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Antioxidant effects of dihydrocaffeic acid in human EA.hy926 endothelial cells

Junjun Huang[a], Tomas de Paulis[b], James M. May[a,*]

[a]Department of Medicine, 715 Preston Research Building, Vanderbilt University School of Medicine, Nashville, TN 37232-6303, USA
[b]Department Psychiatry, Vanderbilt University School of Medicine, Nashville, TN 37232-6303, USA

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Abstract

Dihydrocaffeic acid (DHCA) is a metabolite of caffeic acid with potent antioxidant properties. Since DHCA has been detected in human plasma following coffee ingestion, we tested the hypothesis that DHCA protects the endothelium from oxidative stress in a model in human-derived EA.hy926 endothelial cells. During culture for 16–24 hours, the cells accumulated DHCA against a concentration gradient to low millimolar concentrations. In α-tocopherol-loaded cells, DHCA spared α-tocopherol during overnight culture in a dose-dependent manner. In response to oxidant stress induced by a water-soluble free radical initiator, both α-tocopherol and DHCA diminished oxidation of *cis*-parinaric acid that had been incorporated into the cells, although their antioxidant activities were not additive. DHCA also decreased intracellular oxidation of dihydrofluorescein due to redox cycling by menadione. This suggests that the protective effects of DHCA were caused by scavenging of intracellular reactive oxygen species. DHCA also increased nitric oxide synthase activity in a dose-dependent manner in cultured cells, which was associated with a comparable increase in endothelial nitric oxide synthase protein. Although the DHCA concentrations required for these effects are higher than those likely to be present in plasma or the interstitium, these results indicate that DHCA can function as an intracellular antioxidant.

Keywords: Dietary polyphenols; Endothelial nitric oxide synthase; Menadione; Oxidant stress; α-Tocopherol

1. Introduction

Dietary plant polyphenols and flavonoids have been suggested to prevent colorectal cancer [1] and atherosclerosis [2], at least in part by providing defense against the oxidant stress thought to initiate and perpetuate these diseases. Of the polyphenols, chlorogenic acid (CGA) and its derivatives have long been of interest because of their relatively high content in coffee [3], fruits, vegetables, and wine that characterize the so-called "Mediterranean" diet [4]. Their benefits have been associated with their ability to function as antioxidants. For example, caffeic acid (CA), which corresponds to the hydroxycinnamic acid or "aglycone" portion of of CGA (Fig. 1), is an excellent scavenger of reactive species containing both nitrogen (e.g., peroxynitrite) and oxygen (e.g., superoxide) [5,6]. At least part of the antioxidant effectiveness of CA may also relate to its ability to chelate transition metals, including iron and copper [7].

In biological systems, CA has shown promise as an antioxidant in vitro and at the cellular level. In a cell-free system, CA at 5 μmol/L completely protected human low-density lipoprotein (LDL) from peroxidative damage due both to a free radical initiator and to copper ions [7]. In addition, this effect was associated with sparing of α-tocopherol (α-TOC) in LDL [8]. The mechanism of the sparing effect of CA on α-TOC was attributed to direct recycling of α-TOC by CA, since the α-tocopheroxyl radical did not appear until after CA was consumed [8]. At the cellular level, CA has been shown to protect cultured human endothelial cells from apoptosis induced by oxidized LDL [9]. The IC_{50} (8.3 μmol/L) of the effect of CA on apoptosis was similar to that found to protect LDL [9]. Although

Abbreviations: AAPH, 2,2'-azobis(2-amidinopropane) dihydrochloride; CA, Caffeic acid; CGA, Chlorogenic acid; c-PnA, cis-parinaric acid; DHCA, Dihydrocaffeic acid; eNOS, Endothelial nitric oxide synthase; KRB, Krebs-Ringer buffer; LDL, Low density lipoprotein; a-TOC, a-tocopherol.

* Corresponding author. Tel.: +1 615 936 1653; fax: +1 615 936 1667.
E-mail address: james.may@vanderbilt.edu (J.M. May).

