HUMAN LIVER CARBOXYLESTERASES: PURIFICATION OF THESE TWO ENZYMES, PROPERTIES IN RELATION TO COCAINE AND ALCOHOL METABOLISM, AND SPECIFICITIES

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ABSTRACT: The purpose of this work was: 1) investigation of cocaine metabolism in association with alcohol intake thoroughly. Carboxylesterases specificities with cocaine and cocaethylene metabolites were determined by incubating one of the two purified enzymes with benzoylecgonine, ecgonine methyl ester, ecgonine ethyl ester, norcocaine and norcocaethylene; 2) determination of each enzyme affinity for various xenobiotics. The first step consisted in isolating carboxylesterases 1 and 2 from human liver obtained by autopsy.

INTRODUCTION

Abuse of cocaine in combination with alcohol is widespread [6]. If conjunction of alcohol with cocaine enhances euphoria and a sense of well-being, risks of morbidity and mortality increase [5].

In human, two liver carboxylesterases, designated hCE-1 and hCE-2, are implicated in metabolism of cocaine [1, 2, 8]. Carboxylesterase 1 catalyses hydrolysis of the methyl ester linkage of cocaine forming benzoylecgonine and methanol (Figure 1). Hydrolysis of the benzoyl ester group of cocaine to ecgonine methyl ester and benzoate is catalysed by human liver carboxylesterase 2 and serum cholinesterase [11]. Benzoylecgonine and ecgonine methyl ester are known to be toxicologically inactive metabolites. In the presence of ethanol, carboxylesterase type 1 also catalyses the ethyl transesterification of cocaine to form cocaethylene plus methanol [1, 2]. Cocaethylene is equal to cocaine in production of hepatotoxicity in mice [10]. The oxidation of the N-methyl group of cocaine

by the cytochrome P450 system is a minor pathway [9]. Nevertheless, norcocaine is the precursor of molecules more potently hepatotoxic than cocaine [4]. Hence, the duration and the magnitude of the wanted stimulant effects as well as the toxic effects of cocaine intake alone or simultaneously with alcohol depend on activity of these two enzymes.

Fig. 1. Metabolic pathway of cocaine in association with alcohol. The metabolism of cocaine, cocaethylene and its metabolites involves at least three different classes of reaction: hydrolysis, transesterfication and N-demethylation.

EXPERIMENTAL

Chemicals

Benzoylecgonine and cocaethylene used as standards of reference (1 g/ l) were obtained from CIL Cambridge Isotope Laboratories Inc. (Innerberg, Switzerland). Cocaine • HCl was obtained from Siegfried (Zofingen, Switzerland) and benzoic acid from Merck (Darmstadt, Germany). Cocaine•HCl and benzoic acid reference solutions (1 g/l) were prepared in methanol or acetonitrile. The internal standard lidocaine was purchased from Sigma Chemical Co. (St. Louis, MO) and diluted in methanol to 2 g/l. Ethanol was obtained from Merck (Darmstadt, Germany).

Benzamidine • HCl, PMSF, EDTA, DTT, glycerol and Triton X-100 were obtained from Sigma Chemie (Buchs, Switzerland). Mono Q HR 5/5 and Superdex 200 PC 3.2/30 columns and Concanavalin A Sepharose 4B were purchased from Pharmacia (Dübendorf, Switzerland). Centricon concentrator (30-kDa membrane) came from Millipore (Le Mont-sur-Lausanne, Switzerland).

All the reagents were of analytical grade.

Human tissue

One specimen of human liver was obtained by autopsy. The donor (male, 53-year-old) was not known as a drug or an ethanol abuser.

Homogenization of tissue

Frozen tissue was homogenized in a Waring Blender with Krebs-Henseleit-sodium bicarbonate pH 7.4 buffer containing 2 mM EDTA, 1 mM DTT, 1 mM benzamidine, 1 mM PMSF, 1% Triton X-100 and 20% glycerol.

Centrifugation

Liver supernatant was prepared by ultracentrifugation at 150,000 g for 60 minutes at 4°C.

Purification procedure

The whole purification procedure was performed with a SMART System® from Pharmacia. This procedure required three steps: 1. Ion-exchange chromatography (Mono Q HR 5/5 column); 2. Affinity chromatography on Concanavalin A Sepharose 4B (Mono Q HR 5/5 column); 3. Gel filtration (Superdex 200 PC 3.2/30 column).

Liver supernatant was dialyzed against 20 mM Tris • HCl pH 7.4 containing 2 mM EDTA, 1 mM DTT, 1 mM benzamidine and 1 mM PMSF (Buffer A) and concentrated in a Centricon concentrator. The sample was loaded onto an ion-exchange column and bound proteins were eluted with a 20 ml linear gradient of Buffer A to Buffer A + 1 M NaCl.

Analytical techniques

Reverse phase-high performance liquid chromatography diode array detection

Analysis of cocaine, cocaethylene, benzoylecgonine and benzoic acid were carried out by HPLC-DAD. An isocratic mode was used for separation [CH_3CN: KH_2PO_4 10 mM pH 3.0; 30: 70]. The column was a Merck LiChrospher® 60 RP-select B (25 cm long, 4 mm i.d. and 5 µm particle size) maintained at 40°C. The flow rate was 1.0 ml/min and the detection was performed at 230 nm with a bandwidth of ± 10 nm.

Calibration curves were linear between 0 and 40 mg/l. The internal standard was lidocaine (100 mg/l).

Headspace GC/FID

Methanol and ethanol were analysed by Headspace GC-FID. Dioxane (1%) was used as internal standard.

