Caffeic acid phenethyl ester possesses potent cardioprotective effects in a rabbit model of acute myocardial ischemia-reperfusion injury

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Tan, Jiangning, Zhizhong Ma, Ling Han, Ruyu Du, Liming Zhao, Xing Wei, Dongming Hou, Brian H. Johnstone, Martin R. Farlow, and Yansheng Du. Caffeic acid phenethyl ester possesses potent cardioprotective effects in a rabbit model of acute myocardial ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 289: H2265–H2271, 2005; doi:10.1152/ajpheart.01106.2004.—Although great achievements have been made in elucidating the molecular mechanisms contributing to acute myocardial ischemia/reperfusion (I/R) injury, an effective pharmacological therapy to protect cardiac tissues from serious damage associated with acute myocardial infarction, coronary arterial bypass grafting surgery, or acute coronary syndromes has not been developed. We examined the in vivo cardioprotective effects of caffeic acid phenethyl ester (CAPE), a natural product with potent anti-inflammatory, antitumor, and antioxidant activities. CAPE was systemically delivered to rabbits either 60 min before or 30 min after surgically inducing I/R injury. Infarct dimensions in the area at risk were reduced by 2-fold (P < 0.01) with CAPE treatment at either period. Accordingly, serum levels of normally cytosolic enzymes lactate dehydrogenase, creatine kinase (CK), MB isoenzyme of CK, and cardiac-specific troponin I were markedly reduced in both CAPE treatment groups (P < 0.05) compared with the vehicle-treated control group. CAPE-treated tissues displayed significantly less cell death (P < 0.05), which was in part due to inhibition of p38 mitogen-activated protein kinase activation and reduced DNA fragmentation often associated with caspase 3 activation (P < 0.05). In addition, CAPE directly blocked calcium-induced cytochrome c release from mitochondria. Finally, the levels of inflammatory proteins IL-1β and TNF-α expressed in the area at risk were significantly reduced with CAPE treatment (P < 0.05). These data demonstrate that CAPE has potent cardioprotective effects against I/R injury, which are mediated, at least in part, by the inhibition of inflammatory and cell death responses. Importantly, protection is conferred when CAPE is systemically administered after the onset of ischemia, thus demonstrating potential efficacy in the clinical scenario.

apoptosis; caspase 3; p38 mitogen-activated protein kinase; tumor necrosis factor-α

EARLY INTERVENTION leading to rapid restoration of blood flow to cardiac tissues is the single best strategy to limit cardiac myocyte death resulting from acute ischemia (2, 17), yet reestablishing blood flow to the areas at risk can negatively impact recovery from ischemia due to reperfusion injury. Permanent injuries to myocardium due to ischemia-reperfusion (I/R) injury includes cardiac contractile dysfunction (24), arrhythmias (9), and irreversible myocyte damage, together with both apoptotic and necrotic cell death, leading to fibrosis and degraded ventricular function (34, 38). Reperfusion of the ischemic zone may exacerbate cell damage by accelerating the apoptosis process (8). Caspase 3 appears to play an important role in I/R-induced cell death (1). Additionally, recent reports demonstrate that p38 mitogen-activated protein kinase (MAPK) functions as a prodeath signaling protein in both cultured myocytes and as well as in intact hearts (15, 29). p38 MAPK may be involved in the caspase 3-indepedent cell death (32), and cotreatment with inhibitors of p38 MAPK and caspase 3 showed synergistic protection from cell death (18, 39), suggesting that to achieve optimal protection it is necessary to identify a compound that can simultaneously block both of these two death pathways. Furthermore, inflammatory cytokines also stimulate cell death. Reperfusion injury promotes release of inflammatory cytokines, such as interleukins and TNF-α (20, 38), which activate leukocytes to generate free radicals that induce myocardial injury via lipid peroxidation, calcium overload, and apoptosis (3, 23, 26, 33). Therefore, inhibition of cell death and inflammation pathways holds great promise for minimizing cell death, thus reducing subsequent loss of myocardium.

