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Mitochondrial complex I dysfunction induced by cocaine and cocaine plus morphine in brain and liver mitochondria

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HIGHLIGHTS

- Brain and liver mitochondrial respiration is differentially affected by the drugs.
- Cocaine-induced inhibition of complex I is more evident in brain mitochondria.
- Dependence on complex I may explain differences in brain and liver mitochondria.
- The drug combination had a greater effect on brain state 3 than the drugs per se.
- In other parameters the drug combination had similar effects to cocaine per se.

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ABSTRACT

Mitochondrial function and energy metabolism are affected in brains of human cocaine abusers. Cocaine is known to induce mitochondrial dysfunction in cardiac and hepatic tissues, but its effects on brain bioenergetics are less documented. Furthermore, the combination of cocaine and opioids (speedball) was also shown to induce mitochondrial dysfunction. In this work, we compared the effects of cocaine and/or morphine on the bioenergetics of isolated brain and liver mitochondria, to understand their specific effects in each tissue. Upon energization with complex I substrates, cocaine decreased state-3 respiration in brain (but not in liver) mitochondria and decreased uncoupled respiration and mitochondrial potential in both tissues, through a direct effect on complex I. Morphine presented only slight effects on brain and liver mitochondria, and the combination cocaine+morphine had similar effects to cocaine alone, except for a greater decrease in state-3 respiration. Brain and liver mitochondrial respirations were differentially affected, and liver mitochondria were more prone to proton leak caused by the drugs or their combination. This was possibly related with a different dependence on complex I in mitochondrial populations from these tissues. In summary, cocaine and cocaine+morphine induce mitochondrial complex I dysfunction in isolated brain and liver mitochondria, with specific effects in each tissue.

1. Introduction

Cocaine abuse has been associated with mitochondrial dysfunction and impairment of energy metabolism in brain (Lehrmann et al., 2003), liver (Devi and Chan, 1997) and heart (Yuan and Acosta Jr., 2000) cells. The mitochondrial respiratory chain seems to mediate acute cocaine toxicity, as suggested by the partial resistance to cocaine toxicity in cells lacking functional mitochondria (rho-zero), when compared with rho-plus cells (Cunha-Oliveira et al., 2006). In addition, the neurotoxicity of cocaine was previously shown to involve mitochondrial dysfunction and activation of the mitochondrial apoptotic pathway (Cunha-Oliveira et al., 2006, 2010), and exposure to cocaine was found to down-regulate mitochondrial gene expression, including several subunits of NADH dehydrogenase (complex I), in rat cingulate cortex (Dietrich et al., 2004).

Cocaine may interact directly with mitochondria and other intracellular targets (Heard et al., 2008), after entering the cell due to its positive charge at physiological pH (Cunha-Oliveira et al., 2008). Studies in hepatic mitochondria showed that in vivo cocaine administration decreased state 3 respiration, the respiratory

Abbreviations: BSA, bovine serum albumin; Coc+Mor, combination of cocaine and morphine; $\Delta\Psi$, mitochondrial transmembrane potential; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; RCR, respiratory control ratio; TPP^+, tetraphenylphosphonium.

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control ratio (RCR) and the activity of complexes I, II/III, and IV (Devi and Chan, 1997). However, much less is known about the direct effects of cocaine on the bioenergetics of brain mitochondria.

Cocaine effects may be altered if cocaine is used together with other drugs, such as opiates, a popular drug combination known as speedball (European Monitoring Center for Drugs and Drug Addiction, 2009) that has been reported to be more rewarding in rats than cocaine or heroin alone (Ranaldi and Munn, 1998). The popularity of this drug combination may be due to the reduction of the unwanted side-effects, due to complementary mechanisms of action, and/or from enhanced effects of the combination (Leri et al., 2003). Speedball has serious health consequences. The use of cocaine in combination with heroin is generally associated with a mental illness and may aggravate underlying psychological problems, such as bipolar disorder (European Monitoring Center for Drugs and Drug Addiction, 2008). In addition, speedball abusers present more severe psychopathology in comparison with other cocaine addicts, and are more likely to fail in drug abuse treatment (Bandettini Di Poggio et al., 2006).

