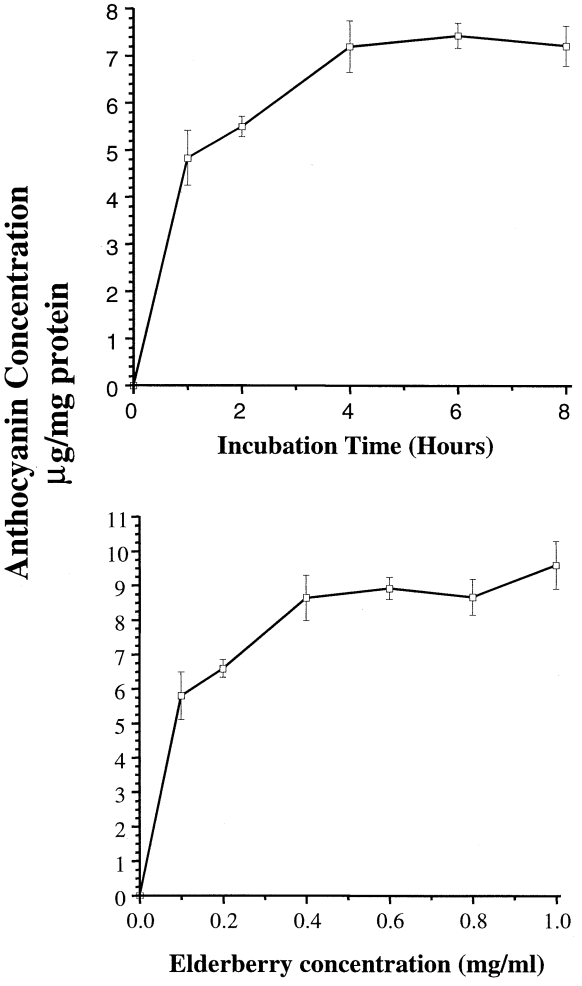

Fig. 3. Effect of time and dose on total elderberry anthocyanin uptake (μg.mg^{-1} protein) into bovine aortic endothelial cells. Values represent the mean ± SEM of three independent experiments. Concentrations are expressed as cyanidin 3-glucoside equivalents.

Protective effects of elderberry extract on BAEC against different oxidative stressors

Following supplementation with elderberry extract for 4 h at 0.05, 0.1 and 0.5 mg.ml^{-1}, BAEC were exposed to different oxidative stressors for 2 h. The % cytotoxicity of control cells not supplemented with extract increased markedly in a concentration-dependent manner following exposure to the different stressors used. The results show that H_2O_2 at 75, 150, and 300 μM had a dramatic effect upon cell viability. However, EC supplemented with elderberry extract (0.05, 0.1, 0.5 mg.ml^{-1}) were afforded significant protection ($p < .001$) against all H_2O_2 concentrations examined (Fig. 4), with % cytotoxicity reduced between 20–40%. No significant differences were observed between different elderberry concentrations, at reducing cytotoxicity at H_2O_2 concentrations investigated.

