



Molecular and Cellular Pharmacology

Structure–activity relationships in the cytoprotective effect of caffeic acid phenethyl ester (CAPE) and fluorinated derivatives: Effects on heme oxygenase-1 induction and antioxidant activities

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ABSTRACT

To determine the relationship between catechol ring modifications and the activity of caffeic acid phenethyl ester (CAPE) as a cytoprotective agent, six catechol ring fluorinated CAPE derivatives were evaluated for their cytoprotective abilities, as well as for their antioxidant and heme oxygenase 1 (HO 1) inducing capacity in a human umbilical vein endothelial cell (HUVEC) model of oxidant stress. To ascertain the involvement of HO 1 induction in the cytoprotective effects of CAPE analogues, their ability to induce HO 1 at 20 μ M was determined by reverse transcriptase polymerase chain reaction, western blotting and the use of HO 1 inhibitor tin protoporphyrin IX. There was significant induction of HO 1 by CAPE derivatives. Inhibition of HO 1 enzymatic activity resulted in reduced cytoprotection. Modification of the catechol ring of CAPE by introduction of fluorine at various positions resulted in dramatic changes in cytoprotective activity. The maintenance of at least one hydroxyl group on the CAPE catechol ring and the phenethyl ester portion was required for HO 1 induction. CAPE and its derivatives were screened for their ability to scavenge intracellular reactive oxygen species generated in HUVECs by measuring 5 (and 6) chlormethyl 2', 7' dichlorodihydro fluorescein diacetate oxidation. The maintenance of 3, 4 dihydroxyl groups on the catechol ring was required for antioxidant activity, but antioxidant activity did not guarantee cytoprotection. Methylation or replacement of one hydroxyl group on the catechol ring of CAPE, however, provided both pro oxidant and cytoprotective activities. These results indicate that the induction of HO 1 plays a more important role in the cytoprotective activity of CAPE derivatives than their direct antioxidant activity.

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1. Introduction

Caffeic acid phenethyl ester (CAPE), a polyphenolic compound concentrated in honeybee propolis, has been reported to exhibit numerous bioactive properties including antioxidant (Son and Lewis, 2002) and anti-inflammatory activities (Michaluart et al., 1999), which may contribute to its protective effects in various pathophysiological processes such as ischemia/reperfusion injury (Tan et al., 2005; Wei et al., 2004) and atherosclerosis (Hishikawa et al., 2005). In an effort to exploit the beneficial effect of CAPE in ischemia/reperfusion injury, we developed an *in vitro* model of oxidant stress

and tested fluorinated derivatives of CAPE (Wang et al., 2006). Subsequent work demonstrated that much of the cytoprotective effect of CAPE against oxidant stress was due to up regulation of heme oxygenase 1 (HO 1) mRNA and this correlated with enzyme production that could be inhibited by tin protoporphyrin (SnPPiX) (Wang et al., 2008).

HO 1 appears to play a critical role in cytoprotection against cellular oxidative stress. In contrast to HO 2, the second isoform of heme oxygenase that is present mainly in the brain and testis, HO 1 is highly inducible by various stimuli that cause cellular stress. As the rate limiting enzyme of heme metabolism, HO 1 exerts its protective effects by maintaining appropriate cellular heme levels and releasing bioactive molecules including biliverdin, free iron, and carbon monoxide. Biliverdin and its reduced form bilirubin have been shown to be potent antioxidants which may contribute to the beneficial effects of HO 1 (Baranano et al., 2002; Stocker et al., 1987). The effects of carbon monoxide on mediating HO 1 protection

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have been reviewed, and they include anti-inflammatory and anti-apoptosis effects that may work through two signal transduction pathways: guanylyl cyclase cyclic GMP and p38 mitogen-activated protein kinase (Otterbein et al., 2003). Our previous studies showed that HO-1 induction may play an important role for CAPE cytoprotection in HUVECs against menadione-caused oxidative injury (Wang et al., 2008). The major mechanism of endothelial cell toxicity caused by menadione is the production of reactive oxygen radicals such as superoxide which serves as a starting point to generate other oxygen radicals including hydrogen peroxide and hydroxyl radical (Kossonjans et al., 1996; Warren et al., 2000). The overproduction of these reactive oxygen species is a major outcome of ischemia/reperfusion injury; therefore, menadione-induced oxidative injury in HUVECs was applied to simulate ischemia/reperfusion injury *in vitro* (Granger et al., 1986).

To study CAPE structure and cytoprotective activity relationships, we synthesized six catechol ring fluorinated CAPE derivatives (F-CAPEs) and evaluated their cytoprotective effects in the HUVEC menadione model (Wang et al., 2006). Fluorine substitution in the design and synthesis of small molecules for potential therapeutic use has become very common (Kirk, 2006). Fluorine is the smallest substitute for hydrogen and minimally alters the steric size of a ligand. The replacement of a hydrogen atom or hydroxyl group by a fluorine atom on an aryl ring can retard metabolic degradation, increase lipophilicity, and exert a profound impact on receptor binding or selectivity, depending upon the position of the fluorine substitution (Park et al., 2001; Haggmann, 2008). Because the hydroxyl groups within the CAPE catechol functional moiety may account for its direct antioxidant activity (Alanko et al., 1999; Lien et al., 1999), we also investigated the effect of substituting one hydroxyl group with a fluorine or methylating one or both catechol hydroxyl groups on the cytoprotection of the resulting derivatives. It has been suggested that the methylation of polyphenolic compounds may result in the loss of their free radical scavenging ability (Deng et al., 2006). Antioxidant activity has often been proposed to account for the beneficial effects of polyphenolic compounds like CAPE, curcumin, and resveratrol in ischemia/reperfusion-induced oxidative damage (Ozer et al., 2005; Shigematsu et al., 2003; Wang et al., 2005). To investigate whether the cytoprotective effect of CAPE was due to a transcription-mediated event or direct chemical antioxidant activity, we evaluated the free radical scavenging abilities of CAPE and F-CAPEs in HUVEC culture, which could be achieved by measuring the intracellular production of reactive oxygen species through a dichlorodihydrofluorescein oxidation-based fluorescence assay (Li et al., 2003). Here, we propose that the cytoprotective effect of CAPE and F-CAPEs on menadione-induced

oxidative injury in HUVECs is mediated mainly through transcriptional activity via induction of HO-1 mRNA and its resultant protein product, rather than through direct antioxidant function.

