Enzymatic Inactivation of N-Nitroso Compounds in Murine Blood Plasma

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ABSTRACT

Murine blood plasma rapidly inactivates nitrosamides and nitroso carbamates but not nitrosoureas. The mechanism of this inactivation in murine blood plasma has been investigated. The vast majority of activity (>97%) was inhibited by serine hydrolase inhibitors. Also, 92% of the activity was inhibited by bis(p-nitrophenyl)phosphate, a selective inhibitor of carboxylesterases. Decomposition products formed after blood plasma action on N-ethyl-N-nitrosoacetamide or N-methyl-N-nitrosoethylcarbamate were separated and identified by gas chromatography. The products formed were consistent with a hydrolytic cleavage of the amido bond. These observations are consistent with the idea that the major active factor(s) in plasma is a carboxylesterase(s).

INTRODUCTION

Chloroethylnitrosoureas have a broad range of antitumor activity against both experimental and human tumors, and several such as 1,3-bis(2-chloroethyl)-1-nitrosourea and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea are used clinically to treat a wide variety of human cancers (14, 21, 22, 26). Although the nitrosoureas are very useful antitumor agents, their usefulness is compromised by their considerable toxicity. In addition to their antitumor activity, nitrosoureas and related N-nitroso compounds are known to be potent mutagens and carcinogens (reviewed in Refs. 18–20, 24, and 25).

Nitrosamides and nitroso carbamates are related to the nitrosoureas by their ability to decompose to alkyl diazotates. The biological effects of N-nitroso compounds, namely mutagenicity, carcinogenicity, and antitumor activity, are considered primarily to be due to the alkylating ability of these diazotates. Oddly enough, although the nitrosoureas, nitrosamides, and nitroso carbamates are all chemically related, they have different biological activities. The nitrosamides and nitroso carbamates are at least 100-fold more potent mutagens than the homologous nitrosoureas in in vitro short-term test systems (3–5, 7, 8). In contrast to the nitrosoureas, the nitrosamides and nitroso carbamates have no in vivo antitumor activity in mice (7, 14, 16).

We have shown previously that murine blood plasma and purified commercial porcine liver esterase are able to rapidly destroy nitrosamides and nitroso carbamates but not the nitrosoureas (3–5). It is likely that the susceptibility of nitrosamides and nitroso carbamates to destruction by murine plasma may explain the lack of antitumor activity of these compounds in mice. In the investigations reported upon here, we have attempted to characterize the activity in mouse plasma responsible for the inactivation of these N-nitroso compounds. The structures of the compounds used in this study are given in Chart 1.

MATERIALS AND METHODS

Source of Drugs and Chemicals. The N-nitroso compounds used in this study were prepared as described previously (5, 7). Carboxylesterase (EC 3.1.1.1) from porcine liver and lipase from porcine pancreas were obtained from Sigma Chemical Co. One unit of esterase activity was defined as the amount of enzyme that will hydrolyze 1 μmol of ethyl butyrate to butyric acid and ethanol per min at pH 8, 25°C. One unit of lipase activity was defined as the amount of enzyme that will produce 1 μeq of fatty acid from triacetin in 1 hr at pH 7.4, 37°C. Human plasma butyryl cholinesterase was obtained from Worthington, and 1 unit of activity was defined as the amount of enzyme that will produce 1 μmol of acetylcholine per min at pH 7.4, 25°C. Cyclohexyl isocyanate and diethyl p-nitrophenyl phosphate were obtained from Aldrich Chemical Co.; all other inhibitors were obtained from Sigma.

Preparation of Mouse Plasma. Mouse plasma was prepared by bleeding female C57BL/6 X DBA/2 F1 (hereafter called B6D2F1) mice from the eye with a heparinized pipet from the lateral tail vein after the animals were fasted for 24 hr. The blood was immediately centrifuged, diluted 10% (v/v) with 0.1 M sodium phosphate buffer at pH 7.4, and stored frozen at −20°C.

N-Nitroso Compound Stability. To measure the stability of these N-nitroso compounds, 2 methods were used. Chemical stability was determined by HPLC analysis. For the HPLC analysis, a solution of N-nitroso compound in 100% ethanol was diluted 1:50 with 0.1 M sodium phosphate buffer at pH 7.4. This was then added to an equal volume of either buffer, 10% plasma, or porcine liver esterase (17 μg/ml, 2.0 units/ml) in the same buffer and incubated at 37°C. Aliquots were removed at intervals and assayed for N-nitroso compound content on an Isco HPLC instrument equipped with a Whatman Partisol ODS3 5-μm column and a fixed wavelength UV detector set at 254 nm. A 70% methanol:water mixture was used as a eluant. The flow rate was 1 ml/min, and all separations were done at room temperature.

