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γ-Glutamyl transpeptidase null mice fail to develop tolerance to coumarin-induced Clara cell toxicity

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ABSTRACT

Coumarin was used as a model Clara cell toxicant to test the hypothesis that tolerance to injury requires increased γ-glutamyl transpeptidase (GGT) activity. Wildtype ($GGT^{+/+}$) and GGT-deficient ($GGT^{-/-}$) mice on a C57BL/6/129SvEv hybrid background were dosed orally with corn oil (vehicle) or coumarin (200 mg/kg). In vehicle-treated mice, Clara cell secretory protein (CC10) expression was distributed throughout the bronchiolar epithelium. After one dose of coumarin, CC10 expression was dramatically reduced and the bronchiolar epithelium was devoid of Clara cells in $GGT^{+/+}$ and $GGT^{-/-}$ mice. In wildtype mice, 9 doses of coumarin produced tolerance, characterized as a renewed bronchiolar epithelium with Clara cells expressing CC10 along with a 40% increase in total glutathione (GSH) and a 7-fold increase in GGT activity in the lung. In contrast, tolerance was not observed in $GGT^{-/-}$ mice. To assess whether changes in whole lung levels of GSH and GGT activity reflect Clara cell specific changes an enriched population of cells was isolated from female wildtype B6C3F1 mice made tolerant to coumarin. Compared to Clara cells from control mice, GSH and GGT activity increased 3- and 13-fold, respectively. Collectively, these data suggest Clara cell tolerance to coumarin toxicity requires increased GGT activity favoring enhanced GSH synthesis.

1. Introduction

Coumarin (1,2-benzopyrone), a natural product found in a variety of plants, is widely used in consumer products as a fragrance ingredient (Lake, 1999). Coumarin has also been evaluated clinically for the treatment of certain malignancies (Egan et al., 1990; Lake, 1999; Marshall et al., 1994). While toxicity in humans is rare, it is well recognized that coumarin is a rat hepatotoxicant. Coumarin is also acutely toxic to the mouse lung, where it selectively targets the non-ciliated bronchiolar epithelial cells, or Clara cells (Born et al., 1998). Additionally, chronic coumarin administration increased the incidence of lung tumors (alveolar/bronchiolar adenomas and carcinomas) in B6C3F1 mice, an effect not observed in F344 rats (NTP, 1993). Other agents known to cause Clara cell necrosis acutely and lung tumors in mice after chronic exposure include naphthalene (Mahvi et al., 1977; NTP, 1992; O'Brien et al., 1985), methylene chloride (Foster et al., 1992; NTP, 1986), trichloroethylene (Fukuda et al., 1983; Maltoni et al., 1986; NTP, 1990; Odum et al., 1992), and styrene (Cruzan et al., 1997, 2001; Green et al., 2001). These chemicals do not cause Clara cell necrosis or lung tumors in rats, suggesting a mechanism that may be unique to the mouse lung (Cruzan et al., 1997, 1998; Green et al., 2001; NTP, 1986, 1990, 1993, 2000).

As the most metabolically-active cell type in the rodent lung, Clara cells are uniquely susceptible to xenobiotic-mediated toxicity (Serabjit-Singh et al., 1988; Widdicombe and Pack, 1982). Cytochrome P450 2F2 (CYP2F2), which is highly expressed in mouse Clara cells catalyzes the formation of reactive epoxide metabolites that contribute to naphthalene-, styrene- and coumarin-induced Clara cell toxicity (Born et al., 2002; Buckpitt et al., 1995; Green et al., 2001; Hynes et al., 1999; Nagata et al., 1990; O'Brien et al., 1985; Ritter et al., 1991). Naphthalene-, styrene-, and coumarin-derived epoxides are detoxified by glutathione (GSH) conjugation (Huwer et al., 1991; Lake, 1984; Pacifici et al., 1981; Warren et al., 1982). However, in the lung, steady-state GSH levels vary widely between individual Clara cells, and it has been demonstrated that this heterogeneity is a significant factor in determining the susceptibility of individual Clara cells to naphthalene toxicity (West et al., 2000a,b).