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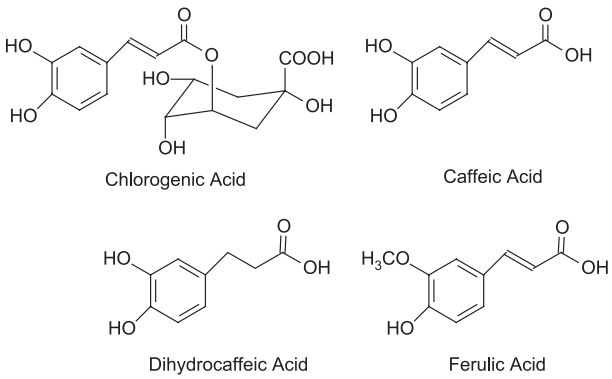

Fig. 1. Chemical structures of chlorogenic acid and its derivatives.

phenolic derivatives such as ferulic acid (Fig. 1), which contain only one phenolic hydroxyl group, also protected LDL, CA was twice as potent in this regard [9]. In these studies, the protective effect of CA was due both to prevention of LDL oxidation and to a direct effect at the cellular level. We also recently reported that CGA, CA, and its reduced form dihydrocaffeic acid (DHCA) (Fig. 1) decreased lipid peroxidation in plasma and liposomes incubated with human erythrocytes, and that this was accompanied by a sparing effect on erythrocyte α-TOC [10]. In our study, CGA protected plasma and erythrocytes at concentrations as low as 5 μmol/L. Of relevance to this result is that a diet of 0.8% by weight CA in rats resulted in nonfasting CA concentrations in plasma of about 5 μmol/L [11].

CA and related compounds may act as antioxidants in endothelial cells, which would have direct relevance to vascular disease and atherosclerosis. For example, CA and flavonols induce endothelium-dependent vasorelaxation in rat thoracic aorta [12]. At the cellular level, CA at the half-maximal concentration of 8 μmol/L prevented apoptosis in endothelial cells induced by oxidized LDL [9]. This was considered due both to prevention of LDL oxidation and to direct protection of the cells. To further investigate the cellular protection afforded by dietary polyphenolics, we studied whether DHCA can prevent the toxic effects of oxidant stress in EA.hy926 endothelial cells. The latter is a permanent cell line derived from human umbilical vein endothelial cells that expresses factor VIII antigen [13], oxidatively modifies human LDL [14], and shows calcium-dependent stimulation of endothelial nitric oxide synthase (eNOS) [15]. DHCA, which is a metabolic product of CA, was chosen for study since it has been detected in human plasma following coffee ingestion [16], and since it has a similar antioxidant potential in human erythrocytes relative to CA [10]. The present results show 1) that DHCA is taken up by EA.hy926 cells; 2) that it spares α-TOC and decreases membrane lipid peroxidation due to a free radical initiator; 3) that it decreases intracellular oxidant stress due to menadione redox cycling; and 4) that it enhances eNOS activity by increasing expression of the protein.

2. Methods and materials

2.1. Materials

Menadione, (d,l)-α-tocopherol (α-TOC) and 3,4-dihydroxyhydrocinnamic acid (DHCA) were obtained from Sigma-Aldrich Chemical Co., (St. Louis, MO). α-TOC was prepared by dissolving it in ethanol to a concentration of 10 mmol/L. *cis*-Parinaric acid (c-PnA) was obtained from Molecular Probes, Inc. (Eugene, OR) and was prepared by dissolving in ethanol to 180 mmol/L. Protein was measured using the BCA kit from Sigma-Aldrich. The 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) was obtained from Wako Chemicals (Richmond, VA).

2.2. Cell culture

Human endothelial EA.hy926 cells were a generous gift from Dr. Cora Edgell (University of North Carolina, Chapel Hill, NC). This cell line is an immortalized hybridoma of the epithelial cell line A549 and cells obtained from human umbilical vein cord [13]. The cells were cultured as previously described [15] and grown to confluence in 5% CO_2 atmosphere at 37°C for 24 hours before use.

2.3. Measurement of cell contents of DHCA, α-TOC, and GSH

DHCA was measured in cell lysates as follows. After rinsing the cell monolayer three times with 2 mL of Krebs-Ringer buffer solution (KRB, 3.3 mmol/L Na_2HPO_4, 0.44 mmol/L KH_2PO_4, 137 mmol/L NaCl, 5.4 mmol/L KCl, and 4.2 mmol/L $NaHCO_3$, pH 7.4), the cells were lysed with 0.2 mL of 25% metaphosphoric acid (w/v), followed by 0.7 mL of phosphate-EDTA buffer. The cells were removed from the plate with a rubber spatula and the lysate was transferred to a microfuge tube. Following centrifugation at 10 000 rpm for 1 minute at 4°C, the supernatant was removed for assay of DHCA, and the pellet was taken for protein determination. DHCA was measured by HPLC with electrochemical detection using a Waters NovaPak C_{18} column and a mobile phase of 17% ethanol and 0.3% glacial acetic acid (v/v) in deionized water [10].

