CAFFEIC ACID PHENETHYL ESTER (CAPE): SCAVENGER OF PEROXYNITRITE IN VITRO AND IN SEPSIS MODELS

Mustafa Kassim,* Marzida Mansor,* Tengku Ain Kamalden,† Ina Ismiarti Shariffuddin,* Mohd Shahnaz Hasan,* Gracie Ong,* Shamala Devi Sekaran,‡ Anwar Suahimi,§ Nazeh Al-Abd,‖ and Kamaruddin Mohd Yusoff†

*Departments of Anesthesiology, †Ophthalmology, ‡Medical Microbiology, ‖Rehabilitation Medicine, and §Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia; and †Department of Molecular Biology and Genetics, Faculty of Arts and Science, Canik Basari University, Samsun, Turkey

Received 17 Dec 2013; first review completed 8 Jan 2014; accepted in final form 18 Mar 2014

ABSTRACT—Excessive free radical production by immune cells has been linked to cell death and tissue injury during sepsis. Peroxynitrite is a short-lived oxidant and a potent inducer of cell death that has been identified in several pathological conditions. Caffeic acid phenethyl ester (CAPE) is an active component of honeybee products and exhibits antioxidant, anti-inflammatory, and immunomodulatory activities. The present study examined the ability of CAPE to scavenge peroxynitrite in RAW 264.7 murine macrophages stimulated with lipopolysaccharide/interferon-γ that was used as an in vitro model. Conversion of 123-dihydrorhodamine to its oxidation product rhodamine-123 was used to measure peroxynitrite production. Two mouse models of sepsis (endotoxemia and cecal ligation and puncture) were used as in vivo models. The level of serum 3-nitrotyrosine was used as an in vivo marker of peroxynitrite. The results demonstrated that CAPE significantly improved the viability of lipopolysaccharide/interferon-γ-treated RAW 264.7 cells and significantly inhibited nitric oxide production, with effects similar to those observed with an inhibitor of inducible nitric oxide synthase (1400W). In addition, CAPE exclusively inhibited the synthesis of peroxynitrite from the artificial substrate SIN-1 and directly prevented the peroxynitrite-mediated conversion of dihydrorhodamine-123 to its fluorescent oxidation product rhodamine-123. In both sepsis models, CAPE inhibited cellular peroxynitrite synthesis, as evidenced by the absence of serum 3-nitrotyrosine, an in vivo marker of peroxynitrite. Thus, CAPE attenuates the inflammatory responses that lead to cell damage and, potentially, cell death through suppression of the production of cytotoxic molecules such as nitric oxide and peroxynitrite. These observations provide evidence of the therapeutic potential of CAPE treatment for a wide range of inflammatory disorders.

KEYWORDS—Caffeic acid phenethyl ester, inflammation, sepsis, peroxynitrite, nitric oxide, macrophage, cell death

