Human Kidney Tubules Detoxify Chloroacetaldehyde, a Presumed Nephrotoxic Metabolite of Ifosfamide

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Abstract. The nephrotoxic effects of the antineoplastic drug ifosfamide have been attributed to its hepatic metabolite chloroacetaldehyde. The effects of chloroacetaldehyde on isolated human kidney cortex tubules metabolizing lactate (a physiologic substrate in human kidneys) were investigated. At concentrations of ≥0.5 mM, chloroacetaldehyde was toxic to the human kidney tubules, as demonstrated by a dramatic decrease in cellular ATP levels and a large increase in lactate dehydrogenase release; chloroacetaldehyde also stimulated pyruvate accumulation and inhibited lactate removal and glucose synthesis. These effects, which were associated with incomplete disappearance of chloroacetaldehyde and extensive depletions of the cellular CoA, acetyl-CoA, and glutathione contents, were prevented by the addition of thiol-protecting drugs (mesna and amifostine). Human kidney tubules were demonstrated to metabolize chloroacetaldehyde at high rates, presumably via aldehyde dehydrogenase, which is very active in human kidneys. Carbon-13 nuclear magnetic resonance spectroscopy measurements indicated that human kidney tubules converted [2-13C]chloroacetaldehyde to [2-13C]chloroacetate, the further metabolism of which was very limited. At equimolar concentrations, chloroacetate was much less toxic than chloroacetaldehyde, indicating that chloroacetate synthesis from chloroacetaldehyde by human kidney tubules represents a detoxification mechanism that could play a role in vivo in preventing or limiting the nephrotoxic effects observed during ifosfamide therapy.

Ifosfamide, which was first synthesized in the late 1960s, is an alkylating agent that belongs to the group of oxazaphosphorines (1,2). Like cyclophosphamide (its structural isomer), ifosfamide is a prodrug whose biotransformation by hepatic cytochrome P450 isoenzymes is required (1,2). Ifosfamide, which has been reported to exhibit greater antitumor activities than cyclophosphamide in certain experimental and human malignancies (1), is used in both pediatric and adult oncology. Unfortunately, this drug may induce renal Fanconi syndrome (3,4), which is never observed after cyclophosphamide administration. In large series, approximately 5% of treated patients experienced this side effect and another 15% developed subclinical tubular dysfunction (5,6). Therefore, the nephrotoxicity of ifosfamide is a limiting factor in high-dose chemotherapy (6–9) and in certain pediatric protocols (10,11).

The pathophysiologic features of ifosfamide-induced renal Fanconi syndrome are not fully understood (12,13). Some authors have proposed that chloroacetaldehyde might be responsible for this nephrotoxicity (14–16). Such a proposal is consistent with the fact that much more chloroacetaldehyde is formed after ifosfamide administration than after cyclophosphamide administration; indeed, Dechant et al. (1) calculated up to 100-fold greater chloroacetaldehyde formation after ifosfamide treatment than after cyclophosphamide treatment. The differences in hepatic metabolism between the two drugs are attributable to their structural differences, which involve a shift of one chloroethyl group from the exocyclic nitrogen to the nitrogen of the oxazaphosphorine ring. The spatial separation of the 2-chloroethyl groups in the ifosfamide molecule slows the rate of activation via ring hydroxylation and decreases the formation of the active metabolite 4-hydroxy-ifosfamide. As a result, side chain oxidation leads to the inactive metabolites 2-dechloroethyl-ifosfamide and 3-dechloroethyl-ifosfamide, with the stoichiometric formation of chloroacetaldehyde (1,13).

The mechanism by which chloroacetaldehyde causes kidney damage is not yet known (12,13). Manifestations of this toxicity include dose-dependent malabsorption of fluid, sodium, glucose, and proteins (16) and impairment of the cellular energy supply, with a severe decrease in the urinary excretion of tricarboxylic acid intermediates (17). In addition, Springate (16) has demonstrated that the renal toxicity of chloroacetaldehyde in vivo is associated with severe glutathione depletion and malondialdehyde accumulation.

In an attempt to gain further insight into the mechanism of the nephrotoxic effects of chloroacetaldehyde, we conducted a study in which we incubated suspensions of human proximal tubular fragments with lactate (a physiologic substrate in human kidneys) (18,19), in the absence or presence of various
concentrations of chloroaetaldehyde. The results obtained clearly demonstrate that chloroaetaldehyde is toxic to human renal proximal tubular cells. They also demonstrate that these cells are capable of converting, at high rates, chloroaetaldehyde into chloroaacetate, a compound that is poorly metabolized and much less toxic than its immediate precursor.