The mutagenicity of these N-nitroso compounds was also followed as an alternate method for determining stability. Mutagenicity tests were performed on Salmonella typhimurium strain hisG46 which detects base substitution mutations. The N-nitroso compound in 3.3% (or 2%) ethanol was diluted into an equal volume of either 0.1 M sodium phosphate buffer (pH 7.4), 10% plasma in 0.1 M sodium phosphate buffer (pH 7.4), butyryl cholinesterase (2 units/ml), or lipase (10 units/ml). These mixtures were preincubated at 37°C, and aliquots were removed at intervals and diluted 1:41 into a suspension of bacteria that had been grown overnight on Oxoid nutrient broth-2, centrifuged, and then resuspended to the same volume in 0.1 M sodium phosphate buffer (pH 7.4). Following a 10-min incubation at 37°C, duplicate 0.1-ml samples were mixed with top agar
ENZYMATIC INACTIVATION OF N-NITROSO COMPOUNDS

R₁ - N - C - R₂
Nitrosamide
ENA  R₁ = CH₃CH₂-  R₂ = -CH₃

R₁ - N - C - O - R₂
Nitrosocarbamate
CNC  R₁ = Cl CH₂CH₂-  R₂ = CH₃CH₂-
MNC  R₁ = CH₃-  R₂ = CH₃CH₂-

Chart 1. Structures of N-nitroso compounds used in this study.

and plated. Plating medium consisted of Vogel-Bonner E medium [27], 1.25% (v/v) liquid Difco nutrient broth, and 1.5% agar, with a top agar containing 0.5% NaCl solution and 0.6% Bacto agar. Since some N-nitroso compounds are photosensitive, all manipulations were performed in subdued light. Plates were hand counted after 48-hr incubation at 37° in the dark. The average number of spontaneous revertants per plate for hisGAG was 9 (an average of greater than 100 plates). Spontaneous revertants were subtracted before the data were plotted. The concentration of N-nitroso compounds used were chosen so as to give about 500 to 600 revertant colonies per plate before the preincubation was begun. This range was chosen to give enough revertants to follow decreases in number with time of treatment of the N-nitroso compound but not enough to cause inaccuracies in counting. When alternate factors were chosen for testing of their effects on the mutagenicity of these N-nitroso compounds, the exact methods are given in the figure legends.

Inhibitor Studies. The CNO-hydrolyzing activity of plasma from female B6D2F₁ mice was measured after incubation at 37° for 30 min with various inhibitors. The inhibitors were diluted into either plasma or esterase from concentrated stocks made in distilled water immediately prior to use. The incubation mixtures consisted of 50 μl of 10% mouse plasma (protein concentration, 5.6 mg/ml) in 0.1 M phosphate buffer (pH 7.4) or 50 μl of porcine esterase (1.0 unit/ml) plus 10 μl of the following inhibitors, with the stock concentrations given in parentheses: CaCl₂ (10⁻³ M); EDTA (2.5 × 10⁻⁴ M); HgCl₂ (10⁻³ M); eserine (2.5 × 10⁻⁴ M); 4-hydroxymercuribenzoate (10⁻³ M); cydohexyl isocyanate (10⁻³ M); diisopropylfluorophosphate (10⁻³ M); dimethyl p-nitrophenyl phosphate (E600, Paraoxon) (10⁻₂ M); or bis(p-nitrophenyl)phosphate (10⁻¹ M, 10⁻² M). After incubation for 30 min at 37°, 40 μl of 1.05 mM CNC were added to the incubation mixture. The reaction mixtures continued to be incubated at 37° while aliquots were removed for HPLC analysis of N-nitroso compound content.

Decomposition Product Analysis. A 250 μM solution of ENA or MNC in normal propyl alcohol was diluted 1:30 with 0.1 M sodium phosphate buffer at pH 7.4. A 50-μl aliquot of this solution was mixed with an equal volume of either phosphate buffer or 10% plasma and incubated at 37° in a sealed vial. Aliquots were removed and analyzed by gas chromatography. Methanol, ethanol, propyl acetate, and ethylpropyl carbonate were determined using a Varian 2700 instrument with a 6-ft Chromosorb 101 column at 200° and a flame ionization detector. N-Ethylethacamide and N-methylethyl carbamate were determined using a Varian 3700 instrument with a 2-ft Tenax column at 150° and an N-P ion-specific detector. Propyl acetate and ethylpropyl carbonate were measured at 2 hr; all other compounds were measured at 5 hr.

