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Effects of Cocaine and Its Oxidative Metabolites on Mitochondrial Respiration and Generation of Reactive Oxygen Species

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ABSTRACT. Cocaine is capable of producing severe hepatocellular necrosis in laboratory animals and humans. The mechanism of cocaine hepatotoxicity is not well understood, but appears to result from the actions of one or more *N*-oxidative metabolites of cocaine. Mitochondria have been proposed as critical cellular targets for cocaine toxicity, and previous studies have found depressed mitochondrial respiration and increased mitochondrial generation of reactive oxygen species (ROS) in animals treated with cocaine. To examine the potential role of cocaine *N*-oxidative metabolites in these effects, mitochondrial respiration and ROS generation were examined in isolated mouse mitochondria treated with cocaine and its *N*-oxidative metabolites—norcocaine, *N*-hydroxynorcocaine, and norcocaine nitroxide. Cocaine, in concentrations of 0.25 or 0.5 mM, had no effect on state 3 respiration, state 4 respiration, respiratory control ratio (RCR), or ADP/O ratio. Norcocaine (0.5 mM) inhibited state 3 respiration, and *N*-hydroxynorcocaine (0.5 mM) inhibited both state 3 and state 4 respiration. Norcocaine nitroxide had the greatest effect on mitochondrial respiration; the lower concentration (0.25 mM) completely inhibited both state 3 and state 4 respiration. Preincubation of mitochondria with cocaine or metabolites increased the inhibitory effect of norcocaine and *N*-hydroxynorcocaine, but not cocaine. Cocaine, norcocaine, and *N*-hydroxynorcocaine (0.1 mM) had no effect on ROS generation during state 3 respiration, and cocaine and norcocaine decreased ROS generation under state 4 conditions. Norcocaine nitroxide interfered with the fluorescence ROS assay and could not be assessed. The results suggest that the effects of cocaine on mitochondrial respiration are due to its *N*-oxidative metabolites. Inhibition of mitochondrial respiration by the *N*-oxidative metabolites of cocaine may be the underlying cause for observed ATP depletion and subsequent cell death. BIOCHEM PHARMACOL **60**;5:615–623, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. cocaine; reactive oxygen species; mitochondrial respiration; hepatotoxicity; norcocaine; *N*-hydroxynorcocaine; norcocaine nitroxide

Several case reports have described hepatic injury from cocaine use, including cases in which cocaine-induced liver damage resulted in mortality [1–5]. In general, clinical response to cocaine in these cases included a rapid rise in serum transaminase levels, and histopathology, when available, revealed hepatocellular necrosis, often accompanied by mild steatosis. Cocaine hepatotoxicity also has been demonstrated in the rat and mouse, with the mouse being the most-studied model. As in humans, cocaine hepatotoxicity in mice is characterized by the development of severe, potentially fatal hepatocellular necrosis [6–8].

The development in mice of hepatotoxicity from cocaine is closely correlated with its oxidative metabolism, and hepatic injury from cocaine generally is regarded as attributable to one or more of its oxidative metabolites [8, 9]. Cocaine in the liver undergoes rapid sequential oxidation of the tropane nitrogen, leading successively to norcocaine, *N*-hydroxynorcocaine, and norcocaine nitroxide. Each of these metabolites has been shown to produce hepatic necrosis in mice [10–12], but the mechanism of their hepatotoxicity remains unclear. Recent evidence suggests that the production of ROS¶ plays an important role in acute cocaine hepatotoxicity, both *in vitro* [9, 13–15] and *in vivo* [16], and uncoupling of both P450-mediated monooxygenase activity [13] and mitochondrial respiration [14, 17] have been hypothesized as possible sources of ROS following cocaine administration. Although it has been shown

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¶ *Abbreviations*: CCCP, carbonyl cyanide 3-chlorophenyl-hydrazone; $\Delta\psi m$, mitochondrial membrane potential; DHR, dihydrorhodamine 123; DMF, *N*,*N*-dimethylformamide; MSH, mannitol–sucrose–HEPES buffer; RCR, respiratory control ratio; and ROS, reactive oxygen species.

that cocaine after *in vivo* administration depresses mitochondrial respiration [17], decreases mitochondrial membrane potential [18], and enhances mitochondrial production of ROS [14], it is not yet clear whether cocaine or one of its metabolites is responsible for these effects.

The objective of this study was to examine and compare the effects of cocaine and its oxidative metabolites on mitochondrial respiration, and to determine whether these metabolites stimulate the release of ROS. For the former objective, mitochondrial respiration was measured in the presence of cocaine, norcocaine, *N*-hydroxynorcocaine, or norcocaine nitroxide. For the latter objective, we adapted and further developed a method based on the oxidative transformation of DHR to the fluorescent product rhodamine 123 [19] to measure ROS production in isolated mitochondria incubated with cocaine or its oxidative metabolites. Data are presented showing that *N*-oxidative metabolites of cocaine reduced the ability of isolated mitochondria to carry out oxidative phosphorylation, but had no effect on ROS production.

MATERIALS AND METHODS

Experiments examining the effects of cocaine and its metabolites on mitochondrial respiration were conducted in the United States, while experiments evaluating effects on mitochondrial ROS generation were conducted in Switzerland. Within practical limits, both phases of the experimentation were conducted in a consistent fashion. Any differences in sources of materials used by the two laboratories are identified in the following sections.

Reagents

CCCP, antimycin A, and DHR were obtained from Fluka AG. Catalase from bovine liver, adenosine 5′-diphosphate from equine muscle, BSA, DMF, succinic acid, and EDTA were purchased from the Sigma Chemical Co. (−)-Cocaine hydrochloride [1*R*-(*exo*,*exo*)-], D-mannitol, and HEPES for experiments in Switzerland were purchased from Fluka AG, while experiments conducted in the United States used Sigma as the source for these chemicals. (−)-Norcocaine was obtained from the National Institute on Drug Abuse (via the Research Triangle Institute). (−)-*N*-Hydroxynorcocaine and (−)-norcocaine nitroxide were synthesized as described previously [12].

