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1-NAPHTHYLISOTHIOCYANATE-INDUCED ELEVATION OF BILIARY GLUTATHIONE

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Abstract—1-Naphthylisothiocyanate (ANIT) has been used for many years to study cholangiolitic hepatotoxicity in laboratory animals. Hallmarks of ANIT hepatotoxicity include portal edema and inflammation with bile duct epithelial and hepatic parenchymal cell necrosis. In rats, ANIT hepatotoxicity is dependent upon hepatic glutathione. Studies *in vitro* have demonstrated that ANIT combines reversibly with glutathione and suggest that intracellular formation and secretion of this glutathione–ANIT conjugate from hepatic parenchymal cells may be responsible for the efflux of glutathione observed upon exposure to ANIT. *In vivo*, glutathione conjugates produced within hepatic parenchymal cells are typically transported into bile for elimination. Therefore, large concentrations of ANIT in bile may result from hepatic parenchymal cell secretion of a reversible glutathione–ANIT conjugate. To investigate this hypothesis, bile and plasma concentrations of ANIT were determined in rats 1, 4, 8, 12 and 24 hr after administration (100 mg/kg, p.o.). Liver and bile glutathione concentrations were also evaluated. Plasma ANIT concentrations ranged between 2 and 5 μM at 1, 4, 8 and 12 hr and were 0.9 μM at 24 hr after administration. ANIT concentrations in bile at 1, 4, 8 and 12 hr were 60, 28, 21 and 22 μM, respectively. Thus, ANIT was concentrated in bile. Hepatic glutathione was not affected by ANIT during the first 12 hr after administration; however, a moderate elevation occurred by 24 hr. In contrast, a marked elevation in bile glutathione concentration (two times control) occurred 1, 4 and 8 hr after ANIT administration. Thus, the early accumulation of ANIT in bile was coincident with an elevation in bile glutathione. These findings support the hypothesis that glutathione functions to concentrate ANIT in bile. The large concentration of this toxicant in bile may be injurious to bile epithelium, a primary cellular target in ANIT hepatotoxicity.

Key words: glutathione; 1-naphthylisothiocyanate; bile; conjugation; liver; metabolism

In rats, ANIT† produces cholestasis, hyperbilirubinemia, and bile duct epithelial cell and hepatic parenchymal cell necrosis within 24 hr after a single administration [1–3]. The mechanism(s) by which ANIT acts to produce these hepatotoxic effects is presently unknown. However, it has been noted that many agents that protect against ANIT hepatotoxicity decrease hepatic glutathione or glutathione-*S*-transferase activity, whereas those that increase ANIT hepatotoxicity increase hepatic glutathione or glutathione-*S*-transferase activity [4]. To understand the role of glutathione in ANIT-induced hepatotoxicity, the effect of glutathione depletion on ANIT hepatotoxicity was examined. Depletion of hepatic glutathione by a variety of agents protects rats against ANIT-induced cholestasis and liver injury [4]. These findings strongly suggest that glutathione participates causally in ANIT hepatotoxicity.

In vitro, ANIT is toxic to freshly isolated hepatic parenchymal cells [5]. In addition, ANIT produces a dose- and time-dependent depletion of intracellular glutathione concomitant with appearance of glutathione in the extracellular medium. A glutathione conjugate, *S*-(*N*-naphthylthiocarbamoyl)-L-glutathione, was identified in the medium of ANIT-treated cell suspensions. This conjugate decomposed in aqueous solution at neutral pH to release reduced glutathione and ANIT. These observations suggested that ANIT can deplete hepatic parenchymal cells of intracellular glutathione *in vitro* via the formation and secretion of a reversible conjugate. Inasmuch as hepatic parenchymal cells *in vivo* readily secrete glutathione conjugates into bile [6], we have investigated the hypothesis that ANIT accumulates in bile concomitant with an elevation of biliary glutathione. To test this hypothesis, the biliary concentrations of glutathione and ANIT were determined in rats after ANIT administration.