Materials and Methods

Preparation of Kidney Cortex Tubules and Incubation Procedures

Fresh normal kidney cortex was obtained from the uninvolved pole of kidneys removed from 18-h-fasted patients because of neoplasms. Specimens of cortex were immediately dissected and placed in ice-cold Krebs-Henseleit buffer gassed with a mixture of 95% O2 and 5% CO2. Kidney tubules were prepared by collagenase treatment, as described previously (20). Incubations were performed at 37°C in a shaking water bath, in 25-m1 hermetically stopped Erlenmeyer flasks, in an atmosphere of 95% O2/5% CO2. The tubular (mainly proximal) fragments obtained were incubated for 30 or 60 min in 4 ml of Krebs-Henseleit medium with 1 mM L-lactate and various chloroaetaldehyde or chloroaacetate concentrations. In all experiments, each experimental condition was assayed in duplicate. Incubations were terminated by the addition of HClO4 (2%, vol/vol, final concentration) to each flask. In experiments in which lactate dehydrogenase (LDH) activity was determined, two aliquots (0.2 ml each) of tubular suspension were collected just before HClO4 addition. In each experiment, zero-time flasks were prepared by adding HClO4 before the tubules. The denatured proteins were removed by centrifugation (4000 × g for 5 min at 4°C), and the supernatant was neutralized with 20% (w/v) KOH and kept on ice until metabolites were assayed. In experiments in which [1-13C]chloroaacetate was used as a substrate, incubation, deproteinization, and measurement of the 13CO2 formed were performed as described previously (21); the medium was then treated as described above.

Analytical Methods

Metabolite Assays. Chloroaetaldehyde levels were determined by using the method described by Bernt and Bergmeyer (22) for acetaldehyde measurements. Alcohol dehydrogenase from yeast, which was used for measurements, was not specific for acetaldehyde as a substrate, and chloroaetaldehyde was immediately transformed into chloroaethanol, with the concomitant oxidation of NADH to NAD+. Glucose, pyruvate, lactate, and alanine concentrations, measured by using standard enzymatic methods, and the dry weight of tubules added to the flasks were determined as described previously (20). ATP levels were determined by using the method described by Lamprecht and Trautschold (23). The sum of CoA and acetyl-CoA was determined by using a kinetic method, as described by Michal and Bergmeyer (24), and glutathione levels (the sum of the reduced and oxidized forms) were assessed as described by Griffith (25).

Measurement of LDH Levels. LDH activity was determined by using the method described by Bergmeyer and Bernt (26). The leakage of LDH from renal cells into the incubation medium was measured in the supernatant obtained from 0.2 ml of tubular suspension collected at the end of the incubation period, after immediate centrifugation for 1 min at 4000 × g, and that value was compared with total LDH amounts measured in a sample in which the tubular suspension had been frozen and thawed three times, to release LDH from renal cells.

Measurement of Aldehyde Dehydrogenase Activity. Pieces of human renal cortex were rinsed in an ice-cold buffer (pH 7.2) containing 0.25 M saccharose, 5 mM Tris-HCl, and 0.5 mM ethylenediaminetetraacetate, weighed, and homogenized in 9 vol of the same buffer containing sodium deoxycholate (30 mg/g wet weight of cortical tissue), in a Potter-Elvehjem glass homogenizer with a motor-driven Teflon pestle. The homogenates were then filtered through a double layer of cheesecloth, for removal of connective tissue. Aldehyde dehydrogenase activity was assayed spectrophotometrically at 23°C, using a Molecular Devices microplate reader, by measuring at 340 nm the reduction of NAD+ in a 50 mM sodium pyrophosphate buffer (pH 8.8), as described by Tottmar et al. (27).

13C Nuclear Magnetic Resonance Techniques

Data were recorded at 125.75 MHz with a Bruker AM-500 WB spectrometer, using a 5-mm broad-band probe thermostated at 20 ± 0.5°C. [2-13C]Glycine was added as an internal standard. The recording conditions were as follows: spectral width, 25,000 Hz; tilt angle, 90 degrees; data size, 32,000; repetition time, 50 s (fully relaxed spectra were obtained); number of scans, 300. Chemical shifts were expressed as parts per million, relative to tetramethylsilane. Proton decoupling was performed during data acquisition, using the standard WALTZ 16 pulse sequence for inverse-gated proton decoupling.

Statistical Analyses

Net substrate utilization and product formation were calculated as the difference between the total flask contents (tissue plus medium) at the start (zero-time flasks) and after the period of incubation. The metabolic rates, reported as means ± SEM, are expressed as micromoles of substances removed or produced per gram dry weight per unit of time, except for ATP, CoA plus acetyl-CoA, and glutathione, the levels of which were expressed as micromoles per gram dry weight. The rates of release of 14CO2 from [1-13C]chloroaacetate were calculated by dividing the radioactivity in 14CO2 by the specific radioactivity of the labeled chloroaacetate, as measured in the medium of zero-time flasks. The amounts of [2-13C]chloroaetaldehyde or [2-13C]chloroaacetate were calculated from the areas of the corresponding peaks, compared with the peak area for [2-13C]glycine, which was added as an internal standard for nuclear magnetic resonance measurements. The results were analyzed by ANOVA for repeated measurements, followed by Scheffe’s test for comparison of the values obtained in the presence and absence of chloroaetaldehyde or chloroaacetate. Probabilities of <0.05 were considered to be significant.