Animals

All experiments were approved by the appropriate institutional authorities. For experiments in Switzerland, male ICR mice [Hsd: ICR (CD-1®); 8 weeks of age] were obtained from Harlan, Horst. The mice were kept in Macrolone cages with wood shavings as bedding at 24 ± 2° and 55 ± 5% relative humidity in a 12-hr light/dark rhythm (7:00 a.m. to 7:00 p.m. light) with free access to water and Nafag 857 mouse pellets (Nafag). For experiments conducted in the United States, male ICR mice, 8 weeks of age, were obtained from Harlan Sprague-Dawley. Mice were housed on corncob bedding in plexiglass cages with free access to food (Teklad Rodent Diet 8604, Harlan Sprague-Dawley) and water. Animal quarters were on a 12-hr light/dark cycle, and maintained at 22 ± 2°, with a relative humidity of 60 ± 5%. All experiments were conducted with mice starved overnight.

Mitochondrial Preparation

Animals were euthanized with carbon dioxide, and each liver was removed quickly. Livers were homogenized immediately in ice-cold MSH/EDTA buffer (210 mM mannitol, 70 mM sucrose, and 5 mM HEPES containing 1 mM EDTA). The mitochondrial pellet was obtained by low-speed centrifugation (1000 *g*) of the homogenate, followed by high-speed centrifugation (10,000 *g*) of the supernatant. The pellet was washed twice in the same buffer. Protein content was determined according to Bradford [20] using BSA as standard protein and adapted for the Cobas Fara autoanalyzer (Roche).

Mitochondrial Respiration

Mitochondria isolated from each liver were used to test the effects of individual metabolites on respiration. Protein was determined in each mitochondrial preparation. Oxygen consumption by isolated mitochondria was measured using a Clarke-type oxygen electrode in a water-jacketed incubation chamber with a capacity of 675 μL (model 202B, Instech Laboratories, Inc.) maintained at 30°. Freshly isolated mitochondria (0.5 mg protein) were introduced into the chamber containing respiration buffer (250 mM sucrose, 2 mM EDTA, 20 mM KH_2PO_4, 4 mM magnesium chloride, pH 7.4) using a microsyringe. Endogenous respiration was recorded for 2 min, and then 30 μL of 150 mM sodium succinate (final concentration 6.66 mM) was injected as substrate to initiate state 4 respiration, which was recorded for 2 min. Five microliters of 12.6 mM ADP (final concentration, 63.1 nM) was injected to initiate state 3 respiration. When state 3 returned to state 4, another 5 μL of ADP was injected to initiate another cycle. Rates of state 3 and state 4 respiration were measured from polarigraphic traces according to Chance and Williams [21]. RCR and ADP/O ratios were calculated according to standard procedures. Mitochondria with normal respiratory function were then used to test the effects of cocaine and cocaine metabolites.

Stock solutions of cocaine (16.65 and 33.3 mM in distilled water) and each metabolite—norcocaine, *N*-hydroxynorcocaine, norcocaine nitroxide (16.65 and 33.3 mM in 95% ethanol)—were prepared just prior to use. For each mitochondrial preparation, the effect of each metabolite on succinate respiration was tested individually. After

at least two cycles of normal state 4 and state 3 respiration with untreated mitochondria (0.5 mg protein), 10 μL of metabolite stock was introduced into the reaction chamber (final concentration of 0.25 or 0.5 mM) during state 3 respiration (immediately following ADP), and the rates of state 3 and the subsequent state 4 respiration were measured. RCR and ADP/O ratios were also calculated. This procedure was repeated with each metabolite. To avoid residual effects on subsequent runs, the reaction chamber was emptied, rinsed with distilled water, and then filled with fresh respiration buffer before testing the next metabolite. Differences in respiration rates before and after treatment were compared for each metabolite. Metabolites evaluated were norcocaine (N = 8 mice), *N*-hydroxynorcocaine (N = 8 mice), and norcocaine nitroxide (N = 6 mice). The effect of the parent compound, cocaine (N = 7 mice), was also tested for comparison. The effect of 95% ethanol (solvent for norcocaine, *N*-hydroxynorcocaine, and norcocaine nitroxide) on respiration was also assessed to eliminate any confounding factors. Addition of 10 μL of ethanol had no effects on oxygen consumption.

In some experiments, mitochondria were preincubated with cocaine or metabolite prior to initiating state 3 or state 4 respiration. For these experiments, freshly isolated mitochondria (0.5 mg protein) were preincubated with 10 μL of 16.6 mM cocaine (N = 8), norcocaine (N = 8), *N*-hydroxynorcocaine (N = 8), or norcocaine nitroxide (N = 6) (final concentration 2.75 mM for each metabolite) for 5 min on ice. The mixture was introduced into the respiration chamber containing respiration buffer, and endogenous respiration was recorded for 2 min after which 30 μL of 150 mM sodium succinate (final concentration 6.66 mM) was injected to initiate state 4 respiration, which was recorded for 2 min. Then 5 μL of 12.625 mM ADP (final concentration, 63.1 nM) was injected to initiate state 3 respiration. State 3 and state 4 respiration rates, RCR, and ADP/O ratio were calculated and compared with those for untreated mitochondria.

Measurement of Mitochondrial ROS Production: DHR Assay

Mitochondrial ROS production was monitored by measuring the formation of the fluorescent dye rhodamine 123 from the oxidation of DHR in the presence of catalase [22]. The fluorescent cation rhodamine 123 formed from the reaction accumulates inside the mitochondria as a function of the $\Delta \psi m$, which results in fluorescent quenching [23]. To completely release the fluorescent dye from mitochondria and, hence, to de-quench the fluorescent signal at the end of the measurement period, the mitochondrial membrane potential was dissipated by the addition of an uncoupling agent, CCCP [24].

