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Caffeine and acetaminophen association: Effects on mitochondrial bioenergetics

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ABSTRACT

Aims: Many studies have been demonstrating the role of mitochondrial function in acetaminophen (APAP) hepatotoxicity. Since APAP is commonly consumed with caffeine, this work evaluated the effects of the combination of APAP and caffeine on hepatic mitochondrial bioenergetic function in mice.
Main methods: Mice were treated with caffeine (20 mg/kg, intraperitoneal (i.p.)) or its vehicle and, after 30 minutes, APAP (250 mg/kg, i.p.) or its vehicle. Four hours later, livers were removed, and the parameters associated with mitochondrial function and oxidative stress were evaluated. Hepatic cellular oxygen consumption was evaluated by high-resolution respirometry (HRR).
Key findings: APAP treatment decreased cellular oxygen consumption and mitochondrial complex activities in the livers of mice. Additionally, treatment with APAP increased swelling of isolated mitochondria from mice livers. On the other hand, caffeine administered with APAP was able to improve hepatic mitochondrial bioenergetic function. Treatment with APAP increased lipid peroxidation and reactive oxygen species (ROS) production and decreased glutathione levels in the livers of mice. Caffeine administered with APAP was able to prevent lipid peroxidation and the ROS production in mice livers, which may be associated with the improvement of mitochondrial function caused by caffeine treatment.
Significance: We suggest that the antioxidant effects of caffeine and/or its interactions with mitochondrial bioenergetics may be involved in its beneficial effects against APAP hepatotoxicity.

1. Introduction

Acetaminophen (paracetamol, *N*-acetyl-*p*-aminophenol, APAP) is a classical non-steroidal anti-inflammatory drug (NSAID) that is widely consumed as an analgesic and antipyretic medication. However, APAP overdose may cause hepatic injury, leading to acute liver failure (ALF) [1]. Liver damage caused by high doses of APAP is associated with *N*-acetyl-*p*-benzoquinone imine (NAPQI) formation, a reactive metabolite that is formed by the cytochrome P-450 (CYP450) enzyme family [2].

One important mechanism involved in APAP overdose is the disruption of hepatic mitochondrial metabolism [3,4]. Mitochondria are the main target of NAPQI [5] which causes functional alterations in calcium metabolism, complexes I and II inhibition and decreased adenosine triphosphate (ATP) levels. The oxidative phosphorylation process results in an increase of ions and metabolic intermediates in mitochondria, causing mitochondrial swelling [6]. These events impair mitochondrial function and ultimately lead to cell death [1]. In this way, the mitochondrial permeability transition is one of the mechanisms involved in APAP-induced impairment of hepatic mitochondria [4].

Conversely, endogenous antioxidants, such as glutathione, and exogenous antioxidant compounds can protect mitochondria against cellular oxidative damage such as APAP-induced stress [7]. In this regard, caffeine, a methylxanthine alkaloid compound present in beverages (coffee, tea, energy drinks), food (chocolate, desserts) and medicines, has long been considered as an antioxidant molecule. Accumulating evidence has suggested a potential antioxidant role for caffeine [8–10]. Chemical studies have proposed the reactive oxygen

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production (ROS) scavenging properties of caffeine, particularly of the hydroxyl radical (OH·) [11–14]. However, the effects of caffeine on mitochondrial bioenergetics have been poorly investigated until now.

Caffeine is also frequently used as an analgesic drug adjuvant in several over-the-counter medications [15,16]. Drugs such as aspirin, diclofenac and APAP are largely combined with caffeine [17] and readily available in drugstores for relief of pathological (or pathology-related) symptoms such as dysregulated body temperature [18].

Studies on the analgesic effects of drugs used for pain relief, such as APAP, have shown that the combination of APAP with caffeine reduces the drug dosage necessary to produce the same analgesic effect when compared to APAP alone [16,17,19]. However, there is a lack of studies that investigate effects on mitochondrial liver function in drugs that combine caffeine with analgesics such as APAP. Usually, studies on caffeine and APAP association focus on the analgesic effects [17,20,21]. In addition, the role of APAP in poisoning mitochondrial metabolism is already known, but the effect of caffeine and APAP association on mitochondria remains unclear [1,22]. Therefore, the aim of the present study is to investigate the effect of APAP and caffeine association on hepatic mitochondrial bioenergetics in mice.

2. Materials and methods

2.1. Animals

Forty male adult swiss albino mice (2 months old, 30–40 g) from our own breeding colony were divided into groups of four or five animals for the biochemical assays. The animals were kept in a separate animal room, with light/dark cycles of 12 h each, at a temperature of 22 ± 2 °C, with free access to food and water. This study was approved by the Ethical and Animal Welfare Committee of the Federal University of Santa Maria, Brazil, under the process number 3208150915/2015.