Reagents

Enzymes, coenzymes, and L-lactate were supplied by Roche (Meylan, France). [2-13C]Chloroaetaldehyde (isotopic enrichment, 99%) was obtained from Eurisotop (Gif-sur-Yvette, France), and [1-13C]chloroaacetate (53 mCi/mmol) was supplied by Isotopchim (Ganagobie-Peyruis, France). Chloroaetaldehyde, chloroaacetate, and mesna (2-mercaptopoethanesulfonate, sodium salt) were from Sigma Chemical Co. (St. Louis, MO). Amifostine (Ethylol) was kindly provided by Schering-Plough (Levallois-Perret, France). The other chemicals used were of analytical grade.

Results

Effects of Various Concentrations of Chloroaetaldehyde in Human Kidney Tubules Metabolizing Lactate

The effects of increasing concentrations of chloroaetaldehyde (1 μM to 5 mM) were studied in human kidney tubules.
incubated with a physiologic concentration of lactate (1 mM). ATP levels were measured as an index of cellular energy metabolism, whereas LDH release into the incubation medium was taken as a marker of cell lysis. As shown in Figure 1, low concentrations of chloroacetaldehyde (1 μM to 0.1 mM) did not alter cellular ATP levels or LDH release. In contrast, higher chloroacetaldehyde concentrations (0.5 to 5 mM) were toxic to the renal tubular cells and caused a dramatic increase in LDH release and a great decrease in ATP levels. The data presented in Table 1 demonstrate that 0.5 mM concentrations of chloroacetaldehyde greatly inhibited lactate uptake and glucose synthesis; the carbon balance, which represents the lactate that has been completely oxidized via the pyruvate dehydrogenase and tricarboxylic acid cycle enzyme reactions, was also decreased. These effects were associated with a large increase in pyruvate accumulation, suggesting that chloroacetaldehyde (0.5 to 5 mM) induced a defect in pyruvate utilization by pyruvate carboxylase and/or pyruvate dehydrogenase. Because pyruvate carboxylase is stimulated by acetyl-CoA and pyruvate dehydrogenase uses CoA as a substrate, the cellular levels of CoA plus acetyl-CoA were measured in the absence and presence of chloroacetaldehyde. As shown in Figure 2, the levels of CoA plus acetyl-CoA remained unchanged after addition of low chloroacetaldehyde concentrations (1 μM to 0.1 mM), but virtually complete depletion of these coenzymes was observed after addition of 0.5, 1, or 5 mM chloroacetaldehyde. Figure 2 also indicates that addition of 0.1 and especially 0.5, 1, and 5 mM chloroacetaldehyde led to dramatic decreases in total cellular glutathione levels (reduced glutathione plus oxidized glutathione).

Prevention of the Adverse Effects of Chloroacetaldehyde by Thiol Compounds

Experiments were performed to study whether the adverse effects of chloroacetaldehyde could be prevented by the addition to the incubation medium of mesna and amifostine, two compounds known to have thiol-protecting properties (1,28,29). As shown in Table 2, the effects of 0.5 mM chloroacetaldehyde on LDH release and the ATP content, as well as on lactate metabolism, were fully prevented by the addition of an equimolar concentration of mesna. Amifostine, preincubated with the tubules for 30 min before the addition of chloroacetaldehyde and used at a 10-fold higher concentration than mesna, led to complete prevention of the effects of chloroacetaldehyde on ATP levels and LDH release (Table 2). It should be noted that neither mesna nor amifostine alone affected the parameters studied (Table 2).

Chloroacetaldehyde Utilization by Human Kidney Tubules

With the aforementioned observation that chloroacetaldehyde depleted cellular thiol compounds, we took advantage of the broad specificity of yeast alcohol dehydrogenase to use the acetaldehyde assay to measure the disappearance of chloroacetaldehyde (Figures 1 and 2 and Table 1). When added at concentrations of 0.001, 0.01, or 0.1 mM at the start of the incubation, chloroacetaldehyde was undetectable after 1 h of incubation with the tubules; in contrast, when added at higher concentrations (0.5, 1, and 5 mM), chloroacetaldehyde was only partly removed by the tubules after 1 h of incubation (results not shown). Because the complete disappearance of chloroacetaldehyde from the incubation medium seemed to be correlated with the absence of toxic effects (Figures 1 and 2 and Table 1), experiments were performed to test whether the toxic effects of 0.5 mM chloroacetaldehyde described above would be suppressed by increases in the amount of kidney tubules in the incubation medium. This was the case, as demonstrated by the data presented in Table 3. Indeed, increasing the amount of tubules from 4 to 10 mg dry weight considerably reduced LDH release and almost completely restored the ATP levels to control values; a further increase in the amount of kidney tubules to 20 mg dry weight completely suppressed the chloroacetaldehyde-induced effects on these two cellular viability parameters.
Table 1. Effects of various concentrations of chloroacetaldehyde on lactate metabolism in human kidney cortex tubules