Caffeic acid phenethyl ester (CAPE), an antioxidant flavonoid, is the active component of the propolis derived from the hives of honeybees. It has antiviral, anti-inflammatory, antioxidant, and immunomodulatory properties, as well as selectively inhibiting the growth of several transformed cell lines but not normal cells (12, 35, 36). It has been demonstrated that CAPE is a potent and specific inhibitor of NF-κB activation (27), lipid peroxidation (21), lipoxygenase activities (37), protein tyrosine kinase (16), and ornithine decarboxylase (42). Recently, it has been shown to exert protective effects in vivo against cell death in a neonatal brain hypoxic-ischemic injury model (40) and stabilize hemodynamics and area at risk in a cardiac I/R injury model (30). However, the molecular mechanism underlying CAPE-induced protection in heart I/R injury is unknown.

In this report, we have found that CAPE effectively blocks I/R injury-induced cell death, even when administered well after induction of ischemia, and has cardioprotective effects that are mediated possibly through suppression of cell death and inflammation.

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MATERIAL AND METHODS

Surgical preparation of rabbits. The study was performed as previously described (31) and approved by AnZhen Hospital, Capital Medical University Animal Care and Use Committee, and was based on National Institutes of Health laboratory standards. New Zealand White rabbits (2.0–3.0 kg) of mixed gender were anesthetized with pentobarbital sodium (45 mg/kg iv). Cardiac electrical activity (electrocardiogram), blood pressure, and heart rate were monitored continuously during surgery and recovery. A midline sternotomy was performed, and the pericardium was opened to expose the heart. The main branch of the left anterior descending coronary artery (LAD) that supplies blood to the anterior-septal wall, left anterior ventricle wall, and apex in the rabbit was ligated temporarily with a 5-0 silk thread for 45 min at the side between one-half to one-third from apex to the atrioventricular groove. Ischemia was evidenced by a rapid change in the color of the left ventricle from red to pale purple, accompanied by marked S-T segment elevations. Such changes in the S-T segments were not noted in sham-operated controls (LAD incised with suture but not ligated). After 45 min ischemia treatments, the ligation was removed to allow for 90 min perfusion (31). Rabbits were injected with a solution of CAPE (Sigma Chemical, St. Louis, MO) in 33% polyethylene glycol of molecular weight 400 (PEG-400, Sigma, stock solution: 1.5 mg/ml) 60 min before (3 mg/kg ip) or 30 min after (15 mg/kg ip) ischemia treatments. In groups without CAPE treatments, rabbits were injected with the same volumes of 33% PEG-400. Blood samples and the hearts were taken at the end of the 90-min reperfusion period. Serum was then prepared from blood samples and stored with heart samples at −80°C for further analyses.

Measurement of infarct and risk area. The area at risk was identified by injecting 1 ml/kg of 2% methylene blue into the left atrium during ligation of the LAD (41). The rabbits were then euthanized by an intraperitoneal overdose of potassium chloride. For measurement of infarct size, the fresh hearts were cut into 2-mm-thick transverse slices that then were incubated in 1% triphenyltetrazolium chloride (TTC) for 20 min at 37°C. TTC-stained viable tissue developed a deep red color, while the infarct area remained unstained. The volumes of infarct myocardium and area at risk were calculated by summing the measured areas on nonstaining tissues by the thickness of each section. For Western blot analysis, the risk area was separated, weighed, and stored at −70°C.

