Biochemical Pharmacology, Vol. 40, No. 8, pp. 1733–1737, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00
Pergamon Press plc

HEPATIC SUBCELLULAR LOCALIZATION OF CRESYLBENZODIOXAPHOSPHORIN OXIDE (CBDP)-SENSITIVE SOMAN BINDING SITES*

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(*Received* 1 *July* 1989; *accepted* 12 *April* 1990)

Abstract—The toxicity of the organophosphorus poison soman (pinacolylmethylphosphonofluoridate) is attributable to its irreversible inhibition of the enzyme acetylcholinesterase. In addition, soman binds irreversibly to a number of noncholinesterase tissue binding sites which appear to be its major means of *in vivo* detoxification. This study was conducted to determine the hepatic subcellular localization of these sites. Subcellular fractions of liver from male Sprague–Dawley rats (200–250 g) were prepared by differential and isopycnic density gradient centrifugation. The binding of [^{14}C]soman to these subcellular fractions was determined in the presence and absence of cresylbenzodioxaphosphorin oxide (CBDP), a compound that binds irreversibly to the noncholinesterase soman binding sites. Crude fractionation of liver homogenates into nuclear, mitochondrial, microsomal, and soluble fractions revealed that 78% of the total CBDP-sensitive binding activity was localized in the nuclear and microsomal fractions. Further purification of these fractions indicated that all of the homogenate binding activity could be accounted for in the purified microsomal fraction. When purified liver microsomes were solubilized and fractionated on linear sucrose gradients, 90% of the CBDP-sensitive soman binding activity cosedimented with carboxylesterase activity which suggests that these binding sites are carboxylesterase.

Soman (pinacolyl methylphosphonofluoridate) is a member of the group of organophosphorus agents whose toxic action is believed to be due to their irreversible inhibition of acetylcholinesterase (AChE), an enzyme whose function is to terminate the action of the neurotransmitter acetylcholine by catalyzing its hydrolysis [1, 2]. Although the inhibition of AChE produces a variety of cholinergic effects by increasing acetylcholine at cholinergic synapses, the inhibition of AChE in brain and diaphragm is particularly important since death from soman intoxication is usually due to respiratory failure [3]. In addition to its inhibition of AChE, soman is detoxified by binding irreversibly to a number of noncholinesterase tissue binding sites [4–9] and by enzymatically catalyzed hydrolysis by organophosphorus acid anhydride hydrolases [10–13]. The importance of the irreversible binding sites as a detoxification route for soman has been suggested by several investigators [4–9, 14–16] and has been established primarily by the use of 2-(*O*-cresyl)-4*H*-1:3:2-benzodioxaphosphorin-2-oxide (CBDP) as an *in vivo* inhibitor of these sites. Pretreatment of animals with CBDP reduces the amount of administered soman required to produce lethality [5, 7, 14, 16], thus demonstrating the importance of noncholinesterase sites in the detoxification of soman. Since initial *in vitro* studies indicated that these noncholinesterase soman binding sites were concentrated in the liver, we decided to determine the hepatic subcellular localization of these sites.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (200–250 g) were obtained from Charles River (Kingston, NY) and maintained on Purina lab chow and tap water *ad lib.* All animals were maintained on a 12-hr day–night cycle (light cycle from 6:00 a.m. to 6:00 p.m.). Rats were fasted for 18 hr before they were used in experiments. Fasting was utilized to increase the yield of hepatic subcellular fractions [17].

Chemicals. Soman was obtained from the Chemical Research, Development and Engineering Center, Aberdeen Proving Ground, MD. Analysis by nuclear magnetic resonance (NMR) spectroscopy showed it to be 96.7% pure. [^{14}C]Soman (pinacolyl [^{14}C]methylphosphonofluoridate; sp. act. 54.5 mCi/mmol) with a purity of greater than 90% by TLC was purchased from Los Alamos National Laboratories (Los Alamos, NM). CBDP (99.5% pure by NMR) was obtained from Starks Associates, Inc. (Buffalo, NY). Sucrose (density gradient ultrapure) was obtained from Schwartz–Mann (Orangeburg, NY). Bovine serum albumin (BSA) and calf thymus deoxyribonucleic acid (DNA) were

* The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. In conducting the research described in this report, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals* (1985) as prepared by the Committee on Care and Use of Laboratory Animals, National Research Council, NIH Publication No. 85-23.

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obtained from the Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade and were obtained from commercial sources.