2. Materials and methods

2.1. Chemicals and reagents

CAPE was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Caffeic acid, menadione sodium bisulfite (menadione), dimethyl sulfoxide (DMSO), and 1 M HEPES buffer were obtained from Sigma Aldrich (Saint Louis, MO, USA). Methyl caffeate and phenethyl dimethyl caffeate (PEDMC) were from LKT Laboratories (St. Paul, MN, USA). Protoporphyrin IX dichloride (SnPPIX) was purchased from Frontier Scientific (Logan, UT, USA). Five (and 6) chloromethyl 2', 7' dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) and 10× Hanks' balanced salt solution (no sodium bicarbonate or phenol red) were obtained from Invitrogen (Carlsbad, CA, USA). Cells to cDNA kit was from Applied Biosystems (Foster City, CA, USA).

2.2. Synthesis of catechol ring fluorinated CAPE derivatives

The chemical structures of the fluorinated CAPE analogues used in this study are shown in Fig. 1. These compounds were synthesized according to a general procedure described previously (Wang et al., 2006).

2.3. Cell culture

HUVECs (Cascade Biologics, Portland, OR, USA) were cultivated on 1% gelatin-coated 75 cm² culture flasks (Corning Incorporated, Corning, NY, USA) in Medium 200 supplemented with 2% fetal calf serum, penicillin (100 units/ml), streptomycin (100 units/ml), and fungizone (0.25 µg/ml) supplied by Cascade Biologics (Zhao et al., 2001). The cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ with medium changes every 2 days until confluence. Prior to an experiment, HUVECs were subcultured with Trypsin/EDTA onto 1% gelatin-coated 48- or 96-well Costar® multiplates (Corning Incorporated, Corning, NY, USA) at 10,000 or 5000 cells/well, respectively, grown to confluence, and kept for 72 h to produce a quiescent cell layer. On the day before the experiment, the medium was changed. Only the second through fifth population doubling levels of cells were used.

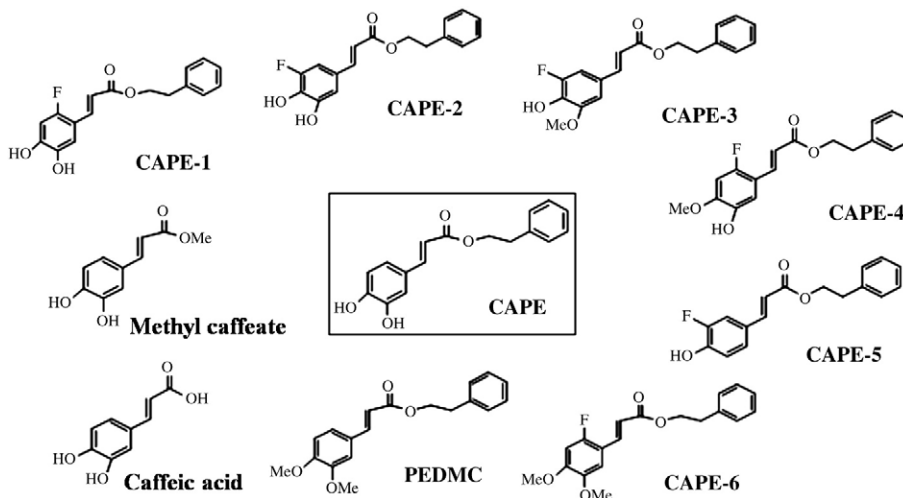


Fig. 1. Chemical structures of CAPE and derivatives.

2.4. Cell viability and toxicity assay

Cell viability was estimated in HUVECs using the Alamar Blue™ assay kit (Biosource International, Camarillo, CA, USA). As previously described (Wang et al., 2006), menadione was used to induce oxidative injury in the cells. Briefly, HUVECs were exposed to a dose of menadione close to its maximum toxicity, causing 80–90% cell death after 24 h incubation. The cells were then incubated with culture medium containing 10% Alamar Blue for 2 h. The 590 nm fluorescence (excitation with 545 nm) was measured using a SpectraMAX® M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The amount of fluorescence produced is proportional to the number of viable cells and all treatments are presented relative to vehicle controls.

2.5. Cell protection assay

CAPE and its derivatives were dissolved in DMSO to 20 mM and diluted 1000 fold with medium. Confluent HUVECs in 48 well plates were pretreated with CAPE and its analogues at 20 μM for 6 h, and the cytotoxic dose of menadione was then added. After 24 h incubation, cell viability was measured using the Alamar Blue assay.

2.6. HO 1 inhibition with SnPPIX

Confluent HUVECs were pretreated with CAPE or F CAPEs at 20 μM in the presence of different doses of HO 1 inhibitor SnPPIX (Abuarqoub et al., 2006) for 6 h before exposure to a toxic dose of menadione for an additional 24 h. SnPPIX was dissolved in 0.1 M NaOH and diluted 1000 fold with medium. Cell viability was measured using the Alamar Blue.

2.7. Cell based antioxidant assay

The antioxidant activities of CAPE or F CAPEs were estimated by measuring the intracellular level of oxygen derived free radical production while exposing HUVECs to the fluorescent probe CM H₂DCFDA (Jones et al., 2002). In brief, HUVECs were seeded onto 96 well plates and grown until confluence. HUVECs were rinsed twice with prewarmed working buffer (Hanks' balanced salt solution containing 10 mM HEPES) and incubated for 30 min at 37 °C in the dark with CM H₂DCFDA (8 μM) in the working buffer. HUVECs were then washed twice with prewarmed working buffer and loaded with the test compounds dissolved in buffer. The fluorescence excited at 480 nm was then recorded at 520 nm using the SpectraMAX® M2 microplate reader at time points of 0, 10, 20, 30, 45, 60, 90, and 120 min. The fluorescent signals were normalized to the initial readings at time zero, and the relative fluorescence was taken to be proportional to the production of reactive oxygen species.