INTRODUCTION

Sepsis is the leading cause of death in critically ill patients in intensive care units. The pathophysiological features of sepsis subsequent to bacterial infection include organ failure and death caused by the dysregulation of the immune response and excessive oxidant and free radical production (1, 2). During sepsis, immune cells such as neutrophils and macrophages release superoxide and/or nitric oxide (NO); NO reacts with the free radical superoxide, leading to the formation of the potent oxidant peroxynitrite, which has a short half-life (3). Although peroxynitrite has numerous functions in host defense and is an important microbicidal compound, it may also have deleterious effects on host tissues (4). In fact, the well-studied adverse effects of excess NO generation on host tissues during the inflammatory response are caused by the formation of peroxynitrite rather than NO itself. Peroxynitrite reacts with a wide range of biological molecules, including amino acids, leading to changes in protein structure and function (4, 5). It also causes chemical cleavage of DNA and a reduction in host defenses by oxidation of cellular thiol pools, lipid peroxidation, and tyrosine nitration. As such, a causative role of peroxynitrite has been implicated in diseases such as asthma, acute lung injury, idiopathic pulmonary fibrosis, inflammatory bowel disease, septic shock, arthritis, psoriasis, multiple sclerosis, systemic lupus erythematosus, Alzheimer disease, acute renal ischemia, and hyperlipidemia (4, 6). One approach to attenuate the toxic effects of peroxynitrite is the use of pharmacological strategies aimed at limiting tissue damage, that is, via inhibition and/or scavenging of peroxynitrite. Caffeic acid phenethyl ester (CAPE) is an active component of honeybee products and has established antioxidant, anticarcinogenic, anti-inflammatory, and immunomodulatory activities in diverse systems (7, 8). However, most importantly, CAPE exhibits antioxidant activity and inhibits lipoxygenases, protein tyrosine kinases, and the activation of the transcription factor nuclear factor-κB (NF-κB). The additional biological effects of CAPE include the inhibition of arachidonic acid release from cell membranes and cyclooxygenase 1 (COX-1) and COX-2 activities (8, 9). In this study, we tested the ability of CAPE to scavenge peroxynitrite in murine macrophages and in two mouse models of sepsis to determine its potential as a therapeutically effective treatment for inflammatory disorders.

MATERIALS AND METHODS

Animals

One hundred sixty male Balb/c mice weighing 20 to 25 g were obtained from the animal experimentation unit in the Faculty of Medicine, University of Malaya. Mice were kept in individual cages under standard conditions (12 h light and 12 h dark). The animals were fed a diet of Purina laboratory chow and given ad libitum access to water. The study was carried out in accordance with the guidelines for animal experimentation of the University of Malaya Animal Ethics Committee under the approved protocols and terms set out in project license ANES/14/07/2010/MKAK (R).
LPS/IFN-γ stimulation of RAW 264.7 cells

Murine RAW 264.7 macrophages maintained in 10% FBS-DMEM were seeded at a density of 2 × 10^5 cells/well in 24-well plates and incubated for 24 h at 37°C. A total of 100 μL of the culture was placed in a 96-well plate with an equivalent L of saline. Both CAPE and the saline control were administered the day before and 2 h before LPS administration by intraperitoneal injection. Endotoxemia was confirmed in the LPS-treated mice at 7 h after the injection. Blood was collected from the four groups (treated and control groups) by cardiac puncture, and the amount of 3-nitrotyrosine, a marker of peroxynitrite, was measured in the serum. Surviving mice (four groups) were administered CAPE or saline for 3 days after LPS administration. Viability was monitored every 12 h for 7 days.

Cecal ligation and puncture

Mice were randomly divided into eight groups (10 mice per group) and injected intraperitoneally with a lethal dose of LPS (25 mg/kg in a final volume of 100 μL). An equivalent volume of saline was administered to the negative control group (12). Treated mice received either 5 or 25 mg/kg of CAPE (total volume, 200 μL), whereas the positive or negative controls were administered 200 μL of saline. Both CAPE and the saline control were administered the day before and 2 h before LPS administration by intraperitoneal injection. Endotoxemia was confirmed in the LPS-treated mice at 7 h after the injection. Blood was collected from the four groups (treated and control groups) by cardiac puncture, and the amount of 3-nitrotyrosine, a marker of peroxynitrite, was measured in the serum. Surviving mice (four groups) were further treated with CAPE or saline for 3 days after treatment. Viability was monitored every 12 h for 7 days.

Measurement of 3-nitrotyrosine levels in RAW 264.7 cells

Lysates were prepared from RAW 264.7 cells collected after treatment as previously described for the stimulation experiments, and the level of 3-nitrotyrosine, a marker of peroxynitrite, was measured. The harvested cells were placed on ice and immediately lysed in RIPA buffer (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing a protease inhibitor cocktail and then centrifuged at 16,000g for 15 min at 4°C. The supernatants (lysates) were transferred to precooled tubes and stored at −80°C until analysis. 3-Nitrotyrosine concentrations in the lysates were measured with a 3-nitrotyrosine enzyme-linked immunosorbent assay (Cell Biolabs, Inc., San Diego, Calif) according to the manufacturer’s instructions and normalized to the amount of protein in milligrams. Protein concentrations were measured using the Bradford method.