Binding assay for CBDP-sensitive soman binding sites. Binding assays were performed in a total volume of 2.0 mL. The assay which contained 0.1 M phosphate buffer (pH 7.4), [^{14}C]soman (0.01 mM, 54.5 mCi/mmol) and tissue at a concentration of approximately 1 mg protein/mL was run in the presence and absence of 0.01 mM CBDP. Assays were performed in triplicate at room temperature for 5 min. Initial results indicated that at this protein concentration the reaction was linear with time. Reactions were stopped by the addition of trichloroacetic acid (TCA) (final concentration 10%, v/v) and subsequently filtered through a Millipore GF/B filter. The filters were rinsed three times with 6 mL each of 10% TCA and then counted in 10 mL of Ultrafluor in a liquid scintillation counter. Counting efficiency was 95%. To determine CBDP-sensitive soman binding, counts bound in the presence of CBDP were subtracted from counts bound in the absence of CBDP.

Preparation of subcellular fractions. The soluble fraction and microsomes were prepared essentially as previously described [10, 18]. In brief, rat livers were removed, placed in beakers on ice, rinsed with ice-cold 0.25 M sucrose, mined with scissors, and then homogenized in 3 vol. of ice-cold 0.25 M sucrose. Homogenization was accomplished by twelve strokes at 400 rpm with a mechanically driven teflon pestle in a glass homogenizer (Arthur H. Thomas, Inc., Philadelphia, PA). After diluting the homogenate to 5% (w/v) with 0.25 M sucrose, crude nuclear and mitochondrial pellets were removed by successive centrifugation at 1000 *g* for 10 min and 10,000 *g* for 10 min in a Sorvall RC-5B refrigerated centrifuge (DuPont Co., Wilmington, DE). The post-mitochondrial supernatant fraction was then centrifuged at 205,000 *g* for 30 min in a Beckman SW41 rotor operated in a Beckman model L8-80 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) to produce a soluble fraction (supernatant) and a microsomal fraction (pellet) [10, 18]. The pellet was washed by suspension and recentrifugation at 205,000 *g* for 30 min in 0.25 M sucrose containing 20 mM Tris–Cl (pH 7.5) to remove adsorbed soluble proteins [19]. Purified nuclei and plasma membranes were isolated as previously described [20, 21].

Sucrose gradient fractionation and analysis of carboxylesterase activity. Soluble liver microsomal carboxylesterase was prepared as described by Maxwell *et al.* [15]. A 5–20% sucrose gradient containing 0.5% Triton X-100 was prepared for each sample. To the top of each gradient tube was added 250 μL of the soluble liver microsomal carboxylesterase, along with 15 μL of β-galactosidase (1 mg/mL) (Boehring Mannheim, Indianapolis, IN) and 10 μL of alkaline phosphatase diluted 1:6 with water (v/v) (Sigma Chemical Co.) as internal markers. The samples were then centrifuged for 18 hr at 70,000 *g* at 5° in an L8-80 Beckman Ultracentrifuge (Beckman Instruments). The gradients were fractionated into 53 fractions each containing 0.21 mL. Each fraction was assayed for carboxylesterase activity [15] and for binding of [^{14}C]soman as previously described (see above).

Table 1. *In vitro* tissue binding of [^{14}C]soman to CBDP-sensitive soman binding sites

Organ or tissue	[^{14}C]soman bound (dpm/mg protein)
Liver	5800 ± 890
Lung	1810 ± 220
Kidney	1350 ± 140
Blood	300 ± 35
Spleen	265 ± 130
Heart	ND
Skin	ND
Muscle	ND
Brain	ND

Values are means ± SE (N = 6). Binding of [^{14}C]soman to the various tissues was determined in the presence and absence of CBDP as described in Materials and Methods. All values reported were at least 2× background (130 dpm/mg protein in the presence of CBDP). ND = not detectable.

Biochemical enzyme assays. Glucose-6-phosphatase was assayed by a modification of the methods of Swanson [22] and deDuve *et al.* [23] as previously described [18]. Alkaline phosphatase was assayed by a modification of the method of DeChatelet and Cooper [24] as previously described [18].

Analytical procedures. Protein was assayed by the method of Lowry *et al.* [25] using bovine serum albumin as a standard. DNA was assayed by the method of Burton [26] using calf thymus DNA as a standard.

RESULTS

The *in vitro* binding of [^{14}C]soman to tissue homogenates varied from high levels in kidney, lung, and liver to the absence of binding in heart, skin, muscle and brain. The average binding of [^{14}C]soman in the presence of CBDP was 130 dpm/mg of protein and was found to be independent of the tissue source. This value was subtracted from tissue binding determined in the absence of CBDP to measure CBDP-sensitive soman binding sites. Of the organs and tissues tested *in vitro*, the liver had the highest specific activity for CBDP-sensitive soman binding sites (Table 1). Subsequent evaluation of the subcellular localization of these sites was performed in the liver because of its high levels of soman binding and well-defined procedures for subcellular fractionation in this organ.

