Inhibition and Stimulation of Long-Chain Fatty Acid Oxidation by Chloroacetaldehyde and Methylene Blue in Rats

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ABSTRACT

The effects of chloroacetaldehyde (CAA) and methylene blue, both alone and together, on mitochondrial metabolism, hepatic glutathione content, and bile flow were investigated in rats. Oxidation of [1-14C]palmitic acid, [1-14C]octanoic acid, and [1,4-14C]succinic acid allowed for the differentiation between carnitine-dependent long-chain fatty acid metabolism, medium chain fatty acid oxidation, and citric acid cycle activity, respectively. CAA, a metabolite of the anticancer drug ifosfamide, which may be responsible for ifosfamide-induced encephalopathy, inhibited palmitic acid metabolism but not octanoic or succinic acid oxidation, depleted hepatic glutathione, and stimulated bile flow. Methylene blue, which is clinically used to either prevent or reverse ifosfamide-associated encephalopathy, markedly stimulated palmitic acid oxidation either in the presence or absence of CAA, but did not affect the oxidation of octanoic and succinic acid or hepatic glutathione. Taken together, this study demonstrates that CAA inhibits palmitic acid metabolism. Methylene blue stimulates long-chain fatty acid oxidation, most likely by facilitating the translocation of fatty acids into mitochondria, and compensates for the CAA effect in vivo.

Ifosfamide, an oxazaphosphorine-alkylating agent effective against a broad spectrum of neoplasms, produces chloroacetaldehyde (CAA) in the course of its metabolism (Sechant et al., 1991). The existence of CAA has been documented in patients receiving ifosfamide (Kaijser et al., 1993; Boddy et al., 1995) and has been suggested as a causative factor contributing to the neurotoxicity associated with ifosfamide chemotherapy (Goren et al., 1986). Clinically, the redox dye methylene blue is used to prevent and reverse ifosfamide-induced encephalopathy (Küpfer et al., 1994; Ferrero et al., 1995), although the mechanism of action is poorly understood. Recently, CAA has been shown to interfere with mitochondrial function by inhibition of long-chain fatty acid oxidation in vitro, an effect reversible with methylene blue (Visarius et al., 1997). Because alterations in mitochondrial function may be an early step in a biochemical chain of events resulting in cellular or systemic dysfunctions, the present study was designed to examine the effects of CAA and methylene blue on mitochondrial function in vivo. Assessment of mitochondrial function was achieved by quantifying the oxidation of [1,4-14C]succinic acid, [1-14C]octanoic acid, and [1-14C]palmitic acid, which served to differentiate between citric acid cycle activity, carnitine-independent, and carnitine-dependent fatty acid oxidation, respectively. The effect of GSH depletion on fatty acid oxidation was also investigated because ifosfamide chemotherapy causes a marked decrease in plasma GSH in patients (Lauterburg et al., 1994) and CAA rapidly depletes GSH from isolated rat hepatocytes (Sood and O'Brien, 1993, 1994). CAA reacts readily with reduced GSH, the major intracellular nonprotein thiol (Marc-hand and Reed, 1989; Jean and Reed, 1992); the resulting GSH depletion might contribute to mitochondrial dysfunction.

Elucidation of the biological actions of CAA is important because it has been identified as a chlorination by-product in finished drinking water supplies (Daniel et al., 1992), produces hepatocellular necrosis (Daniel et al., 1992), and is a reactive metabolite of the large volume industrial chemicals vinyl chloride (Chiang et al., 1997) and ethylene dichloride (McCall et al., 1983) and of the chemotherapeutic (methylcarbamoyl)triazenes (Rouzer et al., 1996) in addition to ifosfamide.

The aims of this investigation were, therefore, to study CAA-induced alterations in hepatic GSH content and test the hypothesis that CAA and methylene blue, both alone and together, could alter mitochondrial fatty acid metabolism in vivo.

