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Renal toxicity of ethylene glycol results from internalization of calcium oxalate crystals by proximal tubule cells

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ABSTRACT

Ethylene glycol exposure can lead to the development of renal failure due to the metabolic formation of calcium oxalate monohydrate (COM) crystals. The renal damage is closely linked to the degree of COM accumulation in the kidney and most likely results from a COM-induced injury to proximal tubule (PT) cells. The present studies have measured the binding and internalization of COM by primary cultures of normal PT cells from humans and from Wistar and Fischer-344 rats in order to examine the roles of these uptake processes in the resulting cytotoxicity. Internalization was determined by incubation of cells with [^{14}C]-COM at 37 °C, removal of bound COM with an EDTA incubation, followed by solubilization of cells, as well as by transmission electron microscopy of COM-exposed cells. COM crystals were internalized by PT cells in time- and concentration-dependent manners. COM crystals were bound to and internalized by rat cells about five times more than by human cells. Binding and internalization values were similar between PT cells from Wistar and Fischer-344 rats, indicating that a differential uptake of COM does not explain the known strain difference in sensitivity to ethylene glycol renal toxicity. Internalization of COM correlated highly with the degree of cell death, which is greater in rat cells than in human cells. Thus, surface binding and internalization of COM by cells play critical roles in cytotoxicity and explain why rat cells are more sensitive to COM crystals. At the same level of COM accumulation after ethylene glycol exposure or hyperoxaluria *in vivo*, rats would be more susceptible than humans to COM-induced damage.

1. Introduction

Acute overdose ingestions of ethylene glycol (EG) can result in a renal failure that is linked with calcium oxalate monohydrate (COM) crystal accumulation in the kidney tissue (Corley et al., 2008; Cruzan et al., 2004; Jacobsen and McMartin, 1986). In these studies, COM accumulation is closely linked with necrotic damage—microscopically, necrotic damage is observed only in the presence of COM, and metabolically, kidney damage is correlated with the highest accumulation of COM. Renal accumulation of COM and renal parenchymal damage also occur in primary hyperoxaluria, i.e., from genetic defects in hepatic enzymes that normally metabolize precursors to other products, thereby shunting towards oxalate production as the major pathway (Milliner et al., 2001). A common animal model for formation of oxalate-containing kidney stones involves the administration of low doses of EG to rats to produce a chronic hyperoxaluria (Green et al., 2005; Asselman et al., 2003). Such doses can produce a large accumulation of COM in the kidney and accompanying renal damage (Corley et al., 2008; Cruzan et al., 2004), but do not produce the acidosis associated with acute EG overdose (Green et al., 2005). Interestingly, there is a large difference in sensitivity of certain rat strains to the renal toxicity of EG, where Wistar rats are much more susceptible than Fischer-344 (F344) rats (Cruzan et al., 2004; Li and McMartin, 2009).

In rats exposed to low levels of EG, the renal damage (a proximal tubule (PT) cell necrosis) is directly related to the amount of accumulation of COM in kidney tissue (Corley et al., 2008; Cruzan et al., 2004). The necrotic damage is most likely due to the accumulation of COM crystals, since, of the various EG metabolites, only oxalate or COM is cytotoxic to kidney cells in culture at relevant concentrations (Guo et al., 2007; Hackett et al., 1995; Scheid et al., 1995). More recent studies have convincingly demonstrated that COM crystals, and not the oxalate ion, are responsible for the cytotoxic effects of COM suspensions (Guo and McMartin, 2005; Schepers et al., 2005). Possible mechanisms for the COM-induced necrotic cell death include generation of oxidative stress (Khand et al., 2002; Scheid et al., 1996; Thamilselvan et al., 1997) as well

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as ATP depletion due to mitochondrial dysfunction (Cao et al., 2004; McMartin and Wallace, 2005). Although cell death could result from effects of COM at the cell membrane, uptake of COM by cells into the cytoplasm would allow for direct interactions with mitochondria or mitochondrial membranes. For example in isolated rat kidney mitochondria, COM, but not the oxalate ion, decreases mitochondrial respiration and directly induces the mitochondrial permeability transition (McMartin and Wallace, 2005), which would lead to a loss of the proton-motive force and to cell death.

