Effects of Caffeic Acid Phenethyl Ester on Endotoxin-Induced Cardiac Stress in Rats: A Possible Mechanism of Protection

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ABSTRACT: Endotoxins (lipopolysaccharides; LPS) are known to cause multiple organ failure, including myocardial dysfunction. The present study aimed to investigate the mechanism of caffeic acid phenethyl ester (CAPE) protection against LPS-induced cardiac stress. Rats were allocated into three groups; group 1 served as a normal control group, group 2 (LPS) received a single intraperitoneal injection of LPS (10 mg/kg), group 3 (LPS + CAPE) was injected intraperitoneally with CAPE (10 mg/kg/day; solubilized in saline containing 20% tween 20) throughout a period of 10 days prior to LPS injection. Rats were maintained 4 h before sacrifice. Caffeic acid phenethyl ester pretreatment normalized LPS-enhanced activities of serum creatine kinase (CK) and lactate dehydrogenase (LDH) as well as glutathione peroxidase (GPx), and myeloperoxidase (MPO) in cardiac tissue. A significant reduction of the elevated levels of serum tumor necrosis factor-alpha (TNF-α) as well as serum and cardiac nitrite/nitrate (NO_x) was achieved after CAPE pretreatment. CAPE also restored malondialdelyde (MDA), reduced glutathione (GSH), and cytosolic calcium (Ca^{2+}) levels in the heart. A marked induction of cardiac heme oxygenase-1 (HO-1) protein level was detected in CAPE-pretreated group. Whereas, LPS-induced reduction of adenosine triphosphate (ATP) and phosphocreatine (PCr) levels was insignificantly changed. Conclusively, the early treatment with CAPE maintained antioxidant defences, reduced oxidative injury, cytokine damage, and inflammation but did not markedly improve energy status in cardiac tissue. The beneficial effect of CAPE might be mediated, at least in part, by the superinduction of HO-1. © 2010 Wiley Periodicals, Inc. J Biochem Mol Toxicol 25:84–94, 2011; View this article online at wileyonlinelibrary.com. DOI 10:1002/jbt.20362

KEYWORDS: **Endotoxins; Cardiac stress; Caffeic acid phenethyl ester; Neutrophil infiltration; NO; TNF-α; HO-1; Calcium; Energy status**

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INTRODUCTION

Over the last few decades, the incidence of heart diseases was progressively increased, contributing 16% of all registered death (Health statistics and health information systems, WHO, 2009). Bacteria or endotoxins are leading and growing causes of cardiovascular failure [1]. Diverse molecular mechanisms of inflammation and cellular damage were involved during endotoxin-induced cardiac stress [2]. Endotoxins (lipopolysaccharides; LPS) initiate a sequence of cellular events that lead to reversible or irreversible damage to cardiomyocytes [3,4]. Impairment of intracellular calcium homeostasis, alterations of excitation/contraction coupling, and enhanced programmed cell death or apoptosis were involved [5].

Additionally, LPS bind to cell membrane proteins of specific cell membrane receptors of different cell types including neutrophils, macrophages, and endothelial cells and thus induce the release of a number of cytokines and mediators. Among them, tumor necrosis factor–alpha (TNF-α), interleukins such as IL-1, IL-6, IL-8 and oxygen free radicals, nitric oxide (NO), and arachidonic acid metabolites are the most important ones in the pathophysiology of endotoxin-induced acute injury [6,7].

Caffeic acid phenethyl ester (CAPE) is an active component of honey bee propolis extracts and has been used in folk medicine for many years. Being an aryl ester, CAPE was subjected to blood esterase degradation. It was distributed extensively into rat tissues and eliminated rapidly as indicated by its high values of volume of distribution and short elimination half-life after systemic administration [8]. Caffeic acid phenethyl ester was known to have antioxidant, anti-inflammatory, antimicrobial, immunomodulatory, and antineoblastic effects [9–11]. It was effective in ameliorating LPS-induced multiple organs failure such as lung injury [12], testicular injury [13], liver injury [14],

brain disorders [15] as well as ischemia-reperfusion injury in rat kidneys [16]. It has been shown to inhibit 5-lipooxygenase catalyzed-oxygenation of linoleic acid and arachidonic acid and to potently induce the inflammatory cell apoptosis through a glucocorticoid receptor independent mechanism [17–19]. Caffeic acid phenethyl ester effectively downregulates a variety of proinflammatory cytokines and mediators by inhibition of the transcription of the nuclear factor-Kappa (NF-κB) [20]. At a concentration of 10 μM, CAPE completely blocks the production of reactive oxygen species (ROS) in human neutrophils and xanthine/xanthine oxidase system [21]. The balance between the production and the catabolism of oxidants by cells and tissues is critical for maintenance of the biological integrity of the tissues [22].

