Ifosfamide-Induced Nephrotoxicity: Mechanism and Prevention

Itzhak Nissim, Oksana Horyn, Yevgeny Daikhin, Ilana Nissim, Bohdan Luhovyy, Peter C. Phillips, and Marc Yudkoff

Abstract

The efficacy of ifosfamide (IFO), an antineoplastic drug, is severely limited by a high incidence of nephrotoxicity of unknown etiology. We hypothesized that inhibition of complex I (C-I) by chloroacetaldehyde (CAA), a metabolite of IFO, is the chief cause of nephrotoxicity, and that agmatine (AGM), which we found to augment mitochondrial oxidative phosphorylation and β-oxidation, would prevent nephrotoxicity. Our model system was isolated mitochondria obtained from the kidney cortex of rats treated with IFO or IFO + AGM. Oxidative phosphorylation was determined with electron donors specific to complexes I, II, III, or IV (C-I, C-II, C-III, or C-IV, respectively). A parallel study was done with 13C-labeled pyruvate to assess metabolic dysfunction. Ifosfamide treatment significantly inhibited oxidative phosphorylation with only C-I substrates. Inhibition of C-I was associated with a significant elevation of [NADH], depletion of [NAD], and decreased flux through pyruvate dehydrogenase and the TCA cycle. However, administration of AGM with IFO increased [cyclic AMP (cAMP)] and prevented IFO-induced inhibition of C-I. In vitro studies with various metabolites of IFO showed that only CAA inhibited C-I, even with supplementation with 2-mercaptoethane sulfonic acid. Following IFO treatment daily for 5 days with 50 mg/kg, the level of CAA in the renal cortex was ~15 μmol/L. Taken together, these observations support the hypothesis that CAA is accumulated in renal cortex and is responsible for nephrotoxicity. AGM may be protective by increasing tissue [cAMP], which phosphorylates NADH:oxidoreductase. The current findings may have an important implication for the prevention of IFO-induced nephrotoxicity and/or mitochondrial diseases secondary to defective C-I. (Cancer Res 2006; 66(15): 7824-31)

Introduction

Ifosfamide (IFO) is an alkylating oxazaphosphorine antitumor prodrug (1, 2), the clinical effectiveness of which is severely limited by a high incidence of nephrotoxicity (3–5), especially in children (6, 7). Neither the biochemical mechanism(s) by which IFO damages the kidney nor the means by which other agents may reverse this damage is clearly understood. Both in patients and in an animal model, IFO induces a persistent Fanconi syndrome and significant proximal renal tubular dysfunction associated with a reduced glomerular filtration rate, glucosuria, aminoaciduria, phosphaturia, and bicarbonaturia (3–8). Nephrotoxicity is a common and potentially serious complication of treatment despite coadministration of the uroprotective agent, 2-mercaptoethane sulfonic acid (MESNA; refs. 3, 4). Recent reports have shown that the frequency of renal damage is remarkably higher than had been appreciated, and IFO-induced nephropathy is likely underestimated because of the inherent flaws in the evaluation methods commonly used and the time dependence of IFO-induced nephrotoxicity (6, 7, 9). This is especially true in cases of late-onset glomerular dysfunction (5, 6, 9).

It has been shown that only metabolites of IFO are toxic to the kidney, whereas the prodrug is not (10). IFO must be oxidized by cytochrome 3A5 (CYP3A5) and CYP2B6 oxidase to acquire an antineoplastic activity (11, 12). The oxidation of IFO occurs via two major routes (a) at the cyclic carbon-4, resulting in the formation of 4-OH-IFO, which is then decomposed to isophoramide mustard (IPM) and acrolein (1, 2); and (b) via the side chain dechloroethylation, which leads to formation of chloroacetaldehyde (CAA). The latter may account for up to 50% of IFO metabolism (1, 2). Because CYP3A5 and CYP2B6 activity is present in kidney and liver (12, 13), CAA can be produced in renal proximal tubules, the site of IFO-induced nephrotoxicity.

In the current study, we sought to determine the initial site of IFO-induced nephrotoxicity. As illustrated in Fig. 1, we hypothesized that the CAA-induced dysfunction of mitochondrial oxidative phosphorylation in renal proximal tubules is at the heart of IFO-induced nephrotoxicity. Disruption of oxidative metabolism impairs energy production, thereby resulting in multiple metabolic abnormalities and cellular damage. We further hypothesized that agmatine (AGM), which we found to increase mitochondrial oxygen consumption and fatty acid oxidation (14, 15), may attenuate or prevent IFO-induced renal damage by maintaining the integrity of the respiratory chain.

