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DIFFERENTIAL EFFECTS OF DAILY ADMINISTRATION OF COCAINE ON HEPATIC AND CEREBRAL GLUTATHIONE IN MICE

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Abstract—Twenty-four hours after acute administration of cocaine HCl (25 mg/kg, i.p.) to male C57BL/6ByJ mice, there was no hepatotoxicity as measured by plasma aspartate aminotransferase (AST) activity. In contrast, daily administration of cocaine (25 mg/kg, i.p.) for 14 days induced marked hepatotoxicity, as characterized by a greater than 400% increase in plasma AST activity when assayed 24 hr after the last injection. Concomitantly, the liver had increased levels of cysteine, γ-glutamylcysteine, glutathione, cysteinylglycine, glutamate, methionine, taurine, and aspartate. The effect appeared to be selective for compounds of the glutathione metabolic pathways, because repeated cocaine exposure did not affect other amino acids such as leucine, isoleucine, phenylalanine, serine, and valine. There was a positive correlation between the magnitude of the elevation of cysteine and the extent of liver damage. Daily cocaine administration did not affect striatal or frontal cortex glutathione. A final cocaine challenge (50 mg/kg, i.p.) did not affect either hepatic or cerebral glutathione metabolism. The increase in hepatic cysteine and glutathione upon daily cocaine administration is a potentially important compensatory mechanism against cocaine-induced hepatotoxicity.

Acute administration of cocaine is hepatotoxic to mice in which the microsomal cytochrome P450 system has been induced, as characterized by increased serum aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1, AST§) activity [1–4] and increased serum alanine aminotransferase (L-alanine:2-oxoglutarate aminotransferase, EC 2.6.1.2) activity [3, 5–9]. In addition, a single high dose of cocaine can cause a reduction in hepatic glutathione in some [2–5, 7–10] but not all mouse strains. It is still under debate whether depletion of glutathione in the liver is a contributing factor in cocaine-induced hepatotoxicity. A role for glutathione in this respect is suggested by the potentiation of cocaine hepatotoxicity following depletion of liver glutathione with diethyl maleate [5], and the protection against liver damage from cocaine offered by cysteine [5, 10]. However, the extent of hepatic glutathione depletion following acute cocaine is much smaller than that associated with liver damage from acetaminophen [4], and sex differences between B6AF1 mice in their sensitivity to cocaine-induced hepatotoxicity are not matched by similar differences in hepatic glutathione depletion [2].

Hepatotoxicity has also been studied in mice treated daily with cocaine [2, 11]. A single low dose of cocaine which fails to induce hepatotoxicity becomes hepatotoxic when administered daily [11]. Although that study does not show a clear correlation between induction of the cytochrome P450 system and extent of hepatotoxicity [11], there is some evidence suggesting that cocaine itself may be an inducer of the P450 system (see Ref. 4). Another possibility is that repeated exposure to cocaine results in a depeletion of glutathione severe enough to compromise the liver. To our knowledge, this possibility has not been addressed experimentally. The present study describes the change in hepatic glutathione following daily cocaine administration to C57BL/6ByJ mice, a strain used in our behavioural experiments with cocaine. The present paper also atempts to examine the underlying mechanism by measuring compounds of the glutathione metabolic pathways. The magnitude of the changes in the concentrations of these compounds in the liver is compared with the extent of hepatotoxicity as measured by plasma AST activity. Finally, the present study reports on glutathione levels in the brain of mice repeatedly exposed to cocaine. This is of interest because of the postulated relationship between glutathione and oxidative metabolites of cocaine [12], and the reported central presence of the oxidative metabolite *N*-hydroxynorcocaine after acute systemic cocaine administration [13, 14].

METHODS

Animals and treatment. Three-month-old male C57BL/6ByJ mice (weighing 24.7 ± 0.3 g) from the Center for Neurochemistry breeding colony were employed for all studies. The mice were kept on a 12 hr light/12 hr dark cycle with food and water available *ad lib.* Cocaine HCl (Sigma Chemical Co.,

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§ Abbreviations: AST, aspartate aminotransferase; SSA, 5-sulfosalicylic acid; and RPHPLC, reversed-phase high performance liquid chromatography.