2.8. Determination of endothelial HO 1 mRNA levels by real time RT PCR

HUVECs were grown in 48 well plates until confluence and pretreated with 20 μM CAPE and its derivatives for 6 h. The cDNA was obtained by reverse transcription of RNA using the Cells to cDNA™ II kit according to the manufacturer's instructions. Real time PCR was performed on a LightCycler™ thermal cycler (Idaho Technology, Salt Lake City, UT, USA) with Roche LightCycler® TaqMan Master for HO 1 and 18S (Roche Diagnostics, Indianapolis, IN, USA). HO 1 and 18S primer sets were from TaqMan® Gene Expression Assays (Applied Biosystems). HO 1 gene was normalized to the expression level of 18S for each sample. Relative quantification was performed with the comparative C_T method. Quantification was based on the number of PCR cycles (C_t) required to cross a threshold of fluorescence intensity, using delta-delta C_T model (User Bulletin #2; Applied Biosystems).

2.9. Polyacrylamide gel electrophoresis and western blotting

Polyacrylamide gel electrophoresis was performed on cells grown on 48 well multiplates and incubated for the indicated time with CAPE or derivatives. The cell layer was rinsed 3 times with PBS, fixed in 1:1 ethanol/acetone for 10 min at –20 °C and air dried. Immediately before electrophoresis the fixed cells were treated with 0.5% Triton X 100, 50 mM Tris (pH 7.5), 2 mM MgCl₂, 40 mM Tris (carboxyethyl) phosphine hydrochloride (Sigma) and 1× in proteinase inhibitors (Halt proteinase inhibitor, ThermoFisher, Houston, TX, USA) containing 1 unit of benzonase (Sigma) for 15 min. After addition of 4× loading buffer at a ratio of 1:4, 10 μl containing approximately 10 μg of protein from each treatment was run on NuPage 4 12% bis Tris gels (Invitrogen) and then transferred to a nitrocellulose membrane using the iBlot system (Invitrogen). Prior to antibody application, the blots were stained with SyPro Ruby blot stain (Invitrogen) according to the supplier's specification to determine that each lane had approximately the same amount of protein. After blocking in 0.2% I Block (Tropix, Bedford, MA, USA), 0.1% Tween 20 (Sigma) and 0.1% thimerosal (Sigma) in PBS, the blots were then incubated with a rabbit anti rat HO 1 antibody (Assay Design, Ann Arbor, MI, USA, 1:1000) for 2 h. Rabbit ABC alkaline phosphatase reagents (Vector Laboratories, Burlingame, CA, USA) were used to label the bands and the alkaline phosphatase visualization was accomplished with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Invitrogen) until the bands became visible. Quantitative analysis was performed with TotalLab TL100 software (Nonlinear Dynamics Ltd, Newcastle, UK) on blots scanned into the computer (KODAK Image Station 2000MM, Carestream Health, Inc., Rochester, NY, USA). In some cases, following staining for HO 1, blots were stripped and reprobed with mouse anti actin (Abcam, Cambridge, MA, USA) to ensure equal loading of protein.

2.10. Statistical analysis

Data are presented as the mean plus standard deviation. Differences among groups were analyzed using one way analysis of variance (ANOVA) followed by post hoc tests of Tukey (equal variances assumed) or Games-Howell (unequal variances not assumed) for multiple comparisons through SPSS statistical software, and $P < 0.05$ was considered significant. All experiments were performed at least 3 times with at least 3 replicates ($n = 3$) per experiment and representative experiments are presented.

3. Results

3.1. Comparison of CAPE and F CAPEs against oxidative stress in HUVECs

The cytotoxic effect of menadione was evaluated in HUVECs, and a dose of 22.5 μM was selected for the cytoprotection assay. We previously reported that most CAPE and F CAPEs showed cytoprotection at doses from 5 to 10 μg/ml (Wang et al., 2006). To compare their beneficial effects at the same molar concentration within that range, CAPE and F CAPEs at 20 μM were examined and shown to ameliorate menadione induced oxidative injury in HUVECs by different levels (Fig. 2A). Of all the fluorinated derivatives, CAPE 1 showed the greatest cytoprotection (approximately 80% of cell viability compared to vehicle control) compared to menadione treated HUVECs (approximately 20% of cell viability compared to vehicle control, $P < 0.05$) although this effect was not significantly different than that of CAPE ($P > 0.05$). CAPE 4 and CAPE 5 each exhibited a protective effect resulting in approximately 60% cell viability compared to vehicle control after menadione exposure ($P < 0.05$). CAPE 3 protected HUVECs relatively weakly with approximately 40% cells surviving after oxidative injury compared to control ($P < 0.05$). CAPE 2 and