C-I (NADH:ubiquinone oxidoreductase) is the largest mitochondrial respiratory chain complex. Defects of C-I commonly cause mitochondrial respiratory diseases and myopathies (16). C-I participates in the production of a proton gradient across the inner mitochondrial membrane and the transfer of electrons from NADH to ubiquinone, thus providing the proton-motive force used for ATP synthesis (17). It is well established that C-I is susceptible to free radical injury (18, 19). Structural investigations suggest that the NADH dehydrogenase moiety may be exposed to the matrix (20), thereby making C-I sensitive to free radical and oxidative injury. CAA, a chemical radical produced both in the liver and kidney of IFO-treated patients, may inhibit C-I activity in renal proximal tubules, where CAA is formed.

If C-I is the primary target of IFO-induced renal injury, then supplementation of an agent that up-regulates C-I might attenuate and even prevent the malfunction of energy metabolism. We recently showed that AGM stimulates oxygen consumption and...
fatty acid oxidation in a liver perfusion system (14, 15). AGM, a metabolite of arginine decarboxylation (21), is widely distributed in mammalian tissue and may act as a hormone affecting multiple metabolic functions (22). We hypothesized that concomitant administration of AGM with IFO may preserve C-I, thereby preventing IFO-induced renal damage. As shown in Fig. 1, AGM may act directly on the NADH:ubiquinone oxidoreductase via augmented production of cyclic AMP (cAMP) and subsequent phosphorylation of C-I. An alternative but not mutually exclusive possibility is that AGM stimulates fatty acid oxidation (15), thus furnishing FADH2 to C-II and bypassing the defective C-I. In addition, recent reports have indicated that AGM inhibits the proliferation of tumor cells (23). Thus, AGM may provide dual benefits during IFO treatment in sustaining normal respiration and inhibiting the proliferation of cancerous cells. Validation of these hypotheses requires the demonstration of inhibition of C-I both in mitochondria isolated from renal cortex of IFO-treated rats and in vitro experiments with mitochondria exposed to metabolites of IFO. We therefore carried out such experiments to assess the oxidative phosphorylation and the protective action of AGM. We determined oxidative phosphorylation with electron donors specific to C-I, C-II, C-III, or C-IV.

Inhibition of C-I would affect diverse aspects of mitochondrial function, including the oxidation of pyruvate via pyruvate dehydrogenase (PDH) as well as flux through the TCA cycle in response to an alteration of the NADH/NAD+ ratio. The PDH complex is controlled by a phosphorylation/dephosphorylation system. Dichloroacetate (DCA), which inhibits PDH kinase (PDK), generates the dephosphorylated and more active form of PDH (24). Thus, an additional series of experiments was carried out with DCA to determine the linkage between IFO-induced nephrotoxicity and flux through the PDH reaction. Increased [NADH] might up-regulate the pyruvate carboxylase (PC) reaction, thereby increasing mitochondrial production of oxaloacetate and aspartate. We evaluated these pathways by using 13C-labeled pyruvate. Gas chromatography–mass spectrometry (GC-MS) was used to determine 13C enrichment and the flux through the PDH reaction and the incorporation of 13C into the TCA cycle and amino acids (Fig. 1).

The results show that inhibition of C-I activity by CAA is the primary lesion of IFO-induced nephrotoxicity. However, this lesion was prevented by concomitant supplementation of AGM.

Materials and Methods

Materials. Chemicals were of analytic grade and obtained from Sigma–Aldrich (Milwaukee, WI). Enzymes and cofactors for the analysis of mitochondrial oxidases, β-hydroxybutyrate, acetoadetate, NADH, NAD+, and citrate were obtained from Sigma (Milwaukee, WI). [U-13C]pyruvate and [1-13C]pyruvate, 99 mol% excess [molar percent enrichment (MPE)] and [13C]thiourea were from Isotec (Miamisburg, OH). 2-Aminothiazole was purchased from Aldrich (Milwaukee, WI). AGM as sulfate salt was purchased from Sigma. IFO was purchased from Bristol-Myers Squibb (Princeton, NJ). MESNA and acrolein were from Sigma and AGM was from Fluka (Milwaukee, WI). IPM and 4-OOH-IFO are generous gifts from ASTA Medica (Frankfurt, Germany).