Detection of 3-nitrotyrosine levels in serum

Blood was collected after 7 h from all groups of mice (CLP and endotoxemia), and the concentration of 3-nitrotyrosine in the serum was measured as previously described.

Statistical analysis

Data were analyzed using GraphPad prism statistical software (version 6; GraphPad Software Inc., La Jolla, Calif). Data are expressed as mean ± SEM. Tukey multiple comparisons test was used to determine the statistical significance of differences between the experimental and control groups. Kaplan-Meier analysis was used to compare survival rates. Statistical significance was set at P < 0.05. The half maximal inhibitory concentration (IC50) was calculated using sigmoidal dose-response nonlinear regression using the same software.
RESULTS

CAFE inhibits peroxynitrite production in vitro

Caffeic acid phenethyl ester inhibited peroxynitrite generation from the synthetic substrate SIN-1 with an IC50 of 0.037 μg/mL, whereas the effect of 1400W (100 μM) was similar to that of the negative (untreated) control (Fig. 1A). Neither CAPE nor 1400W (100 μM) reduced the accumulation of nitrite produced from SIN-1 (Fig. 1B). Moreover, direct incubation of CAPE and peroxynitrite for 15 min inhibited DHR-123 oxidation with an IC50 of 0.05 μg/mL, whereas the results achieved with 1400W were the same as those with the untreated control (Fig. 1C). Taken together, these results suggest that CAPE acts by scavenging peroxynitrite rather than by inhibiting its synthesis.

CAFE inhibits intracellular peroxynitrite production

None of the doses of CAPE and 1400W used in this study was found to be cytotoxic to murine RAW 264.7 macrophages based on a comparison of the viability of the cells after a 24-h incubation with these substances with that of untreated cells (Fig. 2A). In contrast, CAPE and 1400W significantly protected cell viability from the negative effects of the immune response induced with LPS/IFN-γ. In cells induced with LPS/IFN-γ and treated with CAPE and 1400W, viability was more than 88% compared with the less than 74% viability seen in the control cells induced with LPS/IFN-γ but untreated with CAPE (P < 0.001; Fig. 2B).

Both CAPE and 1400W (100 μM) inhibited peroxynitrite production in response to the LPS/IFN-γ induction of RAW 264.7 cells (IC50 CAPE, 0.4 μg/mL; 1400W, 100 μM), as shown by the reduced DHR-123 fluorescence in these cells as compared with that in the untreated cells (Fig. 2C). Moreover, the CAPE reduction of the LPS-induced immune response–mediated accumulation of nitrite was dose dependent. In the positive control, 1400W also blocked nitrite accumulation, whereas no such response was seen in untreated LPS-induced cells (Fig. 2D). After a 24-h incubation of RAW 264.7 cells with LPS/IFN-γ, CAPE and 1400W blocked intracellular peroxynitrite formation, as confirmed by a reduction in the level of 3-nitrotyrosine, an intracellular marker of ONOO− (P < 0.001; Fig. 3).

CAFE inhibits peroxynitrite production in vivo

Caffeic acid phenethyl ester significantly inhibited peroxynitrite accumulation in the endotoxia and CLP models at 7 h after LPS induction and postoperatively, respectively, by inhibiting 3-nitrotyrosine synthesis (P < 0.001 vs. the untreated control in both models; Fig. 4). Survival in the two sepsis models (endotoxia and CLP) was significantly enhanced by CAPE treatment. Compared with the results for untreated mice, doses of 5 and 25 mg/kg of CAPE increased the survival of endotoxemic mice by 52% and 73%, respectively, and of CLP mice by 45% and 64%, respectively (Fig. 5).

