Caffeic Acid Phenethyl Ester (CAPE): Scavenger of Peroxynitrite In Vitro and In Sepsis Models

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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. 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 LPS/IFN-γ, was used as an in vitro model. Conversion of 123-dihydrorhodamine (123-DHR) to its oxidation product 123-rhodamine 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, an in vivo marker of peroxynitrite. The results demonstrated that CAPE significantly improved the viability of LPS/IFN-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 due to 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 due to 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, 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’s 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, i.e., via inhibition and/or scavenging of peroxynitrite. Caffeic acid phenethyl ester (CAPE) is an active component of honeybee products and has established antimitogenic, 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 NF-κB. The additional biological effects of CAPE include the inhibition of arachidonic acid release from cell membranes and cyclooxygenase (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 in order to determine its potential as a therapeutic for inflammatory disorders.

Materials and methods

2.1. Animals

160 Male Balb/c mice weighing (20–25 g) obtained from animal experimentation unit in 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 lab 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).

4.2. Cell culture and reagents

The murine macrophage cell line RAW 264.7 (ATCC No. TIB-71, American Type Culture collection, Rockville, MD, USA) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U penicillin/mL, and 100 U streptomycin/mL in a humidified 37°C, 5% CO₂ incubator. Phenol red-free DMEM, FBS, and antibiotics (penicillin, streptomycin) were purchased from Nacalai Tesque (Kyoto, Japan). LPS, Escherichia coli 0111 B4, CAPE, the inducible NO synthase (iNOS) inhibitor 1400W, and IFN-γ were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. 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 $2 \times 10^6$ cells/well in 24-well plates and incubated for 24 h at 37°C. The cells
were subsequently washed with PBS and resuspended in fresh medium containing a range of concentrations of CAPE (0.125–1 µg/mL) or 100 µM of the iNOS inhibitor 1400W. Untreated cells were included as positive or negative controls in each experiment. After a 1-h incubation at 37°C in a 5% CO₂ atmosphere, LPS/IFN-γ was added to the cultures at concentrations of 1 µg/mL and 35 ng/mL, respectively to increase the induction of iNOS expression and produce high levels of NO, followed by a second incubation for 24 h under the above-described conditions (10). The cells were then processed to assess for viability or for NO or peroxynitrite detection as described below.

2.4. Measurement of viability

The viability of RAW 264.7 macrophages was determined in cultures that were either untreated or were treated with a combination of LPS/IFN-γ and CAPE (0.125–1 µg/mL) or the iNOS inhibitor 1400W (100 µM). Viability was measured in terms of cellular respiration as assessed by the mitochondrial-dependent reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to formazan. Briefly, the cells were cultured, stimulated with LPS, and treated with CAPE or 1400W as described above, after which 100 µL of MTT (5 mg/mL) was added to each well for 1-h incubation under the same conditions. The MTT solution was then removed, and the cells were solubilized in 200 µL DMSO with shaking for 5 min. Absorbance was measured at 550 nm using a microplate reader (GloMax®-Multi Microplate detection, Promega, Madison, WI, USA) (10). All experiments were repeated five times in triplicate.

2.5. NO assay

NO has a short half-life of only a few seconds as it is quickly converted to nitrate (NO₃⁻) and nitrite (NO₂⁻). These products can be measured using the colorimetric Griess
reaction to indirectly determine the NO concentration. RAW 264.7 cells were cultured and treated as described above, after which 100 µL of the culture was placed in a 96-well plate with an equivalent amount of the Griess reagent (50 µL of 1% sulfanilamide in 5% concentrated H₃PO₄ and 50 µL of 0.1% naphthylethylenediamine dihydrochloride in distilled water). The reaction between the Griess reagent and the NO₂ present in the supernatant yields a pink derivative that can be spectrophotometrically quantified from a standard concentration curve prepared from an NO₂ standard (10).

2.6. Dihydrorhodamine-123 oxidation assay

Three models of dihydrorhodamine-123 (DHR-123) were used in this study: incubation with SIN-1 (3-morpholinosydnonimine) as a peroxynitrite donor, incubation directly with peroxynitrite, and incubation with LPS/IFN-γ for live cell stimulation. All models have been previously described (10).

