Calcium Oxalate Monohydrate, a Metabolite of Ethylene Glycol, Is Toxic for Rat Renal Mitochondrial Function

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Ethylene glycol poisoning can produce acute renal failure, requiring long-term hemodialysis to restore function. The mechanism of the renal failure is unknown, but is associated with tubular cell necrosis and ethylene glycol metabolism. The end metabolite of ethylene glycol is oxalic acid, the precipitation of which as calcium oxalate monohydrate (COM) crystals in the tubular lumen has been linked with the renal toxicity. Our recent studies suggest that COM is an intracellular toxicant to normal human proximal tubule cells in culture. The present studies were designed to assess whether COM or ionic oxalate alters mitochondrial function so as to lead to renal cell death. In isolated rat kidney mitochondria, COM produced a dose-dependent decrease in State 3 respiration (40% decrease at 0.05 mM COM with either succinate or glutamate/malate as substrate), without affecting either State 4 respiration or the ADP/O ratio. COM, from 0.01–0.05 mM also dose-dependently increased mitochondrial swelling, which was completely blocked by cyclosporin A. The inhibition of State 3 respiration, however, was not reversed by cyclosporin A administration. Potassium oxalate, at concentrations up to 5 mM did not inhibit mitochondrial respiration or induce swelling. These results suggest that COM, and not the oxalate ion, damages rat kidney mitochondria and induces the mitochondrial permeability transition, which may then lead to renal cell death. Since COM is transported intracellularly by kidney cells, the renal toxicity of ethylene glycol may result from inhibition of mitochondrial respiratory function in proximal tubular cells by COM crystals.

Key Words: mitochondrial permeability transition; ethylene glycol poisoning; renal toxicity; oxalate crystals.

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implied that the adenine nucleotide translocator and the voltage-dependent anion channel are both critical components of the pore (Szewczyk and Wojtczak, 2002). Furthermore, the potent and specific inhibition of the MPT by cyclosporin A (Broekemeier et al., 1989) has been interpreted to suggest that cyclophilin D is a critical component as well. Regardless of its composition, induction of the MPT by a chemical stress results in a depolarization of mitochondrial membrane potential and inhibition of oxidative phosphorylation, which can lead to either an oncotic or apoptotic form of cell death.

Mitochondrial damage is a major mechanism for chemically mediated renal tubular necrosis (Lash and Jones, 1996) and the toxicity of oxalate may result from direct damage to the mitochondria. The oxalate-induced oxidative stress in kidney cells has been shown to result primarily from mitochondrial effects (Khand et al., 2002). In rat liver mitochondria, oxalate (≥ 1 mM) decreases oxygen consumption without altering the ADP/O ratio (Bachmann and Golberg, 1971). However in rat kidney mitochondria (Strzelecki et al., 1989), oxalate at 3 mM does not alter State 3 nor State 4 respiration nor the ADP/O ratio, hence does not affect overall energy production. No studies have directly examined the effects of COM on mitochondrial function, but exposure of rats to ethylene glycol in drinking water, which produces minimal COM accumulation in the kidney, produces a mild decrease in State 3 respiration in kidney mitochondria (Muthukumar and Selvam, 1998).

COM could produce its renal toxicity by inhibiting mitochondrial function in two ways. Studies in cultured kidney cell lines have shown that COM crystals adhere to the plasma membrane of PT cells (Schepers et al., 2003; Verkoelen et al., 1999), then are taken up by an endocytotic process within 30 min (Lieske et al., 1994). Binding and intracellular uptake of COM crystals has been observed in tubular epithelial cells in vivo (Khan, 1995). COM crystals in the cytoplasm could directly affect the mitochondria. The intracellular COM has also been shown to be metabolized (Lieske et al., 1997), potentially releasing the oxalate ion which then could affect the mitochondria. These studies were designed to test the hypothesis that COM is toxic to kidney mitochondrial function, as a potential mechanism of its cytotoxicity.