ROS production was measured during state 3 and state 4 respiration. For measurements during state 4, the reaction mixture consisted of mitochondria (0.5 mg protein), test compound (cocaine, norcocaine, *N*-hydroxynorcocaine at final concentrations of 0.1 mM; antimycin A at a final concentration of 10 μM) or ethanol (0.1%, v/v) (vehicle control), and 1 μM DHR in MSH buffer without EDTA containing BSA (0.1 mg/mL), KH_2PO_4 (500 μM), and catalase (0.04 mg/mL). The final concentration of cocaine and metabolites was 0.1 mM. For state 3 measurements, the reagent mixture was the same except for KH_2PO_4 concentrations (1.5 mM instead of 0.5 mM) and supplementation with 2 mM ADP. The reagent mixtures were preincubated for exactly 3 min in a water bath at 37° and were transferred quickly into an acyl cuvette; fluorescence was recorded at an excitation wavelength of 488 nm and an emission wavelength of 525 nm with an LS-50 fluorescence spectrometer (Perkin Elmer Ltd.). After an initial recording interval of 30 sec, 5 mM succinic acid or a corresponding amount of MSH buffer (for background fluorescence measurement) was added, and recording was continued for exactly 150 sec followed by the addition of 12.5 μM CCCP (from a 5 mM stock solution in DMF). Fluorescence was recorded for another 60 sec. To determine the relative increase in fluorescence over a period of 200 sec, the difference in fluorescence between the time point of 10 sec before the addition of succinic acid/MSH and that of 40 sec after the addition of the uncoupler was calculated. The specific succinate-driven increase in fluorescence was then obtained by subtracting the above-calculated increment in fluorescence of a background measure (addition of MSH buffer instead of succinic acid) from that in the presence of succinic acid. Having for each measure its corresponding background measure was important in order to exclude effects that result from variations in the mitochondrial membrane potential caused by different composition of the incubation mixture (with or without ADP) or "aging" of the mitochondrial suspensions (membrane potential decreases with time after isolation). By the same method, the data were corrected for possible DHR autooxidation. Corresponding background fluorescence measurements were performed as part of each experiment.

Statistical Analysis

To test the effects of cocaine metabolites on mitochondrial respiration, mean values of state 3, state 4, RCR, and ADP/O ratios before and after treatment were compared for each metabolite. ROS generation data were obtained from three independent mitochondrial preparations using at least two measurements for each experimental condition. For statistical analysis, the mean value in each mitochondrial preparation for state 4 ROS production was normalized to 100%, and all values from one preparation were expressed accordingly as a percentage of mean state 4 ROS production. For both sets of experiments, differences among the treatment groups were analyzed by one-way ANOVA with a Scheffé's F test. $P < 0.05$ was considered significant.

TABLE 1. Effect of cocaine or its *N*-oxidative metabolites on mitochondrial respiration

	Cocaine (controls)		Norcocaine (controls)		N-OH-Norcocaine (controls)		Norcocaine nitroxide (controls)	
	0.25 mM	0.5 mM	0.25 mM	0.5 mM	0.25 mM	0.5 mM	0.25 mM	0.5 mM
State 3	66.41 ± 7.3	55.74 ± 5.43	53.98 ± 11.1	15.94 ± 9.20*	50.58 ± 8.2	10.9 ± 7.48*	12.21 ± 8.9*	0.0*
	(66.34 ± 10.1)	(55.85 ± 5.66)	(60.23 ± 11.8)	(49.29 ± 6.51)	(69.29 ± 14.8)	(43.91 ± 4.94)	(50.13 ± 9.8)	(45.55 ± 6.97)
State 4	18.29 ± 2.2	14.48 ± 1.97	17.16 ± 2.0	9.03 ± 4.88	18.57 ± 3.5	4.93 ± 3.43*	3.28 ± 2.4*	0.0*
	(16.92 ± 2.9)	(13.48 ± 2.09)	(14.06 ± 2.3)	(13.75 ± 2.57)	(18.90 ± 4.3)	(13.33 ± 1.57)	(13.58 ± 2.8)	(14.99 ± 2.26)
RCR	3.62 ± 0.5	3.91 ± 0.43	2.97 ± 0.3	1.70 ± 0.22*	2.88 ± 0.5	2.24 ± 0.12	3.65 ± 0.2	NC†
	(3.99 ± 0.4)	(4.37 ± 0.41)	(4.12 ± 0.3)	(4.07 ± 0.59)	(3.68 ± 0.3)	(3.47 ± 0.33)	(3.84 ± 0.2)	(3.05 ± 0.14)
ADP/O	1.87 ± 0.2	1.99 ± 0.11	1.59 ± 0.1	1.14 ± 0.57*	1.84 ± 0.2	0.61 ± 0.40*	0.43 ± 0.3*	NC
	(2.07 ± 0.3)	(1.96 ± 0.17)	(2.15 ± 0.1)	(2.03 ± 0.04)	(2.17 ± 0.3)	(2.22 ± 0.17)	(2.57 ± 0.3)	(2.27 ± 0.10)

Oxygen consumption during state 3 and subsequent state 4 respiration was assessed before and after the addition of the test compound (0.25 or 0.5 mM) into the respiration chamber. Values are means ± SEM (nmol O/min/0.5 mg protein), N = 6–8 reactions/metabolite. Values in parentheses are corresponding untreated control values.

*Significantly different from control mitochondria ($P < 0.05$).

†NC = not calculable; ratio involves division by zero.

RESULTS

Effects of Cocaine or Metabolites on Mitochondrial Respiration

The addition of cocaine, 0.25 or 0.5 mM, to freshly isolated mitochondria during ADP-stimulated state 3 respiration produced no change in oxygen consumption (Table 1). Cocaine addition also produced no change in oxygen consumption during state 4 respiration, and RCR and ADP/O were unaffected. Addition of norcocaine or *N*-hydroxynorcocaine also had no effect on mitochondrial respiration at the lower concentration tested (0.25 mM), but the higher concentration (0.5 mM) of norcocaine decreased oxygen consumption significantly during state 3 respiration and lowered the RCR and ADP/O substantially. *N*-Hydroxynorcocaine at 0.5 mM depressed both state 3 and state 4 respiration, accompanied by significant decreases in ADP/O. The lower concentration of norcocaine nitroxide (0.25 mM) produced effects similar to the higher concentration of *N*-hydroxynorcocaine. Norcocaine nitroxide at 0.5 mM completely eliminated state 3 and state 4 respiration. Collectively, these data suggest increasing ability to inhibit mitochondrial respiration *in vitro* with increasing oxidation of cocaine (i.e. cocaine < norcocaine < *N*-hydroxynorcocaine < norcocaine nitroxide).