2.6.1. Dihydrorhodamine-123 oxidation using SIN-1 - The oxidation of DHR-123 in the presence of SIN-1 spontaneously releases NO and superoxide under physiological conditions. At pH 7.4, SIN-1 is converted to SIN-1A via a base-catalyzed ring opening, during which oxygen undergoes univalent reduction to the radical O₂⁻. SIN-1A then releases NO and is converted to the stable metabolite SIN-1C, while the O₂⁻ reacts with NO to form peroxynitrite (ONOO⁻). The oxidation of DHR-123 by peroxynitrite results in the formation of fluorescent rhodamine-123, which can be measured by fluorometric analysis (GloMax®-Multi Microplate) at an excitation wavelength of 460–530 nm and an emission wavelength of 530–590 nm. In experiments examining the effects of CAPE (0.125–1 µg/mL) on peroxynitrite scavenging, 100 µM SIN-1 was used and the reactions were carried out in PBS, with incubation of the samples for 2 h at 37°C (11). The amount of NO in 100 µL of the supernatant was measured as described above for the NO assay.
2.6.2. DHR-123 oxidation using peroxynitrite - The ability of peroxynitrite to oxidize DHR-123, resulting in its conversion to fluorescent rhodamine-123, was measured directly as previously described (4). Briefly, 10 µM peroxynitrite was mixed with PBS containing 20 µM DHR-123 in the presence or absence of either CAPE (0.125–1 µg/mL) or 100 µM of the iNOS inhibitor 1400W. After a 15-min incubation at room temperature, the fluorescence of the rhodamine-123 reaction product was measured (GloMax®-Multi Microplate) at an excitation wavelength of 460–530 nm and an emission wavelength of 530–590 nm.

4.6.3. DHR-123 oxidation by LPS/IFN-γ-treated RAW 264.7 cells - Cells were cultured, treated with CAPE or 1400W, and stimulated with LPS/IFN-γ, as described above for RAW 264.7 cells, but in the presence of 10 µM DHR-123. After 24 h, 100 µL of the culture suspension was removed and the amount of rhodamine-123 was determined fluorometrically; an additional 100 µL was used for NO measurement, as described above for the NO assay.

2.7. LPS-induced endotoxemia

Mice were randomly assigned to eight groups (10 mice per group) and injected i.p. 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 prior to LPS administration by i.p. 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 d following LPS administration. Viability was monitored every 12 h for 7 d.

2.8. Cecal ligation and puncture

Mice were randomly divided into eight groups (10 mice per group), and cecal ligation and puncture (CLP) was performed as described in (13) with a few modifications. Briefly, the mice were anesthetized (10/group) with ketamine (70 mg/kg, i.m. injection) and xylazine (10 mg/kg). An abdominal midline incision was then performed and the cecum was isolated. After ligation of the cecum at 7 mm from the cecal tip, distal to the ileocecal valve, the ligated cecal stump was perforated by two “through and through” punctures (21-gauge needle). The cecum was then returned to its normal position and the abdomen was closed with a suture thread. Mice were resuscitated by injecting subcutaneously 2 ml of pre warmed 0.9 saline solution using a 25G needle. To analyze the CLP, the mice in the four groups (treated and control groups) were killed at 7 h after the procedure. 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 by i.p. injection the day before and 2 h prior to the CLP procedure. Surviving mice (four groups) were further treated with CAPE or saline for 3 days after treatment. Viability was monitored every 12 h for 7 d.

2.9. Measurement of 3-nitrotyrosine levels in RAW 264.7 cells

Lysates were prepared from RAW 264.7 cells collected after treatment as described above 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% SDS) containing a protease-inhibitor cocktail,
and then centrifuged at 16,000 \( \times g \) 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 nitrotyrosine ELISA kit (Cell Biolabs, Inc, San Diego, CA), according to the manufacturer’s instructions, and normalized to the amount of protein in milligrams. Protein concentrations were measured using the Bradford method.

2.10. 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 described above.

2.11. Statistical analysis

Data were analyzed using Graphpad prism statistical software (version 6; GraphPad Software Inc., La Jolla, California). Data are expressed as the mean ± S.E.M. Tukey's 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 \( p \leq 0.05 \). The half maximal inhibitory concentration (IC\(_{50}\)) was calculated using sigmoidal dose-response non-linear regression utilizing the same software.