<table>
<thead>
<tr>
<th>CAA Concentration (mM)</th>
<th>Lactate Uptake (μmol/g per h)</th>
<th>Pyruvate Accumulation (μmol/g per h)</th>
<th>Alanine Synthesis (μmol/g per h)</th>
<th>Glucose Synthesis (μmol/g per h)</th>
<th>Carbon Balance (μmol/g per h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>415.2 ± 39.2</td>
<td>31.3 ± 8.3</td>
<td>89.8 ± 9.4</td>
<td>110.7 ± 6.6</td>
<td>72.7 ± 24.3</td>
</tr>
<tr>
<td>0.001</td>
<td>364.1 ± 34.8</td>
<td>36.7 ± 8.5</td>
<td>82.8 ± 11.1</td>
<td>100.9 ± 5.0</td>
<td>42.8 ± 25.2</td>
</tr>
<tr>
<td>0.01</td>
<td>365.6 ± 39.6</td>
<td>32.3 ± 6.5</td>
<td>74.8 ± 7.8</td>
<td>103.8 ± 7.4</td>
<td>51.0 ± 27.2</td>
</tr>
<tr>
<td>0.1</td>
<td>421.0 ± 31.1</td>
<td>33.4 ± 8.7</td>
<td>81.1 ± 7.7</td>
<td>121.9 ± 5.6</td>
<td>62.6 ± 24.8</td>
</tr>
<tr>
<td>0.5</td>
<td>108.3 ± 25.7c</td>
<td>88.3 ± 20.1c</td>
<td>86.2 ± 9.7</td>
<td>6.4 ± 1.8c</td>
<td>−78.9 ± 28.6c</td>
</tr>
<tr>
<td>1.0</td>
<td>54.8 ± 19.8c</td>
<td>89.2 ± 18.7c</td>
<td>80.6 ± 6.9</td>
<td>2.3 ± 0.8c</td>
<td>−119.5 ± 21.8c</td>
</tr>
<tr>
<td>5.0</td>
<td>27.1 ± 14.4c</td>
<td>55.6 ± 7.5</td>
<td>64.2 ± 8.4c</td>
<td>1.7 ± 0.8c</td>
<td>−96.0 ± 21.2c</td>
</tr>
</tbody>
</table>

* Each flask contained 4.8 ± 0.6 mg dry weight of tubules. Results are reported as means ± SEM for four experiments performed in duplicate. CAA, chloroacetaldehyde.

The carbon balance was calculated as the difference between lactate uptake and the sum of the pyruvate, alanine, and glucose amounts (expressed in C₃ units) observed. It should be noted that the negative values are probably attributable to inaccurate determination of lactate uptake, which represented a very small proportion of the lactate added under these conditions.

To better characterize the disappearance of chloroacetaldehyde, new experiments were performed using small amounts of kidney tubules. As shown in Figure 3, human kidney tubules readily removed chloroacetaldehyde, with a maximal rate of approximately 400 μmol/g per h being observed at the 0.25 mM substrate concentration. Although chloroacetaldehyde removal was statistically greater after 60 min than after 30 min of incubation for all of the chloroacetaldehyde concentrations studied, it is noteworthy that the rate of chloroacetaldehyde utilization was almost linear with time only for the 0.25 mM substrate concentration. Figure 3 also indicates that the rate of chloroacetaldehyde removal was approximately the same after 30 min of incubation with 0.25, 0.35, or 0.5 mM chloroacetaldehyde but decreased between 30 and 60 min of incubation with substrate concentrations greater than 0.25 mM. It should be noted that, after both 30 and 60 min of incubation, chloroacetaldehyde removal with a substrate concentration of 1 mM was statistically lower than that observed with the other chloroacetaldehyde concentrations. These observations suggest that the decrease in chloroacetaldehyde utilization with time was dependent not only on the incubation period but also on the substrate concentration.

Metabolic Fate of Chloroacetaldehyde in Human Kidney Tubules

As presented in Figure 4, 13C nuclear magnetic resonance analyses performed at the beginning and the end of incubations of human kidney tubules with 1 mM [2,13C]chloroacetaldehyde indicated that only one resonance was observed, at 44.9 ppm, after 60 min of incubation. This resonance corresponded to [2,13C]chloroacetate, the accumulation of which was calculated by comparing its peak area with that of [2-13C]glycine, which was added as an internal standard. After 60 min of incubation, chloroacetate accounted for 76.9 ± 9.7% of chloroacetaldehyde utilization (n = 3). This clearly indicates that chloroacetate was poorly metabolized by human kidney tubules. This finding was verified by incubating human kidney tubules for 60 min with 0.5 mM [1,14C]chloroacetate and measuring the production of 14CO₂, which represented 3.8 ± 0.9% of the chloroacetate present at the beginning of the incubation (n = 4). Therefore, from a quantitative point of view, chloroacetate was the only major product of chloroacetaldehyde metabolism by human kidney tubules.