MATERIALS AND METHODS

Chemicals. 1-Naphthylisothiocyanate, γ-glutamylglutamate, iodoacetic acid, glutathione and glutathione disulfide were purchased from the Sigma Chemical Co. (St. Louis, MO). HPLC reagent grade methanol and acetonitrile (J. T. Baker analyzed) were purchased from VWR Scientific (Chicago, IL). All other reagents were of the highest grade commercially available.

Animals. Male, Sprague–Dawley rats (CF:CD(SD)BR) (Charles River, Portage, MI), weighing 275–425 g, were housed in plastic cages on aspen-chip bedding (Northeastern Products Corp., Caspian, MI) under conditions of controlled

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† Abbreviation: ANIT, 1-naphthylisothiocyanate.

temperature (18–21°) and humidity (55 ± 5%) with a 12-hr light/12-hr dark cycle. Rats were allowed free access to rat chow (Lab Chow No. 5001, Purina Mills Inc., Richmond, IN) and tap water prior to initiation of experimental protocols.

ANIT administration and sample collection. Rats were fasted for 24 hr prior to receiving ANIT (100 mg/kg; 50 mg/mL corn oil, p.o.). At the specified times, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and placed onto heating pads to maintain body temperature. A midline incision was made, and bile was collected for 30 min as described by Dahm and Roth [4]. For glutathione determinations, bile was collected into microcentrifuge tubes containing 250 μL of 10% perchloric acid and 1 mM bathophenanthroline disulfonic acid. For ANIT determinations, bile was collected into empty microfuge tubes. Blood (4.5 mL) was collected from the descending aorta into syringes containing 0.5 mL of 3.8% sodium citrate immediately after bile collection. A section of liver (0.4–0.6 g) was excised and immediately frozen with liquid nitrogen and stored at −70°. The remaining liver was excised and weighed. Separate experiments were done for determination of bile ANIT and glutathione. Plasma total bilirubin was determined as described by Dahm and Roth [4].

Glutathione determinations. Glutathione determinations (reduced and oxidized forms) were made by HPLC using γ-glutamylglutamate as an internal standard as described by Fariss and Reed [7]. Bile samples were spun in a centrifuge to remove precipitated material (15,000 *g*, 1 min). An aliquot of supernatant fluid (0.1 mL) was mixed with 50 μL iodoacetic acid (20 mg/mL) in *m*-cresol (0.2 mM). The pH was then adjusted to greater than 8.0 with $KOH/KHCO_3$. After 30 min, 1-fluoro-2,4-dinitrobenzene (1% in absolute ethanol) was added, and the samples were stored in the dark for 16–24 hr at room temperature. Liver glutathione was determined by homogenizing frozen liver (Homogenizer PT10ST, Brinkmann Instruments, Westbury, NY) in 4.0 mL of 10% perchloric acid containing 1 mM bathophenanthroline disulfonic acid. The homogenate was spun in a centrifuge to remove debris (400 *g*, 10 min), and the supernatant (0.3 mL) was assayed as described for bile.

ANIT determination. Bile samples were prepared by mixing 0.3 mL of bile with 1.2 mL of 40% acetonitrile containing 0.1% glacial acetic acid. Plasma samples were prepared by mixing 0.5 mL of plasma with 1.0 mL of 60% acetonitrile containing 0.1% glacial acetic acid. The mixtures were allowed to stand for 30 min at 4° and then spun in a centrifuge (15,000 *g*, 2 min) to remove the precipitate. Aliquots of the supernatant fluids were injected onto a 4 mm × 250 mm, reverse phase, 5 μm ODSII column (Custom LC, Houston, TX) and eluted (1.0 mL/min) with a linear gradient of acetonitrile containing 0.1% glacial acetic acid (51–90% acetonitrile from 0 to 5.0 min, 90% acetonitrile from 5 to 18 min). Detection of ANIT was performed by measuring the postcolumn effluent absorbance at 308 nm. A standard curve was constructed from the areas of peaks obtained from injection of known amounts of ANIT dissolved in 40% acetonitrile, 0.1% glacial acetic acid. Under these conditions, ANIT had a retention time of approximately 15 min. Identification of ANIT in biological samples was based upon retention time comparisons (i.e. samples with and without added ANIT) and absorbance characteristics [5]. The recovery of ANIT from bile and plasma after addition of a known amount of ANIT to each was 80% for bile and 40% for plasma. The values reported herein have been corrected in accord with the extraction efficiencies.