Results

3.1 CAPE inhibits peroxynitrite production in vitro

CAPE inhibited peroxynitrite generation from the synthetic substrate SIN-1 with an IC\(_{50}\) of 0.037 µg/mL, whereas the effect of 1400W (100 µM) was similar to that of the negative (untreated) control (Figure 1A). Neither CAPE nor 1400W (100 µM) reduced the accumulation of nitrite produced from SIN-1 (Figure 1B). Moreover, direct incubation of CAPE and peroxynitrite for 15 min inhibited DHR-123 oxidation with an IC\(_{50}\) of 0.05 µg/mL, whereas the results achieved with 1400W were the same as those with the untreated control (Figure 1C). Taken together, these
results suggest that CAPE acts by scavenging peroxynitrite rather than by inhibiting its synthesis.

3.2. CAPE inhibits intracellular peroxynitrite production

None of the doses of CAPE and 1400W used in this study were 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 (Figure 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 >88% compared with the <74% viability seen in the control cells induced with LPS/IFN-γ but untreated with CAPE (p < 0.001; Figure 2B).

Both CAPE and 1400W (100 µM) inhibited peroxynitrite production in response to the LPS/IFN-γ induction of RAW 264.7 cells (CAPE: IC_{50} = 0.4 µg/mL; 1400W: 100 µM), as shown by the reduced DHR-123 fluorescence in these cells as compared to that in the untreated cells (Figure 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 (Figure 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 reduction in the level of 3-nitrotyrosine, an intracellular marker of ONOO⁻ (p < 0.001; Figure 3).

3.3. CAPE inhibits peroxynitrite production in vivo

CAPE significantly inhibited peroxynitrite accumulation in the endotoxemia and CLP models at 7 h post-LPS induction and post-operatively, respectively, by inhibiting 3-nitrotyrosine synthesis (p < 0.001 vs. the untreated control in both models; Figure 4). Survival in the two sepsis models (endotoxemia and CLP) was significantly enhanced by CAPE treatment. Compared to 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 (Figure 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. CAPE treatment inhibited peroxynitrite activity in cultured RAW 264.7 cells stimulated with LPS/IFN-γ. Moreover, our results provide evidence that, at low concentrations, CAPE acts as a scavenger of peroxynitrite, since 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 reduction in the level of 3-nitrotyrosine, a marker of peroxynitrite, in two animal models of sepsis, i.e., 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 described above. Various molecules (endogenous and exogenous compounds) can scavenge and react directly or indirectly with peroxynitrite in vitro and 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. During sepsis, increase in the levels of several pro-inflammatory cytokines and chemokines, reactive oxygen species (ROS), cyclooxygenase (COX), and iNOS is seen, accompanied by the activation of the transcriptional activator NF-κB (18). In fact, 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 pre-activation by pro-inflammatory mediators such as tumor necrosis factor (TNF)-α and LPS, and it enhances the production of interleukin (IL)-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, due to 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 (AIF), 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). CAPE is non-toxic, readily bioavailable, and has long been used in traditional medicine. Its activities have been described in several reports and include the inhibition of lipoygenase , 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 anti-atherosclerotic 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). CAPE also reduces mortality and protects against organ failure during sepsis in animals (33). Our results showed that CAPE might have prevented the reduced viability due to 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 IκBα degradation (34). Previous studies have shown that CAPE inhibits the activation of NF-κB induced by various ROS-producing agents in human histiocytic 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 (intercellular adhesion molecule-1 and E-selectin), cytokines (TNF-α and IL-1β), and iNOS (8, 9). The reduction in cytokine levels is particularly important as cytokines play a fundamental role in macrophage activation and are the first mediators of the inflammatory cascade (7, 8). CAPE also inhibits apoptotic cell death and prevents the proliferation and apoptosis of colorectal cancer cells and cerebellar granule neurons in vitro (8, 9). The antiapoptotic 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 lipoxygenase, an additional intracellular source of free radical generation that causes extensive oxidative damage in multiple organ systems (7-9).

Conclusions

CAPE, a potent scavenger of peroxynitrite in vitro and vivo, protects cells from death due to 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.