Inhibition of Human Kidney Cortex Aldehyde Dehydrogenase by Chloroacetaldehyde

Because chloroacetate is an oxidation product of chloroacetaldehyde metabolism, we investigated whether aldehyde dehydrogenase, which is very active in human kidney cortex (30), could be responsible for such an oxidation. In these experiments, aldehyde dehydrogenase activity was measured in human kidney cortex homogenates with 5 mM acetaldehyde or chloroacetaldehyde as the substrate. As demonstrated in Figure 5A, the initial rate of the reaction (for approximately 2 min) with chloroacetaldehyde as the substrate was the same as that of acetaldehyde.
Reversal of the toxic effects of 0.5 mM chloroacetaldehyde by amifostine and mesna in human kidney cortex tubules metabolizing 1 mM lactate

Table 2. Reversal of the toxic effects of 0.5 mM chloroacetaldehyde by amifostine and mesna in human kidney cortex tubules metabolizing 1 mM lactate

<table>
<thead>
<tr>
<th>Condition</th>
<th>LDH Release (μmol/g per h)</th>
<th>ATP Concentration (μmol/g)</th>
<th>Pyruvate Accumulation (μmol/g per h)</th>
<th>Lactate Uptake (μmol/g per h)</th>
<th>Pyruvate Accumulation (μmol/g per h)</th>
<th>Alanine Synthesis (μmol/g per h)</th>
<th>Glucose Synthesis (μmol/g per h)</th>
<th>Carbon Balance (% of Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.2 ± 1.7c</td>
<td>6.4 ± 1.0c</td>
<td>24.8 ± 3.5c</td>
<td>10.1 ± 2.5c</td>
<td>10.8 ± 2.7c</td>
<td>9.1 ± 2.3c</td>
<td>6.8 ± 1.2c</td>
<td>11.1 ± 2.1c</td>
</tr>
<tr>
<td>CAA</td>
<td>7.6 ± 0.7c</td>
<td>1.7 ± 0.6c</td>
<td>100.4 ± 11.5d</td>
<td>8.0 ± 0.6c</td>
<td>22.4 ± 37.1c</td>
<td>7.9 ± 0.3c</td>
<td>6.8 ± 1.2c</td>
<td>13.1 ± 2.1c</td>
</tr>
<tr>
<td>Amifostine (5 mM)</td>
<td>35.0 ± 8.3d</td>
<td>8.0 ± 0.6c</td>
<td>165.6 ± 36.2d</td>
<td>6.4 ± 1.0c</td>
<td>8.0 ± 0.6c</td>
<td>7.9 ± 0.3c</td>
<td>6.8 ± 1.2c</td>
<td>13.1 ± 2.1c</td>
</tr>
<tr>
<td>Amifostine (5 mM) + CAA</td>
<td>35.0 ± 8.3d</td>
<td>8.0 ± 0.6c</td>
<td>165.6 ± 36.2d</td>
<td>6.4 ± 1.0c</td>
<td>8.0 ± 0.6c</td>
<td>7.9 ± 0.3c</td>
<td>6.8 ± 1.2c</td>
<td>13.1 ± 2.1c</td>
</tr>
<tr>
<td>Mesna (0.5 mM)</td>
<td>9.4 ± 2.3c</td>
<td>6.4 ± 1.0c</td>
<td>24.7 ± 22.7c</td>
<td>7.9 ± 0.3c</td>
<td>6.8 ± 1.2c</td>
<td>7.9 ± 0.3c</td>
<td>6.8 ± 1.2c</td>
<td>13.1 ± 2.1c</td>
</tr>
<tr>
<td>Mesna (0.5 mM) + CAA</td>
<td>9.4 ± 2.3c</td>
<td>6.4 ± 1.0c</td>
<td>24.7 ± 22.7c</td>
<td>7.9 ± 0.3c</td>
<td>6.8 ± 1.2c</td>
<td>7.9 ± 0.3c</td>
<td>6.8 ± 1.2c</td>
<td>13.1 ± 2.1c</td>
</tr>
<tr>
<td>Mesna (0.5 mM) + CAA</td>
<td>9.4 ± 2.3c</td>
<td>6.4 ± 1.0c</td>
<td>24.7 ± 22.7c</td>
<td>7.9 ± 0.3c</td>
<td>6.8 ± 1.2c</td>
<td>7.9 ± 0.3c</td>
<td>6.8 ± 1.2c</td>
<td>13.1 ± 2.1c</td>
</tr>
</tbody>
</table>

Each flask contained 5.4 ± 1.0 mg dry weight of tubules. Results are reported as means ± SEM for five experiments performed in duplicate. LDH, lactate dehydrogenase.

b See Table 1 for the carbon balance calculations.

c p < 0.05 versus control.

d p < 0.05 versus chloroacetaldehyde.

Toxicity of Chloroacetate to Human Kidney Tubules

Because the metabolism of chloroacetaldehyde led to the accumulation of chloroacetate, it was important to study the nephrotoxicity of the latter compound. After 60 min of incubation of human kidney tubules in the presence of 0.5 mM [14C]chloroacetate, the intracellular accumulation of this compound reached a value of approximately 12 (results not shown). The data presented in Figure 4 demonstrate that, at equimolar concentrations, chloroacetate was much less toxic than chloroacetaldehyde. Indeed, no significant alterations in LDH release or cellular glutathione contents were observed even in the presence of 5 mM chloroacetate. Again, at equimolar concentrations, the decrease in the cellular levels of CoA plus acetyl-CoA was much less pronounced with chloroacetate than with chloroacetaldehyde; this resulted in less inhibition of lactate metabolism and a slight decrease in the cellular ATP content only with 5 mM chloroacetate.

