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Caffeine restores myocardial cytochrome oxidase activity and improves cardiac function during sepsis

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Abstract

Objective—Impaired mitochondrial function is a potential cause of sepsis-associated myocardial depression. Cytochrome oxidase (CcOX), the terminal oxidase of the electron transport chain, is inhibited in the septic heart. Caffeine increases CcOX activity by increasing cyclic adenosine monophosphate and protein kinase A activity. We hypothesized that caffeine will restore myocardial CcOX activity, increase cardiac function, and improve survival during sepsis.

Design—Prospective randomized controlled study.

Setting—University hospital-based laboratory.

Subjects—One hundred twenty Sprague-Dawley male rats.

Interventions—Sprague-Dawley male rats underwent cecal ligation and double puncture (CLP) or sham operation. At 24 and 48 hours, rats underwent intraperitoneal injection of either caffeine (7.5 mg/kg, the equivalent of 1–1.5 cups of coffee) or equal volume of saline.

Measurements and Main Results—One hour following the 48-hour injection, steady-state CcOX kinetic activity was measured in isolated mitochondria and normalized to citrate synthase activity. Cardiac function was assessed using an isolated rat heart preparation and survival was tracked to 96 hours. CLP significantly decreased myocardial CcOX activity, oxygen consumption, left ventricular pressure, and pressure developed during isovolumic contraction (+dP/dt) and relaxation (−dP/dt). Caffeine restored CcOX activity and increased left ventricular pressure and ±dP/dt toward sham values following CLP. Survival significantly improved following CLP in caffeine-injected animals compared with saline injection.

Conclusion—Caffeine may be a novel therapy to treat sepsis-associated myocardial depression.

Keywords

sepsis; cytochrome oxidase; caffeine; mitochondria; enzyme inhibition; isolated heart; cardiac dysfunction

Sepsis continues to be the major cause of death in critically ill patients and often results in multiple organ failure (1). Sepsis-associated myocardial depression is often profound and can lead to refractory hypotension and cardiovascular collapse (2). Mitochondrial

dysfunction and impaired oxidative phosphorylation have been proposed to cause sepsis-induced organ dysfunction (1, 3-6). Diminished function of any of the electron transport complexes could limit aerobic adenosine triphosphate (ATP) synthesis and lead to bioenergetic failure (5, 7). Cytochrome oxidase (CcOX), the terminal oxidase of the respiratory chain, uses electrons donated by cytochrome c to reduce oxygen to water (8). Coupled with the reduction of oxygen, CcOX pumps hydrogen ions across the mitochondrial inner membrane to the intermembrane space to maintain the hydrogen ion gradient (8). This proton motive force is crucial for ATP synthesis. Myocardial CcOX is inhibited during sepsis (9). This inhibition is competitive and reversible early following cecal ligation and puncture (CLP) and progresses becoming noncompetitive and irreversible during the late phase of sepsis (9). The onset of the hypodynamic phase and mortality in sepsis coincide with maximal competitive CcOX inhibition. Thus, restoring CcOX activity by overcoming the reversible inhibition could potentially improve cardiac function and enhance survival during sepsis.

Caffeine (1,3,7-trimethylxanthine) is a member of the methylxanthine family (10). It is known to inhibit cyclic adenosine monophosphate (cAMP)-phosphodiesterase (10). This leads to increased intracellular cAMP and activation of protein kinase A (10). Subsequently, cAMP-dependent phosphorylation of complex I of the respiratory chain and CcOX stimulates and optimizes the efficiency oxidative phosphorylation (11). Here, we test the hypothesis that caffeine can restore myocardial CcOX activity, improve cardiac performance, and reduce mortality in sepsis. In this study, we administered caffeine once daily (the equivalent of 1-1.5 cups of coffee) to rats beginning 24 hours post-CLP (the onset of the hypodynamic phase when CcOX is competitively inhibited) (9, 10, 12). We then evaluated myocardial CcOX activity and cardiac function at the 48-hour time point and tracked survival to 96 hours.