Materials and Methods

Animals. Male Wistar rats (200–250 g) were bred and housed in the University of Bern vivarium, were allowed access to food and

ABBREVIATIONS: CAA, chloroacetaldehyde; CoA, coenzyme A.
water ad libitum, and were kept on a 12-h light/dark cycle. In animals where the depletion of GSH was desired, diethyl maleate (purity >95%) was injected (0.4 ml/kg i.p.) 15 min before anesthesia (Boyland and Chasseaud, 1970). Anesthetized animals (pentobarbital, 50 mg/kg i.p.) were placed in a supine position on a thermostatically regulated blanket to control body temperature. To measure bile flow, animals received bile duct catheters and bile was collected into preweighed vials for periods of 5 and 10 min throughout the experiments. Small liver biopsies (ca. 200 mg) were taken after bile duct cannulation and/or at the experiments’ termination. Liver was immediately homogenized (1:10, w/v) in phosphate buffer (0.1 mol/l, pH 7.4) on ice and proteins were precipitated with sulfosalicylic acid, 2.5% final concentration.

For the determination of GSH, acid-soluble extracts were neutralized with Tris-HCl buffer (0.2 M, pH 8.5) and derivatized with monobromobimane; GSH was quantified fluorometrically by HPLC (Newton et al., 1981). For the determination of GSSG, acid-soluble extracts of liver homogenate were derivatized with 2,4-dinitrofluorobenzene and measured by HPLC as N,N-bis-dinitrophenyl derivatives (Pariss and Reed, 1987).

In Vivo Oxidation of Labeled Substrates. To collect breath of anesthetized rats, a cylindrical vessel attached to a vacuum pump was placed over the rodent’s head. CAA (250 μmol/kg i.p.), dissolved in sterile saline (0.9%), or an equivalent amount of saline without CAA for control animals, was administered at 0 min. [1-14C]Palmitic acid (3 μCi/kg, 55.0 mCi/mmol) and [1-14C]octanoic acid (0.3 μCi/kg, 55.0 mCi/mmol), both obtained from Amersham (Buckinghamshire, England), were diluted in thistle oil. [1,4-14C]Succinic acid (0.3 mCi/kg, 59 mCi/mmol), both obtained from DuPont-NEN (Boston, MA), was diluted in sterile saline. All tracers were administered i.p. at 5 min. Methylene blue (1 or 10 mg/kg) was administered i.p. 25 min after tracer administration.

To collect 14CO2, resulting from the oxidation of labeled substrates, exhaled air was pulled through successive solutions of ethanol (to dry exhaled breath) and ethanolamine (4 M in ethanol) to trap exhaled 14CO2. The 14CO2 was then quantified by scintillation spectroscopy.

Statistical Analysis. Groups of four animals each were studied unless stated otherwise. Results are presented as mean ± S.E.M. Comparisons between methylene blue-treated animals and either controls or CAA-treated animals were calculated from the time of methylene blue administration to the end of the experiment, effectively from 30 to 90 min. Comparisons between controls and CAA treatment groups were calculated from 0 to 90 min, the time of effect of CAA. The results were analyzed by analysis of variance or, where appropriate, by repeat measure analysis of variance. The results were analyzed by analysis of variance or, where appropriate, by repeat measure analysis of variance followed by the Bonferroni t test for correction for multiple comparisons. All data sets showed normal distribution, justifying the use of parametric statistical tests. Two-tailed probabilities of less than .05 were considered to be significant.