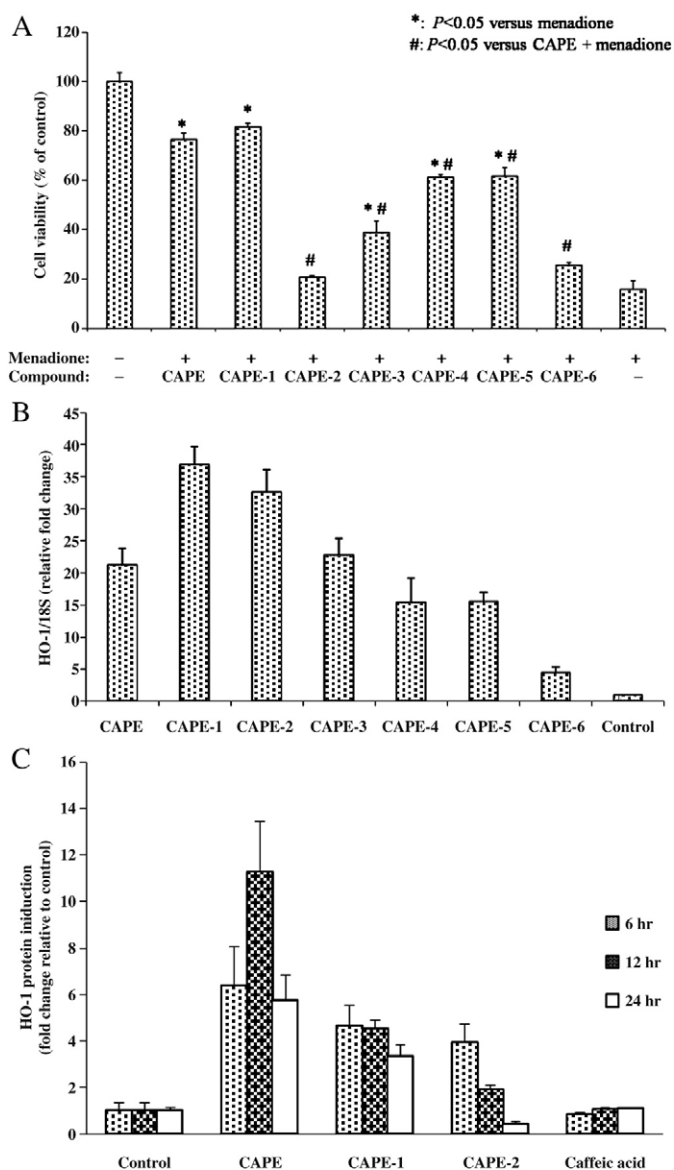


Fig. 2. Cytoprotection, HO-1 mRNA induction, and HO-1 protein expression of CAPE and F-CAPEs in HUVECs. (A) Pretreatment of HUVECs with CAPE and six synthesized derivatives at 20 μ M showed various cytoprotective effects against 22.5 μ M menadione-induced oxidative injury. CAPE-1 exhibited the most cytoprotection among the analogues, which was similar to that of CAPE. CAPE-3, -4, and -5 ameliorated menadione-resulted oxidative stress in HUVECs to some extent, while CAPE-2 and -6 did not protect HUVECs at the dose examined. Values are presented as means plus standard deviation ($n = 3$). (B) HO-1 mRNA induction by CAPE and F-CAPEs in HUVECs was determined through RT-PCR. After 6 h incubation, CAPE and its analogues highly elevated HO-1 gene expression over 15 fold compared to vehicle control excluding CAPE-6. Values are presented as means plus standard deviation ($n = 4$). (C) HO-1 protein expression in HUVECs by CAPE, CAPE-1, CAPE-2, and caffeic acid at exposure time points of 6, 12, and 24 h was determined through western blot. At 20 μ M, CAPE and CAPE-1 consistently induced HO-1 protein production higher than that of control group from 6 to 24 h. However, although CAPE-2 induced HO-1 protein production at 6 h, it increased the turnover of HO-1 protein with time and even inhibited its production at 24 h compared to that of control. Caffeic acid did not induce HO-1 protein within the time period tested.

CAPE 6 had no significant protective effect at the tested dose (20 μ M) ($P > 0.05$).

3.2. HO 1 mRNA and protein expression in HUVECs by CAPE and F CAPEs

HUVECs from the same batch and population doubling level as those for the cytoprotection assay were used to determine HO 1 mRNA abundance levels after a 6 h pretreatment with 20 μ M dose of

CAPE analogues. All of the F CAPEs activated high levels of HO 1 mRNA expression compared to the control group except CAPE 6 (Fig. 2B). Although CAPE 2 induced high levels of HO 1 mRNA expression in 6 h, the western blot data showed that HO 1 protein production was not induced to similar levels as other derivatives at later sampling points (12 or 24 h); additionally, HO 1 protein induced by CAPE 2 dropped to the control level within 24 h (Fig. 2C). It should be noted that CAPE 2 at the same dose was not cytoprotective. Compared to CAPE 2, CAPE and CAPE 1 consistently induced HO 1 protein to a higher level over a period of 24 h and were cytoprotective as well.

3.3. Effects of an HO 1 inhibitor on cytoprotection by fluorinated CAPE analogues against menadione induced oxidative injury in HUVECs

The HO 1 inhibitor SnPPIX was used to determine if the protective effects of CAPE and F CAPEs were due to the induction of HO 1. HUVECs were co incubated with different concentrations of SnPPIX and CAPE or its cytoprotective analogues (CAPE 1, 3, 4, and 5) at 20 μ M. SnPPIX suppressed the cytoprotective effects of CAPE 3, 4, and 5 in a dose dependent manner, and completely abolished CAPE 1 cytoprotective ability at any dose applied (Fig. 3).

3.4. Comparison of antioxidant activity among CAPE and F CAPEs in HUVECs by determining the intracellular production of reactive oxygen species

To elucidate the contribution of antioxidant properties of CAPE and F CAPEs to their potential cytoprotective capabilities, we evaluated their free radical scavenging activity in HUVECs. This was assessed by measuring the cellular level of reactive oxygen species with the fluorescent probe CM H₂DCFDA. These results showed that HUVECs treated with 20 μ M CAPE, CAPE 1, or 2 generated oxygen derived free radicals at a lower rate than the vehicle control (0.1% DMSO), and the corresponding free radical production was significantly lower within a 2 h period compared to the vehicle control (Fig. 4A). HUVECs treated with CAPE 3, 4, and 5, however, produced free radicals at a faster rate than the control group, which resulted in significantly more free radical generation within 2 h (Fig. 4B). CAPE 6 did not change the trend of oxygen radical formation in HUVECs (Fig. 4C). Caffeic acid, a potential CAPE metabolite, and methyl caffeate and PEDMC, which are commercially available CAPE derivatives, were also tested for antioxidant activity in HUVECs. Caffeic acid and methyl caffeate showed similar antioxidant effects compared to CAPE, while PEDMC, like CAPE 6, did not significantly alter free radical production compared to control (Fig. 4). Importantly, none of the commercially available analogues showed any beneficial effects against menadione induced oxidant stress, and they did not elevate HO 1 mRNA compared to CAPE at 20 μ M (Fig. 5). The HO 1 protein expression was also not induced by caffeic acid compared to the control group (Fig. 2C).

4. Discussion

CAPE, a natural polyphenolic compound, contains a well described antioxidant structural moiety, the catechol ring, that largely accounts for its free radical scavenging and metal chelating properties (van Acker et al., 1996). Although many of CAPE's salutary pharmacological effects have been attributed to its antioxidant activity, it is unclear whether this activity results from either the compound's structure or its ability to activate the expression of cytoprotective antioxidant enzymes.