MATERIALS AND METHODS

Induction of Sepsis

The care of the animals in this study was in accordance with National Institute of Health and Institutional Animal Care and use committee guidelines. Under isoflurane general anesthesia (up to 2%), 250 g male Sprague-Dawley rats (Charles River, Boston, MA) underwent CLP with an 18-gauge needle or sham operation as previously described (13). All animals were administered 50 mL/kg saline subcutaneously immediately postprocedure and every 24 hours. Once awake, animals were given access to food and water *ad libitum*.

Caffeine Injection

Twenty-four and 48 hours post-CLP or sham operation, rats received either an intraperitoneal (IP) injection of caffeine (7.5 mg/kg, Sigma-Aldrich, St. Louis, MO) or equal volume saline (1 mL). Rats were killed 1 hour after the 48-hour injection following euthanasia with 150 mg/kg of IP pentobarbital. Four cohorts were evaluated: sham operation (SHAM) saline, SHAM caffeine, CLP saline, CLP caffeine. $n = 5$ per group. The 24-hour time point was chosen for initial intervention based on the onset of the hypodynamic phase when cardiac function begins to deteriorate and the presence of reversible CcOX inhibition (9, 12). Studies were performed at the 48-hour time point because this time point represents the late phase of sepsis when cardiac function is markedly depressed and mortality is 75% (14, 15).

Mitochondrial Isolation

As previously described, cardiac ventricles were harvested and homogenized in ice-cold H medium (70 mM sucrose, 220 mM mannitol, 2.5 mM HEPES, pH 7.4 and 2 mM EDTA)

(9). The homogenate was spun at 1500g for 10 minutes at 4°C. Supernatants were removed and centrifuged at 10,000g for 10 minutes at 4°C. Pellets were resuspended in H medium and centrifuged again at 10,000g for 10 minutes at 4°C. Pellets were again resuspended in H medium and mitochondrial protein concentration determined using the method of Lowry (9).

Cytochrome Oxidase Steady-State Kinetics

CcOX kinetics were assayed by the method of Smith in which the rate of oxidation of ferrocytochrome c was measured by following the decrease in absorbance at 550 nm (9, 16). Assays were executed in a 1-mL reaction volume containing 50 mM PO_4^{2-} (pH 7.0), 2% lauryl maltoside, and 1 μg of mitochondrial protein. Ferrocytochrome c was added at a concentration of 40 mM to initiate the reaction. Specific activity was calculated from mean values of three to four measurements using $21.1 \text{ mM}^{-1}\cdot\text{cm}^{-1}$ as the extinction coefficient of ferrocytochrome c at 550 nm.

Citrate synthase activity was measured via spectrophotometry based on the change in absorbance at 412 nm induced by the cleavage of the thiol ester bond of acetyl-CoA and the utilization of oxaloacetate (Sigma-Aldrich) (17). CcOX activities were normalized to citrate synthase activities.

Steady-State Levels of Cardiac Cytochrome Oxidase Subunit I (the Active Site)

Ten microgram samples of mitochondrial protein were subjected to sodium dodecyl sulfateacrylamide gel electrophoresis and immunoblotting as previously described (9). Blots were labeled with a primary polyclonal antibody to mouse CcOX I (Molecular Probes, Eugene, OR) and secondarily exposed to rabbit anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Mitochondrial protein loading was assessed with a primary monoclonal antibody to mouse porin (VDAC, Molecular Probes) and secondarily exposed to rabbit anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA). The signal was detected with enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ), and density was measured using scanning densitometry. Five animals per group per experiment were evaluated.