Results

Glutathione Status. The livers of CAA-treated animals showed a 61% drop in GSH compared with controls over the 90-min experiment (p < .03, n = 3). The presence of methylene blue neither prevented nor contributed to the CAA-induced GSH depletion as evidenced by the 55% decrease in liver GSH in the CAA plus methylene blue treatment group (Table 1). To exclude the possibility of GSH oxidation, we examined the GSSG content of livers from animals exposed to CAA. At the experiments’ termination, control livers contained 0.40 ± 0.17 μmol GSSG/g liver, whereas 0.32 ± 0.05 μmol GSSG/g liver remained after treatment with CAA. In animals pretreated with diethyl maleate, GSH levels were measured before CAA administration and amounted to 0.16 ± 0.05 μmol GSH/g liver.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial GSH</th>
<th>Final GSH</th>
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<tbody>
<tr>
<td></td>
<td>μmol/g liver</td>
<td>μmol/g liver</td>
</tr>
<tr>
<td>Control</td>
<td>4.4 ± 0.7</td>
<td>3.8 ± 0.4</td>
</tr>
<tr>
<td>Methylene blue control</td>
<td>4.8 ± 0.9</td>
<td>3.8 ± 0.4</td>
</tr>
<tr>
<td>CAA (250 μmol/kg)</td>
<td>4.1 ± 0.6</td>
<td>1.6 ± 0.3*</td>
</tr>
<tr>
<td>CAA plus methylene blue</td>
<td>5.2 ± 0.6</td>
<td>2.3 ± 0.2*</td>
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*p < .03 CAA and CAA plus methylene blue, respectively, versus control.

Effect of CAA on Bile Secretion. Administration of CAA markedly increased bile flow (Fig. 1), where significant differences from basal bile flow lasted 40 min after CAA administration (p < .04, n = 3). Methylene blue rapidly appeared (<1 min after i.p. administration) and was continuously excreted in bile throughout the experiment but did not increase bile flow when administered alone or significantly alter the choleretic response to CAA when administered after CAA. No increase in bile flow was observed after administration of CAA when GSH was depleted by diethyl maleate before the application of CAA.

Effect of CAA on [1-14C]Palmitic Acid Metabolism. CAA impaired palmitic acid oxidation in vivo (Fig. 2). Controls exhaled 3.95 ± 0.13% whereas animals treated with CAA exhaled 1.40 ± 0.09% of the administered tracer dose in 90 min (p < .01). To exclude the possibility that CAA-induced depletion of GSH was responsible for the diminished oxidation of palmitic acid, animals were studied after the depletion of GSH with diethyl maleate. Diethyl maleate-treated animals exhaled 3.83 ± 0.52% of the administered tracer dose as 14CO2, which was not significantly different from control animals, indicating that depletion of GSH could not account for the inhibition of palmitic acid metabolism observed in the CAA-treated animals. When CAA was administered to rats pretreated with diethyl maleate, the oxidation of palmitic acid was again markedly decreased and was not significantly different from the animals receiving CAA alone.
Effect of CAA and Methylene Blue on [1-14C]Octanoic Acid and [1,4-14C]Succinic Acid Metabolism. CAA neither inhibited the oxidation of octanoic acid nor that of succinic acid in vivo (Fig. 4). Octanoic acid controls exhaled 39.28 ± 2.85% whereas the CAA treatment group produced 33.82 ± 1.43% of the administered tracer as 14CO2. Succinic acid controls exhaled 59.04 ± 2.36% whereas the respective CAA treatment group produced 61.02 ± 2.22% of the administered tracer as 14CO2.

Methylene blue did not affect octanoic acid or succinic acid oxidation in vivo (Fig. 5). The octanoic acid plus methylene blue treatment group produced 42.84 ± 0.42% and the succinic acid plus methylene blue group exhaled 59.79 ± 3.54% of the administered tracer as 14CO2.