MATERIALS AND METHODS

**Chemicals.** D-mannitol was purchased from Aldrich Chemical Co. (Milwaukee, WI) and ultra-pure sucrose from ICAP Biomedicals (Aurora, OH). Cyclosporin A (CsA) was a generous gift from Sandoz Pharmaceuticals (East Hanover, NJ). All other reagents were of analytical grade and were purchased from various commercial sources.

**Animals.** Adult male Sprague-Dawley rats, purchased from Harlan Sprague-Dawley (Madison, WI), were maintained in a climate-controlled, AAALAC-accredited animal care facility with free access to food (Purina Rat Chow, Ralston Purina, Richmond, IN) and tap water.

**Preparation of renal mitochondria.** Rats were euthanized with CO2 followed by decapitation. Renal mitochondria were prepared by conventional methods of differential centrifugation (Lash and Sall, 1993). Both kidneys were

rapidly excised, decapsulated, and the cortices were separated from the medulla. The cortex tissue was minced together in ice-cold isolation medium containing 200 mM mannitol, 10 mM sucrose, 5 mM HEPES, 1 mM EGTA, pH 7.4. The tissue was rinsed with isolation medium to remove debris, homogenized with a tightly fitted Potter-Elvehjem homogenizer and centrifuged at 900 × g for 10 min. The supernatant was transferred to new tubes and centrifuged at 10,300 × g for 10 min. The mitochondrial pellet was washed once and resuspended in isolation medium (without EGTA) to a final concentration of 5–15 mg protein/ml. Mitochondrial protein was determined by the method of Bradford (1976) using bovine albumin as standard.

**Mitochondrial respiration.** Mitochondrial respiration (oxygen consumption) was measured polarographically in a thermally jacketed, magnetically stirred, sealed chamber (25 °C) using a Clark-type oxygen electrode. The reaction medium consisted of 0.25 mg mitochondrial protein in 1 ml of respiration buffer (2.5 mM KPi, 100 mM KCl, 50 mM sucrose, 10 μM EGTA, and 10 mM Tris, pH 7.4) containing either 10 mM glutamate and malate or 10 mM succinate plus 1 μM rotenone. Oxygen tension was monitored for 2–3 min (State 2 respiration) prior to adding a known amount of ADP (ranging from 150–400 nmol, confirmed by spectrophotometric determination at 260 nm) to initiate State 3 respiration. The rates of respiration, expressed as n atoms O/min/mg protein, were measured during State 3 and then State 4 (after all the ADP is consumed). The respiratory control ratio, OCR (State 3 rate/State 4 rate), was calculated as a measure of the coupling efficiency of the mitochondrial respiration. Also, the ADP/O ratio was calculated from the amount of ADP added and oxygen consumed during State 3. Respiration parameters were first measured to establish the background values, then the indicated concentrations of oxalate or COM were added by syringe to the chamber and the parameters were measured again with the same mitochondrial preparation. Values were expressed as % of control (treated value × 100/ background value).

**Mitochondrial swelling.** Induction of the MPT was assessed by monitoring mitochondrial swelling as recorded by a progressive decrease in light scattering of mitochondrial suspensions (Beckman DU 7400 spectrophotometer equipped with magnetic stirring assembly). Mitochondria were suspended by stirring in buffer containing 200 mM sucrose, 10 mM MOPS, 1 mM KH2PO4, 10 μM EGTA, pH 7.4 and supplemented with 7 mM succinate and 1 μM rotenone. COM (0.01–0.05 mM) was added to selected cuvettes as indicated and mitochondrial swelling initiated by the addition of CaCl2 (13 μM). The rate and amplitude of the decrease in absorbance at 540 nm was measured as an indication of induction of the MPT and mitochondrial swelling. Confirmation of the MPT was established by complete inhibition of calcium-induced mitochondrial swelling by CsA (0.7 μM CsA).

**Statistical analysis.** Results are presented as the mean ± SEM for the number of experiments indicated in the figure and table legends. Evaluation of statistical significance for most studies was conducted by one-way analysis of variance (ANOVA); if significant at a value of p < 0.05, posthoc analysis for differences among treatment groups was conducted using Tukey’s Test. In one experiment, an n of 2 occurred in one test group, so the data were analyzed for statistical significance by the nonparametric Kruskal-Wallis test; if significant at a value of p ≤ 0.05, then individual Mann-Whitney U-tests were conducted for differences among treatment groups.