Activities determinations

Enzymatic activity

Cocaine methyl and benzoyl esterase activities

Quantitations of benzoylecgonine and benzoic acid (indirect quantitation of ecgonine methyl ester) were used to determine methyl and benzoyl esterase activities, respectively. Supernatant or the active fractions obtained after one of the three purification steps were combined with 116 µM cocaine in Krebs-Henseleit-sodium bicarbonate pH 7.4 buffer (500 µl total volume) and incubated for 60 minutes at 37°C. Assays were quenched with an equal volume of 5% TCA. Precipitated proteins were removed by centrifugation. All supernatants were brought to pH 6.0 by 1 N and 5 N NaOH; the internal standard was added (12.5 µl of lidocaine).

Cocaine ethyl transferase activity

The appearance of cocaethylene was used to measure ethyl transferase activity. The procedure was as described above. The enzymatic reaction was started by addition of 40 mM ethanol.

Protein concentrations were determined with the Bio-Rad protein assay using bovine serum albumin as a standard.

Specific activities were expressed as nanomoles of product formed per hour per milligram of protein.

Chemical activity

Spontaneous hydrolysis of cocaine into benzoylecgonine in aqueous solution at physiological pH was determined by incubation without enzyme.

RESULTS

Based on previous kinetic results, carboxylesterases activities were measured by incubating the liver supernatant (500 µg protein) or the purified esterase (10 µg protein) for one hour, with cocaine alone (116 µM) or in presence of ethanol (40 mM). The cocaine methyl esterase, benzoyl esterase and ethyl transferase activities were determined by measuring the enzymatic hydrolysis (total minus spontaneous) of cocaine to benzoylecgonine, to ecgonine methyl ester and to cocaethylene respectively. While spontaneous hydrolysis occurs in vitro in aqueous solution at neutral to alkaline pHs, this pathway is not prevalent.

The carboxylesterase that catalyses the hydrolysis of cocaine to benzoylecgonine and methanol was partially purified from the 150,000 g human liver supernatant using ion-exchange chromatography (Table I). Cocaine methyl esterase was purified 44-fold (Table II).

Under in vitro conditions, the presence of cocaine ethyl transferase activity in liver supernatant and after the first step of purification, as well as the absence of methyl esterase activity, suggest that alcohol inhibits benzoylecgonine formation. Moreover, ethanol seems to activate cocaine hydrolysis (specific activity is 4-fold greater than in the absence of ethanol). The production of cocaethylene and the absence of benzoylecgonine suggest that these two separates activities are catalysed by the same enzyme, which is in agreement with the literature [1, 2].

DISCUSSION AND CONCLUSION

These preliminary results confirm that, in human liver and under in vitro conditions, the formation of cocaethylene via a cocaine ethyl transferase is alcohol-dependent. Rat liver exhibits cocaine ethyl transferase activity 33-fold greater than that in lung and 600-fold greater than that in kidney and heart [3]. No ethyl transferase activity was identified in brain, spleen or serum. Hence, formation and accumulation of cocaethylene in human   liver

TABLE I. TOTAL AND SPECIFIC COCAINE METHYL AND BENZOYL ESTERASE ACTIVITIES

Purification steps[a]	Total protein [mg]	Cocaine [b]		Benzoylecgonine [b]		Ecgonine methyl ester [b]	
		Total activity [nmol/h]	Specific activity [nmol/h/mg]	Total activity [nmol/h]	Specific activity [nmol/h/mg]	Total activity [nmol/h]	Specific activity [nmol/h/mg]
Supernatant of liver extract	30.60	462.2	15.10	295.0	9.641	167.2	5.464
Ion-exchange chromatography	0.151	88.29	584.7	64.52	427.3	–	–

[a] Partial purification of the hCE-1 from 20 g of human liver.
[b] Carboxylesterases activities with cocaine as substrate were measured as described under « Experimental ».

TABLE II. TOTAL AND SPECIFIC COCAINE ETHYL TRANSFERASE ACTIVITIES

Purification steps	Total protein [mg]	Cocaine [c]		Benzoylecgonine [c]		Ecgonine methyl ester [c]		Cocaethylene [c]	
		Total activity nmol/h	Specific activity [nmol/h/mg]	Total activity [nmol/h]	Specific activity [nmol/h/mg]	Total activity [nmol/h]	Specific activity [nmol/h/mg]	Total activity [nmol/h]	Specific activity [nmol/h/mg]
Supernatant of liver extract Ion-exchange chromatography	30.60	328.	10.72	–	–	64.32	2.102	(1)	(1)
	0.151	0247.8	1641	–	–	–	–	184.2	1220

[c] Carboxylesterases activities with cocaine and ethanol as substrates were measured as described under « Experimental ».
[1] Cocaethylene concentrations were below the detection limit of analytical technique by HPLC-DAD. Ethanol consumption was assessed by Headspace GC-FID. Total and specific activities were 317.0 µmol/h and 10.36 µmol/h/mg, respectively.

is important in a toxicological point of view [7]. Ethyl cocaine could undergo N-demethylation to norcocaethylene and produce cocaine like cytotoxicity (free radicals production) in the rat [3].

Moreover, in the presence of ethanol, the formation of benzoylecgonine from cocaine is inhibited while the synthesis of cocaethylene, a pharmacologically active metabolite, is stimulated [1, 2]. These in vitro effects are consistent with the effects of in vivo ethanol pretreatment of rats that decreases the AUCs for benzoylecgonine in all tissues and serum [3]. In the same study, cocaine methyl esterase activity was identified in kidney, liver, lung, heart and brain, decreasing in this order.

Abbreviations used: hCE-1, human carboxylesterase type 1; hCE-2, human carboxylesterase type 2; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; PMSF, phenylmethylsulfonylfluoride; TCA, trichloroacetic acid.

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