α-TOC was measured in cells that had been treated for 16–18 hours in culture in six-well plates with the α-TOC and other treatments as indicated. Before assay, cell monolayers were rinsed three times with KRB. After removal of residual KRB, the cells were treated with 0.1 mL of a 5 mg/mL solution of pyrogallol, followed by 0.4 mL of 3 % (w/v) sodium dodecyl sulfate. After scraping the cells from the plate with a rubber spatula, the lysate was transferred to glass tube containing 0.7 mL of reagent alcohol (5% methanol, 5% isopropanol, 90% ethanol, v/v). The solution was mixed and 0.6 mL of heptane was added. After thorough mixing, the tube was centrifuged for 3 minutes at 400 × *g* to separate the layers. An aliquot of the upper heptane phase was removed and dried under a stream of nitrogen. The heptane extract was dissolved in 250 μL of

methanol and assayed for α-TOC content by HPLC with electrochemical detection as previously described [17]. The aqueous bottom layer was mixed by vortexing and an aliquot was taken for protein determination.

GSH was measured using the method of Hissin and Hilf [18], which detects only GSH. Intracellular concentrations of DHCA and GSH were calculated based on the measured intracellular water space in EA.hy926 cells of 3.6 ± 1.2 μL/mg protein [19].

2.4. Measurement of intracellular oxidant stress as oxidation of dihydrofluorescein

The method of Wang and Joseph [19] was used to quantify fluorescence due to oxidation of dihydrofluorescein in a microtiter plate reader, with minor modification [20]. After incubations and rinsing as noted, fluorescence was measured every 4 minutes in each well in a microtiter plate reader (Fluostar Galaxy, BMG Labtechnologies, Cork, Ireland) over 36 minutes at 37°C. The excitation wavelength was 480 nm, and the emission wavelength was 520 nm. The fluorescence readings for each well were normalized to the initial reading at time zero.

2.5. Measurement of lipid peroxidation with the c-PnA fluorescence assay

At the incubations of EA.hy926 cells with c-PnA as described, buffer was removed and cells were extracted for 1 hour in the dark with 1.5 mL of isopropanol containing 0.05% (w/v) butylated hydroxytoluene. An aliquot of the isopropanol extract was microfuged to remove debris, and the fluorescence of the supernatant was determined in an Aminco Fluorocolorimeter II (SLM-Aminco, Urbana, IL) using filters appropriate to provide an excitation wavelength of 324 nm and an emission wavelength of 413 nm. Fluorescence levels were normalized to protein content.

2.6. Measurement of NOS activity and protein in intact cells

NOS activity in intact EA.hy926 cells was measured as described by Ghigo et al. [20] with minor modifications [21]. After incubations and washes as noted, 0.5 μCi of L-[^{3}H]arginine was added to a concentration of 9 nmol/L and the cells were incubated for another 15 minutes at 37°C. Subsequent washes, extraction, and measurement of L-[^{3}H]citrulline by chromatography on Dowex AG50WX-8 were carried out as previously described [21]. Values of eNOS were adjusted for changes in the uptake of L-[^{3}H]arginine as recently described [15].

Levels of eNOS protein were measured by Western blotting. Cell homogenates were solubilized in an equal volume of sample buffer (125 mmol/L Tris-HCl, 20% [v/v] glycerol, 4% [w/v] sodium lauryl sulfate, 10% [v/v] mercaptoethanol, and 0.0025% bromphenol blue [w/v], pH 6.8). Samples were incubated for 5 minutes at 37°C, then microfuged for 10 seconds, and the solubilized material was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis according to the method of Laemmli [22]. Transfer to poly(vinylidine difluoride) membrane, was carried out as previously described [23], the blot was probed with a monoclonal antibody to human eNOS (Transduction Laboratories, Lexington, KY), and bands were stained using the ECL system (Amersham). Locations of the bands were noted using pre-stained molecular weight markers.

2.7. Statistical analysis

Data are shown as mean ± SEM. Differences between treatment and control were assessed by paired *t* tests or by one-way or two-way analysis of variance and post-hoc testing by the Dunnett test. SigmaStat 2.0 software (Jandel Scientific, San Rafael, CA) was used for the analyses.

3. Results

The ability of EA.hy926 cells to take up and to retain DHCA was measured after overnight incubation in culture. As shown in Fig. 2, intracellular DHCA progressively increased to concentrations more than 10-fold those initially present in the incubations. This suggests that the cells are able to concentrate DHCA against a gradient. At concentrations >400 μmol/L, there was cell death related to toxicity of DHCA; consequently, for subsequent studies of antioxidant effects, lower concentrations of DHCA were used.