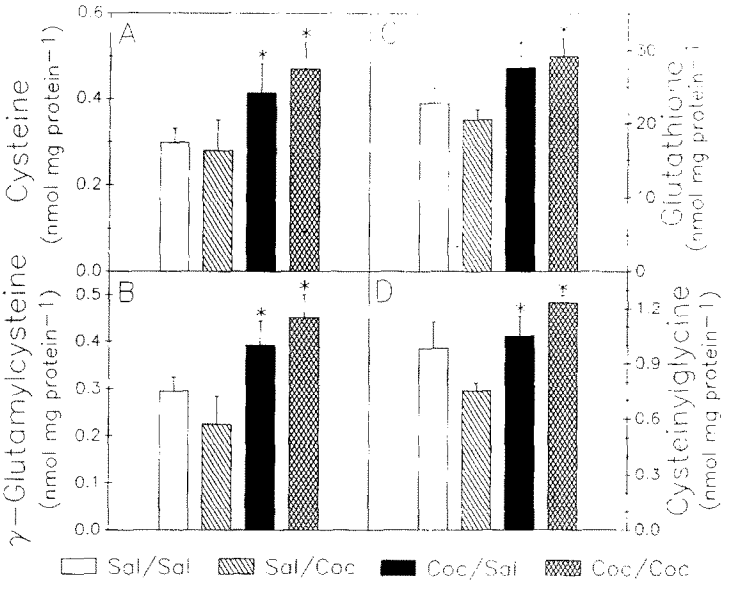

Fig. 1. Effect of daily cocaine with a final cocaine or saline challenge on hepatic cysteine (A), γ-glutamylcysteine (B), glutathione (C), and cysteinylglycine (D) levels. Male C57BL/6ByJ mice received the indicated daily treatment for 2 weeks followed by a final challenge as described in Methods. The animals were killed 1 hr after the final challenge. The results are means ± SE for six animals/group. Statistical evaluation was by two-way analysis of variance with pretreatment as factor A and acute challenge as factor B. Regardless of the challenge, pretreatment with daily cocaine increased cysteine (panel A, $F(1,20) = 6.37$, $P = 0.02$), γ-glutamylcysteine (panel B, $F(1,20) = 10.96$, $P < 0.01$), glutathione (panel C, $F(1,20) = 12.26$, $P < 0.01$), and cysteinylglycine (panel D, $F(1,20) = 8.55$, $P < 0.01$) above levels found in animals pretreated daily with saline (indicated by an asterisk). There were no statistically significant effects in factors B and A × B. Abbreviations used: Sal, saline; and Coc, cocaine.

St. Louis, MO) dissolved in normal saline was administered daily between 9:00 a.m. and 10:00 a.m. at a dose of 25 mg/kg, i.p., in a volume of 0.15 mL/20 g body weight. Control mice received normal saline. After 14 days of treatment, the mice were fasted overnight (food withdrawn at 4:30 p.m.) and subsequently challenged with either 50 mg/kg cocaine HCl or normal saline on day 15. One hour after the challenge the mice were killed by decapitation and exsanguinated. The liver was removed rapidly and homogenized in 5 vol. of 5% (w/v) 5-sulfosalicylic acid (SSA). Simultaneously, the brain was removed, and the striata and frontal cortex were dissected and frozen on dry ice. The brain regions were sonicated subsequently in 0.2 mL of 5% SSA. The SSA homogenates were then prepared for glutathione analysis as described below.

In a separate series of experiments, mice received daily cocaine treatment as described above. Food was withdrawn at 4.30 p.m. on day 14 and the animals were killed on day 15 (24 hr after the last cocaine injection). No challenge was given. The mice were killed by decapitation. Blood was collected in polypropylene tubes containing heparin sodium (Fisher Scientific Co., Fairlawn, NJ, U.S.A.). The liver was rapidly removed and homogenized in 5 volumes of 5% SSA; the resulting homogenate was used for glutathione and amino acid analyses as described below. The plasma was assayed for AST activity as described below.