Discussion

Toxicity of Chloroacetaldehyde to Human Kidney Tubules

Our results demonstrated for the first time that, at concentrations close to those sometimes observed in the plasma and urine of patients treated with ifosfamide (0.11 to 0.22 mM and 0.21 mM, respectively) (31,32), chloroacetaldehyde was toxic to human kidney tubules. This was illustrated by the increase in the leakage of cellular LDH and the decrease in cellular ATP levels (Figure 1). These results confirm that chloroacetaldehyde is a nephrotoxic compound, as demonstrated by other authors with isolated perfused rat kidneys (14,16) and cultured renal cells (15,33).

In the presence of 0.5, 1, or 5 mM chloroacetaldehyde, lactate utilization and glucose synthesis were drastically decreased, whereas pyruvate accumulation was greatly increased. Such pyruvate accumulation may be explained by inhibition of the activity of the two major enzymes involved in pyruvate metabolism, namely pyruvate carboxylase, which initiates the gluconeogenic pathway from pyruvate, and pyruvate dehydrogenase, which catalyzes the first step of pyruvate oxidation. Inhibition of glucose synthesis may have resulted from both the decrease in the levels of ATP, which is needed for glucose synthesis from lactate, and the inhibition of pyruvate carboxylase activity. The cellular ATP concentration decrease in-

with acetaldehyde as the substrate. However, the rate decreased after 2 min of reaction with chloroacetaldehyde, suggesting that chloroacetaldehyde inhibited aldehyde dehydrogenase. To demonstrate this inhibition, aldehyde dehydrogenase activity was measured, with a saturating concentration of acetaldehyde (5 mM), in homogenates of human kidney cortex that had been preincubated for 10 min with increasing concentrations of chloroacetaldehyde (0.05 to 5 mM). As shown in Figure 5B, chloroacetaldehyde inhibited aldehyde dehydrogenase activity in a dose-dependent manner; approximately 50% inhibition was observed with 0.2 mM chloroacetaldehyde and <10% of the control activity remained with the highest concentration of inhibitor used (5 mM).
duced by chloroacetaldehyde may be explained by a reduction of ATP synthesis secondary to the inhibition of pyruvate oxidation.

It should be emphasized that the chloroacetaldehyde-induced inhibition of pyruvate carboxylase and pyruvate dehydrogenase activities is consistent with the decrease in the cellular concentrations of CoA and acetyl-CoA; indeed, CoA is a substrate of pyruvate dehydrogenase, whereas acetyl-CoA is a well established activator of pyruvate carboxylase (34). It is worth noting that cellular depletion of CoA and CoA derivatives was suggested but not demonstrated in previous studies of the cellular toxicity of ifosfamide (17,35). It is also of great interest that our results demonstrated a correlation between the cellular depletion of CoA and acetyl-CoA and the nephrotoxic effect of chloroacetaldehyde (Figures 1 and 2 and Table 1). This emphasizes the importance of these thiol compounds in the mechanism of the nephrotoxic effect of chloroacetaldehyde. The fact that the latter compound has the capacity to bind to cellular thiols was confirmed by the observation that it also induced cellular glutathione depletion (Figure 2). The mechanism by which chloroacetaldehyde might have depleted thiol compounds was studied by incubating water containing glutathione or CoA, alone or in combination with equimolar concentrations of chloroacetaldehyde, for 60 min at 37°C. At the end of the incubation period, glutathione, CoA, and chloroacetaldehyde concentrations were not changed when these compounds were incubated alone but were zero when the compounds were incubated together; under the latter conditions, a stoichiometric amount of chloride was produced (results not shown). Therefore, these results confirm those of Lind et al. (36), indicating that chloroacetaldehyde is a very reactive compound that can bind to thiol compounds in a nonenzymatic chemical manner. Such capacity was further illustrated by prevention of the nephrotoxic effect of chloroacetaldehyde by the addition of mesna and amifostine to the incubation medium (Table 2). Indeed, these compounds, which were designed to protect the cellular thiol groups, competed with the latter for chloroacetaldehyde.

It is also conceivable that chloroacetaldehyde formed adducts with macromolecules such as proteins and nucleic acids. In support of this hypothesis is the observation that the chloroacetaldehyde removed could not be fully accounted for by the production of chloroacetate and CO₂ and by the formation of glutathione adducts (see the Results section and the discussion below), but further studies are needed to test this possibility.

**Metabolism of Chloroacetaldehyde by Human Kidney Tubules**

A major finding of this study was that human kidney tubules were capable of metabolizing chloroacetaldehyde at high rates. Such metabolism was responsible for the major fraction of chloroacetaldehyde disappearance from the incubation medium. A minor fraction was probably represented by binding to thiol groups, mainly those of glutathione (which is present in millimolar concentrations in human renal cells) (Figure 2), and also possibly by protein and nucleic acid adducts. Unlike acetaldehyde utilization (30), chloroacetaldehyde utilization was not concentration dependent and was found to be linear with time at only one concentration (0.25 mM) (see Figure 3 and the corresponding comments in the Results section). The absence of linearity of substrate utilization with time at the 0.1