When rat liver homogenates were fractionated into microsomes, nuclei, mitochondria, soluble fraction and a microsomal wash, 78% of the homogenate activity of the CBDP-sensitive soman binding sites was found in the microsomal and crude nuclear fractions (Fig. 1). Since the crude nuclear fraction was a pellet obtained after centrifugation at 1000 *g* for 10 min of a 5% (w/v) liver homogenate, it would be expected to be contaminated with microsomes and plasma membranes [27]. In an attempt to quantitate more appropriately the amount of these binding sites in the various cell fractions,

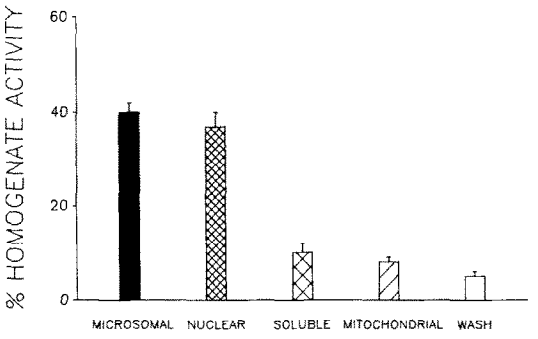

Fig. 1. Percentage of homogenate CBDP-sensitive soman binding sites in crude rat liver fractions. Microsomal and soluble fractions were prepared as described (Materials and Methods). Crude nuclear and mitochondrial fractions were obtained by centrifugation of the homogenate at 1000 *g* for 10 min and 10,000 *g* for 10 min respectively. The values are means ±SE (N = 5). Recovery of activity was 99.8 ± 4.5%.

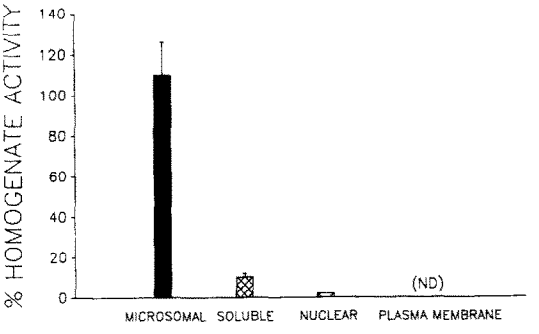

Fig. 2. Percentage of homogenate CBDP-sensitive soman binding sites in purified rat liver fractions. Fractions were prepared and assayed as described (Materials and Methods). The percentage of homogenate activity in the nuclear fraction was corrected for 65% DNA recovery and the percentage in the microsomal fraction was corrected for 31% recovery of the microsomal marker enzyme, glucose-6-phosphatase. Values are means ±SE (N = 6). ND = not detectable.

purified microsomes, nuclei, cytoplasm, and plasma membranes were prepared.

Figure 2 shows the percentage of the homogenate CBDP-sensitive soman binding sites in these purified fractions. Since it is not possible, employing existing techniques, to obtain purified cellular fractions with greater than 30% recovery [28, 29], activities of CBDP-sensitive soman binding sites in the purified fractions shown in Fig. 2 were corrected for recovery by determining the recovery of marker enzymes (glucose-6-phosphatase as a microsomal marker [30] and alkaline phosphatase as a plasma membrane marker [31]) and DNA (as a nuclear marker) for each of these fractions, as shown in Table 2.

Table 3 shows the specific activity of the CBDP-sensitive soman binding sites in each of the purified liver cell fractions. As can be seen, the microsomal fraction had the highest specific activity and was 5.5 times greater than the specific activity of the homogenate. When the purified microsomal fraction was solubilized and subsequently fractionated on a linear sucrose gradient, 90% of the noncholinesterase soman binding activity cosedimented with the major peak of carboxylesterase activity (Fig. 3).

DISCUSSION

Our initial *in vitro* studies indicated that the liver contained a high concentration of CBDP-sensitive soman binding sites (Table 1). Lenz and coworkers [32] had also demonstrated that liver is one of the few tissues in which administration of CBDP reduces the level of *in vivo* radiolabeled soman. Several investigators suggested that these sites were most likely carboxylesterase(s), i.e. aliesterase(s) (EC 3.1.1.1) [5, 7], but this has not been shown unequivocally. It is known, however, that soman toxicity is potentiated by pretreatment of experimental animals with CBDP [4–7], an irreversible inhibitor of carboxylesterase [33, 34], a liver microsomal enzyme [35] which hydrolyzes short chain fatty acids [36] but has no other known function.