**RESULTS**

Isolation procedures yielded well-coupled mitochondria as noted by respiratory control ratios from glutamate/malate about 5–6 and from succinate about 4 (see details in the legends to Tables 1–3). These RCR values are in the range acceptable for mitochondrial functional integrity (Lash and Jones, 1996). Addition of potassium oxalate solutions in concentrations ≥5 mM significantly decreased State 3 respiration (Table 1), without any changes in the State 4 respiration or ADP/O ratio,
**TABLE 1**

<table>
<thead>
<tr>
<th>Oxalate concentration (mM)</th>
<th>State 3 respiration (% of control)</th>
<th>State 4 respiration (% of control)</th>
<th>RCR (% of control)</th>
<th>ADP/O ratio (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>80.1 ± 3.0</td>
<td>90.2 ± 6.2</td>
<td>88.9 ± 6.3</td>
<td>118.4 ± 5.0</td>
</tr>
<tr>
<td>5</td>
<td>72.6 ± 3.1*</td>
<td>84.4 ± 5.0</td>
<td>86.4 ± 4.0</td>
<td>114.1 ± 5.1</td>
</tr>
<tr>
<td>10 (n = 2)</td>
<td>80.5 ± 5.8</td>
<td>92.3 ± 8.0</td>
<td>87.3 ± 72.4</td>
<td>110.8, 100.0</td>
</tr>
<tr>
<td>25</td>
<td>43.8 ± 3.1*</td>
<td>109.8 ± 12.2</td>
<td>41.3 ± 6.5*</td>
<td>104.8 ± 20.2</td>
</tr>
</tbody>
</table>

*Note.* Respiratory parameters were first measured in untreated mitochondria, in which the State 3 and 4 respiration rates were 316.0 ± 16.4 and 56.0 ± 13 natoms O/min/mg, respectively and the RCR and ADP/O ratio were 5.7 ± 0.3 and 2.60 ± 0.10, respectively (n = 11). Then potassium oxalate was added and the parameters were measured in the same mitochondrial suspension, so these data are expressed as percent of control. Values represent the mean ± SEM (n = 3, unless indicated). Analysis of each of the parameters by the Kruskal-Wallis test showed that there were overall significant differences among the concentration groups for the State 3 respiration and for the RCR only (p ≤ 0.05).

*Indicates significant difference from control using posthoc Mann-Whitney U-test, p < 0.05.

**TABLE 2**

<table>
<thead>
<tr>
<th>COM concentration (mM)</th>
<th>State 3 respiration (% of control)</th>
<th>State 4 respiration (% of control)</th>
<th>RCR (% of control)</th>
<th>ADP/O ratio (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005</td>
<td>94.4 ± 4.6</td>
<td>82.4 ± 7.0</td>
<td>115.5 ± 4.6</td>
<td>103.6 ± 5.3</td>
</tr>
<tr>
<td>0.01</td>
<td>92.9 ± 0.7</td>
<td>83.0 ± 3.9</td>
<td>112.3 ± 5.2</td>
<td>99.4 ± 3.5</td>
</tr>
<tr>
<td>0.025</td>
<td>79.4 ± 6.4*</td>
<td>89.1 ± 8.8</td>
<td>90.3 ± 8.4</td>
<td>102.2 ± 3.9</td>
</tr>
<tr>
<td>0.05</td>
<td>73.0 ± 5.7*</td>
<td>102.2 ± 4.4</td>
<td>96.9 ± 6.3</td>
<td>90.2 ± 7.1</td>
</tr>
<tr>
<td>0.05 + CsA</td>
<td>64.5 ± 0.6*</td>
<td>80.2 ± 5.4</td>
<td>83.6 ± 6.8</td>
<td>92.5 ± 6.6</td>
</tr>
<tr>
<td>0.1</td>
<td>39.9 ± 6.2*</td>
<td>103.3 ± 4.8</td>
<td>38.3 ± 6.5*</td>
<td>ND</td>
</tr>
<tr>
<td>0.1 + CsA</td>
<td>35.0 ± 1.3*</td>
<td>89.2 ± 22.9</td>
<td>43.0 ± 9.6*</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Note.* Respiratory parameters were measured in untreated (± CsA, 1 μM) mitochondria, in which the State 3 and 4 respiration rates were 487.8 ± 21.2 and 130.0 ± 5.6 natoms O/min/mg, respectively and the RCR and ADP/O ratio were 3.9 ± 0.1 and 1.95 ± 0.05, respectively (n = 20). Then COM was added and the parameters were measured in the same mitochondrial suspension, so these data are expressed as percent of control. Values represent the mean ± SEM (n = 3). ND = the oxygen consumption tracing after ADP addition became curvilinear, so that ADP/O ratio could not be determined.