Considering the beneficial effects of CAPE, the present study was designed to investigate the mechanism of CAPE protection in LPS-induced cardiac stress in rats. Serum creatine kinase (CK) and lactate dehydrogenase (LDH) were estimated as a measure of cardiac injury. Cardiac levels of malondialdelyde (MDA) and reduced glutathione (GSH) along with glutathione peroxidase (GPx) activity and heme oxygenase-1 (HO-1) protein level (oxidative stress biomarkers) were also determined. In addition, cardiac myeloperoxidase (MPO) activity (a neutrophil infiltration index), serum and cardiac nitrite/nitrate (NO_x) levels (measures of nitric oxide production), as well as TNF-α (a mediator of inflammation) were assessed. Cytosolic calcium (Ca^{2+}) level together with adenosine triphosphate (ATP) and phosphocreatine (PCr) contents (high energy phosphate (HEP) compounds) were estimated to evaluate cardiac contractility and energy status, respectively.

MATERIALS AND METHODS

Materials

Animals

Adult male Wistar albino rats, weighing 170–200 g, were used in this study. They were obtained from the farm of the National Institute for Vaccination, Helwan, Egypt. They were kept under the controlled environmental and nutritional conditions throughout the experimental work.

Chemicals

Endotoxin (lipopolysaccharide (LPS) from *Escherichia coli* serotype 055: B5), CAPE and all biochemical reagents and coenzymes were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MD). Other chemicals were from Analar grade or from the purest grade available. Rat TNF-α and rat HO-1 enzyme-linked immunosorbent assay (ELISA) kits were provided from Assaypro (USA) and Assay Design (Ann Arbor, MI), respectively.

Experimental Design

After 1 week acclimatization period, rats were divided into three experimental groups. Group 1 received a single intraperitoneal dose of saline and served as normal control. Group 2 received a single intraperitoneal dose of LPS (10 mg/kg, prepared in physiological saline) and served as stressed group [1]. Group 3 received a daily intraperitoneal dose of CAPE (10 mg/kg, solubilized in saline containing 20% tween 20) throughout a period of 10 days prior to LPS injection [23,24].

Four hours after LPS injection, rats were killed by decapitation. Blood samples were collected into dry centrifuge tubes and centrifuged at 600× *g* for 15 min. The obtained serum was used for estimation of CK and LDH activities as well as TNF-α and total nitrite/nitrate (NO_x) levels.

Meanwhile, the abdominal cavity of rats was dissected immediately, and the heart was weighed and homogenized in 10 volumes of ice-cold bidistilled water using an UltraTurrax homogenizer. The resultant homogenate (10% w/v) was divided and prepared for the determination of the following: MDA, GSH, HO-1, total nitrite/nitrate (NO_x), calcium (Ca^{2+}), and HEP levels as well as GPx and MPO activities.

Biochemical Assays

Assessment of Cardiac Enzymes

Serum creatine kinase (CK) and LDH activities were assayed using commercially available kits (Stanbio, Texas) based on the method of Rosalki [25] and Buhl and Jackson [26]. Briefly, CK and LDH specifically catalyze the oxidation of lactate to pyruvate and transphosphorylation of ADP to ATP, respectively, with subsequent formation of NADH. The increase in absorbance per min, as a function of NADH production, was measured spectrophotometrically at 340 nm and was proportional to the enzymatic activity.

Cardiac Malondialdehyde

Malondialdelyde level as index of lipid peroxidation was measured according to the method of Uchiyama and Mihara [27]. Malondialdelyde reacts with thiobarbituric acid (TBA) in acid medium giving a pink-colored complex that can be measured spectrophotometrically. Briefly, 1 mL of the homogenate

was mixed with an equal volume of 2.3% KCL solution and centrifuged at 600× *g* at 4°C for 15 min. 0.5 mL of the resulting supernatant was mixed with 3 mL *o*-phosphoric acid (1%), 1 mL TBA solution (0.67%), and heated for 45 min at 100°C. After cooling, 4 mL *n*-butanol were added, mixed vigorously, and centrifuged. The color of the separated butanol layer was measured at 520 nm and 535 nm using 1, 1, 3, 3- tetramethoxypropane as standard.

Cardiac Glutathione

Cardiac GSH was determined as protein-free sulfhydryl content using 5,5-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) that is reduced by the SH-group in GSH to form 5-thio-2-nitrobenzoic acid, which has a stable yellow color measured colorimetrically at 412 nm [28]. In detail, protein precipitation was attained by mixing equal volumes of 10% aqueous homogenate and 7.5% sulfosalicylic acid followed by centrifugation at 600× *g* for 15 min at 4°C [29]. To 0.5 mL of the resulting supernatant, 2 mL phosphate buffer (0.3 M, pH 7.7) and 0.25 mL Ellman's reagent (40 mg% in 1% Na citrate) were added in a microcuvette and the absorbance was measured at 412 nm.

Cardiac Glutathione Peroxidase

The activity of cardiac GPx was determined in the cytosolic fraction of the heart prepared by mixing equal volumes of 10% aqueous homogenate and tris-EDTA buffer, pH 7.6 (100 mM tris and 0.2 mM EDTA) followed by centrifugation at 105,000× *g* at 4°C for 15 min [30]. The resulting supernatant was used for the determination of GPx and protein content.