Results from a previous investigation indicated that CAPE may exert its cytoprotective effect against menadione induced oxidative stress in HUVECs through induction of the enzyme HO 1 (Wang et al., 2008). HO 1 has been identified as a major component of the cellular

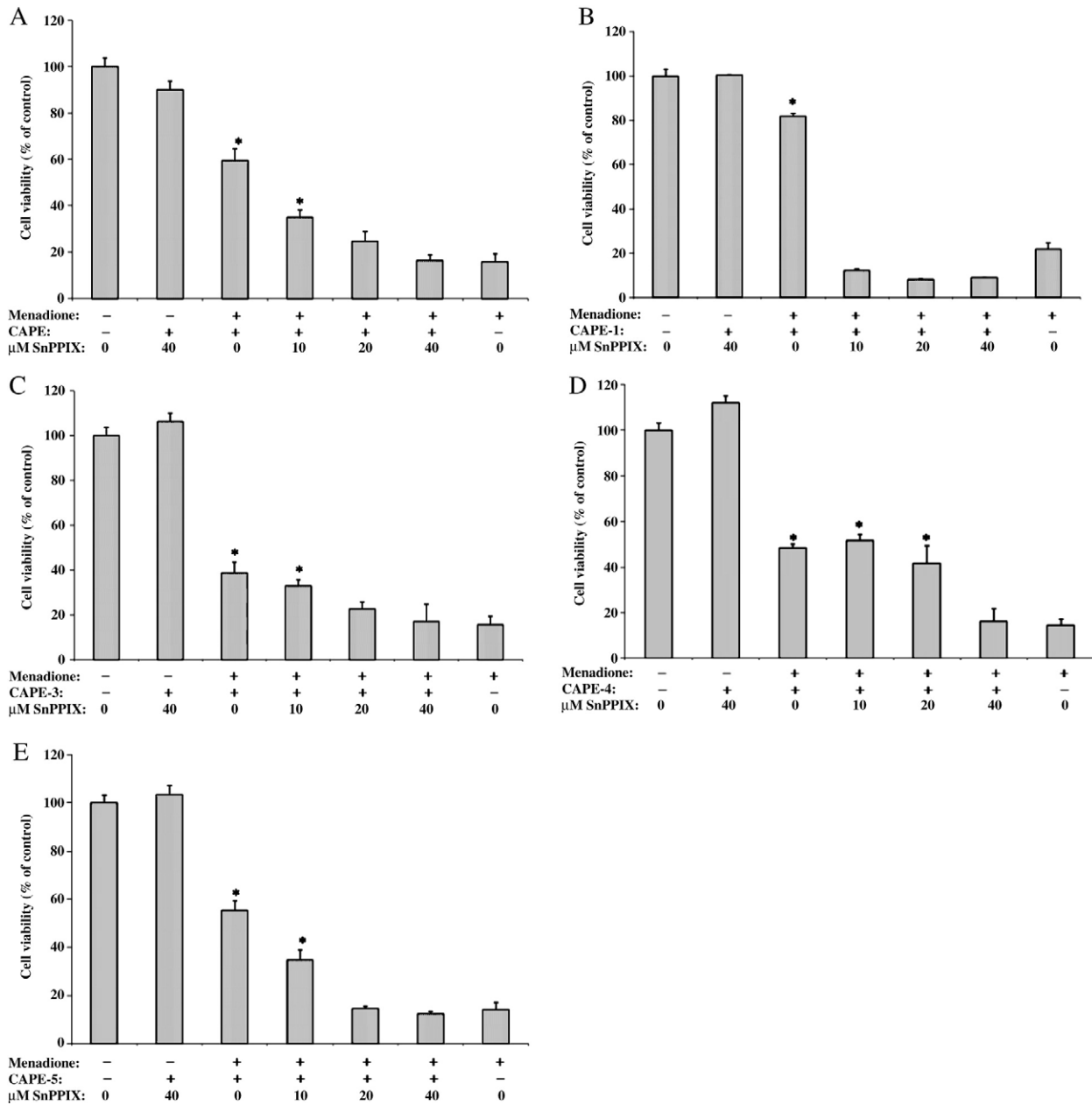


Fig. 3. The effect of HO-1 inhibitor SnPPiX on CAPE and its cytoprotective F-CAPEs at 20 μ M against menadione-mediated oxidative injury in HUVECs. SnPPiX suppressed the cytoprotective effects of (A) CAPE and its analogues (C) CAPE-3, (D) -4, and (E) -5 in a dose-dependent manner against 22.5 μ M menadione-induced oxidative injury, while it completely removed (B) CAPE-1 cytoprotection even at 10 μ M. The incubation of F-CAPEs plus 40 μ M SnPPiX alone with HUVECs was not toxic. Values are presented as means plus standard deviation ($n=3$).

defense system against oxidative stress by generating biliverdin, bilirubin, and carbon monoxide that provide potent antioxidant and anti-inflammatory activities, and by recycling the iron molecule, another end product (Ryter et al., 2006).

In order to elucidate the relationship between CAPE's structure and cytoprotective effects, we synthesized six CAPE catechol ring modified (fluorinated +/- O methylated) derivatives and evaluated their cytoprotective activity in the menadione HUVEC model (Wang et al., 2006). Each derivative yielded a somewhat different cytoprotection profile. In addition, we investigated the molecular mechanism of CAPE cytoprotection by examining whether HO 1 up-regulation was also involved in the beneficial effects of CAPE analogues against oxidative injury *in vitro*. Cytoprotection correlated with HO 1 mRNA up-regulation for most CAPE derivatives. CAPE, CAPE 1, 3, 4, and 5 showed both cytoprotection and high HO 1 mRNA induction, while CAPE 6 neither protected HUVECs nor increased HO 1 expression at

the same level as other analogues. Only CAPE 2 activated HO 1 gene transcription, but did not ameliorate menadione induced oxidative damage in HUVECs. Interestingly, CAPE 2 induction of HO 1 protein synthesis in HUVECs also appeared to influence the turnover of the protein resulting in a more rapid decay. A similar effect has been observed with resveratrol induction of HO 1 in astrocytes (Scapagnini et al., 2004), where resveratrol induced HO 1 mRNA but not the protein product. The requirement for heme oxygenase enzymatic activity in the cytoprotective effect of CAPE and CAPE analogues was determined by the application of SnPPiX, a specific inhibitor of HO 1 activity. Inhibition of HO 1 resulted in a loss of the cytoprotective effect of CAPE and its cytoprotective derivatives (1, 3, 4, and 5) at 20 μ M, which further suggested the direct involvement of HO 1.