*Indicates significant difference from control, ANOVA with Tukey’s test, p < 0.05.

Even at 25 mM. The oxalate ion also decreased the RCR at the 25 mM concentration. Note that these mitochondrial incubation buffers contained no calcium and the mitochondria were isolated in calcium-free, EGTA-supplemented buffers to deplete mitochondrial calcium (Saxena et al., 1995), so that formation of COM would not be likely in the oxalate studies.

COM in a concentration-dependent manner inhibited State 3 respiration from either glutamate/malate (Table 2) or succinate (Table 3) as the substrate. The inhibition of State 3 respiration by COM was not reversed by 1 μM CsA. Effective inhibitory concentrations of COM were ≥0.05 mM (=7 μg/ml) for glutamate/malate and ≥0.025 mM for succinate. COM did not affect State 4 respiration from either glutamate/malate or succinate (Tables 2 and 3). With succinate as substrate, the RCR (State 3/State 4) was decreased by COM (Table 3). COM also did not affect the ADP/O ratio with either glutamate/malate or succinate as the substrate (Tables 2 and 3). These results indicate that COM is operating as an inhibitor of mitochondrial respiration (State 3) and not through an uncoupling mechanism.

The calcium-loading capacity of the mitochondria is considered a sensitive and specific indicator of the mitochondrial sensitivity to induction of the MPT (Starkov and Wallace, 2002). Renal mitochondrial suspensions were titrated with CaCl2 from 10–50 μM to measure the calcium-loading capacity (data not shown). From these results, a concentration of calcium of 13 μM was chosen as an amount that is below the loading capacity of control renal mitochondria, yet is sufficient to support induction of the MPT in response to test agents such as COM. COM, in a concentration-dependent manner, induced mitochondrial swelling in the presence of 13 μM calcium by increasing both the rate and amplitude of the swelling, also with effective concentrations ≥0.05 mM (Fig. 1, swelling rate, amplitude not shown). These effects were completely blocked by CsA, confirming that the COM-induced swelling represents the induction of the MPT, which is known to be specifically inhibited by CsA. Potassium oxalate did not induce the MPT; in fact at concentrations >1 mM, potassium oxalate inhibited mitochondrial swelling (data not shown), presumably because the oxalate ion complexed the calcium that was needed to induce swelling.
et al have linked oxalate ions or COM with cytotoxicity (Bhandari in vivo et al). In contrast, the solubility limit of 1 mM for oxalate suggests that it is not a potent mitochondrial toxicant than is soluble oxalate ion. These results correlate with the two previous studies of the effects of oxalate on respiration in isolated mitochondria. Using rat liver mitochondria, Bachmann and Goldberg (1971) showed that oxalate solutions 1 mM decreased succinate-induced respiration, without affecting the ADP/O ratio. Strzelecki et al. (1989) did not observe a decrease in rat kidney mitochondrial respiration (State 3) with oxalate solutions up to 3 mM. Thus it appears that mitochondrial respiration in the kidney is not affected by the oxalate ion unless its concentrations reach at least 5 mM, although liver mitochondria may be somewhat more sensitive.