Endothelial cell cytotoxicity was also increased fol-

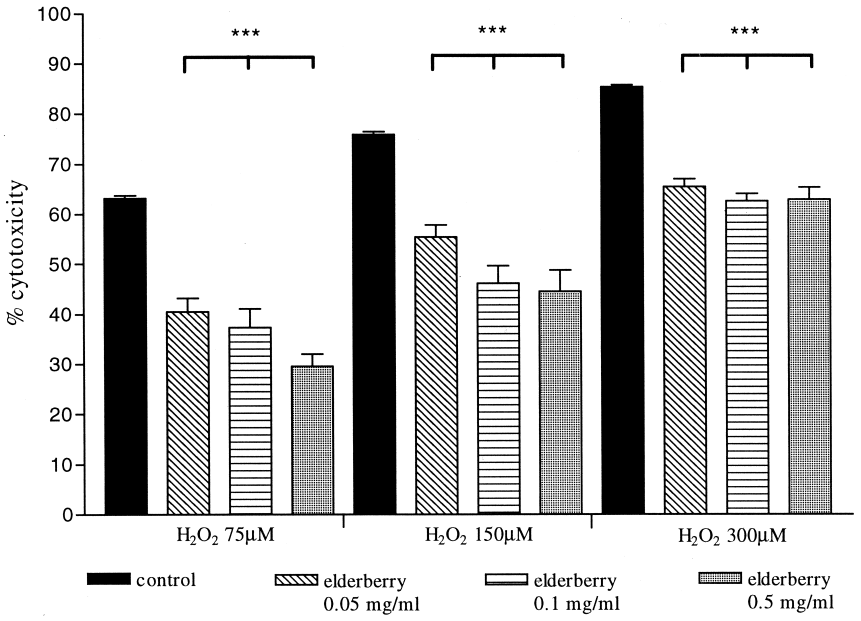

Fig. 4. Protective effects of elderberry presupplementation (0.05, 0.1, or 0.5 mg.ml^{-1}) with bovine aortic endothelial cells for 4 h against H_2O_2 (75 μM, 150 μM, and 300 μM) induced oxidative stress as measured by the MTT assay. *** Represents a significant difference from treatment matched controls (inducers alone) at $p < .001$.

lowing exposure to $FeSO_4$/AA, though it was not as deleterious as H_2O_2 (Fig. 5). Elderberry extract protected EC against $FeSO_4$/AA (250 μM/15 μM) ($p < .05$) at 0.1 and 0.5 mg.ml^{-1} only. With a 10- and 100-fold dilution of $FeSO_4$/AA concentrations, elderberry extract afforded significant protection ($p < .01$) at all three presupple-

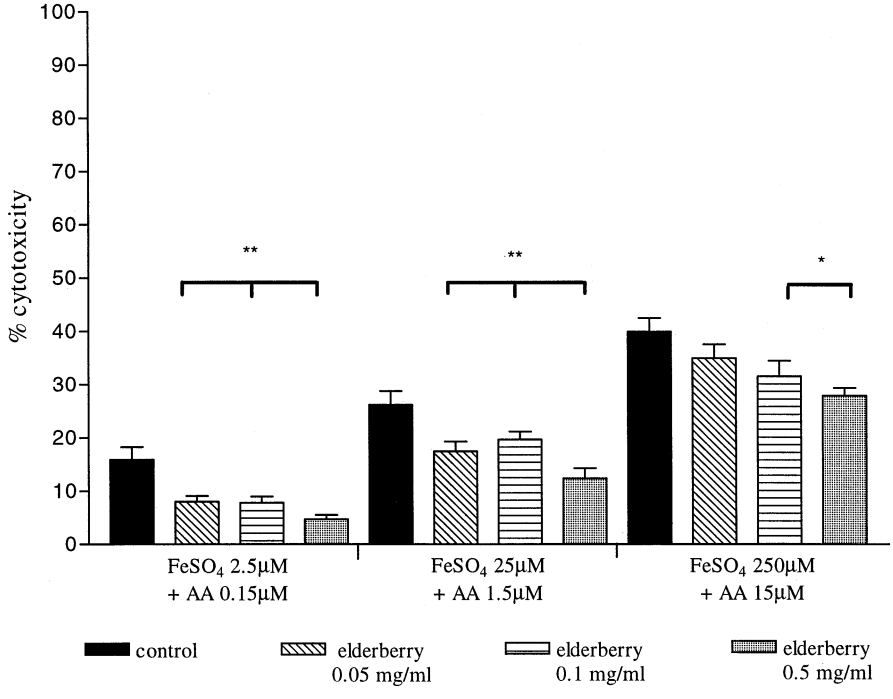

Fig. 5. Protective effects of elderberry presupplementation (0.05, 0.1, or 0.5 mg.ml^{-1}) with bovine aortic endothelial cells for 4 h against $FeSO_4$/Ascorbic acid (2.5 μM/0.15 μM, 25 μM/1.5 μM, and 250 μM/15 μM) induced oxidative stress as measured by the MTT assay. *, ** Represent significant differences from treatment matched controls (inducers alone) at $p < .05$ and .01 respectively.