Discussion

The present study demonstrates that CAA, a metabolite of the alkylating cytostatic drug ifosfamide, which may induce an ifosfamide-related encephalopathy, and methylene blue, currently used to manage ifosfamide encephalopathy, administered either alone or together, affect mitochondrial long-chain fatty acid metabolism in vivo. To localize potential sites of action we examined the influence of CAA and methylene blue on the oxidation of: 1) succinic acid, a substrate for the citric acid cycle; 2) octanoic acid, a medium chain fatty acid and substrate for β-oxidation that may pass through the mitochondrial inner membrane without prior activation; and 3) palmitic acid, a long-chain fatty acid requiring extramitochondrial activation and transport across the mitochondrial membranes via the carnitine system before sequentially gaining access to β-oxidation, the citric acid cycle, and the respiratory chain (Bremer, 1983). Interestingly, the rate of palmitic acid oxidation, but not that of succinic acid or octanoic acid oxidation, was affected by the presence of CAA or methylene blue. Normal rates of succinic acid oxidation after
palmitoyl-L-carnitine oxidation in mitochondria previously palmitic acid in the absence of carnitine and the rate of mitochondria, methylene blue also increases the oxidation of methylene blue induced the uncoupling of oxidative phosphorylation in vivo (Fig. 5) it is unlikely that because methylene blue failed to stimulate octanoic acid and oxidation in vivo (Visarius et al., 1997). However, addition of GSSG did not increase. Rather, the depletion of GSH can explain the effects of CAA on fatty acid oxidation because depletion of GSH by diethyl maleate did not interfere with the oxidation of palmitic acid. Moreover, methylene blue, which partially restored fatty acid oxidation, did not affect adduct formation as evidenced by its lacking effect on bile flow after the administration of CAA and did not prevent the decrease in hepatic GSH. One explanation for the contrasting effects on fatty acid oxidation may be that diethyl maleate, a substrate for GSH transferases, is a more selective depleter of GSH than the more reactive CAA, which also reacts with protein sulphydryls (Sood and O'Brien, 1993) and is likely to deplete CoA.

Adequate cytosolic CoA is required for the activation of long-chain fatty acids, which may then traverse the mitochondrial outer membrane, but not the mitochondrial inner membrane, as acyl-CoA. In the intermembranous space, the acyl-CoA is converted to acylcarnitine, which is subsequently transported across the mitochondrial inner membrane by carnitine translocase (Bremer, 1983). Sequestration of cytosolic CoA by CAA may retard long-chain fatty acid formation, thereby limiting the access of long-chain fatty acids to the mitochondrial matrix and mitochondrial metabolism. In this case, a reduced rate of utilization of substrates dependent on the carnitine system for their import into mitochondria, such as palmitic acid, would be expected, whereas the rate of oxidation substrates capable of entering the mitochondria without extramitochondrial activation would remain unaffected by the presence of CAA. Intramitochondrial CoA may not be substantially affected by the presence of CAA for at least two reasons. First, before gaining access to intramitochondrial CoA, CAA must traverse the cytosol, thereby potentially interacting with cytosolic thiols. Second, in the event that CAA passes through the cytosolic milieu and enters the mitochondria, the much higher intramitochondrial concentration of CoA (approximately 2.5 mM; Robishaw and Neely, 1985) may not be substantially affected by the CAA reaching the mitochondrial matrix. Thus, we would expect mitochondrial β-oxidation and the citric acid cycle to maintain intact function after administration of the chosen dose of CAA.

Taken together, this work describes the capacity of CAA, a metabolite of the widely used antineoplastic drug ifosfamide, to specifically inhibit long-chain fatty acid metabolism and to deplete but not oxidize hepatic GSH in vivo. Dysfunctional changes in fatty acid metabolism have previously been suggested to play a role in chronic fatigue (Gray and Martinovic, 1994) and encephalopathy (Heubi et al., 1987), thus CAA-induced impairment of mitochondrial metabolism may play a role in the chronic fatigue experienced by patients receiving ifosfamide, ifosfamide encephalopathy, or both. Methylene blue rapidly and specifically induces long-chain fatty acid oxidation in vivo, most likely by altering the mitochondrial membrane permeability for long-chain fatty acids. Thus, methylene blue may compensate for the CAA induced dys-
function in mitochondrial metabolism and could become advantageous where the stimulation of long-chain fatty acid metabolism is desired.

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References


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