To determine whether DHCA might spare the antioxidant α-TOC in cell membranes, it was necessary to load EA.hy926 cells with α-TOC, since in culture they contain very low amounts of α-TOC (<0.013 nmol · mg protein $^{-1}$ (Fig. 3). After incubation in culture for 16–20 hours with increasing

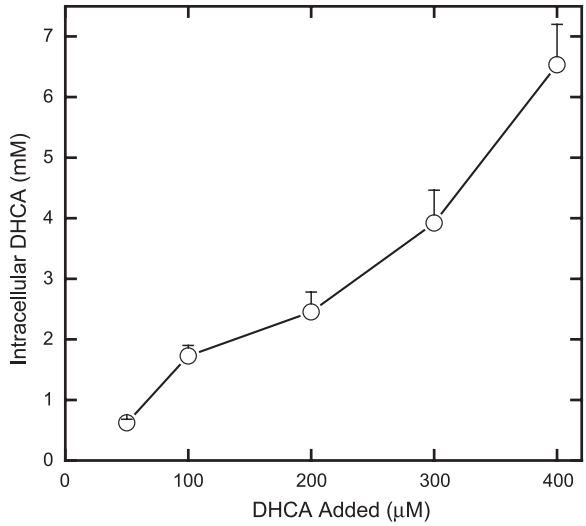

Fig. 2. Uptake of dihydrocaffeic acid (DHCA) by EA.hy926 cells. Confluent EA.hy926 cells in six-well plates were incubated 16–18 hours in culture with the indicated concentration of DHCA. The cells were processed and intracellular DHCA was measured as described in the Methods and materials section. Results are shown as mean ± SEM from four separate plates.

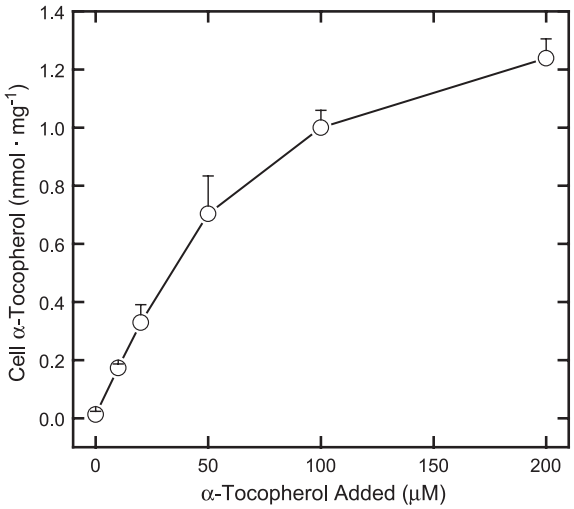

Fig. 3. Loading of EA.hy926 cells with α-TOC. EA.hy926 cells in six-well plates were incubated 16–18 hours in culture with the indicated concentration of α-tocopherol (α-TOC), then prepared for assay of cellular α-TOC as described in the Methods and materials section. Results are shown as mean ± SEM from four separate plates.

amounts of α-TOC, followed by washing to remove any remaining extracellular α-TOC, the intracellular content of α-TOC increased in a saturable manner (Fig. 3). This shows that EA.hy926 are readily loaded with, and also retain α-TOC. To determine whether DHCA spares α-TOC, confluent cells were incubated in culture with increasing concentrations of DHCA in the presence of 20 μmol/L α-TOC for 16–20 hours. After washing the cells three times with the saline buffer, the α-TOC content of the cells was measured. As shown in Fig. 4, increasing concentrations of DHCA spared cellular α-TOC, beginning at a loading concentration of 50 μmol/L DHCA.

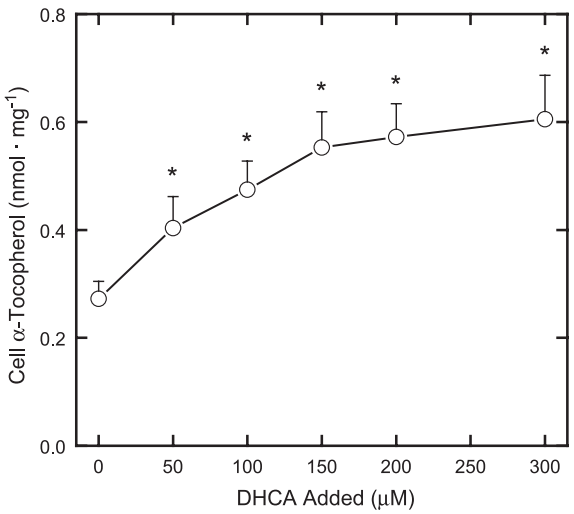

Fig. 4. Protection of cellular α-TOC by DHCA. EA.hy926 cells in six-well plates were cultured in the presence of 20 μmol/L α-TOC and the indicated initial concentration of DHCA. After 16–18 hours the cells were taken for measurement of α-TOC. Results are shown from seven experiments. *P <0.05 by one-way analysis of variance on ranks. Abbreviations as in Figs. 2 and 3.