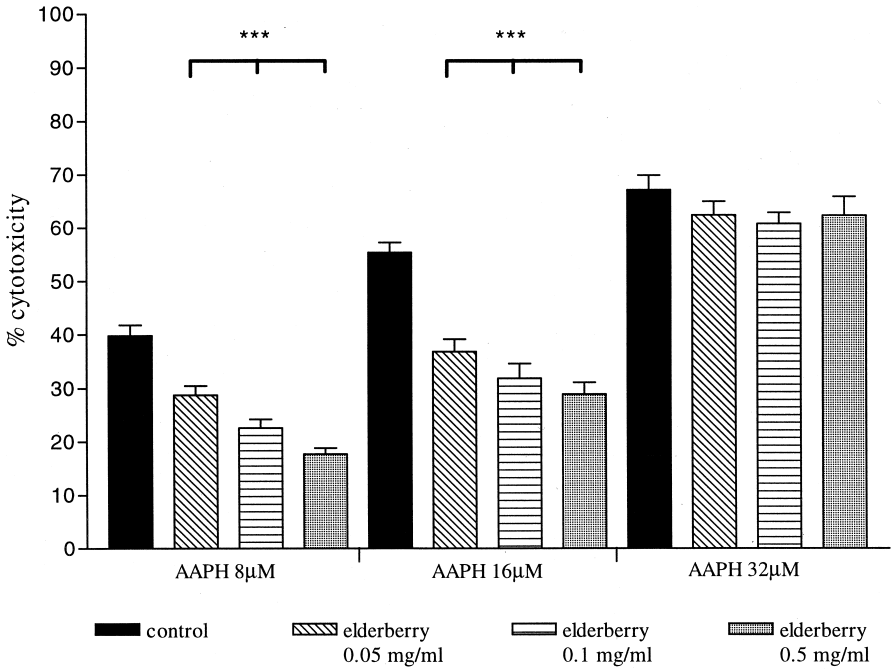

Fig. 6. Protective effects of elderberry presupplementation (0.05, 0.1, or 0.5 mg.ml^{-1}) with bovine aortic endothelial cells for 4 h against AAPH (8 μM, 16 μM, and 32 μM) induced oxidative stress as measured by the MTT assay. *** Represents a significant difference from treatment matched controls (inducers alone) at $p < .001$.

mentation concentrations. The protection afforded to EC was not found to differ between the various elderberry concentrations supplemented at any $FeSO_4$/AA concentration tested.

In contrast, elderberry-supplemented EC were not protected against 32 μM AAPH-induced damage, at any concentration examined (Fig. 6). However, % cytotoxicity was significantly reduced ($p < .001$) with presupplementation at all three extract concentrations against 16 μM and 8 μM AAPH induced oxidative stress. Overall, the greatest protection afforded to the endothelial cells appeared to be from H_2O_2-induced oxidative stress, followed by AAPH at lower concentrations and finally iron/ascorbate-induced oxidative damage.

DISCUSSION

There is no doubting the accumulating evidence describing the in vivo absorption of different dietary flavonoids [7–14]. However there is a growing interest in the use of cell model systems to examine: (i) flavonoid incorporation and distribution and the mechanisms by which this occurs, and (ii) mechanisms of bioactivity, which are sometimes difficult to perform in animal and human feeding studies. More often than not, a disproportionate number of dietary studies merely report an increase in plasma and urine antioxidant activity from which they make correlations with declines in certain pathological conditions [32,33]. However, although these studies provide important information as to possible overall effects following flavonoid consumption, very little insight is gained into the mechanisms by which an increase in plasma antioxidant activity may protect against such conditions, aside from a postulated reduction in sensitivity to oxidative stress.

The use of a cell model however, has shown that flavonoids are able to influence a variety of cell functions. Some examples of these include: modulation of cell signaling [27,34]; altered proliferation and cytotoxicity in cancer cell lines [17,35,36]; protection of DNA integrity [27,37–39]; alterations of immune and inflammatory responses [5,25,40,41]; and modifications in cytokine production [42–45]. Whether or not these parameters can be altered in vivo is another question, but the information they provide is of great value in helping to delineate some of the protective activities observed in clinical/feeding studies.

Unfortunately, in these previous studies no direct evidence is reported describing the biological fate of these compounds. Lenne-Gouverneur and coworkers [29] have shown, using a membrane fluorescent probe, that the membrane biophysical properties were altered following supplementation with flavonoids. Although no attempts were made to isolate these flavonoids from the membrane, the results strongly suggest direct association with(in) the plasma membrane. Further evidence of cel-

lular uptake has come from a study by Boulton and colleagues [28] who were able to isolate ^{14}C radiolabelled quercetin from HepG2 cells (hepatocarcinoma cell line), although again, distinct cellular localization was not investigated. In vivo, studies have also shown flavonoid localization in peripheral tissues, including liver, kidney, and intestinal regions [12–14,46,47]. Like investigations using cell models, the exact cellular localization was not investigated.