Isolated Rat Heart Preparation

Rats were anesthetized with IP pentobarbital (70 mg/kg) and heparinized (1000 U IP). The heart was excised rapidly and the aorta was cannulated. Retrograde perfusion was initiated at constant flow (12 mL·g·min) with a modified Krebs-Henseleit buffer containing NaCl 120 mmol/L, KCl 4.7 mmol/L, MgSO_4 1.2 mmol/L, KH_2PO_4 1.2 mmol/L, CaCl_2 1.25 mmol/L, NaHCO_3 25 mmol/L, and glucose 11 mmol/L. The nonrecirculating buffer was maintained at pH 7.4 equilibrated with 95% oxygen-5% CO_2 at 37°C. Isovolumetric left ventricular pressure (LVP) was measured with a transducer connected to a saline-filled latex balloon inserted into the left ventricle through the left atrium. The balloon was inflated to maintain an end-diastolic pressure of 10 mm Hg. Hearts were immersed in a water-jacketed chamber maintained at 37°C and allowed to stabilize for 30 minutes.

Heart rate, LVP, the maximum rate of positive and negative change in LVP ($\pm dP/dt$), and aortic pressure were collected with an analog-to-digital converter system (Power Laboratory 4SP, ADInstruments, Castle Hill, Australia). All variables were displayed and recorded (Chart ver. 4.12, ADInstruments). Measurements were obtained at coronary flow (CF) rates of 12, 10, 8, 6, and 4 mL·g·min. Coronary inflow oxygen tension (Pao_2) and coronary sinus oxygen tension (PvO_2) were measured (GEM Premier 3000, Instrumentation Laboratory, Belgium). Percent oxygen extraction was calculated as $100 \times (\text{Pao}_2 - \text{PvO}_2 / \text{Pao}_2)$.

Myocardial oxygen consumption ($\text{M } \dot{\text{V}} \text{ O}_2$) was calculated as $\text{CF/g} \times (\text{Pao}_2 - \text{PvO}_2) \times$

oxygen solubility at 760 mm Hg. Oxygen solubility is 24 $\mu\text{L/mL H}_2\text{O}$ at 37°C. Coronary perfusion pressure was calculated as the difference between mean aortic pressure and LV end-diastolic pressure. Coronary resistance was calculated by dividing perfusion pressure by CF.

Survival

We followed survival in a separate cohort of animals in this two treatment parallel-design study to 96 hours. Each animal was injected with either caffeine (7.5 mg/kg IP.) or equal volume of saline (1 mL IP) every 24 hours beginning 24 hours following CLP or sham operation. Twenty animals per group were evaluated based on the probability being 80% that the study will detect a treatment difference at a two-sided 5.0% significance level, if the true hazard ratio is 3 (18).

Statistics

Data are presented as mean $\pm$ SD. Statistical significance was assessed using analysis of variance and post-hoc Tukey's test with $p < 0.05$. Statistical significance in the survival study was assessed using Kaplan-Meier survival curves and Mantel-Cox log-rank test.

RESULTS

Caffeine Overcomes CLP-induced Myocardial CcOX Inhibition and Restores CcOX Kinetic Activity

Myocardial CcOX activity was measured via spectrophotometry in isolated mitochondria and normalized to citrate synthase activity. Consistent with previous findings, normalized myocardial CcOX activity significantly decreased 48 hours following CLP in rats that received saline injection (Fig. 1). Caffeine injection, however, restored CcOX kinetic activity in CLP rats to sham values (Fig. 1). There was no significant difference in CcOX activity between saline- and caffeine-injected sham animals (Fig. 1).

Steady-State Levels of Myocardial CcOX I are Unchanged Following CLP

Immunoblot analysis for CcOX subunit I (the active site) was performed. There was no significant difference in steady-state myocardial CcOX I levels among all groups (Fig. 2). Thus, change in CcOX activity following CLP in saline-injected animals was not on the basis of changes in enzyme levels.