One mechanism accounting for heme oxygenase 1 induction is through the activation of the Keap1/Nrf2/ARE pathway by CAPE and F-CAPEs as Michael reaction acceptors. Under basal conditions, Keap1, a

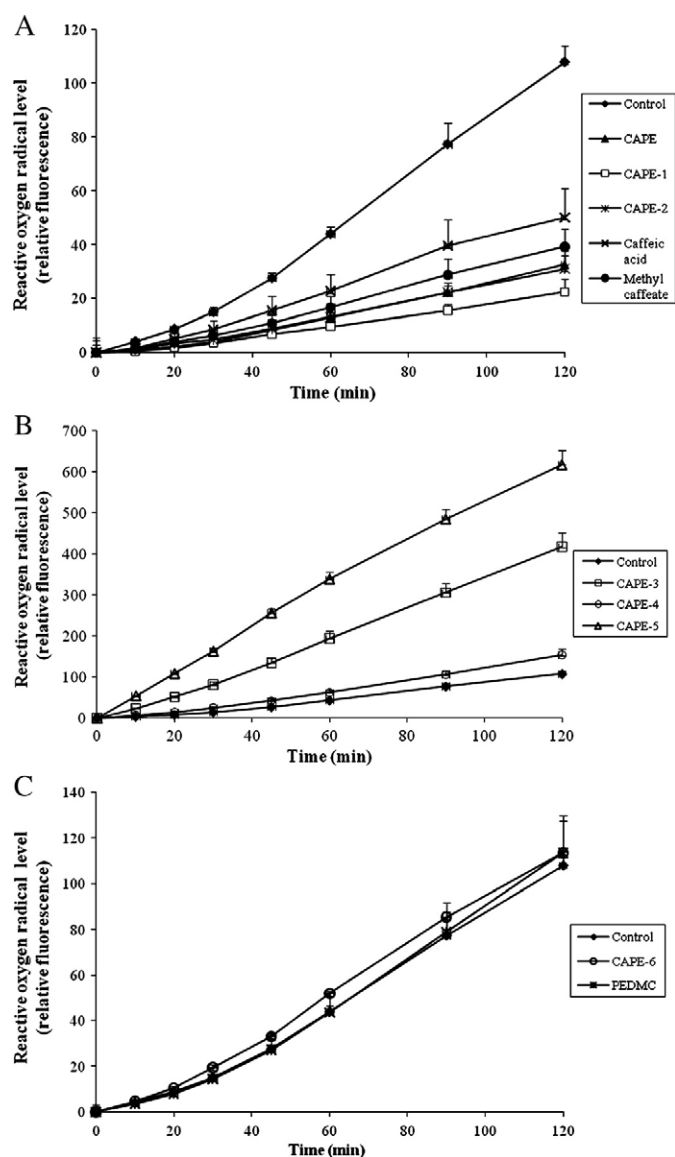


Fig. 4. Direct antioxidant activities of CAPE and analogues in HUVECs demonstrated by measuring the alteration of intracellular levels of reactive oxygen species. Free radical levels after the treatment with caffeic acid, methyl caffeate, CAPE, CAPE-1, and -2 were significantly decreased compared to that of control in a time-dependent manner (A), while the incubation of CAPE-6 and PEDMC generated similar free radical levels as control (C). The treatment with CAPE-3, -4, and -5 significantly increased free radicals to different levels compared to that of control also in a time-dependent manner (B).

cytoplasmic protein associated with the actin cytoskeleton, sequesters Nrf2 in cytoplasm to form a Keap1 Nrf2 complex, thereby repressing its function (Itoh et al., 1999). The inducers with α β unsaturated carbonyl moiety (Michael acceptor functionality) such as CAPE and F CAPEs could directly react with sulfhydryl groups of two cysteine (C273 and C288) residues of Keap1 resulting in the formation of disulfide bonds. This conformational change dissociates the binding of Keap1 to Nrf2, releasing the Nrf2 which can be translocated into the nucleus where it binds to the antioxidant response element (ARE) to accelerate the transcriptional activation of phase 2 antioxidant genes including HO 1 gene (Dinkova Kostova et al., 2002; 2001; Wakabaya shi et al., 2004).

Antioxidant activity has long been proposed as the major beneficial effect of polyphenols due to their scavenging of free radicals. Therefore, we evaluated the direct antioxidant capacity of CAPE, F CAPEs, and commercially available analogues in HUVECs. Intracellular level of reactive oxygen species was reduced in the

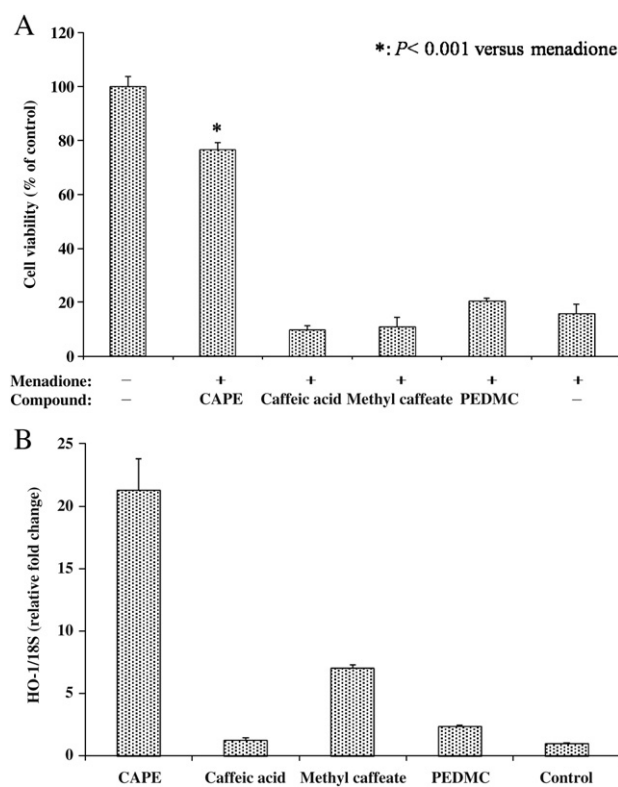


Fig. 5. The cytoprotection and HO-1 induction profiles of 20 μ M caffeic acid, methyl caffeate, and PEDMC in HUVECs compared to CAPE. Neither caffeic acid, methyl caffeate, and PEDMC protected HUVECs from menadione-induced cytotoxicity (A), nor did they induce much HO-1 mRNA (B) compared to CAPE. Values are represented as means plus standard deviation ($n = 3$).