Calcium oxalate monohydrate (COM), but not potassium oxalate, affects mitochondrial function in these studies. An important issue is whether calcium and oxalate ions exist in solution in the COM suspensions used in these studies and thus, whether the effects might be related to the calcium ions. Previous studies with COM suspensions (Guo and McMartin, in press) have demonstrated convincingly that COM does not dissociate to form any oxalate (hence calcium) ions in solution, even during a 6 h incubation. Considering that these studies were conducted in mitochondrial suspensions over a 5 min period, the likelihood of dissociation of COM into ions is negligible.

A key question is whether either COM or oxalate could accumulate to toxic levels in the kidney tubules during ethylene glycol poisoning. Concentrations in the kidney tissue or tubular fluid of humans have never been reported. The most relevant data would be urinary oxalate excretion, which has been reported as 2–5 mM in 19 ethylene glycol-intoxicated humans (Brent et al., 1999). Since the solubility limit of oxalate in calcium-containing solutions would be <1 mM (Burgess and Drusdo, 1993; Hodgkinson, 1981), most of the oxalate in these patients would be COM. In Wistar rats fed ethylene glycol at 1000 mg/kg of diet (Cruzan et al., 2004), total kidney oxalate concentrations reached 0.022 mmol/g of tissue (about 22 mM) within one week. Urine oxalate concentrations were about 5–10 mM at the same time, indicating that kidney oxalate levels are higher than urine oxalate levels. Hence in humans with urine oxalate of 2–5 mM, COM concentrations in kidney tissue should be significantly higher. By 16 weeks in both Wistar and F-344 rats, when kidney damage was severe, kidney oxalate concentrations were markedly higher, about 0.2–1 mmol/g (200–1000 mM). Because of the natural solubility limits for oxalate, most of the total oxalate recovered from the kidneys in these rats is COM, as confirmed by microscopic evaluation (Cruzan et al., 2004).

Studies in ethylene glycol-exposed humans and rats thus indicate that proximal tubular cells in vivo are exposed to COM in concentrations (probably >10 mM) that are well above those that induce the MPT. Numerous studies have indicated that cellular uptake of COM crystals occurs readily by an endocytotic process (Khan, 1995; Lieske and Tobaek, 1993; Lieske et al., 1994), such that accumulation was observed within 30 min. Since a kinetic evaluation of COM uptake by PT cells is lacking, it is not known what concentrations of COM accumulate within the cell. However, since external COM concentrations reach 10–1000 mM, even a small degree of COM uptake could produce COM concentrations in the cell sufficient to inhibit mitochondrial function (0.05 mM). In contrast, the solubility limit of 1 mM for oxalate suggests that it is impossible for soluble oxalate ion to accumulate intracellularly to toxic levels (at least 5 mM).

**FIG. 1.** Mitochondrial swelling in COM-treated mitochondria. Swelling was measured in calcium-treated (13 μM) mitochondria with the indicated concentrations of COM + CsA (1 μM). Data represent the initial rate (ΔAbs/min) of the decrease in absorbance (540 nm). Values represent mean ± SEM, n = 3. *Indicates significant difference from 0 COM and ** indicates significant difference from no CsA, ANOVA with Tukey’s test, p < 0.05.

**DISCUSSION**

The mechanism by which ethylene glycol produces renal failure is not known, although available data suggest that a toxicant-induced proximal tubular necrosis leads to loss of renal function. A major feature of ethylene glycol toxicity is marked accumulation of COM crystals in kidney tissue (Cruzan et al., 2004; Friedman et al., 1962) and oxalate excretion (both ionic and crystalline) in the urine (Brent et al., 1999; Jacobsen et al., 1988). Histopathologic studies have associated COM crystals with cellular damage in vivo (Cruzan et al., 2004; Friedman et al., 1962) and studies in cultured kidney cells have linked oxalate ions or COM with cytotoxicity (Bhandari et al., 2002; Guo and McMartin, in press; Miller et al., 2000; Thamilselvan and Khan, 1998). In most of these studies, it is presumed that the cellular damage results from a membrane irritation effect of COM crystals or an intracellular effect of oxalate ions. The present studies would suggest an alternative explanation, that COM crystals themselves can be cytotoxic via an ability to induce the MPT, thereby leading to a loss of ATP production and eventually to cell death.