Cardiac GPx was determined by following the rate of oxidation of GSH by hydrogen peroxide using NADPH in the presence of GPx. The reaction mixture for four tests was prepared as follows: To 2.59 mL phosphate buffer, pH 7 (0.05 M $KH_2PO_4/Na_2HPO_4 \cdot 2H_2O$ and 0.005 M EDTA), the following solutions were added in turn: 0.1 mL NADPH solution (0.0084 M), one unit of GR, 0.01 mL NaN_3 solution (1.125 M), and finally 0.1 mL GSH solution (0.15 M). Cytosolic fraction (15 μL completed to 25 μL with phosphate buffer, pH 7) was mixed with 0.7 mL of the reaction mixture in a microquartz cuvette. Finally, the enzymatic reaction was initiated by the addition of 25 μL of H_2O_2 (0.0022 M). The conversion of NADPH to $NADP^+$ was followed by recording the decrease in absorbance at 340 nm for 3 min [31].

Cardiac Heme Oxygenase-1 Protein Level

Tissue HO-1 was extracted according to the method described by Takeda et al. [32] and Morsi et al. [33]. 1 mL of 10% aqueous homogenate was mixed with 2.5 mL of tris-HCL buffer, pH 7.6 (10 mM containing 250 mM sucrose and 0.4 mM phenyl methyl sulfonyl fluoride). To separate the mitochondrial pellet, the homogenate was centrifuged at 800× *g* for 10 min and then at 13,500× *g* for 20 min at 4°C. The resulting supernatant was used for the assay of HO-1 protein level. HO-1expression was quantified by means of a commercially available rat HO-1 enzyme-linked immunosorbent assay (ELISA) kit, according to the method of Katori et al. [34]. In detail, 100 μL of standard and samples (tissue samples were diluted 10 times with sample diluents) were added per well. The wells were covered with an adhesive plate sealer and incubated for 1 h. 300 μL of wash buffer was added to all wells four times. The plate was inverted to decant the liquid. 100 μL of the rat HO-1 antibody was added to each well and incubated for 1 h. Again, the plate was washed as described previously. 100 μL of the rat HO-1 conjugate was added to each well and incubated for 30 min. The plate was washed again. Finally, 100 μL of tetramethyl benzidine (TMB) substrate was added to each well and incubated for 15 min. A blue color developed in proportion to the amount of captured HO-1. 100 μL acid stop solution was added to convert the endpoint color to yellow. The intensity of the color was measured at 450 nm using a microtitration plate reader. HO-1 protein level was determined by means of a standard curve constructed using serial dilutions of recombinant rat HO-1 protein.

Cardiac Myeloperoxidase

Tissue MPO was extracted according to the method described by Mancuso et al. [35]. In detail, 0.5 mL of 10% aqueous homogenate was mixed with equal volume of ice-cold potassium phosphate buffer, pH 6 (100 mM), containing 1% hexadecyltrimethylammonium bromide (HTAB), and sonicated for 1 min in an ice-bath. The tubes were centrifuged at 10,000× *g* for 20 min at 4°C. The resulting supernatant was used for the estimation of MPO activity and protein content.

MPO activity was measured according to the method of Renlund et al. [36]. In a macrocuvette, 0.1 mL of supernatant was combined with 2.9 mL of 50 mM phosphate buffer, pH 6, containing 0.167 mg/mL *o*-dianisidine hydrochloride and 0.0005% H_2O_2. The change in absorbance at 460 nm was measured at 1-min intervals for 3 min. Myeloperoxidase activity was determined by means of a standard curve constructed using serial dilutions of horseradish peroxidase. One unit of MPO activity was defined as the amount of enzyme that degrades 1 μmol of peroxide per min at 25°C.

Serum Tumor Necrosis Factor-Alpha

Serum TNF-α level was quantified by means of commercially available Assaymax rat TNF-α ELISA kit [37]. A murine monoclonal antibody specific for rat TNF-α has been precoated onto a microplate. Tumor necrosis factor-alpha in standards and samples was sandwiched by the immobilized antibody and a biotinylated polyclonal antibody specific for rat TNF-α, which was recognized by a streptavidin-peroxidase conjugate. All unbound material was then washed away and a peroxidase enzyme substrate was added. The color development was stopped with an acid stop solution, which converts the endpoint color to yellow. The intensity of the color was measured at 450 nm using a microtitration plate reader. In detail, 50 μL of standard and samples were added per well. The wells were covered with a sealing tape and incubated for 2 h. 200 μL of wash buffer concentrate was added five times. The plate was inverted to decant the liquid at each step. 50 μL of biotinylated TNF-α antibody was added to each well and incubated for 2 h. Again, 200 μL of wash buffer concentrate was added five times. 50 μL of streptavidin-peroxidase conjugate was added per well and incubated for 30 min. 200 μL of wash buffer concentrate was added five times again. 50 μL of chromogen substrate (tetramethyl benzidine) was added per well and incubated for 10 min. 50 μL of acid stop solution (0.5 N HCL) was then added to each well. Tumor necrosis factor-alpha level was determined by means of a standard curve constructed using serial dilutions of recombinant rat TNF-α.