Experimental procedure. Experiments were carried out on male Sprague-Dawley rats (Charles River; 200-250 g), fed Purina rat chow and

Figure 1. Schematic of IFO-induced nephrotoxicity and protection by AGM. We propose that inhibition of C-I by CAA is the basic cause of IFO-induced nephrotoxicity. This cytotoxicity is due to low reactivity of CAA with MESNA, whereas acrolein is completely neutralized by MESNA. CAA may inhibit NADH:oxidoredctase following dephosphorylation of AGDQ, an 18 kDa subunit of C-I. The inhibition of C-I disrupts oxidative phosphorylation, leading to multiple metabolic abnormalities, including elevation of [NADH], decreased PDH, and TCA cycle activity. AGM administration prevents IFO-induced nephrotoxicity by maintaining the integrity of the respiratory chain without diminishing the antitumor action of IFO. This protective action may be mediated via (a) activation of adenylate cyclase and stimulation of production of cAMP, which may increase the activity of C-I following phosphorylation of AGDQ (43), and/or (b) stimulation of fatty acid oxidation (15), and consequently increased production of FADH2, thereby bypassing C-I. Metabolic reactions are as follows: (1) PDH, (2) PC, (3) glutamate-aspartate aminotransferase, (4) glutamate dehydrogenase; C-I, C-II, C-III, C-IV, respiratory chain complexes; C-V, ATP synthetase; Cyt. C, cytochrome c, α-keto, α-ketoglutarate, +, up-regulation by AGM.
Mitochondrial respiratory enzyme activities. A portion of the isolated mitochondrial preparation used for measurement of oxygen consumption (above) was freeze-thawed and then used for measurement of respiratory enzyme activities. Assays and calculations were done as described (27). Activities were determined spectrophotometrically for the following enzymes: rotenone-sensitive NADH:ubiquinone oxidoreductase (C-I), antimycin A–sensitive succinate oxidase (C-II), antimycin A–sensitive duroquinol oxidase (C-III), and sodium azide–sensitive cytochrome c oxidase (C-IV). The activity of each enzyme was determined as a first-order rate during the first 2 to 3 minutes of the reaction. Duroquinol was prepared from duroquinone as described (27). Substrate concentrations and measurement conditions are as outlined in the figure legend.

Metabolic studies with isolated mitochondria. Parallel metabolic studies were done with renal mitochondria isolated from control and treated rats as indicated above. The mitochondrial suspension (2-3 mg protein/ml) was incubated in 25 mL Erlenmeyer flasks sealed with rubber stoppers equipped with plastic center wells. The basic incubation medium (without potassium carbonate), consisted of Tris (50 mmol/L), EDTA (2 mmol/L), KCl (5 mmol/L), MgCl2 (5 mmol/L), and KH2PO4 (pH 7.4). Incubations (2 ml final volume) at 30°C were carried out in a shaking water bath for 10 minutes and with the addition of substrates as indicated below. To determine the effect of IFO treatment on the flux through the PDH reaction and the incorporation of acetyl-CoA into the TCA cycle, mitochondria were incubated with basic medium plus 5 mmol/L/L [1-13C]pyruvate or [U-13C]pyruvate. Two hundred fifty microliters of 0.2 mmol/L NaOH was added to the center wells for collection and measurement of 13CO2 release as indicated below. To determine the effect of CAA on the flux through PDH, a second series of experiments was carried out with mitochondria from untreated rats in the presence of basic incubation medium, increasing concentrations of CAA, and 5 mmol/L/L [1-13C]pyruvate.

At the end of incubation, an aliquot (100 μL) was taken for protein determination, and incubation was stopped with 100 to 150 μL of HClO4 (60%). Metabolite measurements were done in neutralized extracts as indicated (15, 21).

Determination of 13C-labeled metabolites. For measurement of the 13C enrichment in glutamate and aspartate isotopomers, samples were prepared and analyzed with GC-MS as described (15, 21). The production of 13CO2 during mitochondrial incubation was monitored as follows: The CO2 trapped in the center wells with 250 μL of 0.2 mmol/L NaOH was removed and 10 μL were transferred into a sealed tube containing 1 mL of 1 mmol/L NaHCO3. Then, 0.5 mL of 40% phosphoric acid was added to the tubes and left for 20 minutes to liberate CO2. The latter was removed with a sealed syringe and transferred to autosampler tubes for analysis. Isotopic enrichment in 13CO2 was determined by an Isotope Ratio-Mass-Spectrometer (Thermoquest Finnigan Delta Plus), using the m/z 45/44 ratio as indicated (15).