Measurements of lactate dehydrogenase, creatine kinase, creatine kinase-isoenzyme MB, cardiac troponin I, IL-1β, and TNF-α. Serum levels of lactate dehydrogenase (LDH), creatine kinase (CK), CK isofrom MB (CK-MB), and cardiac-specific troponin I (cTnI) were measured with a Dade Dimension RxL clinical chemistry system (Dade Behring). Additionally, tissues from the area at risk of rabbit heart tissue were homogenized in the cold lysing buffer [in mM: 50 Tris-HCl, pH 7.4, 1 EDTA, 10 EGTA, 1 dithiothreitol (DTT), 0.1 PMSF, and 2 µg/µl aprotinin]. After homogenization with 20 strokes of a B-type pestle, lysates were centrifuged at 15,000 rpm at 4°C for 20 min, and protein concentrations in the supernatants were determined (Pierce, Rockford, IL). Extracts were either used immediately or stored at −80°C. Aliquots of protein (30 µg) were incubated with 100 µM caspase 3 substrate (Ac-DEVD-pNA; Calbiochem, La Jolla, CA) in a total volume of 1.0 ml at 37°C.

Caspase 3 assay. Caspase 3 activity assays were performed as described (6). Tissues from the area at risk of rabbit heart were washed three times with PBS, pH 7.2, and resuspended in precooled buffer [in mM: 50 Tris-HCl, pH 7.4, 1 EDTA, 10 EGTA, 1 dithiothreitol (DTT), 0.1 PMSF, and 2 µg/µl aprotinin]. After homogenization with 20 strokes of a B-type pestle, lysates were centrifuged at 15,000 rpm at 4°C for 20 min, and protein concentrations in the supernatants were determined (Pierce, Rockford, IL). Extracts were either used immediately or stored at −80°C. Aliquots of protein (30 µg) were incubated with 100 µM caspase 3 substrate (Ac-DEVD-pNA; Calbiochem, La Jolla, CA) in a total volume of 1.0 ml at 37°C. The colorimetric release of p-nitroaniline from the Ac-DEVD-pNA substrate was recorded every 10 min at 405 nm. Enzymatic activity for caspase 3 was linear over the range of protein concentrations used to calculate specific activity.

Western blot analysis. For Western blot analyses of p38, extracts were dissected from the area at risk of rabbit heart tissue by lysing tissues in a buffer containing 1% Nonidet P-40, 0.1% SDS, 50 mM Tris (pH 8.0), 50 mM NaCl, 0.05% deoxycholate, and protease inhibitor (Roche). Proteins were size fractionated on a 4–12% polyacrylamide gradient gel (SDS-NuPAGE) and transferred onto nitrocellulose (Hybond N, Amersham, CA). The blots were then probed with polyclonal antibodies specific for phosphorylated and nonphosphorylated p38 MAPK (1:500, Cell Signaling Technology, MA), followed by a secondary antibody conjugated with horseradish peroxidase (1:4,000, Jackson Immuno Research Laboratories, PA) and visualized utilizing enhanced chemiluminescence (7).

Mitochondrial isolation and cytochrome c release assay. Mitochondria were isolated from Sprague-Dawley rats (40). Briefly, the procedure involved homogenization in the ice-cold buffer containing 250 mM mannitol, 75 mM sucrose, and 10 µM K-HEPES, pH 7.4, followed by centrifugation at 1,000 g for 10 min. Supernatants were removed to a new tube and centrifuged at 10,000 g for 15 min. Pellets were washed three times with buffer and immediately used in experiments. For in vitro cytochrome c assay, an aliquot of 12.5 µg heart mitochondria (25 µl) was preincubated with CAPE for 5 min after challenge by 200 µM of CaCl2 for 30 min at 30°C. After centrifugation, the supernatant was evaluated by Western blot analysis. For assay of cytochrome c release, cells were washed once with ice-cold PBS and resuspended in 500 µl ice-cold buffer [in mM: 50 Tris-HCl, pH 7.4, 1 EDTA, 1 DTT, complete protease inhibitor (Roche), 250 µM sucrose]. The cells were disrupted by 10 strokes with a pestle in a 7-ml Wheaton douncer. After centrifugation in a microcentrifuge at 1,000 g for 10 min at 4°C, the supernatants were further centrifuged at 12,000 g for 40 min. Aliquots (25 µg) of cytosol were size-fractionated by SDS-PAGE electrophoresis (Nupage, Novex, San Diego, CA) and transferred to a nitrocellulose membrane (Hybond, Amersham, Arlington Heights, IL). The membranes were probed with a monoclonal antibody against residues 93–104 of nonnative cytochrome c (1:500, BD Biosciences, San Diego, CA) and visualized utilizing enhanced chemiluminescence (Amersham).