Serum and Cardiac Total Nitrite/Nitrate

Nitric oxide was measured in serum and tissues according to the method described by Miranda et al. [38] with modification, using zinc sulfate instead of ethanol for protein precipitation. The method employs the reduction of any nitrate to nitrite by vanadium chloride followed by the detection of total nitrite by Griess reagent. The Griess reaction entails formation of a chromophore from the diazotization of sulfanilamide by acidic nitrite, followed by coupling with bicyclic amines such as *N*-1-(naphthyl) ethylene diamine (NEDD). The formed azo derivative can be measured colorimetry at 540 nm. In detail, 0.5 mL each of supernatant, resulting from centrifugation of 10% aqueous homogenate at 21,000× *g* for 15 min, and of serum were treated with 50 μL 30% $ZnSO_4$ for protein precipitation. Then, precipitated protein was removed by centrifugation at 21,000× *g* for 15 min. The resulting supernatant (100 μL for tissue and 150 μL for serum) was diluted to 300 μL with water and treated with 300 μL VCL_3 (0.8% in 1 M HCL), followed by rapid addition of 150 μL sulfanilamide (2% in 5% HCL) followed by 150 μL NEDD (0.1%). The mixtures were then incubated at 37°C for 30 min then cooled. The absorbance of the formed pink chromophore was measured at 540 nm.

Cardiac Calcium

It was assessed in the cytosolic fraction by the atomic absorption technique [39] using a Unicam 929 atomic absorption spectrophotometer (flame type: Air-C_2H_2, fuel flow: 1.4 L/min). The method depends on the fact that free atoms of an element absorb light of a very specific wave length. The amount of light absorbed increases as the number of atoms in the light path increases. Ca^{2+} level was determined by measuring the absorbance at 422 nm.

Cardiac High Energy Phosphate

Simultaneous determination of HEP concentrations (ATP and PCr) in myocardial tissue was achieved by high-performance liquid chromatography (HPLC) according to the method of Teerlink et al. [40], with modification using isocratic elution instead of gradient elution. In detail, 1 mL of the homogenate was mixed with 0.3 mL of ice-cold 4.8 M perchloric acid for protein precipitation. The tubes were then centrifuged at 600× *g* for 15 min at 4°C. 1 mL of the supernatant was mixed with 0.33 mL 2 M $KHCO_3$ and left to allow CO_2 to come off. Again the tubes were centrifuged at 600× *g* for 10 min at 4°C to remove potassium perchlorate precipitates, while the supernatants were used for the estimation of ATP and PCr contents [41].

Analysis was performed by reversed-phase chromatography on a C18 column containing 3 μm particles, employing isocratic elution and UV detection at 254 nm. Separation was achieved using KH_2PO_4 buffer (0.2 M, pH 5) and water/acetonitrile/methanol (50/25/25, v/v/v), in a ratio of 97:3, as a mobile phase (flow rate: 2 mL/min). The peak areas of PCr and ATP were quantified using Shimadzu CR501 Chromatopac integrator and Glass-VP5 integration software with reference to the external standard.

Determination of Protein

The protein content of different fractions, resulting from ultracentrifugation of the homogenate, was determined using the method of Lowry et al. [42], with bovine serum albumin as standard.

Histopathological Examination

Heart specimens were fixed with 10% formaldehyde and processed routinely for embedding in

TABLE 1. Effect of CAPE Pretreatment on Serum CK and LDH Activities in Endotoxin-Stressed Rats

Parameters	*CK (U/L)*	*LDH (U/L)*
Groups		
Control	222.73 ± 9.97	175.81 ± 3.33
LPS	466.87 ± 29.9[a]	456.59 ± 23.02[a]
LPS + CAPE	226.86 ± 19.24[b]	181.34 ± 10.93[b]

Values are expressed as mean ± SE of 8 animals.
[a]Significant difference from control group at $p < 0.05$.
[b]Significant difference from LPS group at $p < 0.05$.

paraffin. Sections of 5 μm were stained with hematoxylin and eosin (H&E) and examined under the light microscope.

Statistical Analysis

Results were expressed as mean ± standard error (SE). Differences among means were tested for statistical significance by one-way analysis of variance (ANOVA). When differences were significant, Tukey-Kramer's test was used for multiple comparisons between groups. Statistical significance was considered when $p < 0.05$.

RESULTS

Biochemical Findings

The results of the present study showed a significant increase by almost 2- to 2.5-fold in serum CK and LDH activities of LPS group compared to normal control group, respectively. Normalization of both enzyme activities was attained following CAPE pretreatment (Table 1).

