Giselli Scaini[1,3], Natália Rochi[1,3], Joana Benedet[1,3], Gabriela Kozuchovski Ferreira[1,3], Brena Pereira Teodorak[1,3], Clarissa Martinelli Comim[2,3], Larissa de Souza Constantino[1,3], Francieli Vuolo[1,3], Leandra Celso Constantino[2,3], João Quevedo[2,3], Emilio Luiz Streck[1,3], Felipe Dal-Pizzol[1,3]

1. Experimental Pathophysiology Laboratory, Health Sciences Post-Graduation Program, Universidade do Extremo Sul Catarinense – Criciúma (SC), Brazil.
2. Neurosciences Laboratory, Health Sciences Post-Graduation Program, Universidade do Extremo Sul Catarinense – Criciúma (SC), Brazil.
3. Instituto Nacional de Ciência e Tecnologia Translacional em Medicina – Porto Alegre (RS), Brazil.

This study was conducted at Experimental Pathophysiology Laboratory – Universidade do Extremo Sul Catarinense – Criciúma (SC), Brazil.

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Corresponding author:
Felipe Dal-Pizzol
Laboratório de Fisiopatologia Experimental
Universidade do Extremo Sul Catarinense
Zip Code: 88806-000 – Criciúma (SC), Brazil.
Fax: + 55 (48) 3431-2539
E-mail: piz@unesc.net

Inhibition of brain citrate synthase activity in an animal model of sepsis

Inibição da atividade da citrato sintase cerebral em um modelo animal de sepse

ABSTRACT

Objective: An extensive body of evidence from experimental studies indicates that sepsis is associated with increased reactive oxygen species production, depletion of antioxidants, and accumulation of markers of oxidative stress. Moreover, mitochondrial dysfunction has been implicated in the pathogenesis of multiple organ dysfunction syndrome (MODS). Citrate synthase is an enzyme localized in the mitochondrial matrix and an important component of the Krebs cycle; consequently, citrate synthase has been used as a quantitative enzyme marker for the presence of intact mitochondria. Thus, we investigated citrate synthase activity in the brains of rats submitted to a cecal ligation puncture model of sepsis.

Methods: At several times points (3, 6, 12, 24 and 48 hours) after the cecal ligation puncture operation, six rats were killed by decapitation. Their brains were removed, and the hippocampus, striatum, cerebellum, cerebral cortex and prefrontal cortex were dissected and used to determine citrate synthase activity.

Results: We found that citrate synthase activity in the prefrontal cortex was inhibited 12, 24 and 48 hours after cecal ligation puncture. In the cerebral cortex, citrate synthase activity was inhibited 3, 12, 24 and 48 hours after cecal ligation puncture. Citrate synthase was not affected in the hippocampus, striatum or cerebellum up to 48 hours after cecal ligation puncture.

Conclusion: Considering that energy impairment due to mitochondrial dysfunction in sepsis has been well described and that oxidative stress plays a crucial role in sepsis development, we believe that energy impairment may also be involved in these processes. If citrate synthase inhibition also occurs in a sepsis model, it is tempting to speculate that a reduction in brain metabolism may be related to the pathophysiology of this disease.

Keywords: Citrate (si)-synthase/metabolism; Sepsis; Mitochondria; Brain; Rats, Wistar; Models, animal

INTRODUCTION

Sepsis and its related complications, such as multiple organ dysfunction syndrome (MODS), are the most frequent causes of morbidity and mortality in intensive care units, contributing to 750,000 cases per year in the United States alone with an average mortality of 29%.[1] Sepsis is a complex syndrome defined as the host's reaction to infection characterized by systemic inflammation[2] and an imbalance between pro- and anti-inflammatory responses to pathogens.[3]

Evidence from the literature has demonstrated that reactive oxygen species (ROS) play an important role in the development of multiple organ

failure and septic shock.[4-8] Treatments that reduce the generation or that prevent or reverse the effects of ROS have shown beneficial effects in a variety of models of endotoxic and septic shock.[9-15]