The present studies demonstrated that COM inhibited State 3 respiration in rat kidney mitochondria in concentrations about 0.05 mM, whereas oxalate ion in solution did not affect State 3 respiration until concentrations reached 5 mM. Thus, on an equimolar basis, crystalline calcium oxalate is a markedly more potent mitochondrial toxicant than is soluble oxalate ion. These results correlate with the two previous studies of the effects of oxalate on respiration in isolated mitochondria. Using rat liver mitochondria, Bachmann and Goldberg (1971) showed that oxalate solutions 1 mM decreased succinate-induced respiration, without affecting the ADP/O ratio. Strzelecki et al. (1989) did not observe a decrease in rat kidney mitochondrial respiration (State 3) with oxalate solutions up to 3 mM. Thus it appears that mitochondrial respiration in the kidney is not affected by the oxalate ion unless its concentrations reach at least 5 mM, although liver mitochondria may be somewhat more sensitive.

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The MPT is an important mechanism for regulating cell death, both apoptotic and necrotic types (Kim et al., 2003). Opening of the permeability transition pore in the inner mitochondrial membrane allows solutes up to 1500 kDa to diffuse across the inner membrane, leading to depolarization, inhibition of oxidative phosphorylation, and to ATP depletion, which produces cell death. In these studies in rat kidney mitochondria, COM decreased State 3 respiration without affecting either State 4 respiration or the ADP/O ratio. The decrease in State 3 respiration was not inhibited by CsA, suggesting that the MPT is not involved in this process. Possible mechanisms for the COM-induced decrease in State 3 respiration include inhibition of a critical component of the electron transport chain, of ATP synthase or of the adenine nucleotide translocator. Because of the unchanged ADP/O ratio, the effect of COM would not appear to be on the phosphorylation system, but rather on the electron transport chain.

Concentrations of COM that affected State 3 respiration also induced the MPT, as indicated by mitochondrial swelling that was completely inhibited by CsA (in contrast to the lack of inhibition of state 3 respiration). This differential sensitivity to CsA suggests that COM-induced inhibition of mitochondrial respiration occurs independently and does not require induction of the MPT. Conversely, the COM-mediated inhibition of State 3 respiration may be the factor responsible for opening the permeability transition pore, leading to the MPT. Since induction of the MPT should lead to ATP depletion, these results suggest that inhibition of mitochondrial respiration is a direct effect of COM that leads to cell injury. Studies in cultured cells have suggested that cytotoxicity from oxalate can be linked with generation of free radicals and ROS (Scheid et al., 1996; Thamilselvan et al., 1999). Since ROS are known to induce the MPT, the mechanism for COM’s effects on the MPT may be secondary to the inhibition of mitochondrial respiration and related to ROS generation (Khand et al., 2002; Muthukumar and Selvam, 1998).

Regardless of the mechanism, the mitochondrial effects of COM can readily explain the cytotoxicity of COM that has been observed in PT cells in culture (Guo and McMartin, in press) as well as the tubular necrosis that occurs in the kidney during ethylene glycol toxicity.

In summary, our working hypothesis is that the ethylene glycol metabolite oxalate accumulates in the renal proximal tubule in sufficient amounts to form COM crystals, which are internalized by the PT cell, where they produce mitochondrial damage that results in cell death, tubular necrosis and the resulting renal failure. We demonstrate in these studies that COM inhibits State 3 respiration in isolated rat kidney mitochondria, which may be responsible for induction of the MPT. Production of the MPT is known to inhibit ATP synthesis by mitochondria and is a major cause of necrotic cell death. Hence, interference with mitochondrial energy production may be an important factor in the renal injury induced by accumulation of COM in kidney tissue during acute and subchronic ethylene glycol exposure.

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