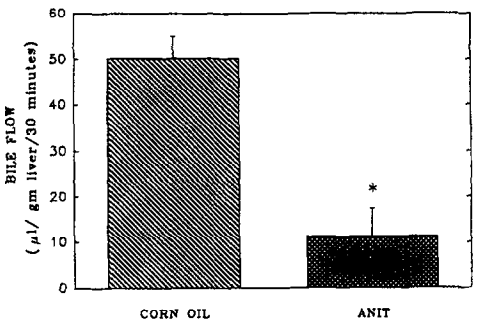

Fig. 1. Bile flow 24 hr after ANIT administration. Rats were fasted for 24 hr prior to administration of ANIT (100 mg/kg, p.o.) or corn oil vehicle. Results are means ± SEM, N = 7–11. Key: (*) significantly different from corn oil control ($P < 0.05$).

HPLC instrumentation. The HPLC system consisted of two Waters HPLC 510 pumps, a Waters 717 WISP autosampler and a Waters 486 variable wavelength detector. Millennium 2010 Chromatography Manager software (Millipore Corp., Milford, MA) was used to control instrument operation and peak integration.

Analysis of data. SigmaStat Statistical Analysis software (Ver. 1.0, Jandel Scientific, San Rafael, CA) was used for the analysis of experimental data. An unpaired Student's *t*-test was used for comparisons between ANIT- and corn oil-treated groups when requirements for parametric analysis were met. Comparisons between groups that did not meet the requirements for parametric analysis were analyzed utilizing the Mann–Whitney Rank Sum test. A value of $P < 0.05$ was the criterion for statistical significance.

RESULTS

ANIT-induced liver injury. ANIT administration to fasted rats produced an 80% reduction in bile flow (Fig. 1) and an 8-fold elevation of plasma total bilirubin (Fig. 2) within 24 hr after administration. These findings are in agreement with previous reports [1, 4].

Plasma and bile ANIT concentrations. The time course for the appearance of ANIT in bile and plasma of treated animals was investigated by sampling at 1, 4, 8, 12 and 24 hr after ANIT administration. ANIT was detected in bile 1 hr after administration (Fig. 3). The concentration of ANIT in bile was greatest at this point (60 μM) and

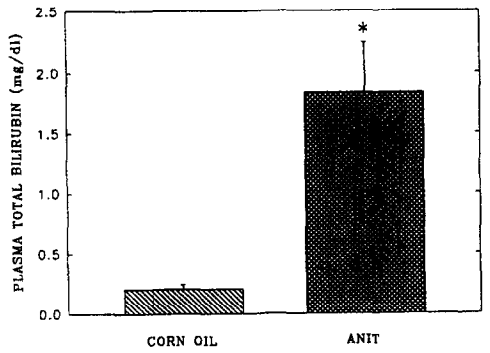

Fig. 2. ANIT-induced hyperbilirubinemia. Plasma total bilirubin was determined 24 hr after administration of ANIT (100 mg/kg, p.o.) or corn oil vehicle to fasted rats. Results are means ± SEM, N = 3–5. Key: (*) significantly different from corn oil control ($P < 0.05$).

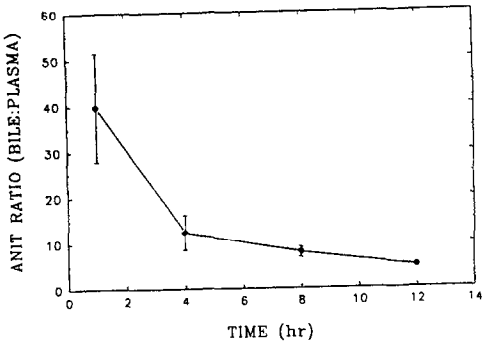

Fig. 5. Bile:plasma ANIT concentration ratio. The bile:plasma ANIT concentration ratio was determined for each rat represented in Figs. 3 and 4. Results are means ± SEM, N = 3–6.