Mitochondrial dysfunction has been strongly implicated in the pathogenesis of multiple organ dysfunction syndrome (MODS) and a wide variety of disease states.[3,16-18] The hypothesis of cytopathic hypoxia postulates that impairment in mitochondrial oxidative phosphorylation reduces aerobic adenosine triphosphate (ATP) production and potentially induces MODS.[19] In this context, some studies have reported deficiencies within the electron transport chain in models of sepsis.[19-22]

Citrate synthase (EC 4.1.3.7) is localized in the mitochondrial matrix and catalyzes the condensation of oxaloacetate and the acetyl group of acetyl coenzyme-A (acetyl CoA), the first step of the Krebs cycle. In this step, oxaloacetate reacts with acetyl CoA and H_2O to yield citrate and CoA. Citrate synthase is inhibited by high levels of ATP, acetyl-CoA and NADH, which are present when the cell energy supply is high. This regulation ensures that the Krebs cycle does not oxidize an excess of pyruvate and acetyl CoA when cellular ATP concentrations are high.[23] In addition, citrate synthase has been used as a quantitative enzyme marker for the presence of intact mitochondria.[24]

Considering that citrate synthase plays an important role in brain energy metabolism and that mitochondrial dysfunction has been implicated in the pathogenesis of MODS, in the present study, we investigated whether sepsis induced by cecal ligation and puncture (CLP) modifies citrate synthase activity in the rat brain.

METHODS

Animals

Male adult Wistar rats (60 days old) were obtained from the Universidade do Extremo Sul Catarinense (UNESC) breeding colony. The animals were housed five per cage with food and water available ad libitum and were maintained on a 12 h light/dark cycle (lights on at 7:00 AM). All experimental procedures involving animals were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care.

Cecal ligation and perforation surgery

The animals were subjected to CLP as previously described by Ritter et al.[25] Briefly, rats were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) given intraperitoneally. Under aseptic conditions, a 3 cm midline laparotomy was performed to expose the cecum and the adjoining intestine. The cecum was tightly ligated with a 3.0-silk suture at its base below the ileocecal valve and was perforated once with a 14-gauge needle. The cecum was then gently squeezed to extrude a small amount of feces from the perforation site and then returned to the peritoneal cavity. The laparotomy was then closed with 4.0-silk sutures. All animals received isotonic saline solution (50 mL/kg s.c.) immediately after the procedure. All animals were returned to their cages with free access to food and water. In the sham-operated group, the rats were submitted to all surgical procedures and received isotonic saline solution (50 mL/kg s.c.) immediately afterwards.

The rats were randomly allocated into the sham and CLP groups just prior to the procedure. At several time points (3, 6, 12, 24 and 48 hours) after the CLP or sham operation, six rats were killed by decapitation, and brain structures (prefrontal cortex, hippocampus, striatum, cerebellum and cerebral cortex) were immediately isolated and stored at -80ºC. All animals showed signs of encephalopathy at 6 h after sepsis (lethargy, mild ataxia, lack of spontaneous movement, and loss of righting reflex) and gradually returned to their normal waking status 24–36 h after CLP.[26]

To minimize the possibility of animals not developing sepsis, the CLP procedure was always performed by the same investigators. In addition, all animals were observed after CLP to determine signs of infection (pyloerection, lethargy, tachypnoea and weight loss), and the number of animals that survived is in accordance with our previous reports.[25]

Tissue and homogenate preparation

Twelve hours after the last injection, the rats were killed by decapitation, the brain was removed, and the prefrontal cortex, hippocampus, striatum, cerebellum and cerebral cortex were homogenized (1:10, w/v) in SETH buffer, pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 IU/ml heparin). The homogenates were centrifuged at 800 × g for 10 min, and the supernatants were kept at –70°C until used for enzymatic activity determination. The maximal period between homogenate preparation and enzyme analysis was always less than 5 days. Protein content was determined by the method described by Lowry et al.[27] using bovine serum albumin as standard.

160

Scaini G, Rochi N, Benedet J, Ferreira GK,
Teodorak BP, Comim CM et al.

Citrate synthase activity

Citrate synthase activity was assayed according to the method described by Srere et al.[28] The reaction mixture contained 100 mM Tris, pH 8.0, 0.1 mM acetyl-CoA, 0.1 mM 5,5'-di-thiobis-(2-nitrobenzoic acid), 0.1% triton X-100, and 2–4 μg supernatant protein. The reaction was initiated with 0.2 mM oxaloacetate and monitored at 412 nm for 3 min at 25°C.

Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey test when F was significant. All analyses were performed using the Statistical Package for the Social Science (SPSS) software. Differences were considered significant when $P<0.05$.
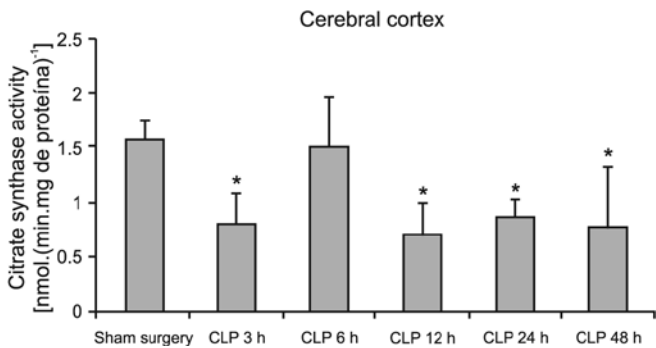
RESULTS

Our results demonstrate that citrate synthase activity in the prefrontal cortex was not affected 3 and 6 hours after CLP. However, the enzyme was significantly inhibited 12, 24 and 48 hours after CLP (Figure 1). As illustrated in figure 2, citrate synthase activity in the cerebral cortex was significantly inhibited 3, 12, 24 and 48 hours after CLP, but the enzyme was not affected 6 hours after CLP. Finally, citrate synthase was not affected in the hippocampus, striatum or cerebellum up to 48 hours after CLP (Figures 3, 4 and 5, respectively).
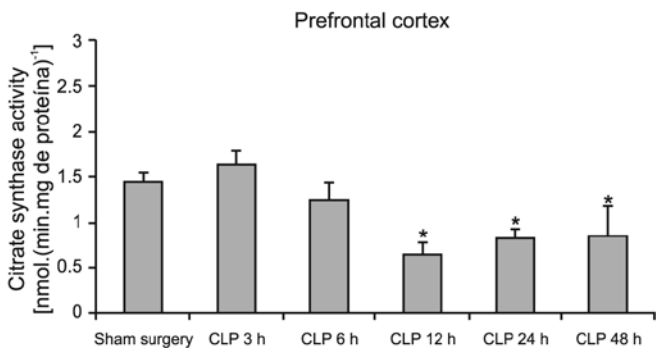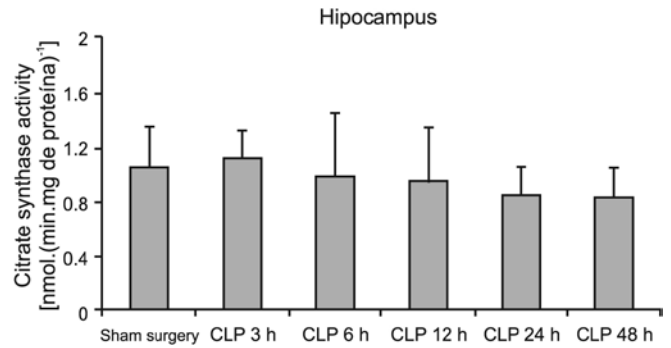
Results are expressed as the mean ± S.D. (n=6). Different from control group, *p<0.05 (One-way ANOVA followed by Tukey test).
Figure 3 - Citrate synthase activity in the hippocampus of rats 3, 6, 12, 24 and 48 hours after cecal ligation puncture (CLP).