Caffeine Improves CLP-induced Cardiac Dysfunction

To assess the effect of caffeine on cardiac function, we used an isolated rat heart preparation and measured heart rate, LVP, and the maximum rate of positive and negative change in LVP ($\pm dP/dt$) over a range of CF rates. There was no significant difference in heart rate between groups (263 ± 30 bpm, sham saline; 276 ± 11 bpm, sham caffeine; 260 ± 17 bpm, CLP saline; 262 ± 11 bpm CLP caffeine; $p =$ not significant). However, hearts from CLP animals demonstrated significant reductions in LVP in saline-injected animals across all CF rates compared with both sham cohorts (Fig. 3). Caffeine significantly improved LVP following CLP compared with saline injection at all flow rates greater than 4 mL·g·min (Fig. 3). LVP in caffeine-injected CLP animals approached saline-injected sham values except at 12 mL·g·min CF where it was significantly lower than both sham cohorts (Fig. 3). Interestingly, caffeine significantly increased LVP in sham-operated animals compared with all other groups at the highest CF rates (Fig. 3).

As with LVP, hearts from CLP animals demonstrated significant reductions in dP/dt in saline-injected animals (Fig. 3). Compared with both sham cohorts, saline-injected CLP

significantly decreased $+dP/dt$ at the majority of CF rates and decreased $-dP/dt$ at all flow rates (Fig. 3). Caffeine significantly improved both $+dP/dt$ and $-dP/dt$ following CLP toward saline-injected sham values at most flow rates (Fig. 3). Again, caffeine significantly increased $+dP/dt$ and $-dP/dt$ following sham operation compared with both CLP groups at all CF rates and significantly increased $+dP/dt$ and $-dP/dt$ compared with saline-injected sham values at the highest flow rates (Fig. 3).

With regard to coronary perfusion pressure, CLP demonstrated significant reductions in both saline- and caffeine-injected cohorts compared with sham controls at almost all CF rates (Fig. 4). Compared with saline-injected CLP, caffeine injection significantly increased coronary perfusion pressure at 12 mL·g·min of CF (Fig. 4). Interestingly, caffeine injection significantly reduced coronary perfusion pressure at the highest flow rate following sham operation compared with saline injection (Fig. 4). Similar changes were seen with regard to coronary resistance (Fig. 4). Caffeine injection significantly increased coronary resistance following CLP and significantly decreased resistance following sham operation compared with their saline injection counterparts, respectively suggesting alternative mechanisms (Fig. 4). Significant reductions in resistance in both CLP groups were consistent with sepsis-induced vasodilation.

To assess *in situ* mitochondrial function, myocardial oxygen extraction and oxygen consumption were measured at 12 mL·g·min CF following stabilization. Consistent with CcOX inhibition, both oxygen extraction and consumption significantly decreased following CLP in saline-injected animals compared with all groups (Fig. 5, A and B). Caffeine, however, significantly increased both oxygen extraction and consumption following CLP compared with saline-injected sham values (Fig. 5, A and B). Following sham operation, caffeine significantly increased myocardial oxygen consumption compared with both CLP cohorts (Fig. 5B).

Caffeine Enhances Survival Following CLP

To assess the effect of caffeine on sepsis-induced mortality, we tracked survival in a separate cohort of animals receiving either a daily injection of caffeine or equal volume of saline. Following CLP, 60% of saline-injected animals survived to 48 hours, 25% survived to 72 hours, and none survived to 96 hours postprocedure (Fig. 6). On the other hand, caffeine injection significantly improved survival post-CLP. Eighty percent of caffeine-injected animals survived to 48 hours, 55% survived to 72 hours, and 40% survived to 96 hours post-CLP (Fig. 6). There was no mortality observed in either sham cohort (Fig. 6).

DISCUSSION

Mitochondrial dysfunction and impaired oxidative phosphorylation may underlie sepsis-induced organ dysfunction (1, 3-6). It has been suggested that CcOX, the terminal oxidase of the electron transport chain, regulates aerobic ATP production (19). Defects in CcOX activity during sepsis could incapacitate bioenergy production and manifest as organ failure. cAMP-dependent phosphorylation of CcOX is known to stimulate and optimize the efficiency of oxidative phosphorylation (11). Therefore, we hypothesized that caffeine, a well-known inhibitor of cAMP-phosphodiesterase, could increase myocardial CcOX function during sepsis and potentially improve cardiac performance and enhance survival.