GC-MS measurement of CAa level in the blood and renal cortex of IFO-treated rats. We developed a GC-MS method to measure the levels of CAa in the blood and kidney cortex of IFO treated rats. The method is a modification of the high-performance liquid chromatography (HPLC) determination of CAa (28). The CAa in blood or neutralized extract of renal cortex was stabilized with formaldehyde and then reacted with 13C-labeled thiourea to form 13C-labeled 2-aminothiazole. The latter was purified on AG-50 column, and subsequently converted into the t-butyldimethylsilyl derivative as described (14, 15). Concentration of CAa was determined using the isotope dilution approach by adding a known amount of the unlabeled 2-aminothiazole to blood or renal cortex and monitoring the m/z 158/157 ratios of the t-butyldimethylsilyl derivative of 2-aminothiazole.

Analytic measurements. The concentration of amino acids was determined by HPLC using precolumn derivatization with o-phthalaldehyde and the level of cAMP, NAD, NADH, acetate, β-hydroxybutyrate, lactate, pyruvate, and citrate as indicated (15, 21).

Calculations and statistical analyses. 13C enrichment in glutamate and aspartate is expressed by MPE, which is the mole fraction (%) of analyze containing 13C atoms above natural abundance (15). The MPE in the M=2 (containing two atoms of 13C) and M = 3 (containing three atoms of 13C) isotopomers of glutamate and aspartate, respectively, was calculated as in
ref. (15). The release of $^{13}$CO$_2$ (nmol/mg protein/min) was calculated by the isotope dilution of $^{13}$CO$_2$ in 1,000 nmol/mL of unlabeled NaHCO$_3$ in the sealed tubes, and normalized to the amount of mg protein/mL. The production of $^{13}$C-labeled amino acid was calculated by the product of (MPE)/100 times concentration (nmol/mg protein/min) from $^{13}$Cpyruvate.

Each series of experiments was repeated three to five times with different mitochondrial preparation as outlined above. Statistical analysis was carried out using In-STAT 1.14 software for the Macintosh. Student’s $t$ test or ANOVA was used to compare two groups or differences among groups as needed. $P < 0.05$ was taken as indicating a statistically significant difference.

Results

Accumulation of CAA in renal cortex of IFO-treated rats. Following IFO injection of 50 mg/kg daily for 5 days, the blood levels of CAA in IFO or IFO + AGM–treated rats were 13 ± 7 μmol/L (mean ± SD, $n = 5$) and in the renal cortex $10 ± 3$ nmol/g wet weight (mean ± SD, $n = 5$). Assuming that the kidney contains 70% water (29), the [CAA] in the kidney is $\sim 15$ μmol/L. The data indicate an insignificant difference in the concentration of CAA between IFO and IFO + AGM groups. The blood concentration of CAA is in agreement with the reported concentrations of CAA in the blood of patients treated with IFO, which is between 10 to 100 μmol/L (28, 30). However, there is no information about the level of CAA in renal cortex of IFO-treated subjects. The current observation is the first demonstration that CAA is accumulated in the renal cortex following IFO treatment. This finding bears directly on our hypothesis that the accumulation of CAA in renal cortex is the major source of IFO-induced nephrotoxicity.

IFO-induced inhibition of oxidative phosphorylation: protection by AGM. Next, experiments were designed to determine the effect of IFO treatment, and thus, accumulation of CAA in renal cortex on oxidative phosphorylation in mitochondria isolated from renal cortex after 6 days of daily treatment with either IFO, IFO + AGM, or IFO + DCA. Data in Fig. 2 show that renal mitochondria obtained from IFO-treated rats manifest $50\%$ ($P < 0.05$) reduction in state 3 (ADP-present) and state 4 (ADP-limiting) respiration compared with control, when the substrate was pyruvate, a C-I-dependent electron donor. In vivo administration of AGM alone significantly increased state 3 respiration compared with control rats. In addition, a concomitant administration of AGM with IFO prevented the IFO-induced inhibition of pyruvate-linked oxidative phosphorylation (Fig. 2). However, administration of DCA alone had no effect on pyruvate-linked oxidative phosphorylation, and a concomitant administration of DCA with IFO failed to prevent the IFO-induced decrease in pyruvate-linked oxidative phosphorylation. In all these measurements, the respiratory control ratio was between 1.6 and 2.1 regardless of the experimental group (Fig. 2), indicating that the current mitochondrial preparation maintained structural integrity. These observations suggest that the inhibition of oxidative phosphorylation by IFO treatment could be an initial biochemical lesion of IFO-induced renal dysfunction. Concomitant administration of AGM prevented this lesion. DCA, which inhibits PDK and activates PDH (24), did not reverse the IFO-induced inhibition of oxidative phosphorylation.