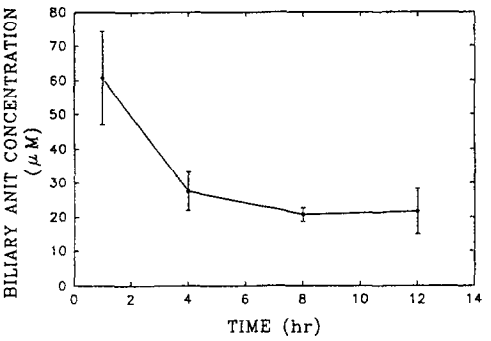

Fig. 3. Bile ANIT concentration after oral ANIT administration. The concentration of ANIT in bile was determined at 1, 4, 8, and 12 hr, as described in Materials and Methods. Results are means ± SEM, N = 3–6.

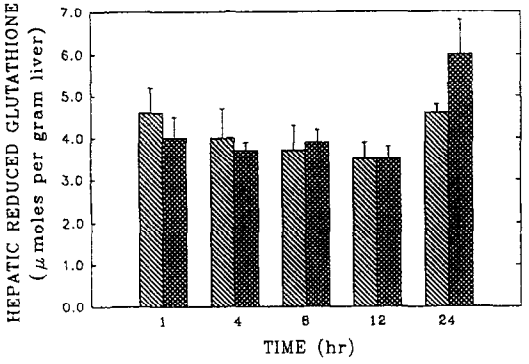

Fig. 6. Reduced glutathione in livers of ANIT-treated rats. Livers from ANIT-treated (100 mg/kg, p.o.; cross-hatched bars) and corn oil-treated (diagonal bars) rats were collected and processed as described in Materials and Methods. Results are means ± SEM, N = 3–8.

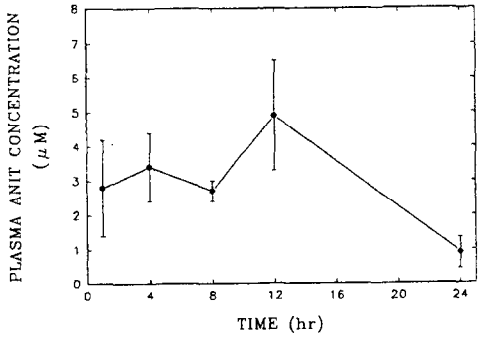

Fig. 4. Plasma ANIT concentration after oral ANIT administration. The concentration of ANIT in plasma was determined at 1, 4, 8, 12, and 24 hr, as described in Materials and Methods. Results are means ± SEM, N = 3–6.

then decreased approximately 50% by 4 hr. The concentration of ANIT in bile at 8 and 12 hr did not differ from that at 4 hr. The lack of bile at 24 hr precluded determination of bile ANIT concentration at this time. ANIT was detected in plasma 1 hr after administration (Fig. 4). Plasma ANIT concentrations were markedly lower than those observed for bile and ranged between 2 and 5 μM from 1 to 12 hr. They then declined to 0.9 μM by 24 hr. The bile:plasma ratio for ANIT concentration was considerably greater than 1 at all times evaluated (Fig. 5).

Liver and bile glutathione. The concentration of reduced glutathione in homogenates of liver was not affected markedly by ANIT during the initial 12 hr (Fig. 6), but tended to be elevated (136%, not statistically significant) at 24 hr after ANIT administration. The effect of ANIT on hepatic glutathione is in agreement with that reported by Dahm and Roth [4] and is believed to be related to the inhibition of bile flow [8]. In contrast, ANIT

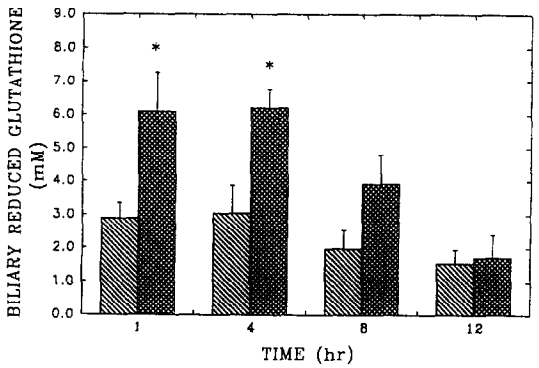

Fig. 7. Reduced glutathione in bile of ANIT-treated rats. Bile from rats treated with ANIT (100 mg/kg, p.o.; cross-hatched bars) or corn oil (diagonal bars) was collected and processed as described in Materials and Methods. Results are means ± SEM, N = 5–8. Key: (*) significantly different from corn oil control ($P < 0.05$).