presence of CAPE, CAPE 1, 2, caffeic acid, and methyl caffeate, all of which maintain the antioxidant catechol moiety. CAPE 6 and PEDMC, which are *O,O*-dimethylcatechols, failed to alter the level of intracellular reactive oxygen species when compared with the vehicle control. Interestingly, the presence of only one hydroxyl group on the aromatic ring resulting from mono methylation of the catechol functionality (CAPE 3 and 4) or replacing one catechol hydroxyl with a fluorine (CAPE 5) led to pro oxidant activity as these analogues afforded more oxygen derived free radicals than the vehicle control. While the mechanistic origins of the differential anti or pro oxidant effects of these CAPE analogues are not clear, the lack of correlation between chemical antioxidant activity and cytoprotective effect indicates that they are not directly related. For example, CAPE 2, which displayed antioxidant activity similar to CAPE, did not provide protection of the human endothelial cells against the detrimental effects of menadione. Similarly, neither caffeic acid nor methyl caffeate afforded cytoprotection, despite their antioxidant activity. Significantly, CAPE 3, 4, and 5, in spite of exhibiting pro oxidant activity in the cell based assay, exhibited some cytoprotection in the HUVEC menadione model. In the case of these CAPE analogues, the loss of cytoprotection by inhibition of cellular HO 1 enzyme activity provides evidence for the important role of HO 1 activation in cytoprotection.

There are interesting structure activity relationships that can be discerned for the three different activities investigated (cytoprotection, HO 1 induction, and intracellular antioxidant ability). In the case of cytoprotection, the presence of a phenolic group appears to be necessary for activity, as does the presence of the phenethyl side chain. Interestingly, while the position of fluorine substitution on the mono methylcatechols CAPE 3 and CAPE 4 has little effect on cytoprotective activity, a catechol *ortho* fluorine substituent is not tolerated (CAPE 2). The structure activity relationship for HO 1

induction closely follows that for cytoprotection, with the exception that catechol ring *ortho* fluorination is tolerated, at least in terms of up regulating transcription, although the effects on protein levels are short lived. In contrast, the structure activity relationship of antioxidant activity is quite distinct. The cell based antioxidant assay clearly indicated that the requirement for a catechol moiety for antioxidant functions and total methylation abolished the free radical scavenging ability of the analogues. The presence of one hydroxyl group and fluorine induced more production of reactive oxygen species; however, their free radical scavenging behavior did not correlate with cytoprotection in the HUVEC menadione model.

In conclusion, the results further confirmed our previous finding that transcriptional activation of antioxidant enzyme HO 1 by CAPE and F CAPEs plays a role in the demonstrated cytoprotective effect, and this appears to be more important than their antioxidant activity in HUVECs under menadione induced oxidative stress. The maintenance of at least one hydroxyl group on the CAPE catechol ring and the phenethyl ester portion is required for HO 1 induction, and fluorine substitution at different positions on the CAPE catechol ring dramatically changed this activity. The induction of HO 1 mRNA but failure to maintain the expression level of the translated product and cytoprotection by CAPE 2 requires further explanation but indicates that there are as yet to be defined effects of CAPE on cellular metabolism.