A common feature of Clara cell toxicants is that following repeated exposure, Clara cells become refractory or tolerant to

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toxicity. This phenotype has been described for several chemicals, including naphthalene, coumarin, methylene chloride, trichloroethylene and 4-ipomeanol (Born et al., 1999; Boyd et al., 1981; Foster et al., 1992; O'Brien et al., 1989; Odum et al., 1992), and Clara cells tolerant to coumarin exhibit cross-resistance to naphthalene (Born et al., 1999). Although the exact mechanisms leading to the development of mouse Clara cell tolerance have yet to be established, it is generally recognized that tolerance is an adaptive response. It was originally suggested that Clara cell tolerance results from a reduction in metabolic activation (Foster et al., 1992; O'Brien et al., 1989; Odum et al., 1992). However, subsequent studies have determined that CYP2F2 expression levels and epoxidation rates are similar in lung fractions from control mice and mice made tolerant to naphthalene or coumarin (Born et al., 1999; West et al., 2002). In contrast, experiments focused on detoxification pathways have demonstrated that tolerance to naphthalene is associated with elevated GSH levels as well as an increase in γ-glutamylcysteine synthetase (GCS) activity in mouse lung terminal bronchioles (West et al., 2002).

Although GCS is rate-limiting in GSH synthesis, γ-glutamyl transpeptidase (GGT), located on the outer surface of the plasma membrane, plays an important role in the extracellular hydrolysis of GSH that is required to provide the amino acids for GSH synthesis. GGT has been used as a marker of Clara cell injury in bronchiolar alveolar lavage fluid (Day et al., 1990) and is also markedly elevated in the lungs from B6C3F1 mice made tolerant to coumarin (Vassallo et al., 2000). In light of these data, and the fact that GGT plays an important role in intracellular GSH synthesis, the purpose of this work was to test the hypothesis that Clara cell tolerance to coumarin requires an increased level of GGT activity. For this work, GGT-deficient ($GGT^{-/-}$) mice generated on a C57BL/6/129SvEv hybrid background were used as a model to determine whether tolerance to coumarin developed in the absence of GGT. Additional studies were conducted to determine if the coumarin-induced increase in GGT activity was specific to Clara cells isolated from B6C3F1 mice.

2. Materials and methods

2.1. Chemicals

Coumarin was purchased from the Sigma–Aldrich Chemical Company and the purity exceeded 99% (Milwaukee, WI). Corn oil was purchased from Acros Organics (Geel, Belgium). All other reagents were HPLC grade or the highest grade available.

2.2. Animals

Breeder pairs of heterozygous mice ($GGT^{+/-}$) (19–30 g) were previously generated on a C57BL/6/129SvEv hybrid background (Lieberman et al., 1996). Mice were bred and offspring were genotyped by polymerase chain reaction as previously reported (Will et al., 2000). Wildtype ($GGT^{+/+}$) (18–32 g) and age-matched GGT-deficient ($GGT^{-/-}$) (16–26 g) mice of both sexes were provided N-acetylcysteine (NAC) ad libitum (10 mg/ml, pH 7 in drinking water) at weaning and throughout the experimental period. Supplementation of NAC in the drinking water helps to restore GSH and cysteine levels and improves the health and longevity of these animals (Lieberman et al., 1996). Female B6C3F1 mice (19–25 g) were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Female B6C3F1 mice were selected because previous work showing Clara cell toxicity and tolerance were demonstrated in this sex and strain (Born et al., 1998, 1999).

All mice were housed in a temperature- and humidity-controlled environment with 12-h light/dark cycles. Animals were allowed rodent chow (Purina Laboratory Rodent Chow; Ralston-Purina, St. Louis, MO) and water ad libitum throughout the studies. All animal procedures were approved by The Procter and Gamble Company (AALAC accredited) and Oregon State University's Lab Animal Resources facility Institutional Animal Care and Use Committee (IACUC).

2.3. In vivo assessment of coumarin-induced Clara cell toxicity and tolerance

Preliminary studies with C57BL/6/129SvEv mice indicated that this strain develops coumarin-induced Clara cell toxicity and tolerance similar to B6C3F1 mice (data not shown) (Born et al., 1998, 1999). $GGT^{+/+}$ and $GGT^{-/-}$ mice, randomized by body weight into treatment groups (n = 7–8), were dosed orally by gavage with corn oil (vehicle) or coumarin (200 mg/kg; 5 ml/kg). This dose was selected because it has been previously shown to cause coumarin-induced lung toxicity and tolerance (Born et al., 1998, 1999). To evaluate acute toxicity, mice received a single dose of coumarin and the biochemical and histological changes were evaluated 24 h after dosing. To produce tolerance, mice were given a total of 9 doses of coumarin with a protocol similar to the NTP bioassay. Mice were dosed Monday–Friday (days 1–5), not dosed Saturday–Sunday with dose days 6–9 representing the 2nd week of dosing (Monday–Thursday). Biochemical and histological changes were evaluated 24 h after the last dose as previously described (Born et al., 1999). Separate groups of mice were used for biochemical, histopathological and immunohistochemical assessments.