Inhibition of oxidative phosphorylation by IFO treatment is located at complex-I: protection by AGM. Because DCA did not prevent the IFO-induced inhibition of pyruvate-linked oxidative phosphorylation, this inhibition may be due to a direct action of IFO and/or one of its metabolites on the following: (a) the transport of metabolites across the mitochondrial membrane; (b) the activity of the electron transport chain, i.e., NADH-oxidoreductase, C-I, C-III, or C-IV; and/or (c) the ATP synthetase reaction. To localize the site of IFO action, we determined oxidative phosphorylation with different substrates, including malate or glutamate (forming NADH oxidized in C-I), succinate (forming FADH$_2$ oxidized in C-II), and $\alpha$-keto-$\gamma$-tetramethyl-p-phenylenediamine (TMPD)/ascorbate (oxidized in C-IV). Data in Figs. 2 and 3 show that IFO-induced inhibition of state 3 respiration is specific to complex-I-linked substrates (pyruvate, glutamate, and malate). IFO treatment did not affect state 3 respiration when C-II- or C-IV-linked substrates were used. Because malate and succinate are shuttled on the same mitochondrial membrane dicarboxylate transporter (31), and because pyruvate, glutamate, or malate use different dehydrogenases to generate NADH (32), the current findings unequivocally show that IFO treatment inhibited the NADH-oxidoreductase activity (C-I). In addition, TMPD donates electrons to the respiratory chain via cytochrome $c$, which damaged mitochondria easily lose (33). The similarly high respiration rates with TMPD/ascorbate in all experimental groups (Fig. 3) indicate that the IFO-induced inhibition of oxidative phosphorylation is not a result of damage to the mitochondrial membrane.

To further examine the effect of IFO treatment on oxidative phosphorylation, the activities of individual components of the electron transport chain were determined using spectrophotometric assays (27). Activity was measured as an apparent first-order rate constant using freeze-thawed mitochondria obtained from each experimental group. Figure 4A shows that the NADH-ubiquinone oxidoreductase, i.e., C-I activity, was reduced by $55\%$ ($P < 0.05$), in mitochondria obtained from IFO-treated rats compared with
Concomitant administration of AGM and IFO preserved the activity of C-I. Measurement of the activity of C-II, C-III, or C-IV shows no significant difference between control, IFO, or IFO + AGM–treated rats (data not shown). Thus, results obtained from both polarographic measurements of oxygen consumption (Figs. 3 and 4) and spectrophotometric measurement of mitochondrial respiratory enzymes (Fig. 4A) point to inhibition of C-I by IFO and/or its metabolites. AGM prevents and/or reverses such inhibition.

Inhibition of C-I is associated with a cascade of metabolic dysfunction in renal cortex: protection by AGM. As expected, IFO treatment increased NADH in renal cortex (Fig. 4C) and diminished NAD (Fig. 4D), whereas AGM prevented these changes. The increase in [NADH] and the decrease in [NAD] in the IFO + AGM group are smaller than in rats treated with only IFO and are statistically insignificant when compared with control rats (Fig. 4). IFO treatment had an insignificant effect on the renal cortical level of acetoacetate, β-hydroxybutyrate, lactate, pyruvate, citrate, or the cellular redox state as calculated by lactate/pyruvate or β-hydroxybutyrate/acetoacetate ratios (data not shown). The absence of increased tissue [lactate] probably reflects high urinary excretion of lactate. We previously showed a marked urinary excretion of lactate in IFO-treated rats (8). An important observation is that [cAMP] was significantly increased in renal cortex after AGM administration with or without IFO (Fig. 4B). The increased [cAMP] may have a key role in the protective action of AGM against IFO-induced inhibition of C-I, as discussed below.