As shown in Table 2, both GPx activity and MDA level in cardiac tissue were significantly increased in LPS group, reaching 176 and 193% respectively of the normal control. Meanwhile, HO-1 protein level was markedly elevated by almost 2.5-fold regarding the normal value. On the other hand, LPS group exhibited a significant reduction in GSH level by 60% as compared with the control group. A restoration of oxidative stress biomarkers (GPx, MDA and GSH) along with a substantial induction of HO-1 by 51% was evident in CAPE-pretreated group as compared to the LPS-stressed group.

Significant increments in serum TNF-α and NO_x levels were observed in LPS group as compared to normal values. Caffeic acid phenethyl ester-pretreated rats showed significant decreases by 19% in both TNF-α and tissue NO_x compared to the LPS-stressed group. Meanwhile, serum NO_x was significantly decreased by only 11% compared to the stressed rats. Myeloperoxidase enzyme activity, as index of neutrophil infiltration, was markedly elevated in LPS group by about threefold regarding the normal value. This indicates that there was a continuous neutrophil influx in response to injury in rats (Figure 1C). Caffeic acid phenethyl ester restored MPO activity as compared to LPS group (Table 3).

Data given in Table 4 revealed that a marked 1.5-fold increase in Ca^{2+} level was associated with LPS group as compared to normal rats. On the other hand, ATP and PCr levels were significantly decreased

TABLE 2. Effect of CAPE Pretreatment on Cardiac Levels of MDA and GSH, GPx Activity as well as HO-l Protein Level in Endotoxin-Stressed Rats

Parameters	*MDA (nmol/g tissue)*	*GSH (μmol/g tissue)*	*GPx (mU/mg protein)*	*HO-l (ng/mg tissue)*
Groups				
Control	43.1 ± 1.6	2.63 ± 0.1	137.4 ± 4.97	28.17 ± 1.11
LPS	83.6 ± 3.6[a]	1.05 ± 0.08[a]	242.2 ± 16.3[a]	68.55 ± 3.56[a]
LPS + CAPE	44.1 ± 2.34[b]	2.57 ± 0.14[b]	158.2 ± 12.3[b]	103.38 ± 8.1[ab]

Values are expressed as mean ± SE of 8 animals.
[a]Significant difference from control group at $p < 0.05$.
[b]Significant difference from LPS group at $p < 0.05$.

TABLE 3. Effect of CAPE Pretreatment on MPO Activity, Serum TNF-α as well as Serum and Cardiac NO_x Levels in Endotoxin-Stressed Rats

Parameters	*MPO (U/mg protein)*	*TNF-α (pg/mL)*	*NO_x (μM)*	*NO_x (μmol/g tissue)*
Groups				
Control	0.69 ± 0.04	3.99 ± 0.23	28.7 ± 0.84	0.23 ± 0.01
LPS	2.21 ± 0.08[a]	10.96 ± 0.6[a]	71.9 ± 2.2[a]	0.68 ± 0.03[a]
LPS + CAPE	0.76 ± 0.05[b]	8.92 ± 0.62[ab]	64.1 ± 2.35[ab]	0.55 ± 0.03[ab]

Values are expressed as mean ± SE of 8 animals.
[a]Significant difference from control group at $p < 0.05$.
[b]Significant difference from LPS group at $p < 0.05$.

TABLE 4. Effect of CAPE Pretreatment on Cardiac Levels of Ca^{2+}, ATP, and PCr as well as their Ratio in Endotoxin-Stressed Rats

Parameters	*Ca^{2+} (μg/g tissue)*	*ATP (μmol/g tissue)*	*PCr (μmol/g tissue)*	*ATP/PCr ratio*
Groups				
Control	78.5 ± 4.23	13.5 ± 0.29	31.3 ± 0.44	0.43 ± 0.004
LPS	110.5 ± 3.9^a	5.78 ± 0.33^a	25.7 ± 0.73^a	0.23 ± 0.014^a
LPS + CAPE	77.2 ± 5.8^b	6.93 ± 0.21^a	28.17 ± 0.56^a	0.25 ± 0.011^a

Values are expressed as mean ± SE of 8 animals.
[a]Significant difference from control group at $p < 0.05$.
[b]Significant difference from LPS group at $p < 0.05$.

reaching 42 and 82% relative to the normal level, respectively. This effect resulted in a significant reduction of their ratio by about two-fold compared to normal values. Caffeic acid phenethyl ester pretreatment normalized Ca^{2+} level, whereas it failed to significantly modulate the decreased levels of HEP compounds.