Our results show for the first time that vascular EC can incorporate anthocyanins into the membrane and cytosol. Interestingly, the proportion of anthocyanins incorporated into the EC was not uniform across the four components in the extract, with concentrations of C3G the highest, despite C3S being supplemented at approximately the same concentration. Under control conditions (no cells), no changes in anthocyanin peak areas were observed following incubation at 37°C over 8 h. Nor were any changes observed during extractions with 1N HCl solution. However, C3S was metabolized to C3G if extractions were performed using 6N HCl (data not shown). Hence, the higher concentrations of C3G as compared with C3S could be due to hindrance of uptake owing to the bulkier glucoside structure of C3S, or to the fact that once within the cells C3S was metabolized/degraded to C3G (Fig. 1). The latter hypothesis would seem more feasible, since both diglycoside components of the elderberry extract, which have two glucoside residues attached to their backbone, were found to be located within the plasma membrane.

We have also shown here that the incorporation of anthocyanins by the EC significantly enhanced their resistance to the damaging effects of reactive oxygen species (ROS). Protection against H_2O_2-induced loss in cell viability was most striking. These findings are consistent with other investigations in which endothelial cell model systems were utilized and where protection against xanthine oxidase-hypoxanthine generated oxyradicals [48], 3-morpholino-sydnonimine-hydrochloride (SIN-1, a nitric oxide donor) [49], and linoleic acid hydroperoxide-induced cytotoxicity [26] by flavonoids were reported. Cyanidin-3-glucoside, one of the major components in elderberry, was also found to elicit protective actions in vivo, reducing susceptibility of serum to lipid peroxidation induced by AAPH and Cu^+ [11], and enhancing protection against hepatic ischemia-reperfusion (I/R)-induced damage [13], indicative of C3G's potent antioxidant properties. These antioxidant properties in vivo against peroxyl radical and transition metals appear to be consistent with our current findings. As such, the findings from this study may have important implications, as endothelial dysfunction has been proposed to be involved in the initiation of vascular disease [50].

It is widely accepted that EC dysfunction is associated with an alteration of the cell redox status [51]. One facet of EC function that appears to be closely regulated by the redox state is the expression of various cell adhesion molecules (CAMs) on the cell surface. Expression of E-selectin mediates binding with neutrophils, monocytes, and memory T-cells [52], whereas intercellular adhesion molecule-1 (ICAM-1) expression facilitates binding to lymphocyte function associate antigen-1 present on all leukocytes [52]. In addition, activation of macrophages produces large concentrations of ROS, which in turn generate a pro-oxidant environment, triggering cellular responses through activation of the nuclear factor- κB (NFκB) transcription factor [53]. NFκB is a pleiotropic regulator of many genes including immune and inflammatory responses and adhesion molecules. The expression of AMs on the EC surface are also inducible in response to inflammatory cytokines such as TNFα and IL-1β, following ischemia/reperfusion [54–56], radiation exposure [57,58], and in pathological conditions such as artherosclerosis and neurodegenerative disorders [59,60]. Interestingly, the expression of these AMs has been shown to be down-regulated by antioxidants, including flavonoids [61–63], resulting in improved endothelial function [64,65].

Clearly the diverse protective effects that dietary flavonoids appear to elicit in vitro and in vivo, have contributed towards the growing interest in the role that these nutrients play in health. Therefore, assessment of their cellular localization will help elucidate their mechanism of action and allow us to exploit their beneficial effect to the fullest. Moreover, dietary studies invariably involve supplementation of whole foods, not individual components; therefore, it seems logical that examination of the ensuing protective roles that follow consumption of fruits and vegetables should involve studies in which the complete profile of the flavonoid families present in foods be examined, and tested in the proportions in which they are supplemented. This will enable determinations of the possible competition kinetics for absorption and of possible synergistic interactions to be identified. The current study has made an initial step in this direction by using a simple elderberry extract, and provided evidence to support the localization of these anthocyanins in the membrane and cytosol, subsequently enhancing resistance against reactive oxygen species. Future work will be directed towards isolating the various flavonoid families from antioxidant rich foods and investigating their absorption and bioactivity in cell model systems.

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