RESULTS

Plasma and Esterase Effects on N-Nitroso Compound Stability. We have shown previously that murine plasma as well as a purified porcine liver carboxylesterase rapidly destroy alkyl nitrosamides and nitrosocarbamates but not nitrosoureas. The majority of activity in murine plasma appears to be enzymatic in nature [3-5]. Chart 2 shows the effect of murine plasma, esterases, and lipase on the mutagenic stability of CNC. In this type of assay, the N-nitroso compounds are mixed with either buffer, plasma, or purified enzymes, and then at various times after mixing, an aliquot is removed, its mutagenicity is determined, and the more the N-nitroso compound decays, the less mutagenic the mixture will be. The mutagenicity of aqueous buffered solutions of N-nitroso compounds (with nothing else added)
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Decreases slowly due to spontaneous decomposition, and these decreases can be characterized by a mutagenic half-life, $t_{1/2}$. The mutantigenic half-life is defined as the time it takes to decrease the mutagenicity of a solution by one-half. In buffer alone, CNC decays with a mutagenic half-life of 330 min. In the presence of 5% mouse plasma or porcine liver carboxylesterase (1 unit/ml), the half-life decreases to 6 or 11 min, respectively. Both plasma and the purified porcine liver carboxylesterase hydrolyze methyl as well as chloroethyl nitrosamines and nitrosocarbamates (3, 5). Purified human plasma butyrylcholinesterase and porcine pancreatic lipase have a negligible effect on CNC (Chart 2).

Inhibitor Studies. Inhibitor studies were performed in order to determine whether esterases play a vital role in inactivation of nitrosamines and nitrosocarbamates. CNC hydrolysis by plasma was measured by HPLC analysis after preincubation of the plasma with various esterase inhibitors. As a control, the effect on CNC hydrolysis by commercial porcine liver esterase was also measured. The many esterases have been classified into 3 groups according to their substrate, activator, and inhibitor characteristics (Table 1).

The plasma-hydrolyzing activity of CNC is strongly inhibited by diisopropylfluorophosphate and diethyl p-nitrophenyl phosphate (Paraoxon). 2 organophosphorus compounds that inactive serine hydrolases (including esterases) by phosphorylation of an active site serine hydroxyl group (Table 2). Cyclohexylisocyanate, also a serine hydrolase inhibitor by virtue of its carboxylation activity, is also a strong inhibitor of the plasma activity. These 3 compounds also inhibited the CNC-hydrolyzing activity of the commercial porcine liver carboxylesterase (a B-esterase) as well (Table 2).

The plasma activity (and purified esterase as well) was not activated by Ca$^{2+}$ nor inactivated by the chelator EDTA. This information along with the complete inactivation by organophosphates rules out the A-esterases as possible mediators of CNC hydrolysis. Eserine, an inhibitor of cholinesterases, does not inhibit plasma activity but, surprisingly, partially inhibits the purified porcine liver carboxylesterase (28%). Heavy metal ions such as Hg$^{2+}$, in addition to 4-hydroxymercuribenzoate, typically inhibit A-esterases, but several B-esterases are inhibited as well (11). Plasma activity may be slightly inhibited by Hg$^{2+}$ (2%), while the purified esterase is inhibited to a large extent (57%). Neither was affected by 4-hydroxymercuribenzoate.

The organophosphate bis(p-nitrophenyl) phosphate is a selective inhibitor of carboxylesterases. Other serine hydrolases such as $\alpha$-chymotrypsin, trypsin, and cholinesterases are not affected (6, 12, 13). Bis(p-nitrophenyl) phosphate inhibited 92% of the activity of mouse plasma on CNC as a substrate. It almost completely inhibited (97%) of the activity of the purified porcine liver carboxylesterase.

Decomposition Products. Products obtained from the reactions of ENA and MNC with mouse plasma were analyzed by gas chromatography (Table 3). In the absence of plasma, the denitrosation products, N-ethylethamide or N-methylthethyl carbamate, increased in the presence of plasma. Both the spontaneous and plasma-catalyzed reactions gave high (78%) yields of alcohols (ethanol from ENA and methanol from MNC) from the alkyl groups on the nitrogen, which is consistent with the liberation of these groups as a diazotate. The addition of plasma greatly increased the production of the esters, propyl acetate and ethylpropyl carbonate, indicating that the plasma acts as an acyl transfer agent.