These results show that DHCA can spare α-TOC that in EA.hy926 cells that would have been otherwise lost in overnight culture in oxygenated medium.

To determine whether sparing of α-TOC or some other antioxidant effect of DHCA is associated with decreased lipid peroxidation, the acute oxidation of c-PnA by the free radical initiator AAPH was measured. c-PnA is a long-chain unsaturated fatty acid that is highly fluorescent but susceptible to oxidation with loss of fluorescence [24,25]. It is first necessary to incorporate c-PnA into cell membranes. The time course of incorporation of 20 μmol/L c-PnA into the EA.hy926 cell membranes showed saturation after 1 hour (results not shown). To test whether DHCA and/or α-TOC prevent oxidation of c-PnA in the cell membrane, EA.hy926 cells were first cultured overnight with either α-TOC or DHCA alone, then loaded with c-PnA (18 μmol/L) for 1 hour, and treated with 40 mmol/L AAPH for 2 hours. c-PnA was extracted with isopropanol and the concentration was measured fluorometrically over 1–2hours of incubation with AAPH. As can be seen in Fig. 5, the percent fall in c-PnA fluorescence over the second hour of incubation with AAPH was about 18% in the control samples for DHCA and α-TOC experiments. Both DHCA and α-TOC retarded the rate of loss of c-PnA fluorescence

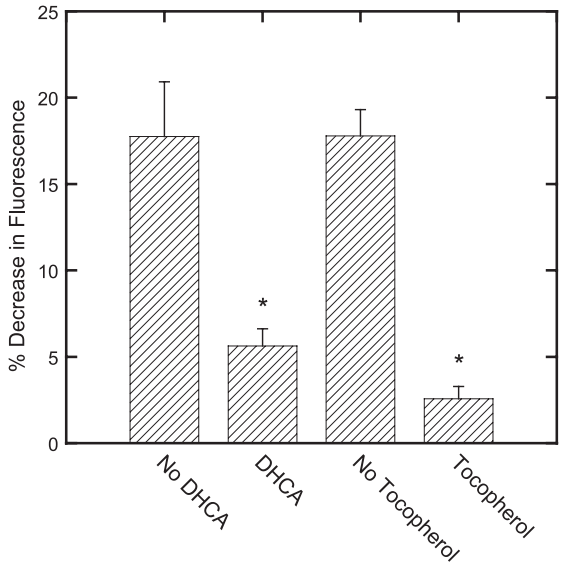

Fig. 5. Protection against 2,2′-azobis(2-amidinopropane (AAPH)–induced oxidation of c-PnA by α-TOC and DHCA. EA.hy926 cells in six-well plates were cultured 16–18 hours with no additions or with 20 μmol/L DHCA or 100 μmol/L α-TOC, as indicated. The cells were rinsed three times in KRB to remove agents and serum, and incubated another 60 minutes in the dark at 37°C in KRB that contained 5 mmol/L D-glucose and 18 μmol/L *cis*-parinaric acid (c-PnA). The cells were again rinsed three times in KRB, followed by incubation for various times at 37°C in KRB containing 5 mmol/L D-glucose and 40 mmol/L AAPH. The medium was removed and the cells were processed for measurement of c-PnA fluorescence as described in the Methods and materials section. Results are shown from six separate plates for each treatment and its respective control as the percent decrease in fluorescence measured over the second hour in incubation. *P<0.05 compared to appropriate control by paired t test. Abbreviations as in Figs. 2 and 3.

to between 3% and 5%. Thus, individually, both antioxidants retard lipid peroxidation in cell membranes in response to an acute oxidant stress due to AAPH. In another series of experiments, we tested whether DHCA could also lower rates of c-PnA oxidation in cells that had been loaded with α-TOC. However, rates of c-PnA oxidation were not significantly different in cells loaded with α-TOC alone compared to cells loaded with DHCA and α-TOC. Together, these results show that relatively high concentrations of DHCA can blunt oxidant damage in EA.hy926 cells due to oxygenated medium and a free radical initiator.