significantly elevated bile glutathione concentration at 1 and 4 hr after administration (Fig. 7). Bile glutathione concentration in ANIT-treated rats at 8 hr tended to be elevated, but the difference was not statistically significant, and it returned to control by 12 hr. Concentrations of oxidized glutathione in bile and liver homogenates were unchanged in response to ANIT treatment (data not shown).

DISCUSSION

The recognition that ANIT-induced hepatotoxicity is dependent upon glutathione and that exposure of isolated hepatic parenchymal cells to ANIT initiates glutathione efflux prompted our examination of the role of glutathione in the excretion of ANIT into bile. The tissue-specific distribution of ANIT and the kinetics of elimination have been investigated by others [9–11]. Capizzo and Roberts [10] determined the distribution of radioactivity into tissues, urine, feces and expired air after administration of radioactively labeled ANIT. The radioactivity in bile and plasma increased continuously throughout the first 24 hr after administration. Bile:plasma ratios of radioactivity, as calculated from their data, yielded values of 3.6 and 4.7 for 4 and 12 hr, respectively. These values are somewhat less than those determined for ANIT in our studies using the same administered dose (Fig. 5). However, their evaluation of total radioactivity included ANIT and any radioactive metabolites of ANIT present within the samples. In contrast, the HPLC analysis employed in our studies was specific for ANIT. Although there may have been metabolites of ANIT present within bile or plasma of ANIT-treated rats, none were observed in our HPLC analysis utilizing a detection wavelength of 308 nm.

ANIT-induced cholestasis and hyperbilirubinemia become apparent 16–24 hr after a single administration of ANIT to rats. Histologically, these functional changes are associated with pronounced damage to bile duct epithelium and modest injury to hepatic parenchymal cells [1, 3, 12–14]. Luminal surface blebbing and other abnormalities in bile duct epithelial cells and hepatic parenchymal cells occur in livers of rats at times prior to the onset of cholestasis and hyperbilirubinemia [13, 14]. At present, it is not known if ANIT acts directly on these cells, or if its effects result indirectly from exposure to factors released by other cells. Our results indicate that the concentration of ANIT in plasma is in the range of 2–5 μM for at least 12 hr after oral administration of a dose of 100 mg/kg. Hepatocytes and other cells in direct contact with plasma may therefore be exposed to ANIT for a considerable length of time. In addition, our results suggest that bile duct epithelial cells are exposed to concentrations as high as 60 μM after ANIT administration. The actual concentration of ANIT in intrahepatic bile may be greater than that which we detected if ANIT is absorbed into cells lining the biliary tree proximal to the common bile duct where bile was collected for analysis. The bile duct epithelial cell degeneration observed after ANIT administration [13, 14] may be in direct response to the large concentrations of ANIT in bile. Accordingly, the concentration of ANIT in bile and resultant exposure of biliary epithelium may be a determinant of cellular targeting in this model. Interestingly, exposure of cultured hepatocytes to 20 μM ANIT for 24 hr was not cytotoxic, whereas concentrations of ANIT greater than 60 μM elicit significant cell death [15]. Whether bile duct epithelial cells are inherently more sensitive to ANIT relative to other cell types remains to be investigated.