It is well established that the PDH reaction is under feedback control by NADH and/or acetyl-CoA (24, 34). We used 13C-labeled pyruvate to elucidate the relationship between the decreased oxidative phosphorylation as well as the [NADH]/[NAD] ratio and PDH flux, TCA cycle activity, and the PC reaction. Figure 5A shows that IFO treatment diminished production of 13CO2 from [1-13C]pyruvate by ~30% (P < 0.05). In contrast, treatment with either AGM or DCA significantly increased 13CO2 formation. Furthermore, concomitant supplementation of AGM or DCA with IFO reversed the IFO-induced decrease in 13CO2 release. The release of 13CO2 represents pyruvate decarboxylation via PDH. Because NADH-induced inhibition of PDH is mediated by PKD

![Figure 3.](image-url) In vivo inhibition of C-I by IFO treatment: protection by AGM. Isolated mitochondria were obtained from renal cortex of IFO-treated and control rats as indicated in the legend to Fig. 2. Mitochondrial suspensions in basic medium were supplemented with either malate (5 mmol/L; C-I), glutamate (5 mmol/L; C-I), succinate (5 mmol/L; C-II), or TMPD (0.24 mmol/L) plus ascorbate (7.2 mmol/L; C-IV). After recording of state 2, ADP (0.3 mmol/L) was added, and state 3 and state 4 were determined. The respiratory rates are oxygen consumption rates at state 3 given in nano–atom O/mg protein/min. Columns, mean of five to seven independent experiments; bars, SD. *, significant difference compared with control.

![Figure 4.](image-url) Effect of IFO or IFO + AGM treatment on the activity of NADH-ubiquinone oxidoreductase, [cAMP] as well as [NADH], and [NAD] in renal cortex. A, activity of NADH-ubiquinone oxidoreductase measured as rotenone-sensitive oxidation of NADH (27). These measurements were done in lysed aliquots (freeze-thaw) of the mitochondrial suspension used for determination of oxidative phosphorylation, as indicated in the legends to Figs. 2 and 3. B to D, level of cAMP, NADH, and NAD, respectively, in extracts of the freeze-clamped renal cortex obtained from treated and untreated rats, as indicated in the legend to Fig. 2. Columns, mean of three to five independent experiments; bars, SD. *, significant difference compared with control.
(24, 34), and because DCA deactivates PDK (24), inhibition of PDH (Fig. 5A) probably occurs secondary to inhibition of C-I and increased [NADH]. Decreased flux through PDH and increased [NADH] would also down-regulate the TCA cycle and stimulate pyruvate carboxylation. We therefore measured the transfer of $^{13}$C from $[U-^{13}$C$]pyruvate to glutamate as a marker of flow of $[1,2-^{13}$C$]acetyl-CoA through the TCA cycle. As shown in Fig. 5B, IFO treatment decreased formation of the M+2 glutamate isotopomer, reflecting diminished TCA cycle flux because glutamate is in equilibrium with $^{13}$C-labeled α-ketoglutarate derived from $[1,2-^{13}$C$]acetyl-CoA (15). AGM alone augmented M+2 glutamate formation and prevented the inhibition by IFO treatment. Figure 5C also illustrates that IFO treatment significantly increased formation of the M+3 isotopomer of aspartate. The latter derives from conversion of $[U-^{13}$C$]pyruvate to $[1,2,3-^{13}$C$]oxaloacetate in the PC reaction (15). The augmented formation of $[1,2,3-^{13}$C$]$ aspartate is in keeping with the notion that the elevation of [NADH] increases pyruvate carboxylation.

CAA is the primary agent responsible for the IFO-induced inhibition of C-I. To determine whether IFO or one of its metabolites is responsible for the inhibition of C-I, we studied the effects of IFO or its metabolites, i.e., CAA, IPM, acrolein, or 4-OOH-IFO, with or without MESNA. In aqueous solution, 4-OOH-IFO is immediately converted to 4-OH-IFO, the primary intermediate metabolite of IFO (35). Neither IFO nor IPM nor 4-OOH-IFO altered state 3 mitochondrial respiration compared with control (data not shown). However, CAA decreased both pyruvate-linked state 3 respiration (Fig. 6A) and the release of $^{13}$CO$_2$ from $[1-^{13}$C$]$pyruvate (data not shown). Such inhibition occurred in a dose-dependent fashion. The IC$_{50}$ values of the CAA effect on state 3 respiration and the PDH reaction are 0.8 and 0.6 mmol/L, respectively. Of special importance is the observation that the supplementation of MESNA with CAA did not prevent the inhibition of oxidative phosphorylation by CAA, whereas the addition of MESNA with acrolein completely reversed such inhibition (Fig. 6A).

Further studies with malate, succinate, or TMPD/ascorbate show that CAA inhibited the oxidative phosphorylation only with C-I-linked substrates (data not shown). Because respiration and phosphorylation are coupled, decreased respiration in mitochondria treated with CAA could result from the inhibition of ATP synthetase. However, the addition of the uncoupler, CCCP to mitochondria treated with CAA did not increase the oxygen consumption (Fig. 6B), thus ruling out a defect in ATP synthetase as the underlying mechanism for the CAA-induced decrease of oxidative phosphorylation.