Data analysis and statistics. Data are expressed as means ± SE. Differences between control and experimental groups were determined using a one-way or two-way ANOVA for repeated measures. Differences between groups were determined using Bonferroni’s post hoc test. A value of P < 0.05 was considered significant. Statistical
analysis was performed using SigmaStat version 2.0 (Statistical Solutions, Saugus, MA).

RESULTS

Treatment with CAPE before or after inducing ischemia blocks apoptosis of cardiac myocytes. To investigate the cardioprotective effects of CAPE on I/R injury-induced cardiac myocyte death in vivo, rabbits were treated with CAPE either 60 min before (3 and 15 mg/kg) or 30 min after (15 mg/kg) the onset of ischemia, followed by 90 min reperfusion. These specific doses were chosen based on previous studies (13, 19) that demonstrated cell protection effects with similar doses of CAPE. Hearts were analyzed by TTC (Fig. 1A) and TUNEL (Fig. 1B) to quantify infarct size and myocyte death (including both apoptotic and necrotic cell death) in the area at risk. I/R-induced infarct area (the percentage of infarcted tissue in the risk zone as assessed by TTC staining) was significantly reduced (P < 0.01) from 38.8% in control hearts to 11.2% in hearts pretreated with CAPE or 18.4% in hearts posttreated with CAPE (Fig. 1A). Similarly, there were significantly fewer TUNEL-positive (i.e., dying) cardiac myocytes within the area at risk of both pre- and posttreated hearts (P < 0.01) (Fig. 1B). Areas at risk in hearts pretreated with CAPE (3 or 15 mg/kg) before I/R injury possessed twofold less (P < 0.01) TUNEL-positive nuclei compared with the vehicle-treated control group (data shown for 3 mg/kg dose only). Importantly, treatment with CAPE (15 mg/kg) 30 min after inducing ischemia also was efficacious at reducing the percentage of dying cells (Fig. 1B). The number of TUNEL-positive nuclei in the area at risk of rabbits hearts given CAPE 30 min after ischemia was reduced by 30% compared with the treatment group (P < 0.05, Fig. 1B). These data indicate that CAPE promotes cardioprotection against I/R-induced myocyte cell death.

CAPE reduces I/R injury-induced serum levels of LDH, CK, CK-MB, and cTnI. We next measured the effect of CAPE treatment on systemic levels of clinical markers of cardiac infarction after I/R injury. As expected, compared with the sham-operated, serum from the I/R group had dramatically higher levels of LDH (2.0 ± 0.3-fold), CK (4.9 ± 0.6-fold), CK-MB (5.0 ± 0.6-fold), and cTnI (34 ± 2-fold) (Fig. 2). Consistent with the cell death data, serum levels of the markers in rabbits that received CAPE before ischemia were significantly reduced compared with vehicle-treated animals (1.3 ± 0.3-fold LDH, 1.8 ± 0.4-fold CK, 1.6 ± 0.3-fold CK-MB, and 3.0 ± 1.0-fold cTnI) (P < 0.01, Fig. 2). CAPE treatment at 30 min after the onset of ischemia also blocked I/R injury-induced increases in serum levels of LDH (1.6 ± 0.2-fold, P < 0.05), CK (3.1 ± 0.3-fold, P < 0.01), CK-MB (3.0 ± 0.4-fold, P < 0.01), and cTnI (12.0 ± 0.4-fold, P < 0.01). Thus CAPE treatment apparently promotes cell integrity as well as inhibiting cell death after I/R injury.