In rats, ANIT-induced hepatotoxicity is dependent upon circulating neutrophils [16]. The mechanism by which neutrophils contribute to ANIT-induced hepatotoxicity and the nature of the signal directing their accumulation into portal tracts are not known. However, upon activation, neutrophils can release a variety of lysosomal constituents and generate reactive oxygen species that could injure or kill nearby cells [17, 18]. In this regard, concentrations of ANIT as low as 35 μM have been shown to stimulate superoxide production by neutrophils *in vitro* [19]. Moreover, exposure of neutrophils to bile potentiates superoxide production in response to a soluble stimulus (12-*O*-tetradecanoic-phorbol-13-acetate) [20]. Accordingly, after ANIT-induced disruption of the biliary tree, neutrophils that have migrated into the portal tract may be exposed to bile containing large concentrations of ANIT. Exposure to this bile may contribute to neutrophil activation and release of substances cytotoxic to adjacent cells. In this manner, neutrophil activation may contribute significantly to the full development of ANIT-induced hepatotoxicity.

ANIT-induced hepatotoxicity in rats is dependent upon glutathione [4]. Although the details of the actions of glutathione have not been elucidated, the results of an investigation with isolated hepatic parenchymal cells lend support to the hypothesis that glutathione functions in directing the transport of ANIT into bile [5]. As reported herein, biliary glutathione concentration increased markedly within 1 hr after ANIT administration and remained

elevated for several hours. During this period, hepatic glutathione concentrations did not change. Even though the increase in concentration of glutathione in bile was substantial, the amount of glutathione appearing in bile during each 30-min collection period never exceeded 5% of the total glutathione present in liver. The liver has considerable capacity to synthesize glutathione, and it appears that increased synthesis by liver cells kept pace with the rate of loss into the bile.

This study revealed increases in both ANIT and glutathione concentrations in bile after oral ANIT administration. The increase in bile glutathione concentration was 35 times greater than the concentration of ANIT in bile at 1 hr. One interpretation of this finding is that 35 mol of glutathione are transported for every mol of ANIT. However, it seems more likely that ANIT and glutathione are transported into the bile in a 1:1 stoichiometry as the glutathione–ANIT conjugate, the decomposition of which releases glutathione and ANIT. Because of its hydrophobic nature, ANIT would be expected to partition into surrounding cells and may also bind covalently to cellular constituents. In contrast, glutathione would be expected to remain within the bile and be excreted or degraded into its constituent amino acids. Therefore, the difference between glutathione and ANIT concentrations in bile likely reflects their disparate abilities to be reabsorbed from bile by cells of the biliary tree.

As a group, isothiocyanates are known to react with a variety of nucleophiles [21]. The reaction with thiols is particularly interesting because the product of the reaction is a dithiocarbamoyl linkage that can decompose, releasing the parent isothiocyanate and thiol. The reactivity of isothiocyanates toward thiols may promote their elimination, given the large concentration of cellular glutathione and its role in phase II metabolism of xenobiotic agents. In this regard, allyl isothiocyanate and benzyl isothiocyanate, two naturally occurring isothiocyanates, are excreted primarily as mercapturic acids in urine after administration to rats [22, 23]. Mercapturic acids represent end products of metabolism of glutathione conjugates. However, the potential for decomposition of the dithiocarbamoyl linkage may represent a hazard in that glutathione conjugation may provide a mechanism for transport of the isothiocyanate to tissues that would not have otherwise been exposed. The stability of glutathione–isothiocyanate conjugates will likely dictate the extent to which specific isothiocyanates reach other tissues. The stability of allyl isothiocyanate and benzyl isothiocyanate conjugates with glutathione is apparently high, since a large percentage of dose is found as the mercapturic acid in urine. In fact, the glutathione conjugates of these isothiocyanates were found to undergo minimal decomposition (<20%) in aqueous solutions at neutral pH [24]. In contrast, the glutathione conjugate of ANIT decomposes almost entirely under similar conditions [5]. This inherent instability may account for the lack of reported ANIT-derived mercapturic acids in urine of exposed animals, as well as the targeting of bile duct epithelial cells.

In conclusion, administration of an hepatotoxic dose of ANIT to rats resulted in concentration of ANIT in bile prior to the onset of cholestasis. Moreover, biliary glutathione concentration increased concomitantly with ANIT concentration. These findings support the hypothesis that ANIT is directed into the bile as a reversible glutathione conjugate, the dissociation of which results in exposure of bile duct epithelium to large concentrations of ANIT.

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