Results are expressed as the mean ± S.D. (n=6). Different from control group, *p<0.05 (One-way ANOVA followed by Tukey test).
Figure 1 - Citrate synthase activity in the prefrontal cortex of rats 3, 6, 12, 24 and 48 hours after cecal ligation puncture (CLP).
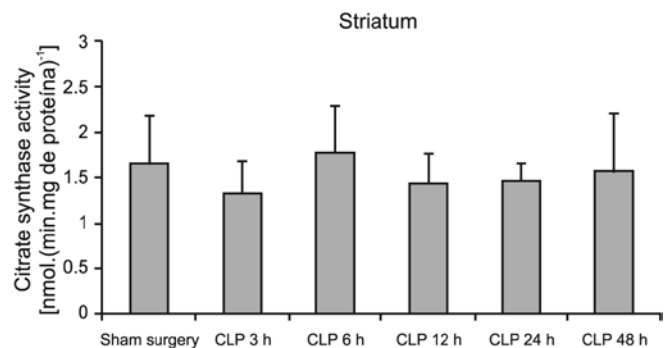
Results are expressed as the mean ± S.D. (n=6). Different from control group, *p<0.05 (One-way ANOVA followed by Tukey test).
Figure 4 - Citrate synthase activity in the striatum of rats 3, 6, 12, 24 and 48 hours after cecal ligation puncture (CLP).

Results are expressed as the mean ± S.D. (n=6). Different from control group, *p<0.05 (One-way ANOVA followed by Tukey test).
Figure 2 - Citrate synthase activity in the cerebral cortex of rats 3, 6, 12, 24 and 48 hours after cecal ligation puncture (CLP).
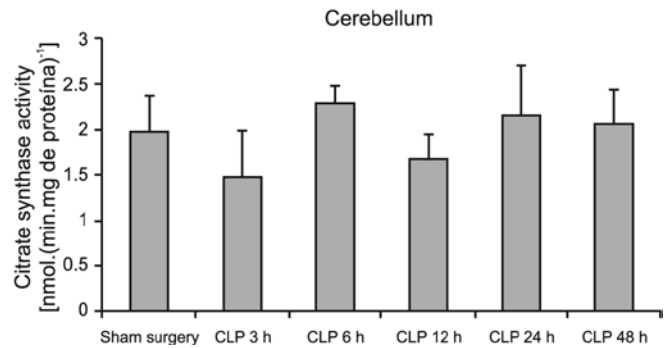
Results are expressed as the mean ± S.D. (n=6). Different from control group, *p<0.05 (One-way ANOVA followed by Tukey).
Figure 5 - Citrate synthase activity in cerebellum of rats 3, 6, 12, 24 and 48 hours after cecal ligation puncture (CLP).

DISCUSSION

Cell death within the central nervous system (CNS) during sepsis has been described in rodent models and humans.[29,30] An extensive body of evidence from experimental and clinical studies indicates that sepsis is associated with increased ROS production, depletion of antioxidants, and accumulation of markers of oxidative stress.

Direct evidence for free radical production in sepsis comes from studies using spin traps to detect hydroxyl radicals and electron paramagnetic resonance spectroscopy to analyze NO production in rats as well as studies detecting ascorbate radical production in septic patients.[31,32] Increased xanthine oxidase (XO) activity, an important ROS producer, has been reported in patients with sepsis.[33] In addition to showing elevated ROS production, studies have also found decreased antioxidant defenses leading to redox imbalance in sepsis patients. Glutathione replenishment by N-acetylcysteine and glutathione has been shown to decrease oxidative stress in patients with sepsis.[34] The increased ROS production and decreased antioxidant levels have been accompanied by increased lipid peroxidation in patients with sepsis compared with controls.[35]

In addition, the respiratory chain is an important pillar in sepsis pathophysiology. Because mitochondria play a critical role in cellular energy production via electron transport chain-dependent synthesis of ATP through oxidative phosphorylation and are the main site of ROS production, inflammatory insult results in mitochondria being damaged functionally and structurally. We previously performed a time-course experiment to evaluate the activities of mitochondrial respiratory chain complexes I, II, III and IV and creatine kinase after CLP; we demonstrated that brain energy metabolism is altered six and twelve hours after CLP.[36] More specifically, we observed that animals submitted to CLP presented decreased mitochondrial respiratory chain activity in complexes I and II but not in complexes III and IV at 24, 48 and 96 h post-CLP. A previous study showed that succinate dehydrogenase activity (an important enzyme in the Krebs cycle) was decreased at 48 and 96 h post-CLP in all analyzed structures.[37] In addition, other works indicate that mitochondrial free radical generation is increased in sepsis.[38,39]