Interestingly, a chemical interaction between cocaine and morphine was found to occur in cocaine–heroin mixtures, involving the formation of a cocaine–morphine adduct (Garrido et al., 2007), which may play a role in the effects of cocaine–heroin combinations, especially when the co-abuse occurs simultaneously and chemical interactions may occur. Thus, besides pharmacodynamic interactions, chemical interactions between drugs could also play a role in speedball neurotoxicity (Cunha-Oliveira et al., 2010). Previously, we used primary cultures of rat cortical neurons exposed to heroin and/or cocaine, administered sequentially or simultaneously (Cunha-Oliveira et al., 2010) to investigate this possibility. The effects of cocaine seemed to predominate over heroin effects when the cells were exposed to a combination of both drugs, but the extent of mitochondrial dysfunction induced by cocaine–morphine combinations was found to differ from that induced by each drug alone, in rat cortical neurons (Cunha-Oliveira et al., 2010). Moreover, the neurotoxic pathways induced by simultaneous co-administration of cocaine and heroin differed from those evoked by a sequential administration of the same drugs, possibly due to chemical interactions between the two drugs (or their metabolites), which may interfere with cell death mechanisms and may affect the capacity of cocaine to interact with its cellular targets, such as mitochondria.

In order to understand the specific effects of cocaine, morphine and cocaine+morphine in brain and liver mitochondria, in this work, we compared their effects on the bioenergetics of mitochondria isolated from each tissue.

2. Materials and methods

2.1. Chemicals

Cocaine hydrochloride and morphine sulfate salt pentahydrate were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA), upon authorization by INFARMED, Portugal (National Authority of Medicines and Health Products). All of the other chemicals were of the highest grade of purity commercially available.

2.2. Animals

Male Wistar-Han rats (9–12 weeks old) were maintained in our animal house, under controlled light (12 h day/night cycle), temperature and humidity, with ad libitum access to food and water. Procedures were approved by the Institutional Animal Care and Use Committee.

2.3. Isolation of liver and brain mitochondria

Animals were sacrificed by cervical displacement and decapitation, following procedures approved by the Institutional Animal Care and Use Committee.

Liver mitochondria were isolated by conventional methods (Moreno et al., 2007), with slight modifications. Briefly, animals were sacrificed and the liver was immediately excised, finely minced, and homogenized in ice-cold medium containing 250 mM sucrose, 10 mM HEPES, 0.5 mM EGTA and 0.1% defatted bovine serum albumin (BSA) (pH 7.4). The homogenate was centrifuged at 900 × *g* (Sorvall RC-5B Refrigerated Superspeed Centrifuge) for 10 min at 4 °C. The resulting supernatant was spun at 10,000 × *g* for 10 min (at 4 °C) to pellet mitochondria. The mitochondrial pellet was washed twice (2 × 10,000 × *g* for 10 min) and suspended in the washing medium (250 mM sucrose, 10 mM HEPES, pH 7.4; EGTA and BSA were omitted from the washing medium). Protein content was determined by the Biuret method (Gornall et al., 1949), using BSA as a standard.

Brain mitochondria were isolated by the method of Rosenthal et al. (1987), with slight modifications, using 0.02% digitonin to free mitochondria from the synaptosomal fraction. Briefly, the whole brain except the cerebellum was rapidly removed, washed, minced, and homogenized at 4 °C in 10 ml of isolation medium (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1 mg/ml defatted BSA, pH 7.4) containing 5 mg of the bacterial protease type VIII. Single brain homogenates were brought to 30 ml and then centrifuged at 740 × *g* (Sorvall RC-5B Refrigerated Superspeed Centrifuge) for 5 min. The pellet, including the fluffy synaptosomal layer, was resuspended in 10 ml of the isolation medium containing 0.02% digitonin and centrifuged at 11,950 × *g* for 10 min. The brown mitochondrial pellet without the synaptosomal layer was then resuspended in 10 ml of medium and centrifuged again at 10,000 rpm for 5 min. The pellet was then resuspended in 10 ml of resuspension medium (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, pH 7.4) and centrifuged at 11,950 × *g* for 5 min. Finally, the mitochondrial pellet was resuspended in 200 μl of resuspension medium. Protein content was determined by the Biuret method (Gornall et al., 1949), using BSA as a standard.