A second set of experiments was conducted in which cocaine and metabolites were preincubated with mitochondria for 5 min prior to the addition of substrate and measurement of respiration. The rationale for this set of experiments was to allow greater opportunity for cocaine or its metabolites to interact with mitochondria before assessment of potential effects. As in the initial set of experiments, the addition of cocaine at 0.25 or 0.5 mM had no significant effect on oxygen consumption during state 3 or state 4 metabolism, nor on RCR or ADP/O (Table 2). However, preincubation with any of the metabolites at 0.25 or 0.5 mM reduced or eliminated oxygen consumption significantly during both state 3 and state 4 respiration (Table 2).

Use of the DHR Assay to Determine Mitochondrial ROS Production

Impairment of mitochondrial respiration might lead to an enhanced production of ROS [25, 26]. Therefore, we

TABLE 2. Effect of pretreatment with cocaine or its metabolites on mitochondrial oxidative phosphorylation

	Cocaine (controls)		Norcocaine (controls)		N-OH-Norcocaine (controls)		Norcocaine nitroxide (controls)	
	0.25 mM	0.5 mM	0.25 mM	0.5 mM	0.25 mM	0.5 mM	0.25 mM	0.5 mM
State 3	100.46 ± 18.7	46.53 ± 4.43	0.0*	0.0*	0.0*	0.0*	0.0*	0.0*
	(66.34 ± 10.1)	(55.85 ± 5.66)	(60.23 ± 11.8)	(49.29 ± 6.51)	(69.29 ± 14.8)	(43.91 ± 4.94)	(50.13 ± 9.8)	(45.55 ± 6.97)
State 4	27.22 ± 0.7	14.70 ± 2.74	0.0*	0.0*	0.0*	0.0*	0.0*	0.0*
	(16.92 ± 2.9)	(13.48 ± 2.08)	(14.06 ± 2.3)	(13.75 ± 2.57)	(18.90 ± 4.3)	(13.33 ± 1.57)	(13.58 ± 2.8)	(14.99 ± 2.26)
RCR	3.36 ± 0.6	3.61 ± 0.46	0.0*	0.0*	NC†	NC	NC	NC
	(3.99 ± 0.4)	(4.37 ± 0.41)	4.12 ± 0.3)	(4.07 ± 0.59)	(3.68 ± 0.3)	(3.47 ± 0.33)	(3.48 ± 0.2)	(3.50 ± 0.14)
ADP/O	2.26 ± 0.4	2.02 ± 0.07	0.0*	0.0*	NC	NC	NC	NC
	(2.07 ± 0.3)	(1.96 ± 0.17)	(2.15 ± 0.1)	(2.03 ± 0.04)	(2.17 ± 0.3)	(2.22 ± 0.17)	(2.57 ± 0.3)	(2.27 ± 0.10)

Mitochondria were preincubated with metabolites (0.25 or 0.5 mM) for 5 min. Respiration supported by succinate was evaluated during state 3 and state 4. Values are means ± SEM (nMol O/min/0.5 mg protein), N = 3–8 reactions/metabolite. Values in parentheses are corresponding untreated control values.

*Significantly different from control mitochondria ($P < 0.05$).

†NC = not calculable; ratio involves division by zero.

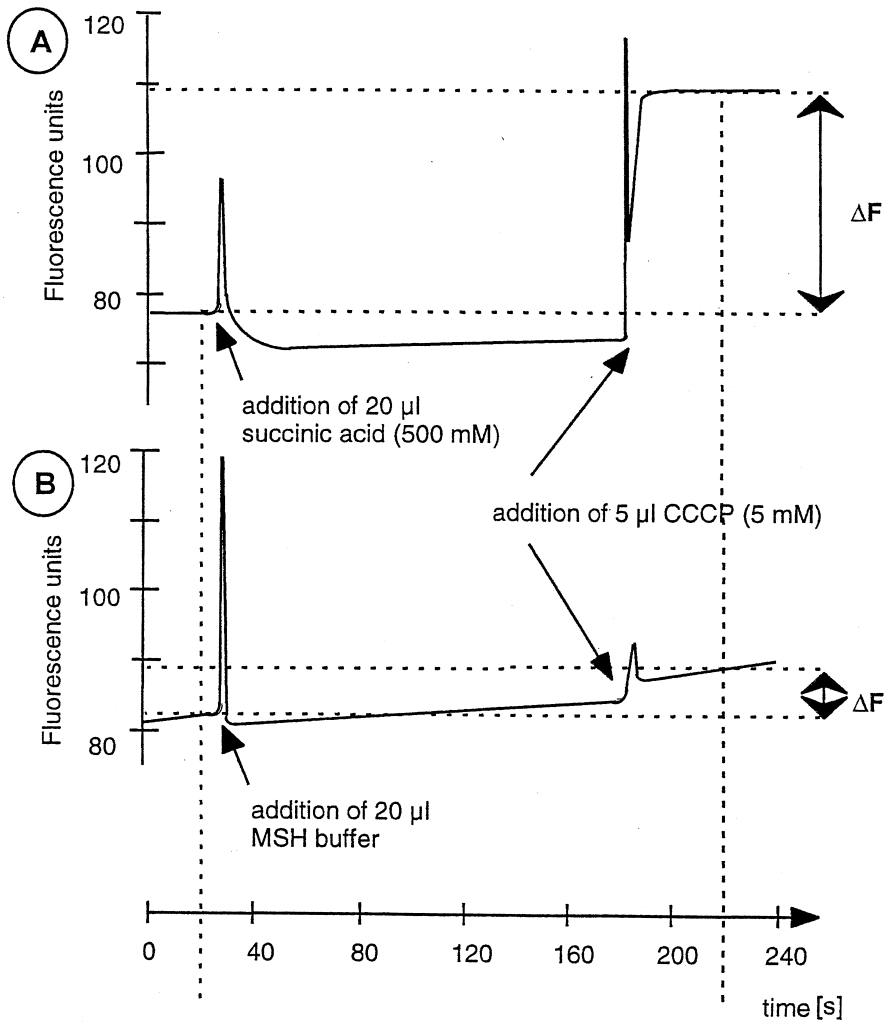

FIG. 1. Representative fluorescence recordings of ROS production in vehicle-treated control mitochondria obtained with the DHR method. Mitochondria were incubated in the presence (A) or absence (B) of succinic acid (5 mM), as described under Materials and Methods. The difference of the increase in fluorescence (ΔF) over the 200-sec recording periods represents the succinic acid-driven ROS production.