To determine whether lower concentrations of DHCA might also decrease endogenous oxidant stress in cells, we next looked at the ability of DHCA to prevent oxidation of dihydrofluorescein during menadione-induced redox cycling. In these experiments, cells were incubated 30 minutes with varying concentrations of DHCA during loading with dihydrofluorescein, rinsed to remove extracellular reagents, and exposed to 40 μmol/L menadione. As shown in Fig. 6, there was a dose-dependent inhibition of this oxidation by pre-incubation with DHCA. A decrease in the rate of oxidation was evident 12.5 μmol/L DHCA. As shown in the inset to Fig. 6, the decrease in the rate of oxidation of dihydrofluorescein followed biexponential decay. This suggests the presence of two processes, one that might reflect DHCA uptake and the other interaction with the oxidant species. Similar findings were obtained in three additional experiments. These results show that a short-term incubation of EA cells with DHCA decreases endogenous oxidant stress generated by menadione. DHCA does not react directly with the quinone menadione, nor does it reduce fluorescein to dihydrofluorescein, so DHCA must be scavenging intracellular reactive oxygen species generated by the redox cycling of menadione. The location of DHCA activation within the cell was confirmed with the use of CGA, which is an ester of CA and quinic acid, which we have previously found does not enter human erythrocytes [10]. In a similar experiment, up to 250 μmol/L CGA failed to blunt menadione-induced intracellular oxidant stress (results not shown). This indicates that DHCA acts intracellularly.

To determine whether DHCA affects endothelial cell function, we tested the ability of the cells to synthesize nitric oxide in an intact cell assay of nitric oxide synthase. A 30 minutes incubation with DHCA concentrations up to 200 μmol/L at 37°C was without effect on eNOS activity (results not shown). On the other hand, when cells were cultured overnight in the presence of increasing concentrations of DHCA, nitric oxide synthase activity progressively increased by about 30% (Fig. 7A, squares). To test whether this effect was due to changes in eNOS protein, we performed Western blots using an antibody specific for eNOS. In the representative blot shown in Fig. 7B, there was an increase in eNOS protein. When the results of three experiments were quantified by densitometry, the tracing shown by the circles in Fig. 7A was obtained. Increases in

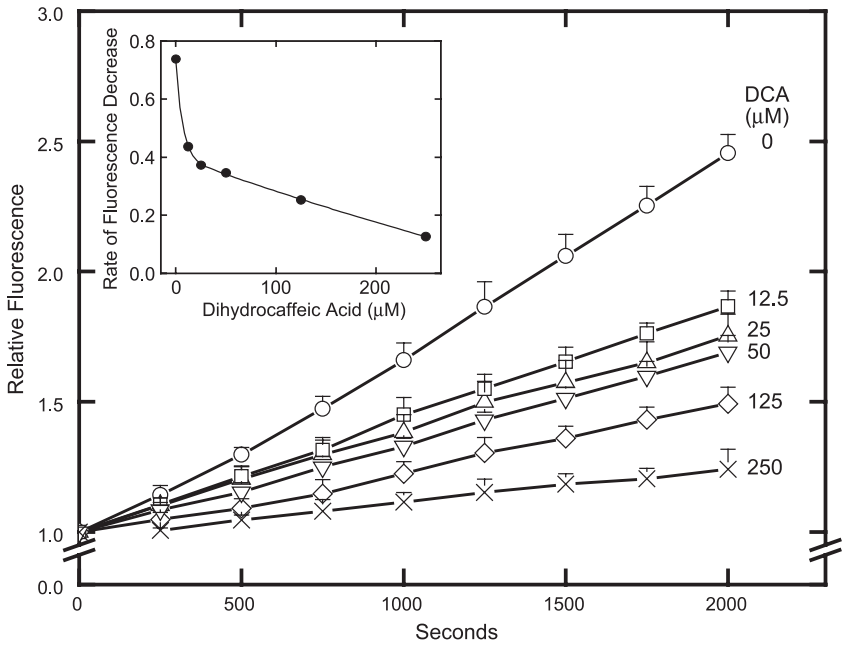

Fig. 6. Inhibition of menadione-induced oxidant stress by DHCA. Confluent EA.hy926 cells in 96-well plates were rinsed with 0.2 mL of KRB to remove serum, and incubated in KRB containing 5 mmol/L D-glucose, 20 μmol/L dihydrofluorescein diacetate, and either the indicated concentration of DHCA or the same concentration of dimethylsulfoxide as present in the corresponding DHCA incubation. After 30 minutes of incubation in the dark at 37°C, the medium was removed and the wells were rinsed three more times with 0.2 mL of KRB and incubated in KRB containing 5 mmol/L D-glucose and 40 μmol/L menadione. The plate was placed in the fluorescence microtiter plate reader and fluorescence was measured as described in the Methods and materials section. Results are shown from a typical experiment of three performed as mean ± SEM of four replicates. The inset shows a bi-exponential fit to the slopes of the respective lines in the main figure. Abbreviations as in Figs. 2, 3, and 5.