Determination of cysteine and cysteine containing peptides. Cysteine, γ-glutamylcysteine, glutathione, and cysteinylglycine were determined by reversed-phase high performance liquid chromatography (RPHPLC) by the method of Fahey and Newton [15] which detects the reduced (sulfhydryl), but not the oxidized (disulfide) form. Briefly, tissue samples were homogenized in 5% SSA and centrifuged. A 0.1-mL aliquot of the resulting supernatant fraction was derivatized with monobromobimane (Thiolyte MB, Calbiochem, La Jolla, CA) and subjected to RPHPLC separation with fluorescence detection. The contribution of intracellular glutathione disulfide was considered negligible since it composes less than 1% of the total glutathione pool [16].

Amino acid analysis. Amino acid analyses were performed using the method of Neidle *et al.* [17]. Briefly, an additional 0.1-mL aliquot of the 5% SSA supernatant fraction was derivatized with 1-naphthylisocyanate (Aldrich Chemical Co., Milwaukee, WI) and subjected to RPHPLC separation with UV and fluorescence detection.

Aspartate aminotransferase activity. Plasma AST activity was assayed using a diagnostic kit (Sigma Chemical Co., Procedure No. 58-UV). One unit of activity was defined as the amount of AST which produced 1 μmol of product/min at 25°.

Protein determination. Protein was determined by the method of Lowry *et al.* [18] using bovine serum albumin as standard.

Statistical analysis. Statistical evaluation was by analysis of variance and correlation analysis. The accepted level of significance was 5%.

Table 1. Effect of daily cocaine administration with a final cocaine or saline challenge on brain glutathione levels in male C57BL/6ByJ mice

Daily treatment/Final challenge	Glutathione (nmol/mg protein) Striatum	Frontal cortex
Saline/Saline	14.0 ± 0.7	16.3 ± 0.7
Saline/Cocaine	14.6 ± 0.4	15.2 ± 0.7
Cocaine/Saline	13.7 ± 0.4	15.3 ± 1.1
Cocaine/Cocaine	14.2 ± 0.8	14.8 ± 0.5

Cocaine was administered as described in Methods. Glutathione was determined by reversed-phase high performance liquid chromatography as described in Methods. The results are means ± SE for six animals/group. Two-way analysis of variance with daily treatment and acute challenge as the two factors did not indicate statistically significant differences.

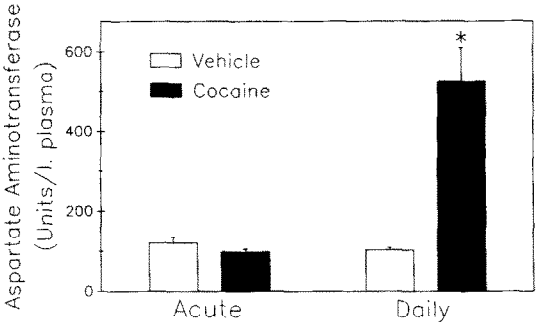

Fig. 2. Effects of acute and daily cocaine on plasma aspartate aminotransferase (AST) activity in male C57BL/6ByJ mice. AST activity was measured 24 hr after the last injection of cocaine. The animal treatment and AST assay were as described in Methods. The results are means ± SE for twelve animals/group. Key: (*) Statistical evaluation was by one-way analysis of variance; for daily cocaine $F(1,22) = 20.05$, $P < 0.001$.

Table 2. Effect of daily cocaine on hepatic amino acid and peptide levels in male C57BL/6ByJ mice

Amino acid or peptide (nmol/mg protein)	Daily treatment Saline	Cocaine
Cysteine	0.56 ± 0.04	0 97 ± 0.07* (+72%)
γ-Glutamylcysteine	0.31 ± 0.01	0.54 ± 0.03* (+73%)
Glutathione	24 ± 1	42 ± 1* (+73%)
Cysteinylglycine	1.03 ± 0.06	1.47 ± 0.08† (+44%)
Glutamate	7.8 ± 0.4	14.5 ± 1.2* (+86%)
Glycine	7.6 ± 0.5	7.4 ± 0.5
Methionine	0.15 ± 0.01	0.23 ± 0.03‡ (+56%)
Taurine	8.3 ± 0.5	12.9 ± 0.9† (+56%)
Aspartate	1.54 ± 0.08	2.1 ± 0.1‡ (+36%)
Tyrosine	0.73 ± 0.07	0.92 ± 0.13
Leucine	0.88 ± 0.07	0.97 ± 0.09