developed a new method to measure ROS production from isolated mitochondria treated with cocaine or its *N*-oxidative metabolites using the fluorescent dye dihydrorhodamine. Fluorescence recording of the reagent mixture alone revealed a slight increase in fluorescence over time (Fig. 1, A and B), which might represent DHR autooxidation or ROS production from mitochondrial respiration using endogenous substrate. The addition of succinic acid resulted in an initial decrease of rhodamine fluorescence (Fig. 1A). This initial decrease was not seen after the addition of MSH buffer (Fig. 1B) and is interpreted as an increase in $\Delta\Psi m$, leading to the accumulation of rhodamine at the inner mitochondrial membrane, which resulted in fluorescence quenching. The addition of the uncoupler CCCP readily caused mitochondrial membrane depolarization accompanied by an abrupt increase in rhodamine fluorescence. Addition of the indicated amount of CCCP was sufficient to cause maximal release of rhodamine because further addition of CCCP did not lead to a further increase in fluorescence (not shown).

The extent of DHR oxidation was higher during state 4 respiration than during state 3 respiration (Fig. 2), reflecting enhanced ROS production in state 4 compared with state 3. This is in accordance with previous reports [27]. The addition of antimycin A, an inhibitor of mitochondrial electron transport, enhanced DHR oxidation in state 3 (Fig. 2) but had no effect on state 4 respiration (data not shown).

Mitochondrial ROS Production in the Presence of Cocaine and Its Oxidative Metabolites

Figure 3 summarizes the results obtained with the DHR assay with mitochondria exposed to 0.1 mM cocaine, norcocaine, or *N*-hydroxynorcocaine. A relatively low concentration of cocaine or its metabolites was chosen to assess ROS production because ROS production in hepatocytes has been shown to be more pronounced at lower cocaine concentrations, decreasing progressively at concentrations higher than 0.1 mM [15]. While none of the three agents affected state 3 DHR fluorescence, both cocaine and norcocaine attenuated mitochondrial DHR fluorescence under state 4 conditions. This effect, however, did not occur with *N*-hydroxynorcocaine. In the presence of nor-

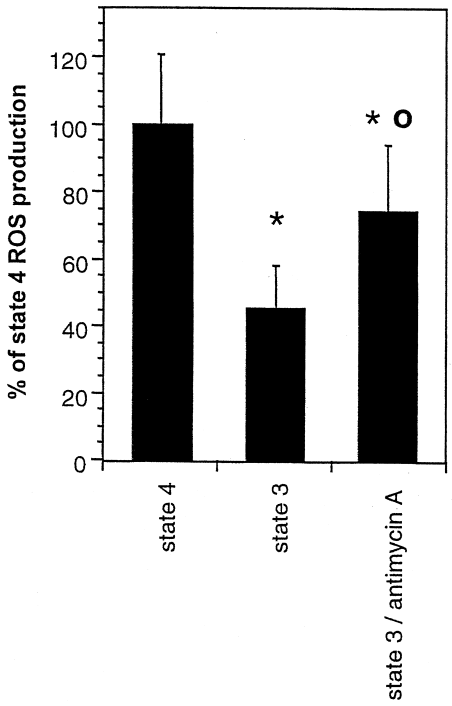

FIG. 2. Dependence of ROS production in isolated mitochondria on the respiration state. Dihydrorhodamine fluorescence was measured in the presence (10 μM) or absence of antimycin A as described under Materials and Methods. Values are means ± SD from 9 independent mitochondrial preparations including 15–21 measurements per treatment group. Key: (*) significantly different ($P < 0.05$) from state 4; and (°) significantly different ($P < 0.05$) from state 3 without antimycin.

cocaine nitroxide, ROS production could not be determined with this method because norcocaine nitroxide interfered with the assay by massively increasing rhodamine 123 fluorescence independent of mitochondrial respiration. These results clearly demonstrated that acute treatment of isolated mitochondria with cocaine, norcocaine, or *N*-hydroxynorcocaine did not result in enhanced ROS production. In contrast, exposure to cocaine or norcocaine led, instead, to a decrease in ROS generation during state 4.

DISCUSSION

Several studies have suggested that hepatic mitochondria may be an important target for cocaine effects. Significantly diminished state 3 respiration and RCR have been observed in mitochondria isolated from cocaine-treated mice [28] and rats [17]. This impairment of mitochondrial respiration provides a logical explanation for decreased cellular ATP that accompanies hepatotoxic doses of cocaine [14, 29] and perhaps explains, at least in part, loss of cell viability from cocaine both *in vivo* and *in vitro*. Results of the study presented here suggest that the effects of cocaine on mitochondrial function are due to one or more *N*-oxidative metabolites. While cocaine alone had no effect to diminish respiration in isolated mitochondria, the *N*-oxidative metabolites norcocaine, *N*-hydroxynorcocaine, and particularly norcocaine nitroxide had significant effects. Although concentration–response data were limited, potency appeared to increase with increasing oxidation of the tropane