2.2. Experimental protocol

The animals were randomly divided into four groups, with three or four animals per group, depending on the analysis: (1) control saline, (2) caffeine, (3) APAP and (4) caffeine plus APAP. The doses of caffeine (20 mg/kg) and APAP (250 mg/kg) used in these procedures were described previously [23,24] and were administered by intraperitoneal injection (i.p.). The APAP dose of 250 mg/kg was chosen because this dose produces mild hepatotoxicity, based on serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and moderate mitochondrial damage is preferable to distinguish the caffeine effect from that of APAP alone to evaluate the effect of the APAP and caffeine combination on mitochondrial bioenergetics. To study the effects of APAP and caffeine association on mitochondrial bioenergetics, the i.p. route of administration was chosen, since both compounds, caffeine and APAP, would not suffer any alteration during its passage through the gastrointestinal tract. The animals were fasted for 16 h [25] before starting the experimental protocol. Fig. 1 portrays the experimental protocol used in this study. The animals were pre-treated with caffeine or saline 30 min before APAP administration. Four hours after APAP administration, animals were sacrificed, and the livers were immediately removed for biochemical analyses according to previous studies [26,27].

2.3. Mitochondria isolation from the livers of mice

Mouse liver mitochondria were isolated at 4 °C by differential centrifugation [28]. After removal, livers were immersed and homogenized proportionally 1/10 (weight/volume) in ice-cold isolation buffer I containing 320 mM sucrose, 1 mM ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 1 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM Trizma-base, pH 7.4. The resulting suspension was centrifuged for 10 min at 2500 × *g* in a Hitachi CR 21E centrifuge. The supernatant was centrifuged at 10,000 × *g* for 10 min. The pellet was re-suspended in 200 μL of isolation buffer I with bovine serum albumin (BSA) containing 320 mM sucrose, 1 mM EDTA, 1 mM EGTA, 0.1% fatty acid free BSA and 10 mM Trizma-base, pH 7.4. The resulting supernatant was decanted, and the final pellet was gently washed and re-suspended in isolation buffer II containing 250 mM sucrose and 10 mM Trizma base, pH 7.4. The pellet was washed three times with isolation buffer II, and finally, the pellet was re-suspended, also in isolation buffer II consisting of intact mitochondria. Isolated mitochondria were used in the mitochondrial swelling assay and mitochondrial reduced glutathione (GSH) content assay.

2.4. ROS production assay

ROS generation was determined spectrofluorimetrically in liver homogenate using 2′7′dichlorodihydrofluorescein diacetate (H_2DCF-DA) (1 mM). The oxidation of H_2DCF to 2′7′dichlorofluorescein (DCF) is used as an index of the peroxide production by cellular components [29]. Briefly, liver homogenate was added to the standard medium, and the fluorescence was determined at 488 nm for excitation and 525 nm for emission, with slit widths of 3 nm.

2.5. Measurement of thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation (LPO) was determined by measuring thiobarbituric acid reactive substances (TBARS) [30]. An aliquot (200 μL) of liver homogenate was mixed with 500 μL thiobarbituric acid (0.6%), 200 μL sodium dodecyl sulfate (SDS) 8.1%, and 500 μL acetic acid (pH 3.4) and incubated at 90 °C for 1 h. TBARS levels were measured at 532 nm using a standard curve of malondialdehyde (MDA), and the results were reported as nmol MDA/mg protein.

2.6. High-resolution respirometry (HRR) assay

For the respirometry determination, mice livers were weighed and homogenized in 2 mL of cold buffer containing 5 mM Tris-HCl, 250 mM sucrose and 2 mM EGTA (pH = 7.4) and transferred into the Oxygraph-2k (O2k, OROBOROS INSTRUMENTS, Innsbruck) 2-mL chambers. Oxygen polarography was performed at 37 °C, and the oxygen flux was recorded in real time using DatLab software. In the present protocol, the liver homogenate (0.1 mg/mL) was added to the chamber containing the mitochondrial respiration medium (MIR05) (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 110 mM sucrose, 0.1 mg/mL fatty acid free BSA). After signal stabilization, the experimental protocol of high-resolution respirometry was performed by sequential addition of 10 mM glutamate, 2 mM malate, 2.5 mM adenosine diphosphate (ADP), 10 mM succinate, 0.5 μM rotenone and 2.5 μM antimycin A [31–33]. We used tissue homogenates for the analysis of mitochondrial respiration instead of isolated mitochondria because the mitochondrial isolation process can remove > 60% of the mitochondrial population compared to tissue homogenate, impair the mitochondria structure and functionality, and disrupt the mitochondrial network [34].

2.7. Mitochondrial swelling assay

Measurements of mitochondrial swelling were performed using an RF-5301 Shimadzu spectrofluorometer at 600 nm with 1.5-nm slits for excitation and emission. Mitochondria (0.1 mg protein) were incubated in buffer II containing 250 mM sucrose and 10 mM Trizma base at pH 7.4 [35]. This mitochondrial swelling assay requires the use of isolated mitochondria in order to obtain more reliable results [36].