**Figure 5.** IFO-induced inhibition of C-I is associated with a cascade of metabolic dysfunction in renal cortex: protection by AGM. Experimental details are as indicated in the legend to Fig. 2. **A,** PDH flux as indicated by the release of $^{13}$CO$_2$ from $[1-^{13}$C$]$pyruvate. A portion of mitochondrial preparation used to measure oxidative phosphorylation (Fig. 2) was incubated at 30°C for 10 minutes with basic incubation medium supplemented with 5 mmol/L $[1-^{13}$C$]$pyruvate. Collection and determination of $^{13}$CO$_2$ is as described in Materials and Methods. Flux through the PDH reaction was estimated by the release of $^{13}$CO$_2$ and expressed as mmol/mg protein/min. **B** and **C,** production of $^{13}$C-labeled amino acids from $[U-^{13}$C$]pyruvate. Isolated mitochondria obtained from renal cortex of treated and untreated rats (see legend to Fig. 2) were incubated at 30°C for 10 minutes with basic medium plus 5 mmol/L $[U-^{13}$C$]$pyruvate. Extracts of mitochondrial incubation were analyzed with GC-MS and HPLC for determination of $^{13}$C enrichment (MPE) and amino acid concentrations (nmol/mg protein), respectively, as detailed in Materials and Methods. **B,** the M+2 isotopomer of glutamate (glutamate containing two atoms of $^{13}$C); **C,** the M+3 isotopomer of aspartate (aspartate containing three atoms of $^{13}$C). $^{13}$C-isotopomer appearance (nmol/mg protein) is the product of amino acid concentration (nmol/mg protein) and $^{13}$C enrichment (MPE/100). Columns, mean of four to five independent experiments; bars, SD. * significant difference compared with control.
Figure 6. Inhibition in vitro of oxidative phosphorylation by CAA or acrolein: effect of MESNA. A, isolated mitochondria obtained from renal cortex of untreated rats were used for determination of oxidative phosphorylation with 5 mmol/L pyruvate as C-I-linked substrate with or without 1 mmol/L CAA and 2 mmol/L MESNA. Comparative experiments were done with 0.25 mmol/L acrolein with or without 2 mmol/L MESNA. B, effect of CAA on coupled and uncoupled oxidative phosphorylation. Isolated mitochondria from untreated rats were added to a polarographic chamber containing basic medium plus 0.3 mmol/L ADP and 5 mmol/L pyruvate with or without 1 mmol/L CAA. Following determination of ADP-dependent respiration without the addition of CICCP (−), the uncoupled respiration was determined after addition of 10 mmol/L CICCP (+). Columns, mean of three to five independent experiments; bars, SD. *, significant difference compared with control.

Discussion

The present study was undertaken to test the hypothesis that IFO-induced renal injury derives from inhibition of oxidative phosphorylation, and this inhibition may be prevented by coadministration of AGM. Our data show that impairment of NADH-oxidoreductase is the most dramatic consequence of IFO treatment (Figs. 2-4). Furthermore, the demonstration that coadministration of AGM minimized or prevented the inhibition of C-I activity and preserved oxidative phosphorylation provides solid experimental evidence supporting the cytoprotective action of AGM against IFO-induced renal injury. Taken together, the premise of these findings may have important clinical implications for the prevention of nephrotoxicity associated with IFO treatment in cancer patients.

As evident by the elevated [NADH] in renal cortex of IFO-treated rats (Fig. 4B), the malfunction of complex-I is not due to depletion of the mitochondrial NADH pool, but to a direct inhibitory action of CAA on NADH-oxidoreductase. The current findings strongly support a key role for CAA inhibition of NADH-oxidoreductase activity in the pathogenesis of the IFO-induced nephrotoxicity, despite the addition of MESNA (Fig. 6A). This mode of action may explain previous observations indicating that CAA depleted cellular reduced glutathione and ATP, disturbed Ca²⁺ signaling, lipid peroxidation, and, ultimately, cell necrosis and death (8, 35–39).

The cytotoxic effect of CAA is likely due to low or no reactivity with MESNA (Fig. 6A). This conclusion is in agreement with the clinical phenomenon showing that IFO induced nephrotoxicity despite coadministration of MESNA (3, 4).