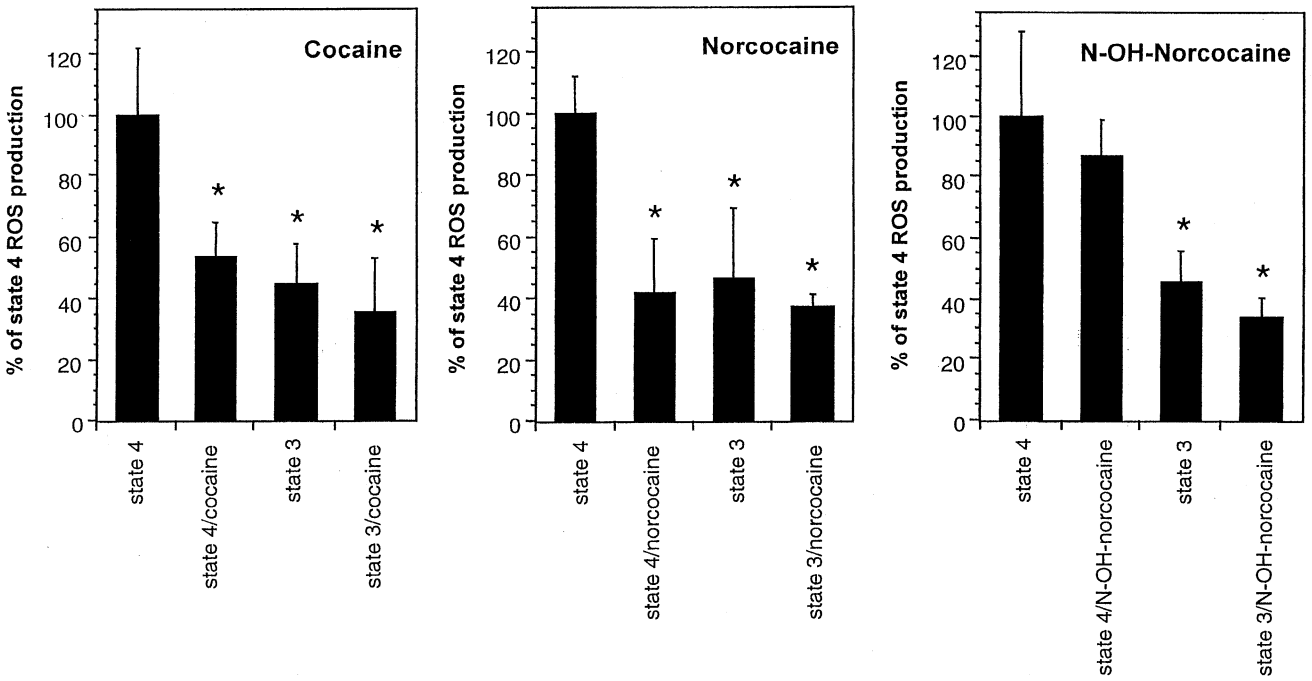

FIG. 3. Effect of acute exposure of isolated mitochondria to cocaine, norcocaine, or N-hydroxynorcocaine on mitochondrial ROS production. Increases in DHR fluorescence were measured in isolated mitochondria as described under Materials and Methods. Mitochondria were incubated in the presence of 100 μM cocaine, norcocaine, or N-hydroxynorcocaine. Values are means ± SD from 3 independent mitochondrial preparations including 6–8 measurements per treatment group. The three test compounds were run in separate series of experiments. Key: (*) significantly different ($P < 0.05$) from state 4.

nitrogen of cocaine, consistent with previous observations regarding the relative potency *in vivo* of cocaine and metabolites in producing hepatocellular necrosis [8, 10].

Boyer *et al.* [30] also found norcocaine nitroxide to be much more potent than cocaine or norcocaine in disrupting mitochondrial respiration. In isolated mitochondria from mice, norcocaine nitroxide caused a complete loss of acceptor control at a concentration of 0.5 mM, while no effects on either site I (β-hydroxybutyrate) or site II (succinate) RCR were observed at cocaine or norcocaine concentrations of less than 2.5 mM. León-Velarde *et al.* [28] found inhibition of state 3 respiration and RCR in isolated mouse mitochondria from treatment with cocaine at a concentration of 2 mM. While no effect from cocaine was observed in the present study at concentrations up to 0.5 mM, the observations of Boyer *et al.* [30] and León-Velarde *et al.* [28] suggest that at very high concentration (*ca.* 2 mM or greater), even cocaine is capable of disrupting mitochondrial respiration. Powers *et al.* [31] found no effect of cocaine, norcocaine, or *N*-hydroxynorcocaine on RCR or ADP/O ratios in isolated mouse mitochondria in concentrations up to 2 mM. The reason underlying the stark contrast between these observations and ours is unclear. It may be significant to note that no morphological changes in mitochondria following cocaine treatment were observed in their mouse model ($B6AF_1$/J hybrid), with the exception of severely injured cells, whereas others [32] have observed changes in mitochondrial structure as a relatively early event in cocaine hepatotoxicity in another mouse strain (DBA/2Ha). Thus, the differences could result from the use of different mouse strains.

Preincubation of mitochondria with cocaine for 5 min before assessment of the effects on respiration had no effect, but preincubation increased markedly the inhibition by norcocaine and *N*-hydroxynorcocaine. In fact, with preincubation, norcocaine and *N*-hydroxynorcocaine achieved effectiveness in inhibiting state 3 and state 4 respiration equivalent to that of norcocaine nitroxide. One explanation for this observation is that the toxicity of each of the cocaine *N*-oxidative metabolites to mitochondria is greatly enhanced by continuous exposure. Under these conditions, at the concentrations tested, each was maximally effective and the inherent differences in potency seen with very short exposure were no longer apparent. It is also possible that the mitochondrial effects of these metabolites are produced principally or exclusively by norcocaine nitroxide, and preincubation of mitochondria with precursor metabolites (norcocaine and *N*-hydroxynorcocaine) provides greater opportunity for formation of the toxic species. There have been several reports of mitochondrial metabolism leading to bioactivation of drugs and chemicals, including quinones, nitroaromatic compounds, and nitroimidazoles [33]. Many of these bioactivation reactions are the result of reduction rather than oxidation, however, and currently there is no evidence that *N*-oxidation of cocaine occurs in mitochondria.

Several lines of evidence indicate an important role of ROS in cocaine hepatotoxicity [9, 13, 34]. Mitochondria are normally the major source of ROS in the cell, and it is reasonable to postulate that increased ROS in response to cocaine could arise through mitochondrial effects. However, exposure of isolated mouse hepatic mitochondria to cocaine, norcocaine, or *N*-hydroxynorcocaine did not cause an increase in ROS production. Rather, cocaine or norcocaine decreased ROS generation during state 4. There exist several explanations for this observation. On one hand, decreased ROS generation during cocaine or norcocaine exposure could be caused by uncoupling of oxidative phosphorylation. Uncoupling of state 4 respiration has been reported to result in decreased production of ROS both *in vitro* [35] and *in situ* [36]. However, in view of the finding that mitochondrial respiration was not increased, this possibility appears unlikely. Another possible explanation for the observed effects on ROS generation would be a total inhibition of electron influx into the mitochondrial respiratory chain, or an inhibition of electron transfer, prior to the sites of electron leakage [37], ubiquinone or NADH-dehydrogenase [38], since the increased reduced status of the elements of the mitochondrial electron transport chain and the increased mitochondrial membrane potential in state 4 would normally facilitate the "leakage" of electrons out of the respiratory chain [39]. However, a block at complex II would be unlikely to lead to the reported results under succinic acid-stimulated respiration. In conclusion, our results do not support the hypothesis of enhanced ROS production due to disturbed mitochondrial respiration. They rather confirm the conclusions [13] that microsomal uncoupling of monooxygenase (cytochrome P450), rather than mitochondria, may be an important source of enhanced ROS production observed after cocaine treatment.