Consistent with previous work, CLP induced a significant impairment in myocardial CcOX activity (9). A daily injection of caffeine beginning 24 hours post-CLP (roughly equivalent to the human consumption of a single cup of coffee) restored CcOX function to sham values at 48 hours. Increased oxygen extraction and myocardial oxygen consumption in the isolated rat heart preparation corroborated this finding *in situ*. These data strongly suggest that

caffeine stimulated oxidative phosphorylation during sepsis and were associated with significant improvements in cardiac function and survival.

It is important to note that, despite caffeine-induced restoration of CcOX function following CLP, LVP was significantly less than sham values at the highest CF rate. In addition, although caffeine considerably improved survival to 96 hours post-CLP, mortality remained significantly high. This may be explained in a number of ways. First, depressed LVP in the setting of restored oxygen consumption suggests that caffeine may have potentially uncoupled oxidative phosphorylation. In other words, electron transport and oxygen consumption were dissociated from ATP production. Therefore, bioenergetic capacity would not be restored entirely and limited ATP synthesis could continue to impair cardiac function. Another explanation is the multifactorial nature of sepsis. It is likely that impaired CcOX activity by itself is not solely responsible for sepsis-induced organ failure. Thus, abrogation of myocardial CcOX inhibition during sepsis may not completely restore cardiac function. In addition, cardiac dysfunction likely contributes to sepsis-induced mortality but may not be the sole cause. Thus, marked improvement in myocardial function may not necessarily equate with marked improvements in survival.

Although caffeine-enhanced myocardial CcOX activity during sepsis and cardiac performance following both CLP and sham operation, the exact mechanisms of action have not been elucidated. The most likely explanation for the effect on CcOX function is that caffeine inhibited cAMP-phosphodiesterase, leading to protein kinase A-mediated phosphorylation of CcOX in the septic heart (10). However, the key elements in this pathway were not evaluated in this study making this explanation mere speculation. Therefore, other mechanisms need to be considered with regard to cardiac function and survival.

Caffeine is known to increase circulating epinephrine levels and has sympathomimetic action mediated by cAMP (20, 21). It is unlikely, however, that a resultant increase in epinephrine led to the observed improvements in cardiac function. This is because we evaluated cardiac function in an *ex vivo* preparation, thereby removing potential neurohumoral mediators. Certainly, increased epinephrine could have enhanced cardiac performance *in vivo*, contributing to increased survival. Because of the limitations of using an isolated heart preparation, the effect of caffeine on sepsis-induced myocardial depression *in vivo* is unknown. However, the sympathomimetic action of caffeine could certainly account for the observed results.

Another mechanism to consider is the effect of caffeine on calcium. Caffeine increases intracellular calcium by stimulating release from calcium stores and activating the ryanodine receptor (22, 23). This results in increased cardiac inotropic state. It has been demonstrated that LVP and $\pm dP/dt$ significantly increase with calcium chloride therapy in this sepsis model (24). Thus, this plausible mechanism of caffeine needs to be explored. Next is the anti-inflammatory effect of caffeine. Mediated through cAMP/protein kinase A activation, caffeine inhibits production of proinflammatory cytokines, such as tumor necrosis factor- α and interleukin-12, and promotes production of anti-inflammatory cytokines, such as interleukin-10 (10). This could improve survival in sepsis. However, caffeine administration at such a late time point in sepsis argues somewhat against this mechanism as an explanation for our findings.

Finally, one of the most important mechanisms to consider is antagonism of adenosine. Adenosine, an endogenously produced nucleoside, is important for vasoregulation (25). Activation of the A_{2A} adenosine receptor causes coronary vasodilation (26). Caffeine nonspecifically antagonizes adenosine receptors and is believed to attenuate A_{2A} receptor-

mediated coronary vasodilation (10, 27). The increase in coronary perfusion pressure and resistance in caffeine-treated CLP animals compared with saline-injected cohorts suggests antagonism of the A_{2A} receptor. However, reduced coronary perfusion pressure and resistance in caffeine-treated sham animals compared with saline-injected controls suggests an alternative mechanism. It is quite possible that the observed effects of caffeine following sham operation were due to cAMP-induced coronary vasodilation (28). This suggests different mechanisms of cardiovascular action in healthy states vs. sepsis and may have implications for the use of caffeine as a therapeutic agent.