Production of ROS perpetuates and propagates mitochondrial injury, leading to mitochondrial swelling and diminution of cytochrome c levels in the mitochondria.[40] Consequently, derangements in mitochondrial function primarily affect cells that have a high energy demand, such as neurons,[41] and brain energy impairment has been linked to neuronal death and neurodegeneration.[42] In this context, the brain may be one of the first organs affected during sepsis, and encephalopathy is a frequent association but infrequently recognized.[43,44] Acute encephalopathy has demonstrated in animal models of polymicrobial sepsis, and human sepsis survivors present cognitive impairment that could be a secondary effect of CNS damage.[26] In addition, survivors from critical care units, including sepsis patients, may show persistent brain-related morbidity, including neurocognitive deficits and development of psychiatric disorders.[45-49]

In the present study, we demonstrate that citrate synthase activity is inhibited in the prefrontal cortex and cerebral cortex of adult rats after sepsis induced by CLP. Considering that energy impairment resulting from mitochondrial dysfunction in sepsis has been well described and that citrate synthase has been used as a quantitative enzyme marker for the presence of intact mitochondria, we therefore believe that energy impairment may also be involved in these processes. If the inhibition of citrate synthase also occurs in a sepsis model, it is tempting to speculate that a reduction of brain metabolism may be related to the pathophysiology of this disease.

Prefrontal cortex lesions are associated with social disinhibition; impulse dyscontrol; organizational, planning, and attentional dysfunctions; dysfluency and slowing of spontaneous behaviors.[50] Konarska et al.[51] reported that regional deficits in the frontal lobe, particularly in the anterior cingulate and the orb to the frontal cortex, consistently delineate subjects with central nervous system disorders from the general population. We hypothesize that mitochondrial dysfunction may be related to CNS damage in sepsis.

CONCLUSION

In conclusion, we have demonstrated that citrate synthase is decreased by CLP in the prefrontal cortex and cerebral cortex. These data corroborate those from other studies, suggesting that mitochondrial dysfunction is implicated in the pathogenesis of CLP.

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162

Scaini G, Rochi N, Benedet J, Ferreira GK,
Teodorak BP, Comim CM et al.

RESUMO

Objetivo: Um amplo corpo de evidência oriundo de estudos experimentais indica que a sepse se associa com um aumento da produção de espécies de oxigênio reativo, depleção de antioxidantes, e acúmulo de marcadores de estresse oxidativo. Além disto, a disfunção mitocondrial foi implicada na patogênese da síndrome de disfunção de múltiplos órgãos. A citrato sintase é uma enzima que se localiza no interior das células, na matriz mitocondrial, sendo uma etapa importante do ciclo de Krebs; esta enzima foi utilizada como um marcador enzimático quantitativo da presença de mitocôndrias intactas. Assim, investigamos a atividade da citrato sintase no cérebro de ratos submetidos ao modelo sepse com de ligadura e punção do ceco.

Métodos: Em diferentes horários (3, 6, 12, 24 e 48 horas) após cirurgia de ligadura e punção do ceco, seis ratos foram sacrificados por decapitação, sendo seus cérebros removidos e dissecados o hipocampo, estriato, cerebelo, córtex cerebral e córtex pré-frontal, e utilizados para determinação da atividade de citrato sintase.

Resultados: Verificamos que a atividade de citrato sintase no córtex pré-frontal estava inibida após 12, 24 e 48 horas da ligadura e punção do ceco. No córtex cerebral, esta atividade estava inibida após 3, 12, 24 e 48 horas da ligadura e punção do ceco. Por outro lado a citrato sintase não foi afetada no hipocampo, estriato e cerebelo até 48 horas após a ligadura e punção do ceco.

Conclusão: Considerando-se que é bem descrito o comprometimento da energia decorrente da disfunção mitocondrial na sepse, e que o estresse oxidativo desempenha um papel essencial no desenvolvimento da sepse, acreditamos que o comprometimento da energia pode também estar evolvido nestes processos. Se a inibição da citrato sintase também ocorre em um modelo de sepse, é tentador especular que a redução do metabolismo cerebral pode provavelmente estar relacionada com a fisiopatologia desta doença.

Descritores: Citrato (si)-sintase/metabolismo; Sepse; Mitocôndrias; Ratos Wistar; Modelos animais

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