Mitochondria isolated from rats treated with a single dose of cocaine have been observed to have significantly increased rates of ROS generation [14]. This effect was inhibited significantly by pretreatment of the rats with the cytochrome P450 inhibitor SKF 525A, suggesting that the effect to increase ROS generation was due to one or more *N*-oxidative metabolites. In attempting to reconcile these observations with those of the present study, there are several possibilities. One is simply that the role of mitochondrial ROS generation in cocaine toxicity may differ between rats and mice—with cocaine stimulating mitochondrial ROS generation in rats (per Ref. 14), but not in mice, as suggested by observations in the present study. A second possibility is that the cocaine metabolite exclusively responsible for stimulating ROS generation is norcocaine nitroxide. While cocaine, norcocaine, and *N*-hydroxynorcocaine produced no effect or a decrease in ROS generation *in vitro*, norcocaine nitroxide could not be tested due to interference with the assay. It cannot be ruled out that this cocaine metabolite, in contrast to the others, is able to stimulate ROS production. A third possibility is that the acute exposure of mitochondria to cocaine metabolites *in vitro* does not adequately represent the situation *in vivo*. For example, chronic treatment of rats with the uncoupling

agent 2,4-dinitrophenol, followed by isolation of liver mitochondria from these rats, resulted in enhanced mitochondrial ROS production when respiration was blocked with antimycin A [26]. Additional studies will be required to sort out these possibilities, but the results suggest nevertheless that if mitochondrial ROS generation is important at all in cocaine hepatotoxicity in the mouse, it is not through the direct effects of cocaine, norcocaine, or *N*-hydroxynorcocaine.

The concentrations of cocaine and *N*-oxidative metabolites used in this and other studies of mitochondrial effects of cocaine *in vitro* are rather high compared with those that are associated with cocaine hepatotoxicity *in vivo*. For example, a recent study of hepatic concentrations of cocaine and its metabolites after a hepatotoxic dose measured peak concentrations of about 5 and 2.5 μg/g liver for cocaine and norcocaine, respectively [40]. Assuming for the sake of approximation a hepatic water content of 70–80%, this corresponds to an average hepatic concentration of about 25–30 μM for cocaine and 10–12 μM for norcocaine. While these concentrations are clearly much lower than those employed in the *in vitro* studies (250 and 500 μM), strict comparisons of concentrations are difficult. Hepatic cocaine and metabolite concentrations measured after cocaine treatment reflect average tissue concentrations. Localized conditions of higher concentrations, particularly of cocaine metabolites, may exist in subcellular compartments such as mitochondria. Also, it should be recognized that effects observed *in vitro* in these experiments resulted from exposure to cocaine or metabolites for only a few minutes. Lower concentrations may be capable of producing the same effects when exposure is for a longer period, as would occur *in vivo* after a cocaine dose.

This study represents the first systematic examination of the effects of cocaine and its *N*-oxidative metabolites on both mitochondrial respiration and mitochondrial ROS generation. It suggests that the ability of cocaine to depress mitochondrial respiration observed in previous *in vivo* studies is due to its *N*-oxidative metabolites, and finds that norcocaine nitroxide is particularly toxic in this regard. The hepatotoxicity of norcocaine nitroxide in mice has been demonstrated recently, and was found to include significant alteration of mitochondrial morphology [12]. The role of increased mitochondrial ROS generation from cocaine and metabolites in cocaine hepatotoxicity is less clear. Direct examination of the effects of cocaine and its *N*-oxidative metabolites on mitochondrial ROS generation suggests that mitochondria are not the source of increased ROS from cocaine. ROS from cocaine may instead arise from effects on other organelles, particularly oxidative metabolism in the endoplasmic reticulum [13].