2.4. Tissue collection for biochemical assessment

Under sodium pentobarbital anesthesia (50 mg/kg; ip) mice were exsanguinated. Two small pieces of lung (<100 mg) were removed and homogenized with either five volumes of 5% sulfosalicylic acid for total glutathione analysis or five volumes of Tris–HCl buffer (0.1 M, pH 8.5) for GGT analysis. Total protein in the BALF and lung homogenates was determined by the Bradford assay with bovine serum albumin as the standard following solubilization in 0.1 N NaOH (Bradford, 1976). All samples were stored at −80 °C until time of use.

2.5. Tissue collection for histopathological assessment

Mice were exsanguinated under sodium pentobarbital (~50 mg/kg; ip) anesthesia, after which the lungs, with the trachea and heart attached, were removed and perfusion fixed using Karnovsky's fixative at a constant pressure of 25 cm via the trachea. For histopathology, serial sections from lung blocks were cut at 5 μm thickness, stained with hematoxylin and eosin (H&E) and examined by light microscopy (magnification 60×).

2.6. Tissue collection for immunohistochemical assessment

Lungs were fixed by inflation with 1% paraformaldehyde. After immersion in 1% paraformaldehyde for 1 h, the tissues were transferred to 70% ethanol for processing overnight and embedding in paraffin the next day. Paraffin sections (5 μm thick) were stained for Clara cell secretory protein (CC10) using the avidin–biotin-peroxidase complex (ABC) method (Vector Laboratories, Burlingame, CA). All tissues were blocked for endogenous peroxidase activity for 10 min in 3% hydrogen peroxide, rinsed, and then blocked for proteins by incubating for 20 min in a Universal Blocking Reagent (Biogenex, San Ramon, CA). For CC10 staining, the primary antibody was an anti-rat CC10 antiserum (Singh and Katyal, 1984; kindly provided by Dr. Gurmukh Singh, University of Pittsburgh). The antiserum was diluted 1:10,000 and allowed to incubate on the slides for 1 h at room temperature. Following incubation with the primary antibody, the Elite Rabbit kit (Vector Laboratories) for the secondary antibody and the ABC steps were followed as per the manufacturer's directions. Both stains were developed for 5 min using Vector's 3,3′-diaminobenzidine kit with nickel enhancement. The sections of CC10 were counterstained with Mayer's hematoxylin (Biogenex, San Ramon, CA).

2.7. Assessment of GSH and GGT activity in isolated Clara cells

Female B6C3F1 mice, randomized by body weight into treatment groups (n = 4), were dosed orally by gavage with corn oil (vehicle) or coumarin (200 mg/kg) (5 ml/kg). To induce tolerance, mice received 9 doses of coumarin (5 days/week), and Clara cells were isolated 24 h after the last dose (Born et al., 1999). This dose was selected because it has been previously shown to cause coumarin-induced lung toxicity and tolerance (Born et al., 1998, 1999).

Clara cells were isolated according to the method of Bolton et al. (1993) and modified by replacing elastase with dispase. A Nycodenz gradient was used according to the method of Viscardi et al. (1992) instead of IgG panning (Ford and Rickwood, 1982). Cells were collected and counted on a hemocytometer. Cell viability and Clara cell purity were determined by erythrosine B exclusion and nitro blue tetrazolium staining, respectively (Devereux and Fouts, 1980).

Following isolation, Clara cells were centrifuged at 700g for 10 min. The medium was removed and the cells were resuspended in 0.5 ml of 0.9% saline. This process was repeated two more times and following the third spin, the cells were resuspended in either 5% sulfosalicylic acid (10^6 cells/ml) for GSH analysis or Tris–HCl (100 mM, pH 8.5) (10^6 cells/0.1 ml) for GGT analysis. Following a brief vortex and sonication (10 s), the cells were either analyzed for protein and GGT activity or they were centrifuged at 14,000 rpm for 10 min and the supernatant was analyzed for protein and GSH. Total protein was determined by the Bradford assay with bovine serum albumin as the standard following solubilization in 0.1 N NaOH (Bradford, 1976).

2.8. Total glutathione determination

Total glutathione, representing reduced GSH, oxidized glutathione (GSSG), and glutathione–protein mixed disulfides, was quantified in whole lung homogenates