2.4. Mitochondrial respiration

Mitochondrial oxygen consumption was monitored polarographically at 30 °C with a Clark-type oxygen electrode (Yellow Spring Instrument, Model YSI5331) connected to a Kipp and Zonen recorder accordingly to Estabrook (1967), in a thermostatic water-jacketed chamber with magnetic stirring. The reactions were carried out at 30 °C in 1 ml of standard respiratory medium with 1 mg of liver mitochondria or 0.5 mg of brain mitochondria. For liver mitochondria the reaction medium was composed of 130 mM sucrose, 50 mM KCl, 2.5 mM $MgCl_2$, 2.5 mM KH_2PO_4, 5 mM HEPES and 0.01 mM EGTA (pH 7.4). For brain mitochondria the reaction medium was composed of 100 mM sucrose, 100 mM KCl, 2 mM KH_2PO_4, 5 mM HEPES and 0.01 mM EGTA (pH 7.4). Liver or brain mitochondria were incubated, for 1 min, in 1 ml of respiratory medium in the absence or presence of the drugs, alone or combined at 0.2–1 mM, and energized with 10 mM glutamate/5 mM malate or 5 mM succinate. When succinate was used as respiratory substrate, the medium was supplemented with 2 μM rotenone. State 3 was initiated with ADP (150 nmol/mg protein for brain mitochondria and 125 nmol/mg protein for liver mitochondria), followed by addition of oligomycin (1 μg/ml) plus ADP (150 nmol/mg protein for brain mitochondria and 125 nmol/mg protein for liver mitochondria). Uncoupled respiration was initiated by the addition of 1 μM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP). Respiration rates were calculated assuming an oxygen concentration of 240 nmol O_2/ml in the experimental medium at 30 °C. The respiratory state 2 (oxygen consumption in the absence of substrate), state 3 (oxygen consumption in the presence of substrate and ADP), state 4 (oxygen consumption after ADP phosphorylation) and RCR (state 3/state 4) were calculated according to Chance and Williams (1956). ADP/O ratio was expressed by the ratio between the amount of ADP added and the oxygen consumed during state 3 respiration.

2.4.1. Assessment of mitochondrial respiratory activity through complexes I and II

For the assessment of direct effects of the drugs and their combination over mitochondrial complexes I and II, independently of mitochondrial physiology (Moreira et al., 2012), after isolation, mitochondrial preparations were frozen in liquid nitrogen and kept at −80 °C until further use. At the day of the experiments, the preparations were thawed and sonicated 4 times for 10 s, on ice, with even intervals. Oxygen consumption was measured in the oxygen electrode in 1 ml reaction medium, using 1 mg and 0.8 mg protein, respectively, of liver or brain mitochondrial fractions. To evaluate the direct effect of the drugs on complex I-driven respiration, 1 mM NADH was added. Complex II-driven respiration was assessed independently, by adding 5 mM succinate in the presence of 2 μM rotenone. KCN was added at the end of the experiment, to confirm oxygen consumption through complex IV.

2.5. Membrane potential ($\Delta\Psi$) measurements

The mitochondrial transmembrane potential ($\Delta\Psi$) was monitored by evaluating transmembrane distribution of the lipophilic cation TPP^+ (tetraphenylphosphonium) with a TPP^+-selective electrode prepared according to Kamo et al. (1979) using $Ag/AgCl_2$-saturated electrode as reference. TPP^+ uptake was assessed from the decreased TPP^+ concentration in the medium sensed by the electrode. The difference between the potential of the selective electrode and the reference electrode was measured with an electrometer and recorded continuously in a Kipp and Zonen recorder. The voltage response of the TPP^+ electrode to log [TPP^+] was linear with a slope of 59 ± 1, in a good agreement with Nernst equation.

To monitor $\Delta\Psi$ associated to mitochondrial respiration, 1 mg (liver) or 0.5 mg (brain) mitochondria were incubated in the standard respiratory medium