Cocaine was administered as described in Methods. Cysteine, γ-glutamylcysteine, glutathione, and cysteinylglycine were determined by reversed-phase high performance liquid chromatography following pre-column derivatization with monobromobimane as described in Methods. Glutamate, glycine, methionine, taurine, aspartate, tyrosine, and leucine were determined by reversed-phase high performance liquid chromatography following pre-column derivatization with 1-naphthylisocyanate as described in Methods. The results are means ± SE for twelve animals/group. Statistical evaluation was by one-way analysis of variance.

* $P \leq 0.0001$ compared to saline.
† $P < 0.001$ compared to saline.
‡ $P < 0.01$ compared to saline.

RESULTS

Effects of daily cocaine administration with a final cocaine or saline challenge on hepatic cysteine and cysteine containing peptides. Twenty-four hours after cessation of daily cocaine administration (25 mg/kg for 14 days) gross pathological changes (roughened pigskin-like appearance) were observed (data not shown) consonant with those reported by Shuster *et al.* after a single dose of cocaine [1]. Daily administration of cocaine increased hepatic glutathione measured on day 15 (Fig. 1C). A cocaine challenge (50 mg/kg) 1 hr before the mice were killed on day 15 did not affect this increase in hepatic glutathione and was without effect in animals that had received daily administration of saline (Fig. 1C). Cysteine (Fig. 1A), γ-glutamylcysteine (Fig. 1B), and cysteinylglycine (Fig. 1D) were also increased in mice that received daily administration of cocaine; again there was no effect of a cocaine challenge on the final (15th) day.

Effects of daily cocaine administration with a cocaine or saline challenge on cerebral glutathione. Glutathione was determined in both the striatum and frontal cortex of mice receiving repeated administration of cocaine (or saline) (once daily for 2 weeks) and a final cocaine (or saline) challenge on day 15. As shown in Table 1, neither striatal nor frontal cortex glutathione was affected by daily administration of cocaine. In addition, animals receiving a final challenge of cocaine had the same levels of glutathione as animals challenged with saline.

Effect of daily cocaine administration on hepatic amino acids and cysteine containing peptides. In a separate series of experiments, repeated cocaine

administration (once daily for 2 weeks) increased hepatic cysteine, γ-glutamylcysteine, glutathione, cysteinylglycine, glutamate, methionine, taurine, and aspartate without affecting hepatic glycine, tyrosine, or leucine (Table 2). Hepatic serine, valine, isoleucine, and phenylalanine were not affected by daily cocaine administration (data not shown).

Effects of acute and daily cocaine on plasma aspartate aminotransferase activity. A single 25 mg/kg injection of cocaine HCl did not affect plasma AST activity when assayed 24 hr later (Fig. 2). Daily administration of this same dose for 14 days was hepatotoxic as evidenced by the greater than 400% increase in plasma AST activity when assayed 24 hr after the last daily injection (Fig. 2). In addition to the elevation of plasma AST activity (Fig. 2) and hepatic amino acids and cysteine containing peptides (Table 2) upon daily administration of cocaine, there was a direct correlation between the degree of hepatotoxicity (plasma AST activity) and the hepatic concentration of cysteine ($r = 0.696$, $N = 12$, $P < 0.02$; data not shown) in animals receiving daily cocaine.