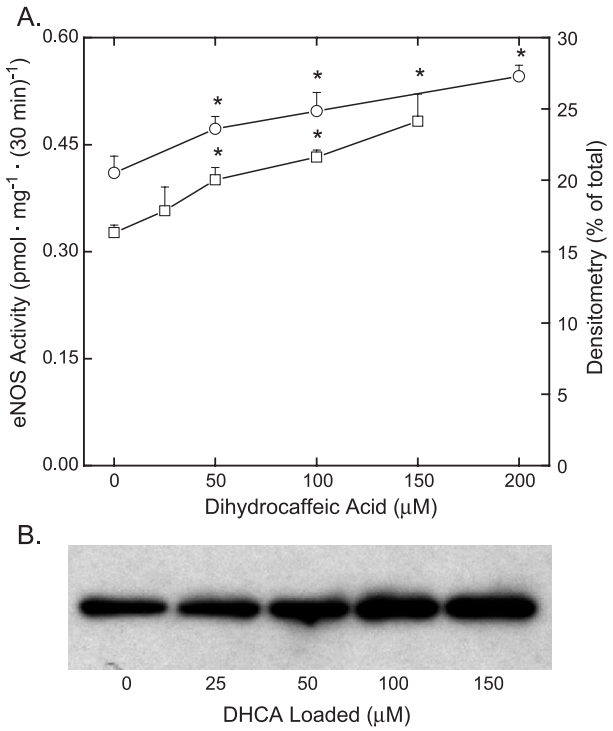

Fig. 7. Stimulation of endothelial nitric oxide synthase (eNOS activity) and protein expression by DHCA. (*Panel A*:) eNOS protein and activity. The averaged densitometer readings for three Western blots are shown by the circles, expressed as a percent of the total density measured for all bands. For assay of eNOS activity in intact cells, EA.hy926 cells in six-well plates were incubated 16–18 hours in culture in the presence of the indicated DHCA concentration. The cells were rinsed three times in KRB, and the eNOS assay was carried out as described in the Methods and materials section. Results are shown by the squares as means ± SEM from three experiments. *$P<0.05$ compared to cells not treated with DHCA by one-way analysis of variance. (*Panel B*) eNOS protein expression. EA.hy926 cells in six-well plates were incubated for 16–18 hours in culture with the indicated concentration of DHCA, rinsed to remove medium, then taken for electrophoresis and Western blotting. A representative blot is shown. Abbreviations as in Figs. 2 and 5.

eNOS protein expression were of a similar magnitude as observed for eNOS activity.

4. Discussion

In this work we found that human-derived endothelial cells take up DHCA during overnight incubation in culture, and that they retain it under steady-state conditions against a concentration gradient (Fig. 2). This differs from human erythrocytes in short-term (1-hour) incubations, in which neither CA nor DHCA accumulated to concentrations higher than those present in the incubation medium [10]. The present results suggest that DHCA may be transported into endothelial cells via an active transport mechanism that is absent in erythrocytes. A similar situation exists for ascorbic acid, in which human erythrocytes differ from nucleated cells in that they do not accumulate ascorbic acid against a concentration gradient [26]. The probable reason for this latter difference is that erythrocytes lack the sodium- and energy-dependent vitamin C transporter that is present in nucleated cells [27]. The mechanism for DHCA transport in endothelial cells may also involve an energy-dependent transport system. It has been shown, for example, that bovine aortic endothelial cells possess such a transporter for the flavonoid morin, and that ATP-dependent morin uptake is inhibited 73% by 100 μmol/L CA [28]. Further studies are needed to evaluate the kinetics and sodium/energy dependence of the transport activity for DHCA, and whether it might correspond to the flavonoid transporter type.

Once DHCA enters endothelial cells, our results show that it protects the cells against oxidant stress and modestly enhances eNOS activity, the latter representing an important function of these cells. We have previously shown that ascorbic acid spares loaded α-TOC and diminishes oxidation of c-PnA in the cultured rat hepatocyte line H4IIE [29]. We observed similar results in EA.hy926 cells in the present work with DHCA. The α-TOC content of EA.hy926 cells in culture was measurable but very low, necessitating supplementation to allow its study. When cells were loaded overnight with α-TOC, we found that the extent of oxidation of cPnA in response to the free radical initiator AAPH was significantly decreased compared to cells not loaded with α-TOC (Fig. 5). This effect is predicted based on the ability of α-TOC to prevent endothelial dysfunction [30] and damage due to hydrogen peroxide [31] in endothelial cells. We also found that culture of EA.hy926 cells overnight with both α-TOC and DHCA increased the amount of α-TOC in the cells in a dose-dependent manner (Fig. 4). This sparing of α-TOC accords with our previous results in H4IIE cells at [29], and with the observations of Laranjinha et al. [8] and Laranjinha and Cadenas [32], who showed that CA can recycle α-TOC in human low-density lipoprotein. Whether the sparing of α-TOC occurred in the medium or in the cell membrane cannot be determined from our data. Even in the absence of α-TOC loading, overnight loading of cell with DHCA blunted the oxidation of cPnA in response to ROS generated acutely by AAPH (Fig. 5). This effect was comparable in size to that observed with α-TOC, and occurred in cells in which extracellular DHCA had been removed. The latter finding supports an intracellular effect of DHCA, assuming little leakage of DHCA out of the cells during incubation with AAPH. Although DHCA might act to some extent through recycling of α-TOC, we believe that the observed sparing of α-TOC and protection of c-PnA from oxidation are more likely due to scavenging of ROS that would otherwise oxidize α-TOC or unsaturated fatty acids in the cell membranes.