References

- Abuqarqoub, H., Foresti, R., Green, C.J., Motterlini, R., 2006. Heme oxygenase-1 mediates the anti-inflammatory actions of 2'-hydroxychalcone in RAW 264.7 murine macrophages. *American Journal of Physiology. Cell Physiology* 290, C1092–C1099.
- Alanko, J., Riutta, A., Holm, P., Mucha, I., Vapaatalo, H., Metsa-Ketela, T., 1999. Modulation of arachidonic acid metabolism by phenols: relation to their structure and antioxidant/prooxidant properties. *Free Radical Biology & Medicine* 26, 193–201.
- Baranano, D.E., Rao, M., Ferris, C.D., Snyder, S.H., 2002. Biliverdin reductase: a major physiologic cytoprotectant. *Proceedings of the National Academy of Sciences of the United States of America* 99, 16093–16098.
- Deng, D., Zhang, J., Cooney, J.M., Skinner, M.A., Adaim, A., Jensen, D.J., Stevenson, D.E., 2006. Methylated polyphenols are poor “chemical” antioxidants but can still effectively protect cells from hydrogen peroxide-induced cytotoxicity. *FEBS Letters* 580, 5247–5250.
- Dinkova-Kostova, A.T., Massiah, M.A., Bozak, R.E., Hicks, R.J., Talalay, P., 2001. Potency of Michael reaction acceptors as inducers of enzymes that protect against carcinogenesis depends on their reactivity with sulfhydryl groups. *Proceedings of the National Academy of Sciences of the United States of America* 98, 3404–3409.
- Dinkova-Kostova, A.T., Holtzclaw, W.D., Cole, R.N., Itoh, K., Wakabayashi, N., Katoh, Y., Yamamoto, M., Talalay, P., 2002. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proceedings of the National Academy of Sciences of the United States of America* 99, 11908–11913.
- Granger, D.N., Hollwarth, M.E., Parks, D.A., 1986. Ischemia-reperfusion injury: role of oxygen-derived free radicals. *Acta Physiologica Scandinavica Supplement* 548, 47–63.
- Hagmann, W.K., 2008. The many roles for fluorine in medicinal chemistry. *Journal of Medicinal Chemistry* 51, 4359–4369.
- Hishikawa, K., Nakaki, T., Fujita, T., 2005. Oral flavonoid supplementation attenuates atherosclerosis development in apolipoprotein E-deficient mice. *Arteriosclerosis, Thrombosis, and Vascular Biology* 25, 442–446.
- Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J.D., Yamamoto, M., 1999. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes and Development* 13, 76–86.
- Jones, W., Li, X., Qu, Z.C., Perriott, L., Whitesell, R.R., May, J.M., 2002. Uptake, recycling, and antioxidant actions of alpha-lipoic acid in endothelial cells. *Free Radical Biology & Medicine* 33, 83–93.
- Kirk, K.L., 2006. Fluorine in medicinal chemistry: recent therapeutic applications of fluorinated small molecules. *Journal of Fluorine Chemistry* 127, 17.
- Kossenjans, W., Rymaszewski, Z., Barankiewicz, J., Bobst, A., Ashraf, M., 1996. Menadione-induced oxidative stress in bovine heart microvascular endothelial cells. *Microcirculation* 3, 39–47.
- Li, H., Burkhardt, C., Heinrich, U.R., Brausch, I., Xia, N., Forstermann, U., 2003. Histamine upregulates gene expression of endothelial nitric oxide synthase in human vascular endothelial cells. *Circulation* 107, 2348–2354.
- Lien, E.J., Ren, S., Bui, H.H., Wang, R., 1999. Quantitative structure–activity relationship analysis of phenolic antioxidants. *Free Radical Biology & Medicine* 26, 285–294.
- Michaluart, P., Masferrer, J.L., Carothers, A.M., Subbaramaiah, K., Zweifel, B.S., Koboldt, C., Mestre, J.R., Grunberger, D., Sacks, P.G., Tanabe, T., Dannenberg, A.J., 1999. Inhibitory effects of caffeic acid phenethyl ester on the activity and expression of cyclooxygenase-2 in human oral epithelial cells and in a rat model of inflammation. *Cancer Research* 59, 2347–2352.
- Otterbein, L.E., Soares, M.P., Yamashita, K., Bach, F.H., 2003. Heme oxygenase-1: unleashing the protective properties of heme. *Trends in Immunology* 24, 449–455.
- Ozer, M.K., Parlakpınar, H., Vardi, N., Cigremis, Y., Ucar, M., Acet, A., 2005. Myocardial ischemia/reperfusion-induced oxidative renal damage in rats: protection by caffeic acid phenethyl ester (CAPE). *Shock* 24, 97–100.
- Park, B.K., Kitteringham, N.R., O'Neill, P.M., 2001. Metabolism of fluorine-containing drugs. *Annual Review of Pharmacology and Toxicology* 41, 443–470.
- Ryter, S.W., Alam, J., Choi, A.M., 2006. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiological Reviews* 86, 583–650.
- Scapagnini, G., Butterfield, D.A., Colombrita, C., Sultana, R., Pascale, A., Calabrese, V., 2004. Ethyl ferulate, a lipophilic polyphenol, induces HO-1 and protects rat neurons against oxidative stress. *Antioxidants Redox Signaling* 6, 811–818.
- Shigematsu, S., Ishida, S., Hara, M., Takahashi, N., Yoshimatsu, H., Sakata, T., Korthuis, R. J., 2003. Resveratrol, a red wine constituent polyphenol, prevents superoxide-dependent inflammatory responses induced by ischemia/reperfusion, platelet-activating factor, or oxidants. *Free Radical Biology & Medicine* 34, 810–817.
- Son, S., Lewis, B.A., 2002. Free radical scavenging and antioxidative activity of caffeic acid amide and ester analogues: structure–activity relationship. *Journal of Agricultural and Food Chemistry* 50, 468–472.
- Stocker, R., Yamamoto, Y., McDonagh, A.F., Glazer, A.N., Ames, B.N., 1987. Bilirubin is an antioxidant of possible physiological importance. *Science* 235, 1043–1046.
- Tan, J., Ma, Z., Han, L., Du, R., Zhao, L., Wei, X., Hou, D., Johnstone, B.H., Farlow, M.R., Du, Y., 2005. Caffeic acid phenethyl ester possesses potent cardioprotective effects in a rabbit model of acute myocardial ischemia–reperfusion injury. *American Journal of Physiology* 289, H2265–H2271.
- van Acker, S.A., van den Berg, D.J., Tromp, M.N., Griffioen, D.H., van Bennekom, W.P., van der Vijgh, W.J., Bast, A., 1996. Structural aspects of antioxidant activity of flavonoids. *Free Radical Biology & Medicine* 20, 331–342.
- Wakabayashi, N., Dinkova-Kostova, A.T., Holtzclaw, W.D., Kang, M.I., Kobayashi, A., Yamamoto, M., Kensler, T.W., Talalay, P., 2004. Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers. *Proceedings of the National Academy of Sciences of the United States of America* 101, 2040–2045.
- Wang, Q., Sun, A.Y., Simonyi, A., Jensen, M.D., Shelat, P.B., Rottinghaus, G.E., MacDonald, R.S., Miller, D.K., Lubahn, D.E., Weisman, G.A., Sun, G.Y., 2005. Neuroprotective mechanisms of curcumin against cerebral ischemia-induced neuronal apoptosis and behavioral deficits. *Journal of Neuroscience Research* 82, 138–148.
- Wang, X., Stavchansky, S., Bowman, P.D., Kerwin, S.M., 2006. Cytoprotective effect of caffeic acid phenethyl ester (CAPE) and catechol ring-fluorinated CAPE derivatives against menadione-induced oxidative stress in human endothelial cells. *Bioorganic and Medicinal Chemistry* 14, 4879–4887.
- Wang, X., Stavchansky, S., Zhao, B., Bynum, J.A., Kerwin, S.M., Bowman, P.D., 2008. Cytoprotection of human endothelial cells from menadione cytotoxicity by caffeic acid phenethyl ester: the role of heme oxygenase-1. *European Journal of Pharmacology* 591, 28–35.
- Warren, M.C., Bump, E.A., Medeiros, D., Brauhut, S.J., 2000. Oxidative stress-induced apoptosis of endothelial cells. *Free Radical Biology & Medicine* 29, 537–547.
- Wei, X., Zhao, L., Ma, Z., Holtzman, D.M., Yan, C., Dodel, R.C., Hampel, H., Oertel, W., Farlow, M.R., Du, Y., 2004. Caffeic acid phenethyl ester prevents neonatal hypoxic–ischemic brain injury. *Brain* 127, 2629–2635.
- Zhao, B., Bowden, R.A., Stavchansky, S.A., Bowman, P.D., 2001. Human endothelial cell response to gram-negative lipopolysaccharide assessed with cDNA microarrays. *American Journal of Physiology. Cell Physiology* 281, C1587–C1595.