DISCUSSION

Peroxynitrite is a strong oxidant and is therefore a potent inducer of cell death (4). Its breakdown or pharmacological inhibition is therapeutically beneficial in a variety of pathological conditions, for example, vascular diseases, ischemia-reperfusion injury, circulatory shock, inflammation, pain, and neurodegeneration (4, 14). The results of the current study clearly confirm that CAPE is a potent scavenger of peroxynitrite both in vitro and in vivo. Caffeic acid phenethyl ester treatment inhibited peroxynitrite activity in cultured RAW 264.7 cells stimulated with LPS/IFN-γ. Moreover, our results

![Fig. 1. A. Effect of CAPE on peroxynitrite production from SIN-1, a synthetic peroxynitrite donor, after a 2-h incubation. The reaction was carried out in PBS (pH 7.4) in the presence of the fluorescent indicator DHR-123. Caffeic acid phenethyl ester significantly reduced DHR-123 oxidation in a dose-dependent manner, with an IC50 of 0.065 μg/mL, compared with the effects of SIN-1 alone or SIN-1 + 1400W. B. Effect of CAPE on nitrite production from SIN-1 (100 μM). After a 2-h incubation of CAPE and 1400W with SIN-1, there was no significant reduction in the amount of nitrite formed under any of the tested conditions. C. Direct effect of CAPE on the level of peroxynitrite (ONOO−) after a 15-min incubation in the presence of DHR-123. Caffeic acid phenethyl ester dose-dependently prevented peroxynitrite from oxidizing DHR-123 (IC50, 0.05 μg/mL), whereas the addition of 1400W produced the same results as the untreated control. Each value indicates the mean ± SEM value from five independent experiments. Statistical significance of Tukey multiple comparisons test was set at P < 0.001 (***).](https://shockjournal.org/article/Fig1.png)
provide evidence that, at low concentrations, CAPE acts as a scavenger of peroxynitrite because its incubation with SIN-1 resulted in significantly lower levels of this oxidant. Similarly, the direct incubation of CAPE with peroxynitrite resulted in the inhibition of DHR-123 oxidation. The antioxidant activity of CAPE also caused a reduction in the level of 3-nitrotyrosine, a marker of peroxynitrite, in two animal models of sepsis, that is, CLP and endotoxemia. In addition, in septic mice treated with CAPE, the survival rate significantly increased. Scavengers and neutralizers of peroxynitrite are required for the treatment of many diseases as previously described. Various molecules (endogenous and exogenous compounds) can scavenge and react directly or indirectly with peroxynitrite in vitro and in vivo, for example, uric acid and ebselen (4). In vitro, the specific interaction between peroxynitrite and DHR produced a highly fluorescent oxidation product without interfering with NO and superoxide, providing an excellent indicator of peroxynitrite scavenging. The potency of peroxynitrite scavenging was inversely proportional to the amount of fluorescent product. Our in vitro results demonstrated that CAPE strongly scavenged peroxynitrite.

In vivo, at physiological pH, peroxynitrite is protonated to generate peroxynitrous acid or can react with CO2 to form nitrosoperoxycarbonate. Both species can nitrate phenols such as tyrosine. The appearance of 3-nitrotyrosine is considered a biomarker of peroxynitrite-mediated protein oxidation and has been detected in organs after ischemia/reperfusion injury and sepsis (15, 16). In previous studies, scavengers and decomposition of peroxynitrite significantly reduced 3-nitrotyrosine levels in blood and organs (17). Our in vivo results showed that CAPE is a potent inhibitor of protein oxidation by peroxynitrite and reduces 3-nitrotyrosine levels in sepsis models.