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**Table 3. Toxicity of 0.5 mM chloroacetaldehyde in relation to the amount of human kidney cortex tubules present in each flask (4, 10, or 20 mg dry weight)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>4 mg Dry Weight of Tubules</th>
<th>10 mg Dry Weight of Tubules</th>
<th>20 mg Dry Weight of Tubules</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CAA</td>
<td>Control</td>
</tr>
<tr>
<td>ATP concentration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µmol/g)</td>
<td>6.7</td>
<td>0.2</td>
<td>6.5</td>
</tr>
<tr>
<td>LDH release</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(% of total LDH)</td>
<td>8.4</td>
<td>37.0</td>
<td>5.9</td>
</tr>
</tbody>
</table>

*Results are reported as means for two experiments performed in duplicate.*

---

**Figure 3.** Time course of the utilization of various concentrations of chloroacetaldehyde by human kidney cortex tubules. Bars represent means ± SEM for four experiments performed in duplicate. Each flask contained 1.4 ± 0.1 mg dry weight of tubules. ■, 30 min; ▣, 60 min.
mM concentration can be simply explained by the fact that chloroacetaldehyde concentrations tended to be very low at the end of the incubation period, but this argument cannot be put forward to explain the lack of linearity with time at concentrations higher than 0.25 mM (Figure 3). The hypothesis that, at these concentrations (0.35, 0.5, and 1.0 mM), chloroacetaldehyde exerted an inhibitory effect on aldehyde dehydrogenase is supported by the data presented in Figure 5. Therefore, chloroacetaldehyde seems to inhibit its own oxidation in human kidney.

Another major finding of this work, which is in agreement with the involvement of aldehyde dehydrogenase, was that chloroacetaldehyde was mainly converted to chloroacetate (Figure 4). It should be emphasized that the complete metabolic elimination of chloroacetaldehyde by human kidney tubules was associated with the absence of nephrotoxicity of this compound (Figures 1 and 2 and Tables 1 and 3). Therefore, our results strongly suggest that the metabolism of chloroacetaldehyde by human kidney tubules represents a detoxification mechanism, as demonstrated by Joqueviel et al. (37) in isolated perfused rabbit hearts.

**Toxicity of Chloroacetate to Human Kidney Tubules**

The complete elimination of chloroacetaldehyde by human kidney tubules led concomitantly to both suppression of the toxic effects of this compound and accumulation of an almost stoichiometric amount of chloroacetate. This strongly suggests that chloroacetate was not toxic to the human tubules. In fact, the results presented in Table 4 demonstrate that, at any given concentration, chloroacetate was much less toxic than chloroacetaldehyde (see Figures 1 and 2 and Table 1 for comparisons). The lower toxicity of chloroacetate with respect to lactate metabolism is in agreement with the relatively minor decrease in the cellular levels of CoA plus acetyl-CoA, compared with that observed in the presence of equimolar concentrations of chloroacetaldehyde (Figure 2 and Table 4). This clearly indicates that chloroacetate was much less reactive with thiol compounds than was chloroacetaldehyde and that, in contrast to suggestions by other authors (17,35), little chloroacetyl-CoA accumulated. The relatively poor reactivity of chloroacetate with cellular thiol groups is further demonstrated by the lack of a decrease in cellular glutathione levels (Table 4). In agreement with this finding is the observation that incubation of glutathione and chloroacetate without kidney tubules did not result in glutathione disappearance or chloride formation (results not shown).

**Involvement of Chloroacetaldehyde in the Nephrotoxicity Observed In Vivo during Ifosfamide Therapy**

It might be tempting to conclude from our results that chloroacetaldehyde is not nephrotoxic at clinically relevant concentrations (0.005 to 0.2 mM); indeed, our data demonstrate that a 0.5 mM concentration but not a 0.1 mM concentration of chloroacetaldehyde is toxic to isolated human kidney tubules. However, it should be remembered that, in our experiments, the exposure of isolated human kidney tubules to chloroacetaldehyde was short (60 min). Our results clearly

\[ \text{Figure 4. } ^{13}\text{C nuclear magnetic resonance spectra (125.75 MHz) of neutralized perchloric acid extracts obtained from human kidney cortex tubules incubated with 1 mM [2-}{^{13}\text{C}]\text{chloroacetaldehyde for 0 or 60 min. CAA, chloroacetaldehyde; CAT, chloroacetate; GLY, [2-}{^{13}\text{C}]\text{glycine (added as an internal standard). The chemical shifts, expressed in parts per million, are presented with reference to tetramethylsilane. It should be noted that the surfaces (surf) of the glycine peaks are different at 0 and 60 min.} \]
demonstrated that, when exposure of human kidney tubules to chloroacetaldehyde was rapidly suppressed by increases in the amounts of tubules, no nephrotoxicity was observed. This means that both the chloroacetaldehyde concentration and the duration of exposure of human kidney tubules to chloroacetaldehyde are important factors that contribute to the nephrotoxicity of this compound. Therefore, the following question arises: are human proximal tubules in vivo chronically exposed to chloroacetaldehyde during ifosfamide therapy? The answer is yes, because plasma chloroacetaldehyde levels of 0.005 to 0.2 mM have been measured among patients treated with ifosfamide (2,31,32). The latter observation, together with the in vitro results of this study, does not allow exclusion of the possibility that chloroacetaldehyde contributes to the renal proximal tubular cell damage observed after the administration of ifosfamide.