In order for COM to accumulate in renal tissues, COM must adhere to tubular cell membranes, otherwise the newly formed crystals would be swept from the lumen by the natural flow (Finlayson and Reid, 1978). The binding of COM to epithelial layers provides an initiation spot for further binding and hence enlargement of crystals. Studies in transformed kidney cell lines have indicated that COM can bind to the plasma membrane of PT-like cells (Schepers et al., 2003; Verkoelen et al., 1999), while binding to distal tubular/collecting duct-like cells occurs only when the latter have been damaged (Verkoelen et al., 1998). Further studies have shown that COM crystals can be taken up into transformed kidney cells by an endocytotic process, with cytoplasmic appearance of crystals within 30–60 min (Lieske et al., 1994; Lieske and Toback, 1993). No studies have examined whether normal kidney cells are able to bind or internalize COM crystals, nor has the internalization of COM by cells been quantitated. Thus, the present studies have quantitatively examined the binding and internalization of COM by normal PT cells from humans and rats. Secondarily, these studies have compared the binding and internalization by cells from Wistar and F344 rats in order to examine whether these processes play roles in the strain difference in susceptibility to EG-induced damage. Through these studies, the key relationship between the internalization of COM and the amount of cell death produced by COM has been demonstrated.

2. Methods

2.1. Materials

Dulbecco's modified Eagle's medium (DME), Ham's F-12 medium (F-12) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). Purified bovine collagen type I was purchased from Inamed BioMaterials (Fremont, CA). Sodium oxalate (NaOX), calcium chloride ($CaCl_2$), trypsin–EDTA, collagenase, DNase and triiodothyronine (T3) and rat tail collagen were purchased from Sigma Chemical. The media supplements insulin, transferrin, selenium (collectively as ITS), hydrocortisone (HC), and epidermal growth factor (EGF) were purchased from BD Biosciences (Bedford, MA). [^{14}C]-oxalic acid (5 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO).

2.2. Preparation of COM and [^{14}C]-COM

Equal volumes of 10 mM $CaCl_2$ and 10 mM NaOX were mixed at room temperature to prepare COM crystals (Kohjimoto et al., 1996). The crystals were washed with deionized water one time per day. After 3 days, the crystals were dried at 60 °C and then stored at room temperature. [^{14}C]-COM crystals were prepared by mixing an equal volume of 10 mM $CaCl_2$ with 10 mM NaOX that had been spiked with 290,000 dpm of [^{14}C]-oxalate per ml. The resulting labeled crystals were centrifuged at 3200 × *g*, washed 3 times with deionized water, and resuspended at 1470 μg/ml. Prior to use in experiments, COM crystals were resuspended in incubation buffer by gentle sonication to minimize crystal aggregation and produce a uniform particle size. The average size of the COM crystals prepared in this manner is 2 ± 0.6 (SD) μm (Verkoelen et al., 1995).

2.3. Cell culture

These studies were conducted with cultures of normal human and rat PT cells (HPT and RPT, respectively), since PT cell necrosis is a major feature of the renal toxicity associated with EG exposure (Corley et al., 2008; Cruzan et al., 2004). HPT cells were isolated from normal human kidney cortex tissue, obtained by nephrectomy due to tumor or trauma; tissue was judged normal in macroscopic appearance and then separated by the surgical pathologist. These studies were approved by the Institutional Review Board for Human Research at LSU Health Sciences Center-Shreveport. Some tissue samples were obtained locally after informed consent was obtained using an approved procedure; some tissue samples were provided by the Cooperative Human Tissue Network, which is funded by the National Cancer Institute. Outer strips of cortical tissue were used to isolate HPT cells by a collagenase-DNase digestion, filtration and centrifugation technique that produces a suspension of primarily PT cells (Todd et al., 1996). To further limit the growth of contaminating cells and to enrich the population of PT cells, the suspensions were cultured on collagen-coated flasks in a serum-free mixture of DME/Ham's F-12 (50/50) media with added growth factors (ITS, EGF, HC, T3 and L-glutamine) until confluence at 5–7 days, then subcultured to 24-well plates (0.5×10^6 cells/well) for experiments (Todd et al., 1996). HPT cells prepared in this way represent a population of cells that retain the properties of the PT as indicated by enzyme activities, transport functions, hormonal responses and immunohistochemistry (Detrisac et al., 1984; Blackburn et al., 1988). The viability of these cells is routinely >95%, determined using uptake of ethidium homodimer (Guo et al., 2007).