**Fig. 2.** A, Effects of CAPE on the viability of RAW 264.7 cells. Cells were untreated or treated for 24 h with CAPE or with 1400W at the concentrations shown. Cell viability was assessed in an MTT assay as described in Materials and Methods. The number of surviving cells is expressed as a percentage of the control untreated cells (no addition of CAPE or 1400W). B, Effects of CAPE on the viability of RAW 264.7 cells induced with LPS/IFN-γ. Cells were untreated or treated for 1 h with CAPE or 1400W at the concentrations shown before a 24-h treatment with LPS (1 μg/mL) and IFN-γ (35 ng/mL). Cell viability was assessed in an MTT assay, as described in Material and Methods. The number of surviving cells is expressed as a percentage of the control (untreated cells, or without CAPE, 1400W, or LPS/IFN-γ). C, Effect of CAPE on peroxynitrite produced from RAW 264.7 cells induced with LPS/IFN-γ for 24 h in the presence of DHR-123. After a 1-h incubation with CAPE, DHR-123 oxidation was significantly lower (IC50, 0.4 μM) than that for the untreated control, whereas 1400W (100 μM) completely inhibited DHR-123 oxidation. D, Effect of CAPE on nitrite production in RAW 264.7 cells induced with LPS/IFN-γ for 24 h. A 1-h incubation with CAPE resulted in a significant dose-dependent inhibition of nitrite production, whereas 1400W treatment almost completely blocked nitrite production as compared with the positive control (LPS/IFN-γ). Each value indicates the mean ± SEM from five independent experiments. Statistical significance of Tukey multiple comparisons test was set at P ≤ 0.05 (**) and P ≤ 0.001 (***)

**Fig. 3.** Effect of CAPE on intracellular peroxynitrite in RAW 264.7 cells induced for 24 h with LPS/IFN-γ. Caffeic acid phenethyl ester and 1400W, at the indicated concentrations, significantly inhibited 3-nitrotyrosine production compared with the results for the untreated control (LPS/IFN-γ). Data points correspond to the mean ± SEM of three independent experiments. Tukey multiple comparison test P values (P ≤ 0.001 [***]) are compared with untreated control (LPS/IFN-γ).
During sepsis, an increase in the levels of several proinflammatory cytokines and chemokines, reactive oxygen species (ROS), COX, and iNOS is seen, accompanied by the activation of the transcriptional activator NF-κB (18). Indeed, ROS production is critical for NF-κB activation. Conversely, several antioxidants that downregulate NF-κB are protective in animal models of injury (19). Peroxynitrite activates NF-κB directly, that is, without preactivation by proinflammatory mediators such as tumor necrosis factor-α (TNF-α) and LPS, and it enhances the production of interleukin 8 and TNF-α via an NF-κB–mediated pathway in a variety of cells. The DNA-binding activity of NF-κB is also enhanced by peroxynitrite (20, 21). The mechanism underlying this interaction is thought to involve nitration of the tyrosine 181 residue of IκBα, an NF-κB inhibitor, leading to its dissociation and subsequent NF-κB activation (22). Persistent oxidative damage is caused by increased production of free radical species such as NO. These toxic compounds lead to severe cellular injury via a number of mechanisms, including the generation of reactive derivatives such as peroxynitrite (ONOO-). Among the numerous cellular targets of these agents are lipids, DNA, and proteins (23). Cellular exposure to high concentrations of peroxynitrite leads to rapid necrotic-type cell death because of acute and severe cellular energetic derangements (14, 24), whereas low concentrations of peroxynitrite result in programmed cell death (apoptosis) mediated by the activation of caspase-3, caspase-2, caspase-8, and caspase-9 (25, 26). Recent studies have demonstrated that peroxynitrite stimulates the release of mitochondrial apoptosis–inducing factor, which triggers DNA fragmentation processes (27), along with other mitochondrial proapoptotic factors. This, in turn, leads to cytochrome C–dependent apoptosis in the cytosol through the peroxynitrite–dependent oxidation of mitochondrial permeability transition pore components. The key role of peroxynitrite in promoting mitochondrial dysfunction is clearly exemplified in experimental...
sepsis, where peroxynitrite-mediated inhibition of mitochondrial respiration, in a process associated with mitochondrial protein nitration, is prevented by NOS inhibitors and Mn-porphyrin therapy (peroxynitrite decomposition) (28, 29). Furthermore, some of the physiological modulators of peroxynitrite reactivity have been shown to exert beneficial effects in animal models of inflammation and reperfusion injury (1, 5, 24, 30).