DISCUSSION

Nitrosamides and nitrosocarbamates are readily inactivated in blood plasma, whereas the nitrosoureas are negligibly affected (3–5). The major activity contributing to the decomposition of nitrosamides and nitrosocarbamates is enzymatic. There are several lines of direct and indirect evidence that lead us to the conclusion that this activity or activities either are an esterase or have esterase-like properties: (a) the activity is catalytic, since activity capable of hydrolyzing greater than $5 \times 10^{-4}$ M nitroso compound is inhibited by $10^{-5}$ M inhibitors; (b) purified commercial porcine liver carboxylesterase rapidly hydrolyzes methyl and chloroethyl nitrosamines and nitrosocarbamates, while leaving the nitrosoureas virtually untouched; (c) analysis of decomposition products shows the transfer of acyl groups to the acceptor normal propyl alcohol; and (d) inhibitor studies show sensitivity to serine hydrolase inhibitors as well as to a specific inhibitor of carboxylesterases.

Inhibitor studies show the plasma activity to be completely (>97%) inhibited by cyclohexyl isocyanate and the organophosphates diisopropylfluorophosphate and diethyl p-nitrophenyl phosphate (Table 2). This strongly implies the activity is a serine hydrolase. Studies with a specific inhibitor of carboxylesterase [bis(p-nitrophenyl)phosphate] show at least 92% of the plasma activity to be inhibitable and, therefore, presumably due specifically to carboxylesterase(s) action. The profile of plasma activity in the presence of different inhibitors resembles most closely that of the B-esterases (carboxylesterases) (Tables 1 and 2).

The decomposition products obtained after the reaction of ENA or MNC with mouse plasma were analyzed by gas chro-

<table>
<thead>
<tr>
<th>Esterase</th>
<th>Substrates</th>
<th>Activators</th>
<th>Inhibitors</th>
<th>Active site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholinesterase</td>
<td>Choline esters</td>
<td>Ca$^{2+}$</td>
<td>Eserine</td>
<td>Serine</td>
</tr>
<tr>
<td>A-Esterases</td>
<td>Aromatic esters</td>
<td>Mg$^{2+}$</td>
<td>Organophosphates*</td>
<td>Cysteine</td>
</tr>
<tr>
<td>B-Esterases (carboxylesterases)</td>
<td>Aliphatic esters</td>
<td>Ca$^{2+}$</td>
<td>EDTA, Hg$^{2+}$</td>
<td>Serine</td>
</tr>
</tbody>
</table>

*Organophosphate inhibitors, e.g., diisopropylfluorophosphate and Paraoxon.

†4-HMB, 4-hydroxymercuribenzoate.

‡Some B-esterases are also inhibited by Hg$^{2+}$ and 4-hydroxymercuribenzoate as well.

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Table 2
Inhibitor effects on CNC hydrolysis by mouse plasma and purified porcine liver carboxylesterase

See "Materials and Methods" for details. Rates were corrected for spontaneous hydrolysis.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Mouse plasma</th>
<th>Esterase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca²⁺ (10⁻⁴ M)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EDTA (2.5 x 10⁻⁴ M)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hg²⁺ (10⁻⁴ M)</td>
<td>2</td>
<td>57</td>
</tr>
<tr>
<td>4-Hydroxymercurobenzoate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Eserine (2.5 x 10⁻⁴ M)</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td>Cyclohexylisocyanate (10⁻⁴ M)</td>
<td>98</td>
<td>99</td>
</tr>
<tr>
<td>Didesopropylfluorophosphate</td>
<td>10⁻⁴ M</td>
<td>97</td>
</tr>
<tr>
<td>Diethyl p-nitrophenyl phosphate (10⁻⁴ M)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Bis(p-nitrophenyl)phosphate (10⁻⁴ M)</td>
<td>32</td>
<td>96</td>
</tr>
<tr>
<td>Bis(p-nitrophenyl)phosphate (10⁻⁴ M)</td>
<td>85</td>
<td>97</td>
</tr>
<tr>
<td>Bis(p-nitrophenyl)phosphate (10⁻⁴ M)</td>
<td>92</td>
<td>ND³</td>
</tr>
</tbody>
</table>

³Plasma was from female B6D2F, mice. Plasma concentration in the final reaction mixture was 0.5%.