It is clear that this work raises more questions than it answers. However, it demonstrates that caffeine, given in a dose equivalent to the average daily human consumption, results in restoration of myocardial CcOX activity, improved cardiac function, and enhanced survival during sepsis. Although further investigation is necessary, it is possible that caffeine may be developed as a novel therapy for sepsis and sepsis-associated myocardial depression.

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Figure 1.

Myocardial cytochrome oxidase (*CcOX*) kinetic activity. Steady-state *CcOX* kinetic activity was measured in isolated mitochondria and normalized to citrate synthase activity. Saline- and caffeine-injected groups are presented. Values are expressed as means $\pm$ SD, $n = 5$ per group. * $p < 0.03$ vs. sham-operated cohorts (*SHAM*) saline and cecal ligation and puncture (*CLP*) caffeine.

Figure 2.

Steady-state levels of cytochrome oxidase (*CcOx*) I protein. A representative Western blot of CcOx subunit I is depicted. Saline- (*sal*) and caffeine-injected (*caff*) groups are demonstrated. Porin was used as the mitochondrial protein loading control. Graphical representation of relative densities is shown below the blot. Values were normalized to porin density and are expressed as means $\pm$ SD. Sham saline values were set arbitrarily to one. $n = 5$ per group. $p =$ not significant. *SHAM*, sham operation; *CLP*, cecal ligation and puncture.

Figure 3.

Left ventricular function. Left ventricular developed pressure (*LVP*) and the maximum rate of positive and negative change in *LVP* ($\pm dP/dt$) were measured over a range of coronary flow rates using an isolated rat heart preparation. Saline- (*sal*) and caffeine-injected (*caff*) groups are demonstrated. Values are expressed as means $\pm$ SD. $n = 5$ per group. * $p < 0.01$ vs. all other groups, ‡ $p < 0.05$ vs. all other groups, † $p < 0.05$ vs. sham-operated (*SHAM*) groups, @ $p < 0.05$ vs. cecal ligation and puncture (*CLP*) groups, # $p < 0.05$ vs. *CLP sal*, $p > 0.01$ vs. *SHAM caff*.

Figure 4.

Coronary artery perfusion pressure and resistance. Perfusion pressure was measured over a range of coronary flow rates using an isolated rat heart preparation. Resistance was calculated by dividing perfusion pressure by flow. Saline- (*sal*) and caffeine-injected (*caff*) groups are demonstrated. Values are expressed as means $\pm$ SD, $n = 5$ per group. * $p < 0.05$ vs. sham-operated (*SHAM*) groups, ‡ $p < 0.05$ vs. cecal ligation and puncture (*CLP*) *caff*, † $p < 0.05$ vs. *SHAM* groups, # $p < 0.05$ vs. *SHAM sal*.

Figure 5.

Myocardial oxygen extraction and consumption *in situ*. Percent oxygen extraction and myocardial oxygen consumption were calculated from the difference between coronary inflow oxygen tension (PaO_2) and coronary sinus oxygen tension (PvO_2) in an isolated heart preparation. Saline- and caffeine-injected groups are presented. Values are expressed as means $\pm$ SD. $n = 5$ per group. * $p < 0.04$ vs. all groups, † $p < 0.04$ vs. sham operation (SHAM) caffeine. CLP, cecal ligation and puncture.

Figure 6.

Kaplan-Meier survival curves. Animals were either injected with intraperitoneal caffeine or equal volume of saline at the 24-hour time point and every 24 hours. Survival was tracked to the 96-hour time point. Saline- (*sal*) and caffeine-injected (*caff*) groups are demonstrated. Values represent percent survival. $n = 20$ per group. * $p < 0.001$ vs. cecal ligation and puncture (CLP) *sal*, † $p < 0.01$ vs. sham-operated (SHAM) groups.