We therefore fractionated liver to determine the hepatic subcellular location of the CBDP-sensitive soman binding sites. Our results indicated that the majority of the CBDP-sensitive soman binding sites are localized in the microsomal fraction of the liver (Fig. 2), a subcellular localization similar to the previously reported location of carboxylesterase [35]. In addition, when purified liver microsomes were solubilized and subsequently fractionated on linear sucrose gradients (Fig. 3), 90% of the CBDP-sensitive soman binding sites cofractionated with carboxylesterase activity. This result suggests that the CBDP-sensitive soman binding sites are carboxylesterase or that soman binds irreversibly to a microsomal fraction with the same molecular weight as carboxylesterase.

Recent studies have suggested that carboxylesterases located in the liver may not be important in the *in vivo* detoxification of soman since soman toxicity at doses <1 LD_{50} is increased several-fold by pretreatment with amounts of CBDP which have no effect on liver carboxylesterase activity [37]. Soman toxicity is very highly correlated with the inhibition of plasma [14, 37] and lung [14] carboxylesterase. Thus, the liver sites may only bind soman during acute exposure when multiple LD_{50} doses are administered which saturate plasma and lung binding sites.

However, liver may be important as the site of synthesis of the CBDP-sensitive soman binding sites found in plasma. It is known that carboxylesterase is synthesized on liver microsomes [38]. Its synthesis can be induced by phenobarbital [39, 40] with a subsequent increase of carboxylesterase activity in the serum [40]. Increased levels of carboxylesterase in the serum have been shown to correlate with decreased soman toxicity [14, 37]. Therefore, it appears that these CBDP-sensitive soman binding sites may be synthesized on liver microsomes and secreted to the serum where they detoxify soman. If a non-toxic method to induce the synthesis of these sites or increase the release of previously

Table 2. Recovery of markers in purified microsomes, plasma membranes, and nuclei

Marker	Specific activity	Protein (mg/g liver)	Total activity	Recovery %
Glucose-6-phosphatase*				
Microsomes	7.57 ± 0.29	15.5 ± 0.84	117	
Homogenate	2.24 ± 0.09	170 ± 6.4	384	31
Alkaline phosphatase†				
Plasma membranes	22.5 ± 1.3	0.994 ± 0.01	22	
Homogenate	0.666 ± 0.06	170 ± 6.43	113	20
DNA				
Nuclei		5.9 ± 0.5		66
Homogenate		9.0 ± 0.8		

Fractions were prepared and assays were performed as described in Materials and Methods. Values are means ±SE (N = 6).

* Values for specific activity are expressed in μmol of inorganic phosphate released per 20 min per mg protein.

† Values for specific activity are expressed in nmol of *p*-nitrophenol formed per min per mg protein.

synthesized binding sites to the serum could be found, it may be possible to decrease significantly the toxicity of soman *in vivo*..

Table 3. Specific activity of CBDP-sensitive soman binding sites

Fraction	Specific activity (dpm/mg protein)
Homogenate	5880 ± 390
Microsomes	32,400 ± 1,690
Mitochondria	7360 ± 480
Soluble	3070 ± 165
Nuclei	825 ± 170
Plasma membranes	ND

Values are means ±SE (N = 6). Fractions were purified fractions prepared as described in Materials and Methods except for the mitochondrial fraction which was a 10,000 *g* for 10 min pellet of a 5% (w/v) liver homogenate. All values reported were at least 2× background (130 dpm/mg protein in the presence of CBDP). ND = not detectable.

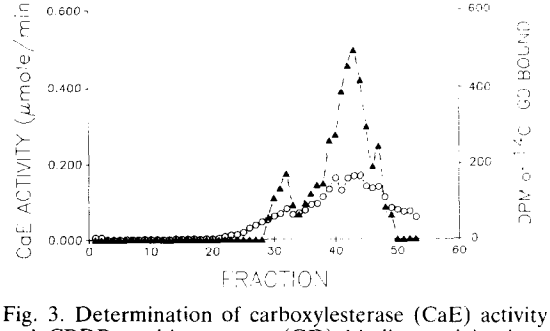

Fig. 3. Determination of carboxylesterase (CaE) activity and CBDP-sensitive soman (GD) binding activity in a linear sucrose gradient. Solubilized purified microsomes were layered over and centrifuged into a linear sucrose gradient as described (Materials and Methods). Fractions were collected from the gradient and assayed for carboxylesterase activity (– – – ○ – – –) and CBDP-sensitive [^{14}C]soman binding activity (– – – ▲ – – –).

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