DISCUSSION

Upon acute systemic administration of cocaine to mice, *N*-hydroxynorcocaine can be detected in the brain at a concentration two to three times that in the liver [13, 14]. Although brain microsomes are capable of forming norcocaine and *N*-hydroxynorcocaine and the toxic free radical norcocaine nitroxide from cocaine and *N*-hydroxynorcocaine, and of generating lipid peroxyl radicals *in vitro* [13, 19, 20], it appears that upon systemic cocaine administration, the centrally occurring *N*-hydroxynorcocaine originates in the periphery [13]. Although the oxidative pathway of cocaine metabolism is implicated in hepatotoxicity, it remains to be seen whether centrally occurring norcocaine metabolites are potentially toxic to the brain. Cocaine toxicity in the nervous system is still a subject of debate, especially in the case of monoaminergic systems [21–23]. The presence of norcocaine nitroxide in the brain may be one possible mechanism leading to free radical induced neurotoxicity; however, to the best of our knowledge, norcocaine nitroxide has not been detected in the brain following the systemic administration of cocaine. The present study clearly shows that glutathione pathways in the striatum and frontal cortex did not respond to repeated cocaine exposure in contrast to those in the liver. This may be related to the fact that the activity of brain microsomes is extremely low as compared to liver microsomes, so that the oxidative steps generating norcocaine metabolites are occurring mostly outside the brain [13]. In addition, the present study shows that cerebral glutathione is unchanged in male C57BL/6ByJ mice as previously observed in BALB/cBy mice [13] upon administration of a single high dose (50 mg/kg) of cocaine.

The liver, like the brain, of male C57BL/6ByJ mice did not show any change in glutathione upon acute cocaine exposure (Fig. 1C), coinciding with a lack of hepatotoxicity (Fig. 2). According to the theory linking glutathione depletion with oxidative cocaine metabolism [12], the oxidative steps deplete NADPH; because NADPH is an essential co-factor for the reduction of glutathione disulfide to glutathione by glutathione reductase (NADPH:glutathione disulfide oxidoreductase, EC 1.6.4.2), the result is a decrease in glutathione and a decrease in glutathione peroxidase (glutathione:hydrogen peroxide oxidoreductase, EC 1.11.1.9) activity [12]. This then leads to lipid peroxidation, and, ultimately, cell death. There are several observations that weaken this theory. For example, hepatic glutathione depletion can occur without cocaine-induced hepatotoxicity; thus, female B6AF1 mice, induced on pine bedding, show a reduction of hepatic glutathione (as do males), but have no liver damage (in contrast to males) after acute cocaine treatment [2], and administration of cocaine to uninduced B6C3/F1 mice lowers liver glutathione but causes no hepatotoxicity [9]. The present study adds another piece of evidence: mice exposed repeatedly to cocaine show signs of hepatotoxicity and have *increased* rather than decreaseed levels of hepatic glutathione (Fig. 1, Table 2). In addition, there was an increase in hepatic cysteine, the magnitude of which was correlated positively with the magnitude of hepatotoxicity. Two possibilities should be considered. First, the liver attempts to compensate for the repeated insults by increasing the reducing environment for scavenging free radical cocaine metabolites, and the greater the damage, the greater is the compensatory response. In this context it is of interest that pretreatment with cysteine, a precursor of glutathione, has been shown to be beneficial in preventing cocaine-induced hepatotoxicity [10]. Second, the increase in hepatic glutathione is a non-specific effect that results from increased protein breakdown occurring during the insult and leads to an increase in one of the constituent amino acids, cysteine, the rate-limiting precursor for glutathione synthesis (see Fig. 3).

What is the underlying mechanism for the increase in hepatic glutathione upon repeated cocaine exposure? Glutathione is synthesized from its constituent amino acids by two successive enzymatic reactions, as shown in Fig. 3 (reactions A and B). In the first reaction, catalyzed by γ-glutamylcysteine synthetase [L-glutamate:L-cysteine γ-ligase (ADP-forming), EC 6.3.2.2], glutamate and cysteine are combined forming γ-glutamylcysteine in an ATP-requiring manner. The second reaction (Fig. 3, reaction B) adds glycine to form glutathione; this reaction is catalyzed by glutathione synthetase [γ-L-glutamyl-L-cysteine:glycine ligase (ADP-forming), EC 6.3.2.3] and also requires ATP. Transpeptidation of glutathione by γ-glutamyltransferase [(5-glutamyl)-peptide:amino acid 5-glutamyl transferase, EC 2.3.2.2; Fig. 3, reaction C] forms cysteinylglycine and a γ-glutamyl-amino acid, which is "recycled" to glutamate by the successive reactions of γ-glutamylcyclotransferase [(5-L-glutamyl)-L-amino acid 5-glutamyltransferase (cyclizing), EC 2.3.2.4; Fig. 3, reaction D] and the ATP-requiring enzyme 5-oxoprolinase [5-oxo-L-proline amidohydrolase (ATP-hydrolyzing), EC 3.5.2,9; Fig. 3, reaction E]. Cysteinylglycine is "recycled" by dipeptidase (dipeptide hydrolyase, EC 3.4.13.11; Fig. 3, reaction