**Declaration of competing interests**
There are no competing interests to declare.

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Figure legends

Figure 1: (A) Effect of CAPE on peroxynitrite production from SIN-1, a synthetic peroxynitrite donor, following a 2-h incubation. The reaction was carried out in PBS (pH 7.4) in the presence of the fluorescent indicator DHR-123. CAPE significantly reduced DHR-123 oxidation in a dose-dependent manner, with an IC$_{50}$ of 0.065 µg/mL, compared to 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. CAPE dose-dependently prevented peroxynitrite from oxidizing DHR-123 (IC$_{50}$ = 0.05 µg/mL), while the addition of 1400W produced the same results as the untreated control. Each value indicates the mean ± S.E.M. value from five independent experiments. Statistical significance of Tukey's multiple comparisons test was set at p ≤ 0.001 (***)

Figure 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 percent 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 prior to 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 percent 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 (IC$_{50}$ = 0.4 µg/mL) than that for the untreated control, while 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, while 1400W treatment almost completely blocked nitrite production as compared with the positive control (LPS/IFN-γ). Each value indicates the mean ± S.E.M. from five independent experiments. Statistical significance of Tukey’s multiple comparisons test was set at p ≤ 0.05 (**) and p ≤ 0.001 (***)

**Figure 3:** Effect of CAPE on intracellular peroxynitrite in RAW 264.7 cells induced for 24 h with LPS/IFN-γ. CAPE 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 ± S.E.M of three independent experiments. Tukey’s multiple comparisons test P values (p ≤ 0.001 (***) are compared with untreated control (LPS/IFN-γ).

**Figure 4:** **(A)** Effect of CAPE on serum peroxynitrite concentrations in a mouse model of endotoxemia. Mice (4 groups n =10/group) were administered LPS (3 groups) or saline (one group = control group) by ip injection and killed at 7 h. CAPE, at the indicated concentrations, was administered the day before and 2 h prior to LPS administration. Inhibition of serum 3-nitrotyrosine was significant in the treated group as compared with the untreated group (LPS). **(B)** Effect of CAPE on serum peroxynitrite concentrations in a CLP model of sepsis. Mice were (5 groups n=10/group); CLP groups (3 groups) and sham-operated (one group) received CAPE or saline by ip injection and killed at 7 h as indicated in the Methods section. The control group (normal mice) was used two times in A and B. Significant inhibition of 3-nitrotyrosine was achieved as compared with the
results for the untreated group (CLP). Each value indicates the mean ± S.E.M. Statistical significance of Tukey's multiple comparisons test was set at p ≤ 0.001 (**).

**Figure 5:** (A) CAPE is protective against LPS-induced endotoxemia. Survival curves for LPS-induced death in mice (3 group n=10/group) pretreated with vehicle alone (LPS) or CAPE (5 or 25 mg/kg). One group (n=10) as a negative control, mice were pretreated and challenged with vehicle alone (saline). Survival rates were monitored for 7 d. Kaplan-Meier analysis was used to compare survival rates. (B) CAPE is protective against the CLP model of polymicrobial sepsis. Survival curves for CLP-induced death. Mice (3 group n=10/group) were left untreated or pretreated with CAPE (CLP + CAPE 5 or 25 mg/kg), or vehicle alone (CLP). Sham-operated (Sham group n=10) mice were used as a negative control. Survival rates were monitored for 7 d. Kaplan-Meier analysis was used to compare survival rates. Statistical significance was set at p ≤ 0.05 (**) and p ≤ 0.001 (**).
Figure 1
Figure 4

A

B

Nitrotyrosine (nM)

Nitrotyrosine (nM)

Control
LPS (25 mg/kg)
LPS + CAPE (5 mg/kg)
LPS + CAPE (25 mg/kg)

Control
Sham
CLP
CLP + CAPE (5mg/kg)
CLP + CAPE (25 mg/kg)
Figure 5

A

Percent survival

Time (h)

LPS
LPS+CAPE 5 mg/kg
LPS+CAPE 25 mg/kg
Control

B

Percent survival

Time (h)

Sham
CLP
CLP+CAPE 5 mg/kg
CLP+CAPE 25 mg/kg