The level of CAA in the blood of patients receiving IFO treatment can be as high as 100 μmol/L (30), although much CAA that is produced in liver can be degraded in the blood and may not reach the kidney. CYP3A5 and CYP2B6 activities are present in kidney and liver (12, 13). The calculated [CAA] in rat renal cortex is ~15 μmol/L following daily injection of 50 mg/kg IFO for 5 days. This [CAA] is ~40-fold lower than the observed IC₅₀ for CAA inhibition of C-I, i.e., 0.6 to 0.8 mmol/L. However, the inhibitory action of CAA is time and dose dependent (39). The current value for IC₅₀ was obtained following 5- to 10-minute exposure of isolated mitochondria to an increasing concentration of CAA, whereas kidneys of patients treated with IFO may be exposed to CAA for the duration of the therapy, i.e., days and/or weeks, similar to the current in vivo studies (Figs. 2-5).

The decreased respiration rate is specifically related to C-I-linked substrates. This is evident by little improvement in state 3 respiration with the addition of malate and/or glutamate (Fig. 3). Furthermore, the minimal changes in state 3 respiration with C-II- or C-IV-linked substrates (Fig. 3) suggest that there is little or no loss of cytochrome c activity, and that the respiration functions of complexes II, III, IV, and V remain largely intact in IFO-treated rats. Similarly, experiments with and/or without the addition of CICCP indicate that the ATP synthetase reaction is unaffected (Fig. 6B). The extent of C-I inhibition required to affect ATP synthesis and cellular functions has been reported to vary widely in mitochondria from different cell types (40). The current observations indicate ~50% inhibition of state 3 respiration for complex-I-linked substrates in mitochondria isolated from IFO-treated rats (Figs. 2-4). This value is in the range of the biochemical threshold of 25% to 80% previously indicated in various cell types and biochemical insults (40).

The defective C-I activity may initiate a metabolic cascade that exacerbated mitochondrial dysfunction following IFO treatment. In addition to the curtailment of ATP synthesis, dysfunction of C-I may lead to elevated production of superoxide radicals, causing mitochondrial DNA mutations, lipid peroxidation, and protein denaturation (35–38). The current study shows that the IFO-induced inhibition of NADH-oxidoreductase was associated with a significant elevation of [NADH], inhibition of the flux through the PDH reaction, and incorporation of acetyl-CoA (derived from pyruvate) into the TCA cycle (Figs. 4 and 5). However, AGM cotreatment restored mitochondrial functions to a control level.

The current findings raise the question, How does AGM prevent the CAA-induced inhibition of NADH-oxidoreductase? AGM, which is taken up by mitochondria (41), may elicit effects on multiple metabolic and physiologic events that are independent of one another (14, 15, 22, 42). Our recent report suggests that the stimulation of β-oxidation by AGM may be mediated via elevation of cAMP (15). In addition, other reports suggest that...
cAMP-dependent protein kinase A (PKA) increases the activity of C-I following phosphorylation of AIDQ, an 18 kDa subunit of C-I, which is tightly associated with the inner membrane (43). As illustrated in Fig. 1, CAA may inhibit NADH:oxidoreductase following dephosphorylation of the AQQ complex subunit, and AGM prevents this inhibition via phosphorylation of AQQ secondary to elevated tissue [cAMP] (Fig. 4B). This possibility is in accord with a prior study showing that forskolin, which stimulates adenylate cyclase, negates the inhibitory action of CAA on Na+/Ca2+ exchange in renal proximal tubule cells and suggests that the action of CAA is through interaction with cAMP-PKA signaling (36). An alternative but not exclusive possibility is that the stimulation of β-oxidation by AGM furnishes more FADH2, which is oxidized in C-II, thus bypassing the defective C-I.

In summary, the current findings show a strong protection by AGM against CAA-induced inhibition of NADH:oxidoreductase. This action, together with its action against proliferation of tumor cells (23), make AGM a potentially useful selective cytoprotective agent against IFO-induced renal injury without compromising the antitumor activity of IFO. AGM, which is present in various tissues and human colon (22, 42), may interfere with both polyamine and DNA synthesis resulting in the inhibition of cell growth (23, 44). Thus, AGM and IFO may provide a synergistic action against tumor proliferation. In addition, the possibility that the deficiency of C-I activity could be negated by AGM is intriguing not only for the gene for the human mitochondrial dicarboxylate carrier: evolution of the carrier family. Biochem J 1999;344: 953–60.


Ifosfamide-Induced Nephrotoxicity: Mechanism and Prevention

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