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References

1. Perino LE, Warren GH and Levine JS, Cocaine-induced hepatotoxicity in humans. *Gastroenterology* **93**: 176–180, 1987.
2. Wanless IR, Dore S, Gopinath N, Tan J, Cameron R, Heathcote EJ, Blendis LM and Levy G, Histopathology of cocaine hepatotoxicity. Report of four patients. *Gastroenterology* **98**: 497–501, 1990.
3. Kanel GC, Cassidy W, Shuster L and Reynolds TB, Cocaine-induced liver cell injury: Comparison of morphological features in man and in experimental models. *Hepatology* **11**: 646–651, 1990.
4. Silva MO, Roth D, Reddy KR, Fernandez JA, Albores-Saavedra J and Schiff ER, Hepatic dysfunction accompanying acute cocaine intoxication. *J Hepatol* **12**: 312–315, 1991.
5. Radin DR, Cocaine-induced hepatic necrosis: CT demonstration. *J Comput Assisted Tomogr* **16**: 155–156, 1992.
6. Evans MA and Harbison RD, Cocaine-induced hepatotoxicity in mice. *Toxicol Appl Pharmacol* **45**: 739–754, 1978.
7. Kloss MW, Rosen GM and Rauckman EJ, Cocaine-mediated hepatotoxicity. *Biochem Pharmacol* **33**: 169–173, 1984.
8. Roberts SM, James RC and Harbison RD, Cocaine-induced hepatotoxicity. In: *Cocaine. Pharmacology, Physiology, and Clinical Strategies* (Eds. Lakoski JM, Galloway MP and White FJ), pp. 15–33. CRC Press, Boca Raton, 1992.
9. Boelsterli UA and Göldlin C, Biomechanisms of cocaine-induced hepatocyte injury mediated by the formation of reactive metabolites. *Arch Toxicol* **65**: 352–360, 1991.
10. Thompson ML, Shuster L and Shaw K, Cocaine-induced hepatic necrosis in mice. The role of cocaine metabolism. *Biochem Pharmacol* **28**: 2389–2395, 1979.
11. Evans MA, Role of protein binding in cocaine-induced hepatic necrosis. *J Pharmacol Exp Ther* **224**: 73–79, 1983.
12. Ndikum-Moffor FM, Schoeb TR and Roberts SM, Liver toxicity from norcocaine nitroxide, an N-oxidative metabolite of cocaine. *J Pharmacol Exp Ther* **284**: 413–419, 1998.
13. Boelsterli UA, Wolf A and Göldlin C, Oxygen free radical production mediated by cocaine and its ethanol-derived metabolite, cocaethylene, in rat hepatocytes. *Hepatology* **18**: 1154–1161, 1993.
14. Devi BG and Chan AWK, Cocaine-induced peroxidative stress in rat liver: Antioxidant enzymes and mitochondria. *J Pharmacol Exp Ther* **279**: 359–366, 1996.
15. Diez-Fernandez C, Zaragoza A, Alvarez AM and Cascales M, Cocaine cytotoxicity in hepatocyte cultures from phenobarbital-induced rats: Involvement of reactive oxygen species and expression of antioxidant defense systems. *Biochem Pharmacol* **58**: 797–805, 1999.
16. Aoki K, Ohmori M, Takimoto M, Ota H and Yoshida T, Cocaine-induced liver injury in mice is mediated by nitric oxide and reactive oxygen species. *Eur J Pharmacol* **336**: 43–49, 1997.
17. Devi BG and Chan AWK, Impairment of mitochondrial respiration and electron transport chain enzymes during cocaine-induced hepatic injury. *Life Sci* **60**: 849–855, 1997.
18. Masini A, Gallesi D, Giovannini F, Trenti T and Ceccarelli D, Membrane potential of hepatic mitochondria after acute cocaine administration in rats—The role of mitochondrial reduced glutathione. *Hepatology* **25**: 385–390, 1997.
19. Emmendörfer A, Hecht M, Lohmann-Matthes ML and Roesler J, A fast and easy method to determine the production of reactive oxygen intermediates by human and murine phagocytes using dihydrorhodamine 123. *J Immunol Methods* **131**: 269–275, 1990.
20. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.

21. Chance B and Williams GR, The respiratory chain and oxidative phosphorylation. *Adv Enzymol* **17**: 65–130, 1956.
22. Henderson LM and Chappell JB, Dihydrorhodamine 123: A fluorescent probe for superoxide generation? *Eur J Biochem* **217**: 973–980, 1993.
23. Emaus RK, Grunwald R and Lemasters JJ, Rhodamine 123 as a probe of transmembrane potential in isolated rat-liver mitochondria: Spectral and metabolic properties. *Biochim Biophys Acta* **850**: 436–448, 1986.
24. Palmeira CM, Moreno AJ and Madeira VMC, Mitochondrial bioenergetics is affected by the herbicide paraquat. *Biochim Biophys Acta* **1229**: 187–192, 1995.
25. Cadenas E and Boveris A, Enhancement of hydrogen peroxide formation by protonophores and ionophores in antimycin-supplemented mitochondria. *Biochem J* **188**: 31–37, 1980.
26. Dryer SE, Dryer RL and Autor AP, Enhancement of mitochondrial cyanide-resistant superoxide dismutase in the livers of rats treated with 2,4-dinitrophenol. *J Biol Chem* **255**: 1054–1057, 1980.
27. Boveris A, Oshino N and Chance B, The cellular production of hydrogen peroxide. *Biochem J* **128**: 617–630, 1972.
28. León-Velarde F, Huicho L and Monge C, Effects of cocaine on oxygen consumption and mitochondrial respiration in normoxic and hypoxic mice. *Life Sci* **50**: 213–218, 1992.
29. Boyer CS and Petersen DR, Hepatic biochemical changes as a result of acute cocaine administration in the mouse. *Hepatology* **14**: 1209–1216, 1991.
30. Boyer CS, Shirey JJ and Petersen DR, Effects of cocaine and its oxidative N-demethylation products on isolated mouse liver mitochondrial respiration. *Hepatology* **8**: 1235, 1988.
31. Powers JF, Alroy J and Shuster L, Hepatic morphologic and biochemical changes induced by subacute cocaine administration in mice. *Toxicol Pathol* **20**: 61–70, 1992.
32. Gottfried MR, Kloss MW, Graham D, Rauckman EJ and Rosen GM, Ultrastructure of experimental cocaine hepatotoxicity. *Hepatology* **6**: 299–304, 1986.
33. Rotilio G, Mavelli I, Rossi L and Ciriolo MR, Biochemical mechanism of oxidative damage by redox-cycling drugs. *Environ Health Perspect* **64**: 259–264, 1985.
34. Göldlin C and Boelsterli UA, Reactive oxygen species and non-peroxidative mechanisms of cocaine-induced cytotoxicity in rat hepatocyte cultures. *Toxicology* **69**: 79–91, 1991.
35. Boveris A and Chance B, The mitochondrial generation of hydrogen peroxide. *Biochem J* **134**: 707–716, 1973.
36. Okuda M, Lee HC, Kumar C and Chance B, Comparison of the effect of a mitochondrial uncoupler, 2,4-dinitrophenol and adrenaline on oxygen radical production in the isolated perfused rat liver. *Acta Physiol Scand* **145**: 159–168, 1992.
37. García-Ruiz C, Colell A, Morales A, Kaplowitz N and Fernández-Checa JC, Role of oxidative stress generated from the mitochondrial electron transport chain and mitochondrial glutathione status in loss of mitochondrial function and activation of transcription factor nuclear factor-κB: Studies with isolated mitochondria and rat hepatocytes. *Mol Pharmacol* **48**: 825–834, 1995.
38. Forman HJ and Boveris A, Superoxide radical and hydrogen peroxide in mitochondria. In: *Free Radicals in Biology* (Ed. Pryor WA), pp. 65–90. Academic Press, New York, 1982.
39. Liu SS, Generating, partitioning, targeting and functioning of superoxide in mitochondria. *Biosci Rep* **17**: 259–272, 1997.
40. Roberts SM, Harbison RD and James RC, Inhibition by ethanol of the metabolism of cocaine to benzoylecgonine and ecgonine methyl ester in mouse and human liver. *Drug Metab Dispos* **21**: 537–541, 1993.