Normal rat proximal tubule (RPT) cells were isolated by collagenase-DNase digestion of kidney cortex tissue, obtained under pentobarbital anesthesia, from untreated Wistar and F344 rats (to be able to study the potential strain differences in cell sensitivity). The animal protocols were approved by the Institutional Animal Care and Use Committee (Louisiana State University Health Sciences Center-Shreveport) and were in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*. Isolated cells were cultured on rat tail collagen-coated flasks in a serum-free mixture of DME/F-12 (50/50) media with growth factors (ITS, EGF, HC, T3 and glutamine) until confluence at about 5–7 days (Sikka and McMartin, 1996), then subcultured for experimentation into 24-well plates (1×10^6 cells/well).

2.4. PT cell treatment to assess COM binding

Binding of COM to cells was measured by techniques similar to those described (Lieske et al., 1994; Lieske and Toback, 1993). In brief, confluent cultures of PT cells (HPT and RPT from Wistar and F344 rats) were rinsed twice with ice-cold incubation buffer (20 mM HEPES, pH 7.4, containing (in mM) 107 NaCl, 5.3 KCl, 1.9 $CaCl_2$, 1.0 $MgCl_2$, 7.0 D-glucose and 26 $NaHCO_3$). Cells were incubated with 0.5 ml of [^{14}C]-COM (147 μg/ml, 440 μg/ml, or 735 μg/ml) at 4 °C for 5 min. At this temperature, COM bound externally to HPT cells, but was not internalized due to the inhibition of endocytosis at 4 °C (McMartin et al., 1992). Toxicity of COM was also minimal in these binding studies, because of the low temperature incubation. After treatment, the cells were rinsed 3× with 1 ml of cold phosphate-buffered saline (PBS) to remove unbound or loosely bound COM. The cells were then solubilized with 0.25 ml of 0.5% Triton X-100 for 1 h to release the remaining [^{14}C]-COM, which was determined by scintillation analysis. COM binding has been shown to reach a maximum within 1 min (Lieske et al., 1994); preliminary studies showed that binding to our PT cells was maximal within 3 min, so 5 min incubations were done to produce maximally saturated binding.

2.5. PT cell treatment to quantitate COM internalization

PT cells (HPT and RPT from Wistar and F344 rats) were exposed to 0.5 ml of [^{14}C]-COM (74 μg/ml, 147 μg/ml, 294 μg/ml, or 440 μg/ml) for 0.5–8 h at 37 °C, at which temperature both binding and internalization may occur. Lower concentrations of COM were used in these internalization studies than in the binding studies in order to minimize the potential for cell toxicity, which could have led to artifactual internalization. After treatment, the cells were rinsed 3× with 1 ml of cold PBS to remove unbound COM, and then treated with 0.2 ml of EDTA (4 mM) for 8 min to remove the bound COM. After the EDTA treatment, the cells were rinsed 3× with cold PBS to complete the removal of bound radiolabel. Finally, the cells were solubilized with 0.25 ml of 0.5% Triton X-100 and the amount of internalized label was determined by scintillation analysis.

To validate that this method removes the COM that is bound to the surface of the PT cell, in order to be able to subsequently quantitate the amount of COM that is internalized, confluent cultures of HPT cells were rinsed twice with ice-cold incubation buffer and incubated with 0.5 ml of [^{14}C]-COM (735 μg/ml) at 4 °C for 5 min. As noted above, these incubation conditions saturated the binding sites on PT cells, without inducing any internalization, thus enabling this test of the ability to remove surface binding. Cells were rinsed 3× with 1 ml of cold PBS to remove loosely bound COM and then incubated with 0.2 ml of ice-cold EDTA (Na salt, 4 mM) for up to 10 min to remove the surface-bound COM. Control incubations to measure the total amount of COM binding to the cells contained 0.2 ml of PBS instead of EDTA. This concentration of EDTA should solubilize COM (Guo and McMartin, 2005) and the anionic nature of EDTA minimizes its uptake across the plasma membrane. As such, the external EDTA treatment removes only the surface-bound COM by solubilizing the crystals at their binding sites. After the EDTA treatment, the cells were rinsed 3× with cold PBS to complete the removal of EDTA-solubilized label. The total amount of surface-bound [^{14}C]-COM remaining after the EDTA treatment was determined by Triton solubilization and scintillation analysis, as above.

2.6. PT cell treatment to visualize COM internalization

HPT cells were grown to confluency on Thermanox plastic coverslips (Nunc) in 24-well plates and then rinsed with PBS. Cells were exposed in duplicate to COM (74 μg/ml, 147 μg/ml, 441 μg/ml or 735 μg/ml for 10 and 30 min and 1, 2, 4 and 6 h or