The fact that DHCA scavenges intracellular ROS is supported by the results with menadione-induced oxidation of dihydrofluorescein. Dihydrofluorescein oxidation to fluorescein (which is detected by its intense fluorescence) has been shown to be a sensitive method for detecting intracellular ROS [33], most of which probably derives from superoxide. In the absence of an oxidant stress, EA.hy926 cells generate little ROS when measured by this method.

Therefore, we used the redox cycling agent menadione to generate intracellular ROS. Menadione is known to generate primarily superoxide within the cells during redox cycling [34,35]. We recently showed in EA.hy926 cells that the resulting ROS oxidize intracellular dihydrofluorescein, and that the oxidation product fluorescein is detected primarily after it leaks from the cells during incubation in the fluorescence microtiter plate reader [15]. In that work, intracellular ascorbic acid blunted oxidation of dihydrofluorescein. In the present studies, similar results were found in cells loaded acutely with DHCA (Fig. 6). This establishes a mechanism by which DHCA can also scavenge much lower concentrations of endogenous ROS.

The finding that intracellular DHCA enhanced eNOS activity and protein expression by about 30% (Fig. 7) suggests that it also has effects on the transcriptional regulation of eNOS. Ascorbic and lipoic acids have been found to enhance calcium-dependent eNOS activity by as much as 2-fold [20,36]. In the case of ascorbate, this increase is not associated with increased eNOS protein expression [36] but with sparing tetrahydrobiopterin, an important cofactor for eNOS that is readily oxidized [36–39]. Increased tetrahydrobiopterin availability then maintains eNOS activity at optimal levels. Although DHCA may enhance nitric oxide release by a different mechanism than ascorbate, our results provide a mechanism for the observation that CA and other polyphenols enhance endothelium-dependent vasorelaxation in rat thoracic aorta [12].

The question arises as to the physiologic relevance of the present findings based on the expected plasma and interstitial concentrations of DHCA after ingestion. Perhaps the richest dietary source of DHCA and related compounds is coffee [40]. Depending on the type, coffee may contain as much as 18% 5-CGA and related derivatives such as CA [3,41]. In a study of ileostomy patients, CA and CGA were administered orally and the unabsorbed derivatives were collected in ileostomy fluid [42]. Although 95% of a 500-mg dose of CA was absorbed, only 11% of a 1-g dose of CGA was absorbed [42]. In another study in which coffee containing 96 mg of CGA was administered to normal volunteers, no CGA could be detected in plasma [43]. Based on these results, it was suggested that most or all of the CGA that appeared to be absorbed in the ileosotomy study was hydrolyzed to caffeic and quinic acids [43]. On the other hand, oral administration of 700 μmol/kg (200 mg/kg) of CA to rats resulted in plasma levels of free CA of 1.5 μmol/L, and as much as 30 μmol/L as glucuronide and 10 μmol/L as sulfate derivatives [44]. Increases in CA levels were noted with a similar time scale in humans after administration of 168 mg in a cup of brewed coffee, typically peaking after 1 hour at about 1 μmol/L as glucuronide conjugates, with free CA about 1% of total [43]. Although DHCA has not be directly quantified in human plasma, it has been identified in plasma from coffee drinkers compared to non–coffee drinkers [16]. Whereas CA and DHCA concentrations in plasma may not exceed 1 μmol/L after coffee consumption, the intracellular accumulation of DHCA noted in the present study could provide adequate amounts to exert an antioxidant effect. Our studies, although performed at much higher DHCA concentrations, were designed to demonstrate proof-of-principle of whether DHCA could function as an antioxidant in endothelial cells.

In conclusion, we have found that DHCA, one of the major human metabolites of CA-containing dietary constituents, is taken up and able to augment the antioxidant activity of α-TOC in human endothelial cells.

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