Table 3
Products of spontaneous and plasma-catalyzed decomposition of N-nitroso compounds in the presence of propyl alcohol

<table>
<thead>
<tr>
<th>Nitroso compound</th>
<th>Product</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spontaneous</td>
<td>Plasma catalyzed</td>
</tr>
<tr>
<td>ENA</td>
<td>Ethanol</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>Propyl acetate</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>N-Ethylacetamide</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>85</td>
</tr>
<tr>
<td>MNC</td>
<td>Ethanol</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>Ethylpropyl carbonate</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>N-Methylpropyl carbonate</td>
<td>5</td>
</tr>
</tbody>
</table>

matography. Denitrosation to yield N-ethy lacetamide (from ENA) or N-methylpropyl carbamate (from MNC) accounted for less than 5% of the products and was not increased by plasma. With normal propyl alcohol added to the incubation media as an acyl acceptor, the plasma-catalyzed decomposition of ENA yielded propyl acetate and ethanol as products. Propyl acetate production was dependent upon added plasma. MNC decomposed in plasma with added normal propyl alcohol to yield ethylpropyl carbonate, methanol, and ethanol. Again, the ethylpropyl carbonate was not formed without plasma in the reaction mixture; however, both ethanol and methanol were formed in buffer alone (due to spontaneous decomposition). Transfer of the acyl groups from ENA and MNC to the acceptor normal propyl alcohol (due to spontaneous decomposition). Transfer of the acyl groups from ENA and MNC to the acceptor normal propyl alcohol occurred in high yield considering that the normal propyl alcohol at approximately 0.3 M is competing with water at approximately 50 M. Acyl transfer by esterases from amino acid esters and aromatic amides to alcohols has been shown by others previously (9, 10, 15, 28).

A reaction scheme for MNC is shown in Chart 3. While ENA has only one way in which to hydrolyze, MNC has 2 potential paths. In path A, MNC reacts with the esterase to release a diazotate and generate an ethoxycarbonylated enzyme. In a second step, the enzyme transfers the ethoxycarbonyl group to either water or normal propyl alcohol to produce the ethylpropyl carbonate found. If one assumes that the ratio of "acyl" group transferred to water to that transferred to normal propyl alcohol is independent of the nature of the acyl group, the similarity of the yield of propyl acetate from ENA to the yield of ethylpropyl carbonate from MNC indicates most of the reaction is by Path A. In Path B, MNC reacts with the esterase to release ethanol and generate a nitrosocarbamylated enzyme. Since the N-methyl-N-nitrosopropyl carbamate generated by this path is unstable and a substrate for the esterase, some contribution from this pathway cannot be ruled out. It is possible that nitrosocarbamates in which —O—P₂ (Chart 1) is a better leaving group may decompose primarily by Pathway B.

Esterases are a large group of heterogeneous enzymes with broad, overlapping substrate specificities (Table 1). Most animal tissues and blood plasma contain multiple esterases. Despite extensive study, little is known about the in vivo role of esterases. Our results, presented here and elsewhere (3-5), suggest that one class of esterases, the carboxylesterases, could comprise an important host defense mechanism against endogenously formed mutagens and potential carcinogens. For example, the half-life of CNC in buffer is 330 min, and this is reduced to 9 min in 5% mouse plasma. If the rate of inactivation is proportional to plasma concentration for CNC as it appears to be for inactivation of N-(2-chloroethyl)-N-nitrosacetamide (5), the CNC half-life in 100% plasma would be reduced some 700-fold. Augustinsson (2) has compared the profile of esterases, carboxylesterases, and cholinesterases in 27 different plasma from vertebrates. What is most striking about his results is that 7 mammals, including human, pig, and cow, have no detectable plasma carboxylesterases; the characteristic esterases in mammalian plasma are esterases. Interestingly enough, plasma from these 3 species lacking pronounced carboxylesterase activity (cow, pig, and human) contain very little if any CNC-hydrolyzing activity (3, 4) compared to mouse plasma. If the carboxylesterases comprise host defense mechanisms against endogenous toxic agents of biological importance, there would appear to be striking interspecies differences that could have significant impli-
cations regarding selection of test species for assessment of particular classes of carcinogens and/or chemotherapeutic agents.

This project originally arose out of a continuing study involving the evaluation of antitumor activity of analogues of currently used nitrosoureas. It was shown earlier that simple alkyl nitrosamides and nitrosocarbamates possess no antitumor activity in mice in vivo (7). The data presented here suggest that one of the reasons for this observation may be rapid detoxification in murine blood plasma. The majority of this detoxification in blood plasma is due to serine hydrolase activity, most probably a carboxylesterase.

REFERENCES


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