Histopathological Findings

As shown in Figure 1, the cardiac muscle fibers of the control sections revealed no histopathological alterations. Fibers are grouped in bundles with connective tissues in between. Blood capillaries are found in

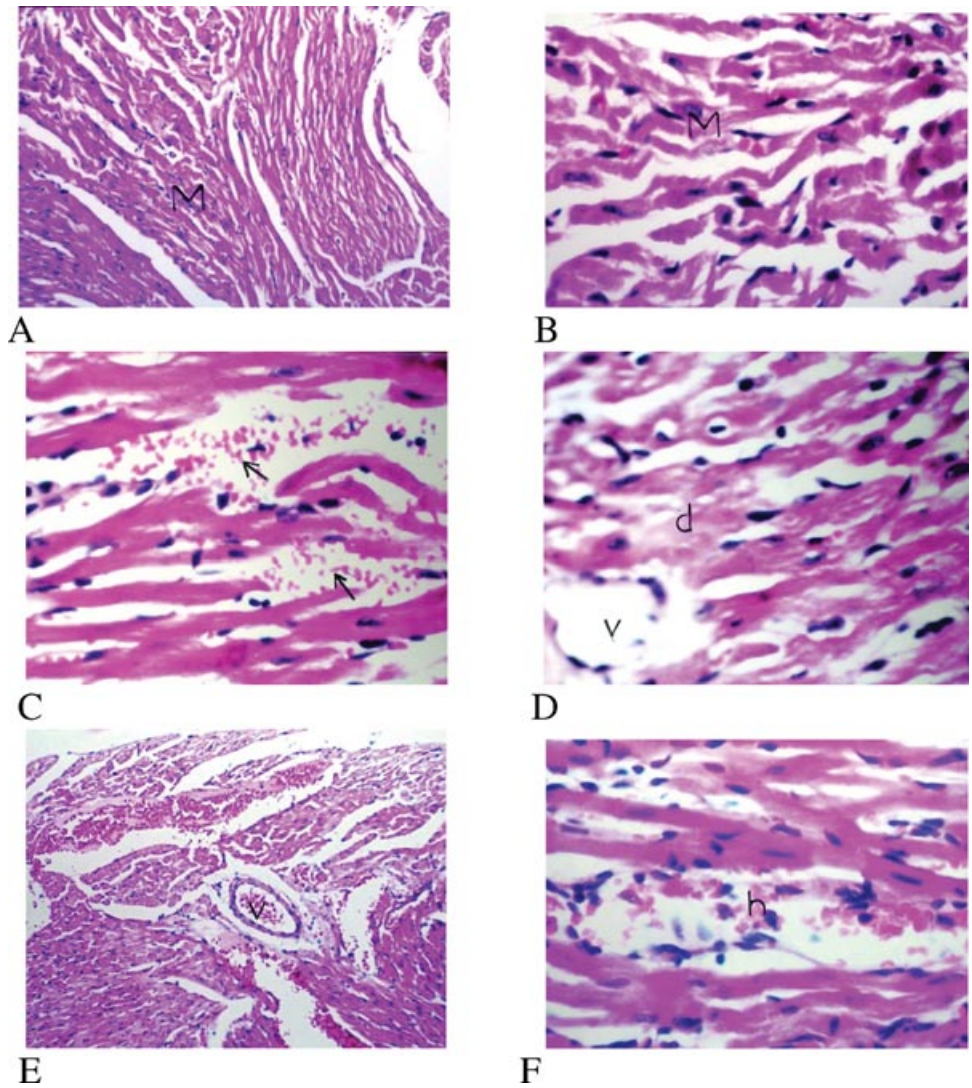

FIGURE 1. A photomicrograph of cardiac muscle fibers (A&B) control group (H&E, 40 × and 160 × respectively), (C&D) LPS-treated group (H&E 160 ×), (E&F) LPS + CAPE group (H&E, 40 × and 64 × respectively).

connective tissues and between the cardiac fibers. Each muscle fiber has an acidophilic cytoplasm and a central nucleus (A, B). In contrast, the animals receiving LPS were greatly affected. Focal inflammatory cell infiltration was noticed in between the cardiac bundles associated with dilatation in the blood vessels. Focal hemorrhages, swelling, edema, and degeneration were detected in between the myocardial bundles (C, D). Administration of CAPE showed mild congestion in the blood vessels with mild perivascular edema and focal hemorrhages in between the bundles (E, F).

DISCUSSION

Persistent oxidant damage caused by the increased production of free radical species along with recurrent inflammation triggered by cytokines characterizes the development of numerous pathologies [43]. Investigators have suggested that this oxidative damage may be a major cause of organ failure and mortality associated with endotoxemia [44] and that the administration of antioxidants may be an adjuvant to conventional therapy in its management [45]. According to previous studies, caffeic acid was a superior antioxidant compared to *p*-coumaric and ferulic acids in inhibiting oxidation of low-density lipoprotein [46] and also in quenching radicals [47]. It has been demonstrated that CAPE has antioxidant, antiinflammatory, immunomodulatory, and anticarcinogenic properties [16]. The present study was thus designed to investigate the efficacy of CAPE pretreatment in ameliorating endotoxin-induced cardiac stress in rats. As shown in Table 1, CAPE pretreatment significantly protected rats from LPS-induced elevated CK and LDH levels. The epidemiological study that relates the low incidence of heart diseases and the generous intake of food and beverage–containing phenolic compounds strongly supports our finding [48].