CAPE blocks I/R injury-induced activation of caspase 3 and p38 MAPK phosphorylation, as well as calcium-induced cytochrome c release. To better understand the mechanisms underlying CAPE protection against I/R-induced cardiac myocyte death, mechanisms underlying the decreased frequency of cell death were examined. Because intracellular calcium concentrations may also play a role in I/R injury and mitochondrial cytochrome c release is a prerequisite for commitment to apoptosis by activating caspase 3, mitochondria isolated from rat hearts were treated with calcium to stimulate cytochrome c release. CAPE concentrations as low as 20 μM blocked cytochrome c release (Fig. 3A). Additionally, levels of caspase 3 activity (Fig. 3B) and p38 MAPK phosphorylation (Fig. 3, C and D) were compared between groups. Both caspase 3 activity and p38 MAPK phosphorylation were markedly increased in the group after I/R injury, and such inductions were inhibited by CAPE treatment. Thus we suggest that CAPE may protect myocytes by inhibiting at least two separate cell death pathways.

CAPE blocks I/R injury-induced expression of IL-1β and TNF-α in affected cardiac tissues. Because IL-1β and TNF-α have been proposed to mediate (at least in part) I/R injury-induced cardiac myocyte death (20, 38), we measured both
IL-1β and TNF-α in homogenates obtained from the areas at risk of rabbit hearts (Fig. 4). Both proteins were markedly upregulated in the heart tissues of vehicle-treated control animals vs. the sham-operated group. Induction of IL-1β and TNF-α was reduced by CAPE pretreatment ($P < 0.05$ for IL-1β and $P < 0.01$ for TNF-α). However, when CAPE was administered 30 min postischemia, a reduction of TNF-α ($P < 0.05$), but not IL-1β, was detected. These data indicate that CAPE is also a potent inhibitor of inflammatory processes.

Fig. 2. Clinical markers of heart injury were significantly inhibited by CAPE treatments. I/R injury-induced serum levels of lactate dehydrogenase (LDH; A), creatine kinase (CK; B), CK isoenzyme MB (CK-MB; C), and cardiac-specific troponin I (cTnI; D) in sham-operated (sham), IRI, IRI-CAp, and IRI-CAa groups ($* P < 0.05$, $** P < 0.01$; $n = 7$ in each group; values are means ± SE). Group designations as described in Fig. 1.

Fig. 3. CAPE treatment blocks cell death at multiple levels, including calcium-induced cytochrome c release, caspase 3 activity, and p38 MAPK activation. A: calcium-induced heart mitochondrial cytochrome c release is inhibited in a dose-dependent manner by the addition of CAPE. B: caspase 3 activity in sham-operated, IRI, and IRI-CAp (1-way ANOVA, $*** P < 0.001$). C: phosphorylation of p38 MAPK kinase in the hearts of sham-operated, IRI, IRI-CAp, and IRI-CAa rabbits. Top row was probed with antibody specific for phosphorylated p38 MAPK (p-p38), and bottom row was probed with an antibody recognizing all forms of p38 MAPK. D: densitometric quantifications of p-p38 were compared between CAPE-treated and untreated groups ($n = 3$ each group, $** P < 0.01$, $*** P < 0.001$). Group designations as described in Fig. 1.
DISCUSSION

Our data demonstrate that CAPE effectively protects the heart from injury due to I/R. Although the experiments reported herein were short term, the significant reduction in cell death observed over this period suggests that the protection will have long-term benefits, such as reduced scarring and heart failure. Importantly for translation to the clinic, a significant protective effect was observed when drug was administered after the onset of ischemia (i.e., after the initial insult leading to cell damage), although a higher dose (15 mg/kg) was used compared with pretreatment (3 mg/kg). This result suggests that a certain serum level of CAPE immediately after posttreatment is important for CAPE to exert its protective effect. However, we have yet to determine the optimal dose required for either period of drug administration, which will depend on the pharmacokinetic profile of CAPE, as well as the route of administration, both of which remain to be the focus of future studies.