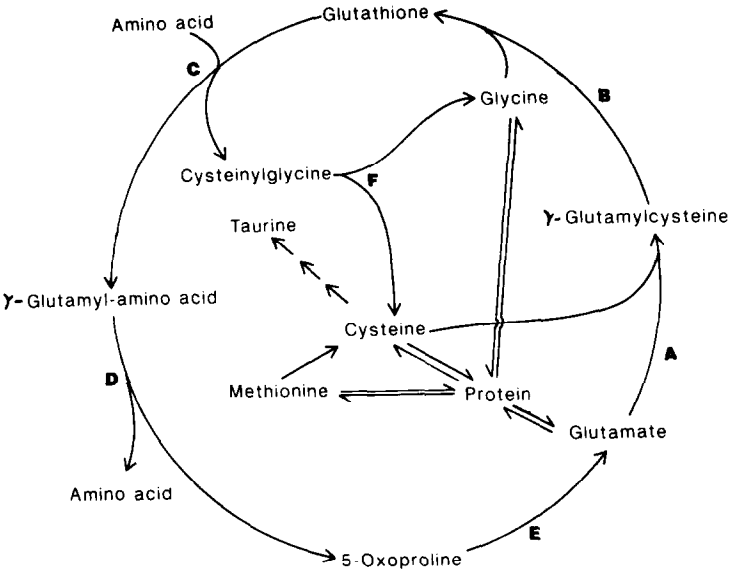

Fig. 3. Pathways of cysteine and glutathione metabolism. Adapted from Meister and Anderson [16].

F] (see also Meister and Anderson [16]). With the exception of glycine (not a rate-limiting substrate for glutathione biosynthesis), all compounds that are part of the glutathione pathways are increased upon repeated cocaine administration: cysteine, γ-glutamylcysteine, glutathione, cysteinylglycine, glutamate, methionine, and taurine (a metabolite of cysteine) (Fig. 1, Table 2). Aspartate, a potential amino group donor for glutamate by transamination, was also elevated. These increases may not be the result of a geneal protein breakdown accompanying loss of hepatic tissue because amino acids such as tyrosine, leucine, valine, isoleucine, serine, and phenylalanine were not affected (Table 2). Rather, the elevation of hepatic cysteine may be the result of an accelerated formation from methionine, triggered by repeated cocaine exposure in an unknown fashion. The observed increase in hepatic cysteine, γ-glutamylcysteine, glutathione, and cysteinylglycine are compatible with an increased flux through the γ-glutamyl cycle (Fig. 3) as a result of repeated cocaine exposure. The significant correlation between cysteine and the extent of hepatotoxicity suggests some linkage between these two phenomena.

An alternative explanation for these results may be offered if one considers that the animals used in this study were fasted overnight before they were killed. It is common practice to fast mice overnight to standardize the experimental conditions [24]. Singhal *et al.* [24] reported that hepatic glutathione levels of male Swiss–Webster mice decrease by approximately 50% after such a fast. The fall in hepatic glutathione as a result of an overnight fast may be suppressed in daily cocaine-treated animals. There is no experimental evidence in the literature arguing for or against this possibility. Since all experiments reported in this paper were conducted on animals fasted overnight, a direct comparison to fed animals cannot be made.

In contrast to the lack of hepatotoxicity observed 1 day after a single 25 mg/kg dose of cocaine, daily exposure to this dose of cocaine for 2 weeks caused hepatotoxicity (Fig. 2); however, hepatic glutathione was increased, not decreased, under these conditions (Fig. 1C and Table 2). The positive correlation between the increase in hepatic cysteine, a precursor of glutathione, and the extent of hepatotoxicity supports the idea that there is a connection between the two events. It is possible that the increase in hepatic glutathione serves as a compensatory mechanism against free radical damage from oxidative cocaine metabolism.

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