Assessments of lipid peroxidation, GSH content as well other antioxidant enzymes in cardiac tissue have been always used as markers for tissue injury and oxidative stress. Herein, cardiotoxicity and oxidative damage induced by LPS are manifested by a significant increase in cardiac GPx activity and MDA level along with a significant decrease of GSH content. The elevated GPx activity of LPS group implied a reduced redox status (GSH/GSSG ratio) and might be in order to guard against the encountered oxidative stress. Caffeic acid phenethyl ester pretreatment restored GPx activity (Table 2). The same oxidative trend was obtained by Mohamadin et al. [49] in attenuation of oxidative stress induced by hyperthyroidizm in rats treated with CAPE.

Primary targets of ROS attack are the polyunsaturated fatty acids in the membrane lipids, causing lipid peroxidation which may lead to disorganization of cell structure and function. The heart is highly susceptible to oxidative stress due to its inherent decreased detoxifying natural antioxidants [50]. The association between elevated cardiac MDA and lowered cardiac GSH levels, found in the present study, strongly proved the oxidative damage caused by LPS. This is in agreement with a previous study [51]. Pretreatment with CAPE has markedly modulated the oxidative damage induced by LPS through its ability to restore GSH and MDA levels. The 3,4-dihydroxyl configuration on the catechol ring of CAPE and similar polyphenolic compounds provides the key functional group for their free radical scavenger activity [52]. Caffeic acid phenethyl ester was reported to neutralize the toxicity of the hydroxyl radical, singlet oxygen, and possibly, the peroxyl radical and the superoxide anion [13]. Caffeic acid phenethyl ester is also effective in protecting nuclear DNA, membrane lipids and, presumably, cytosolic proteins from oxidative damage [16]. Thus, it is possible that the interference of CAPE with free radicals generation is related to a decline of oxidative stress and consequently lipid peroxidation in LPS-stressed rats. Another explanation of this statistically significant increase in GSH levels is the effect of CAPE upon the enzymes involved in GSH synthesis [53]. Singhal et al. [54] have demonstrated that low concentrations of polyphenolics can increase the activity of γ-glutamyl cysteinyl synthetase and other GSH-linked detoxifying enzymes, whereby maintain the level of GSH during oxidative stress.

Heme oxygenase-1 (HO-l) is a ubiquitous and redox-sensitive inducible stress protein [55]. HO-1 catalyzes the degradation of heme to generate carbon monoxide, free ferrous iron, and biliverdin; the latter is rapidly converted to bilirubin by biliverdin reductase [56]. A substantial body of evidence demonstrates that increased carbon monoxide and bilirubin contribute to modulate important physiological processes within the cardiovascular, immune, and nervous systems [57]. Lipopolysaccharides (LPS) were reported to induce HO-1 expression in cultured vascular smooth muscle cells and several organs of endotoxemic rats [58], suggesting that HO-1 may be involved in the pathogenesis of endotoxic shock. Results of the present study confirmed these previous studies. Interestingly, CAPE pretreatment markedly induced HO-1 as compared to LPS group. The major signaling transduction involved in HO-1 induction by polyphenolic compounds was attributed to the activation of NF-E_2 related factor-2 (Nrf2). Under normal conditions, Nrf2 is inactive in cytoplasm because it is bound to keap1. It has been suggested that polyphenolic antioxidants

interact with the thiol groups of keap1, which releases Nrf2 from Nrf2/keap1 complex. The free Nrf2 is subsequently translocated into the nucleus, binds to the antioxidant response element (ARE) and accelerates the transcriptions of HO-1 [59]. Thus, the overall concept emerging from this and other studies is that the induction of HO-1 is an essential step in the cellular adaptation to stress inflicted by pathological events.

The observed increase in cardiac MPO activity in endotoxin-treated rats is an indication of the injurious state as a result of neutrophil infiltration. When neutrophils are stimulated, they release MPO in phagosomes and extracellular space. This enzyme catalyzes the formation of hypochlorus acid (HOCl), a toxic agent for cells initiating oxidative injury [60]. Caffeic acid phenethyl ester significantly attenuated neutrophil infiltration as evidenced by the remarkable reduction in MPO activity. Such effect has been related to the scavenging of HOCl by CAPE [21].

Additionally, enhanced neutrophil infiltration might be attributed to the reported increases in TNF-α in endotoxin-induced cardiac injury [3]. The inhibitory effect of CAPE on TNF-α production (Table 3) has been reported previously [20]. Caffeic acid phenethyl ester is known to be a specific inhibitor of the transcription factor NF-κB, one of the most ubiquitous transcription factors that regulate gene expression involved in cellular proliferation, inflammatory responses and cell adhesion. Inhibition of NF-κB consecutively resulted in down regulation of many proinflammatory enzymes and cytokines and showed potential for therapeutic interventions [61]. Hence, suppression of MPO activity was attributed to the reported reduction of TNF-α by CAPE that in turn would inhibit its stimulatory effect on neutrophil adhesion to endothelium [62].