The mode of CAPE protection appears to be through significantly attenuating cardiac myocyte death, as demonstrated both histologically and biochemically by reduced infarct dimensions and less cell death in the area at risk. The integrity of cells as a consequence of the insult is much less compromised with CAPE treatment, as evidenced by the decreased levels of the intracellular enzymes (particularly cTnI) detectable in systemic blood. It should be noted that in this study we cannot rule out the possibility that the decrease in intracellular enzymes detected in the serum results in the reduction in I/R-induced apoptosis/inflammation. Additional work will be required to delineate role(s) of these enzymes in the I/R-induced apoptosis/inflammation.

From our results, the mechanism by which CAPE protects cardiac myocytes from I/R injury is possibly through suppressing both inflammatory signaling and cell death. Systemic levels of inflammatory cytokines IL-1β and TNF-α were significantly lower in animals treated with CAPE. TNF-α and IL-1β have been implicated in I/R injury-induced cardiac myocyte death (20, 38). TNF-α also has been implicated in the pathogenesis of many cardiovascular diseases other than I/R injury, including heart failure, cardiac allograft rejection, and sepsis-associated cardiac dysfunction (25). Myocardial ischemia induces degranulation of resident mast cells (11) and cleavage of membrane-bound TNF-α by the TNF-α cleavage enzyme TACE (20), both inducing the release of TNF-α in the ischemic myocardium (14). The released TNF-α negatively impacts myocardial function, either by directly inducing oxidative stress, calcium dyshomeostasis, disruption of excitation-contraction coupling, and myocyte apoptosis, or by indirectly triggering the inflammatory cascade that accounts for subacute I/R injury. Additionally, it has been suggested that IL-1β plays a potentially important role in myocardial I/R injury and overexpression of IL-1 receptor antagonist provides cardioprotection by reducing inflammation-mediated myocardial damage after I/R injury in vivo (38). In this study, we have shown that CAPE inhibits both inflammatory factors after I/R injury in vivo.

The potent anti-inflammatory effects of CAPE are complemented by direct inhibition of cell death pathways in cardiac myocytes by preventing p38 MAPK phosphorylation and caspase 3 activation, both of which were reported to play important roles in I/R-induced cell death. Overexpression of caspase 3 in the heart significantly increases infarct size (4); conversely, treatment with a caspase inhibitor reduces infarct size (28). Using a complementary in vitro system, we have shown that CAPE directly blocks calcium-induced cytochrome c release from mitochondria. In apoptosis, release of cytochrome c from mitochondria to cytoplasm activates caspase 3, leading to the inevitable fate of cell death (22). Although there are conflicting data regarding whether p38 MAPK exerts pro- or anti-cell death roles, most recent reports support that p38 MAPK functions as a prodeath signaling effector in both cultured myocytes as well as the intact heart. Interestingly, because p38 MAPK may be involved in caspase 3-independent cell death (32) and cotreatment with inhibitors of p38 MAPK and caspase 3 showed synergistic protection from cell death, CAPE may exert more potent cardioprotection than inhibitors of either caspase 3 or p38 MAPK alone. Furthermore, it should be noted that CAPE pretreatment reduced phosphorylated p38 MAPK levels even below sham. The physiological significance of this phenomenon remains to be determined.

In this study we clearly show that CAPE has potent cardioprotective activities that occur through simultaneously blocking apoptotic and inflammatory pathways. The next step will be to determine whether CAPE treatment after acute myocardial infarction elicits a lasting protective effect. These studies will be best carried out in a large animal porcine model, which possesses multiple advantages over rodent or rabbit models in that the physiology and anatomy of porcine heart more closely mimics that of human, and as a result, clinical devices for measuring heart function are easily applied.
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