Figure 5. (A) Activity of aldehyde dehydrogenase in human kidney cortex homogenates, with either 5 mM acetaldehyde or 5 mM chloroacetaldehyde as the substrate. OD, OD at 340 nm. (B) Inhibition of aldehyde dehydrogenase (ALDH) activity in human kidney cortex homogenates (measured with 5 mM acetaldehyde as the substrate) by increasing concentrations of chloroacetaldehyde preincubated for 10 min before the start of the reaction. Bars represent mean ± SEM for four experiments. *, P < 0.05 versus control (i.e., 0 mM chloroacetaldehyde).

### Table 4. Effects of various concentrations of chloroacetate on 1 mM lactate metabolism in human kidney cortex tubules

<table>
<thead>
<tr>
<th>Chloroacetate Concentration (mM)</th>
<th>LDH Release (% of Total LDH)</th>
<th>ATP Concentration (µmol/g)</th>
<th>Lactate Uptake (µmol/g per h)</th>
<th>Pyruvate Accumulation (µmol/g per h)</th>
<th>Alanine Synthesis (µmol/g per h)</th>
<th>Glucose Synthesis (µmol/g per h)</th>
<th>Carbon Balanceb</th>
<th>GSH + GSSG Concentration (µmol/g)</th>
<th>CoA + Acetyl-CoA Concentration (mmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.6 ± 2.3</td>
<td>9.3 ± 0.6</td>
<td>256.8 ± 22.4</td>
<td>26.6 ± 6.8</td>
<td>71.6 ± 10.3</td>
<td>66.8 ± 7.6</td>
<td>25.1 ± 29.8</td>
<td>12.2 ± 3.2</td>
<td>286.0 ± 30.1</td>
</tr>
<tr>
<td>0.001</td>
<td>9.3 ± 4.4</td>
<td>8.0 ± 0.4</td>
<td>231.1 ± 16.2</td>
<td>29.9 ± 7.7</td>
<td>65.9 ± 7.3</td>
<td>59.7 ± 6.9</td>
<td>18.5 ± 24.8</td>
<td>10.6 ± 1.9</td>
<td>246.0 ± 18.9</td>
</tr>
<tr>
<td>0.01</td>
<td>8.2 ± 4.4</td>
<td>8.7 ± 0.4</td>
<td>240.5 ± 18.9</td>
<td>27.8 ± 5.8</td>
<td>61.4 ± 5.8</td>
<td>62.7 ± 7.5</td>
<td>23.9 ± 21.4</td>
<td>9.8 ± 1.6</td>
<td>271.0 ± 34.9</td>
</tr>
<tr>
<td>0.1</td>
<td>9.3 ± 5.9</td>
<td>8.7 ± 0.7</td>
<td>246.4 ± 18.7</td>
<td>33.6 ± 8.4</td>
<td>66.6 ± 10.2</td>
<td>67.1 ± 6.1</td>
<td>12.0 ± 30.6</td>
<td>10.4 ± 2.2</td>
<td>220.1 ± 19.5</td>
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<tr>
<td>0.5</td>
<td>7.7 ± 4.5</td>
<td>8.5 ± 0.6</td>
<td>257.5 ± 24.1</td>
<td>46.4 ± 9.3</td>
<td>70.8 ± 11.9</td>
<td>51.6 ± 11.5</td>
<td>-4.8 ± 25.5</td>
<td>10.6 ± 2.4</td>
<td>155.3 ± 3.1</td>
</tr>
<tr>
<td>1.0</td>
<td>12.0 ± 6.3</td>
<td>8.1 ± 0.3</td>
<td>173.5 ± 22.9c</td>
<td>60.5 ± 8.9c</td>
<td>73.2 ± 10.4</td>
<td>29.6 ± 10.3c</td>
<td>-19.3 ± 15.0</td>
<td>9.9 ± 2.6</td>
<td>141.5 ± 10.1c</td>
</tr>
<tr>
<td>5.0</td>
<td>9.2 ± 4.4</td>
<td>6.2 ± 0.88</td>
<td>100.4 ± 8.7c</td>
<td>117.1 ± 10.5c</td>
<td>64.9 ± 12.2</td>
<td>3.4 ± 1.3c</td>
<td>-88.3 ± 16.0c</td>
<td>7.6 ± 1.7c</td>
<td>112.6 ± 9.5c</td>
</tr>
</tbody>
</table>

*a Each flask contained 7.0 ± 1.4 mg dry weight of tubules. Results are reported as means ± SEM for four experiments performed in duplicate. GSH, reduced glutathione; GSSG, oxidized glutathione.

*b See Table 1 for the carbon balance calculations.

*c P < 0.05 versus control (0 mM chloroacetate).
Acknowledgments

L.D. was supported by grants from the Association pour la Recherche sur le Cancer and the Académie Nationale de Médecine. We thank Professor J. M. Dubernard and his co-workers and Dr. R. Bouvier for collaboration.

References