Many of the natural flavonoids found in fruits, vegetables, and teas are potent antioxidants and NF-κB inhibitors. One of the most potent lipophilic antioxidants is CAPE, which is rapidly absorbed and metabolized by plasma esterases (7, 9, 31). Caffeic acid phenethyl ester is nontoxic, readily bioavailable, and has long been used in traditional medicine. Its activities have been described in several reports and include the inhibition of lipoxigenase, protein tyrosine kinases, and lipid peroxidation (7, 9). Thus, CAPE is an antioxidant, an anti-inflammatory agent in the prevention of reperfusion injury, an immunostimulant, and an antiatherosclerotic agent. In rats, CAPE pretreatment reduces brain infarction and cerebral vasospasm, which is consistent with its defensive role against neurovascular inflammation and oxidative stress that occur secondary to injury, as demonstrated in a neonatal brain hypoxic-ischemic injury model and a cardiac ischemia reperfusion injury model (32). Caffeic acid phenethyl ester also reduces mortality and protects against organ failure during sepsis in animals (33). Our results showed that CAPE might have prevented the reduced viability caused by LPS treatment and increases survival rate in sepsis models. These results are in agreement with previous in vitro and in vivo studies demonstrating the protective effect of CAPE against cell death and multiorgan failure. The mechanism underlying CAPE-mediated cytoprotection has been investigated in several systems, both in vitro and in vivo, particularly with respect to the oxidants NO and peroxynitrite. As determined in the present study and elsewhere, CAPE protects cells against these cytotoxic molecules by scavenging peroxynitrite and inhibiting iNOS (34). Peroxynitrite scavenging, such as that performed by CAPE, completely blocked the activation of NF-κB (34). Unlike other antioxidants, CAPE specifically suppresses NF-κB binding to DNA, without affecting IkBα degradation (34). Previous studies have shown that CAPE inhibits the activation of NF-κB induced by various ROS-producing agents in human histiocyte cells and coronary artery endothelial cells (35). By inhibiting peroxynitrite formation and, therefore, NF-κB activation, CAPE prevents the downstream expression of NF-κB–dependent genes, including adhesion molecules (intracellular adhesion molecule 1 and E-selectin), cytokines (TNF-α and interleukin-1β), and iNOS (8, 9). The reduction in cytokine levels is particularly important because cytokines play a fundamental role in macrophage activation and are the first mediators of the inflammatory cascade (7, 8). Caffeic acid phenethyl ester also inhibits apoptotic cell death and prevents the proliferation and apoptosis of colorectal cancer cells and cerebellar granule neurons in vitro (8, 9). The ant apoptotic effects of CAPE rely on its ability to block ROS formation and inhibit caspase activity (7–9). Previous studies have shown that CAPE has numerous other beneficial antioxidant effects; it blocks ROS production in human neutrophils and inhibits the xanthine/xanthine oxidase system and formation of lipoxyn- genase, an additional intracellular source of free radical generation that causes extensive oxidative damage in multiple organ systems (7–9).

**CONCLUSIONS**

Caffeic acid phenethyl ester, a potent scavenger of peroxynitrite in vitro and in vivo, protects cells from death caused by free radical (NO and peroxynitrite) production during the immune response as well as protects animals from sepsis. Based on the multiple beneficial activities of CAPE, including its natural antioxidant activity, further studies should be directed at developing its pharmacological properties for the treatment of a wide range of inflammatory conditions. Thus, administration of CAPE in sepsis patients might offer a suitable new therapeutic tool for the treatment of septic shock, multiple organ failure, and other microbe-mediated diseases in humans, in whom “out-of-control” inflammation often leads to fatal outcomes.

**REFERENCES**