Nitric oxide is associated with inflammatory reaction and is produced by inducible nitric oxide synthase (iNOS) in certain cells activated by various proinflammatory agents such as lipopolysaccharide (LPS), TNF, interleukin-1 (IL-1), and interferon-γ (INF-γ). Excessive production of NO has been suggested to contribute to loss of peripheral vascular tone, depression of myocardial contractility, and hypotension [63]. Ishiwata et al. [64] reported that the reaction products of iNOS increased in vascular endothelial cells, vascular smooth muscle cells, and cardiomyocytes 4 h after endotoxin administration. Increased serum NO_x might represent the summation of NOS activity throughout the time period studied, because NO secreted by these cells rapidly decomposes into more stable products. In agreement with Nagaoka et al. [65] and Atik et al. [66], CAPE pretreatment significantly reduced the elevated levels of NO_x (Table 3). This NO inhibitory effect of CAPE might be related to its ability to block the activation of iNOS. Montpied et al. [15] attributed the CAPE effect on NO production to the decrease of NF-κB binding to NOS-2 gene promoter. Interestingly, ROS have been shown to decrease c-AMP responsive element binding protein (CREB) activity, a known antiinflammatory substance involved in the cellular death induced by ROS and strongly affects NOS-2 expression [67]. Caffeic acid phenethyl ester, by decreasing ROS due to its antioxidant properties, might increase the CREB nuclear concentration that in turn will suppress NOS-2 expression leading to a lowering of NO synthesis [68].

In the present study, LPS induced a significant increase in cytosolic Ca^{2+} concentration. Liu and Wu [69] suggested that impairment of Ca^{2+} transport was due to defective phosphorylation of the sarcolemma, reduced number of Ca^{2+} pumps, and an altered membrane phospholipase A activation after endotoxin treatment. Moreover, Fukui et al. [70] demonstrated that endotoxin induces structural changes and decreases Ca^{2+}-ATPase activity in vascular endothelial cells and cardiomyocytes. As shown in Table 4, CAPE pretreatment inhibited the increase in cytosolic Ca^{2+} concentration triggered by endotoxin. The exact mechanisms of the effect of CAPE on abnormal cardiac Ca^{2+} level are not fully known. Caffeic acid phenethyl ester has been reported to modulate ion channels. Caffeic acid phenethyl ester increased the Ca^{2+}-activated K^+ current and slightly suppressed the voltage-dependent L-type Ca^{2+} current [71]. Another report stated that CAPE by its ability to quench ROS and to preserve oxidation of SH groups, essential for proper functioning of the membrane bound enzymes, could attenuate depression in sarcoplasmic reticulum Ca^{2+} transport as well as heart dysfunction associated with LPS group [72]. Additionally, high concentrations of CAPE, as previously demonstrated, were able to abolish intracellular Ca^{2+} increase triggered by phenylephrine or by KCL in aortic smooth muscle cells, and this effect was preserved after removal of extracellular Ca^{2+}, confirming an effect of CAPE on both Ca^{2+} influx and Ca^{2+} release from intracellular stores [73].

Quantification of creatine compounds and adenine nucleotides in myocardial tissue has great importance for determining changes in its energetic state [74]. A significant reduction of ATP and PCr contents as well as ATP/PCr ratio was observed in endotoxin-stressed rats compared to normal control group. It is likely that the reduced energetic state in response to endotoxemia leads to a reduced maximal rate of sustainable ATP turnover in the heart. That is, the range of ATP turnover rates over which the creatine kinase reaction can temporally buffer ATP may be reduced by virtue of the lower PCr and adenine nucleotide pools [75]. In the current study, CAPE did not significantly alter the levels of ATP and PCr compared to LPS group (Table 4). This could be explained on the basis that elevated levels of

these HEP compounds were masked by their increased consumption in CAPE-stimulated ion channel activity.

Reductions in ATP and PCr by increased NO were in agreement with other studies [76,77]. Nitric oxide has been recognized to affect cellular respiration. Indeed, NO reversibly inhibits O_2 uptake by binding to cytochrome oxidase in rat skeletal muscle [78] and heart mitochondria [79], implying a likely transitory impairement of ATP availability for muscle contraction. In addition, NO reacts with superoxide anion ($O_2^{-\cdot}$) to form peroxy nitrite ($ONOO^-$), which irreversibly inhibits several mitochondrial enzymes such as aconitase, NADH, and succinate dehydrogenases, and superoxide dismutase [80]. Thus, the large quantities of NO synthesized by iNOS during endotoxemia could initiate a sequence of reactions leading to nitration of mitochondrial proteins, mitochondrial dysfunction, and inhibition of ATP production as observed in the current study.

In conclusion, the study indicates that CAPE is beneficial as a protective agent against LPS-induced cardiac stress. The protection is probably due to multiple mechanisms involving free radical scavenger properties, attenuating lipid peroxidation, and increasing the antioxidant status of cardiac tissue. Caffeic acid phenethyl ester also prevents cytokine damage and blocks inflammatory pathways. The study reveals a potential novel aspect in the mode of action of CAPE, that is, the ultimate stimulation of HO-1 pathway is likely to account for the established and powerful antioxidant/antiinflammatory properties of CAPE. However, further studies are warranted to elucidate the effect of CAPE on energy status in cardiac tissue.

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