4,4′-Methylenedianiline-Induced Hepatotoxicity Is Modified by N-Acetyltransferase 2 (NAT2) Acetylator Polymorphism in the Rat

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ABSTRACT
4,4′-Methylenedianiline (MDA) is widely used in the manufacturing of polyurethane foam, epoxy resins, and polymers. Exposure to MDA induces liver damage in humans and rats. MDA undergoes N-acetylation catalyzed by N-acetyltransferase 1 (NAT1) and 2 (NAT2) in the liver. Both human and rat NAT2 are polymorphic, and human NAT2 genetic polymorphism modifies the frequency and/or severity of drug and xenobiotic toxicity in human populations. Recombinant expression of rat Nats in Escherichia coli showed that MDA was acetylated by both recombinant rat Nat1 and Nat2 and was catalyzed at substantially higher rates by rapid acetylator Nat2 compared with slow acetylator Nat2. Rapid acetylator F344 rat liver cytosols catalyzed the N-acetylation of MDA at significantly higher rates than those from slow acetylator Wistar-Kyoto (WKY) inbred rats. To test the effect of NAT2 genetic polymorphism on hepatotoxicity from acute MDA exposure, we compared hepatotoxicity in rapid (F344) and slow (WKY) Nat2 acetylator inbred rats that were administered MDA. Based on the results of dose-response studies ranging up to 150 mg/kg MDA administered by intragastric gavage, the effect of a moderately hepatotoxic dose (37.5 mg/kg) was compared in rapid versus slow acetylator rats. Plasma alanine transaminase enzyme activities were approximately 5-fold higher (p < 0.05) in rapid versus slow acetylator rats after MDA treatment, and necrotizing hepatitis with portal damage consisting of bile ductular necrosis, portal expansion, and inflammation was clearly more prominent. These results suggest that acetylator phenotype is an important factor for susceptibility toward MDA hepatotoxicity.

4,4′-Methylenedianiline (MDA) is a primary aromatic amine used extensively in a variety of industrial synthetic processes. It is an important intermediate in the production of 4,4′-methylene diphenyl disocyanate (MDI) and rigid polyurethane foams, and it is a component of epoxy-hardening agents (National Institute for Occupational Safety and Health, 1976). Individuals exposed to MDI are in turn exposed to MDA, because it is a major metabolite of MDI (Sepai et al., 1995; Dalene et al., 1996). Because of widespread use and large-scale production of both MDI and MDA, the potential for human exposure and intoxication from MDA is significant. Humans exposed to MDA accidentally or occupationally develop jaundice, cholangitis with cholestasis, toxic hepatitis, and skin rash; i.e., originally termed the “Epping Jaundice” (Kopelman et al., 1966; McGill and Motto, 1974).

Short-term oral administration of MDA to rats causes necrotizing cholangitis with periportal necrosis (Bailie et al., 1993). In addition, MDA is genotoxic (McQueen and Williams, 1990), forms DNA adducts in the liver (Schutze et al., 1996), and induces DNA damage in primary cultures of rat and human hepatocytes (Martelli et al., 2002). MDA is carcinogenic in both rats and mice with primary tumor sites in the liver, kidney, and thyroid (Lamb et al., 1986). Based on rodent carcinogenicity, MDA is reasonably expected to be carcinogenic in humans (National Toxicology Program, 2005).

An industrial outbreak of occupational MDA exposure suggested that MDA hepatotoxicity was modified by individual susceptibility (McGill and Motto, 1974). N-Acetyl-MDA is a...
major metabolite in urine samples of workers exposed to MDA (Robert et al., 1995; Schutze et al., 1995) or MDI (Sepai et al., 1995; Dalene et al., 1996). Both the N-acetyl and the \(N,N'\)-diacetyl-MDA metabolites have been identified in urine after administration of MDA (Tanaka et al., 1985) or MDI (Gledhill et al., 2005) to rats. Whereas N-acetyl-MDA represents more than half of all MDA metabolites in human urine in MDA-exposed workers, the \(N,N'\)-diacetyl-MDA metabolite represents \(<3\%\). Furthermore, the individual ratio of N-acetyl-MDA to total MDA in workers exposed to MDA (Robert et al., 1995) or MDI (Sepai et al., 1995) varied widely, suggestive of genetic polymorphism in the N-acetylation of MDA in human populations.

N-acetyltransferases 1 (NAT1) and 2 (NAT2) catalyze the N-acetylation of aromatic amines (Hein et al., 1993). Genetic polymorphism in N-acetyltransferase 2 (NAT2) segregates humans and other mammals, such as rats, into rapid and slow acetylators (Hein et al., 1997). Homozygous rapid (F344) and slow (WKY) acetylator inbred rats have been characterized as an animal model for investigations of the N-acetylation polymorphism (Hein et al., 1991a,b; 1997). NAT1 and NAT2 genes from rapid and slow acetylator rats each contain an intronless 870-bp open reading frame (Doll and Hein, 1995). Slow acetylator WKY inbred rats are homozygous for the Nat2*21A allele that possesses four single nucleotide polymorphisms, G361A (Val121Ile), G399A (silent), G522A (silent), and G796A (Val266→Ile), as compared with the Nat2*20 allele in the F344 rapid acetylator inbred rat. Recombinant Nat2 20 exhibits significantly higher N-acetyltransferase activities than recombinant Nat2 21A in an Escherichia coli JM105 expression system (Doll and Hein, 1995; Hein et al., 1997). In contrast, Nat1-coding regions from rapid and slow acetylator rats are identical to each other and their recombinant proteins expressed in E. coli strain JM105 have equivalent N-acetyltransferase activity (Doll and Hein, 1995).

The Nat2 genetic polymorphism in the rat model has previously been shown to modify metabolism and toxicity of aromatic amines (Feng et al., 1997; Jiang et al., 1999). Because MDA is subject to N-acetylation in the rat, we hypothesized that the Nat2 genetic polymorphism would modify the hepatotoxicity of MDA. We tested this hypothesis in the rapid and slow acetylator rat model.

### Materials and Methods

#### Animals
Female F344 (rapid acetylator) and WKY (slow acetylator) rats (9–10 weeks of age) were purchased from Harlan (Indianapolis, IN) and used within 1 week of arrival. Preliminary dose-response studies were conducted on F344/WKY hybrids maintained as a colony at the University of Louisville. All procedures were approved by the University of Louisville Institutional Animal Care and Use Committee.

#### Expression of Recombinant Rat Nat1 and Nat2
Rapid and slow acetylator Nat2 were recombinantly expressed in E. coli JM105 as described previously (Doll and Hein, 1995). In brief, bacteria harboring rat Nat1- or Nat2-containing plasmids were grown overnight in Luria-Bertani medium containing 100 \(\mu\)g/ml ampicillin at 37°C. Fresh Luria-Bertani ampicillin broth was reinoculated, and Nat1, Nat2-expressing bacteria were grown to an approximate absorbance of 0.5 at 600 nm. Isopropyl \(\beta\)-D-thiogalactopyranoside (1 mM) was added to the broth for induction, and the cultures were grown for an additional 3 h. The cells were harvested by centrifugation at 5000g for 10 min. Cell pellets were suspended in 1/20 volume of homogenization buffer (20 mM sodium phosphate, pH 7.4, containing EDTA (1 mM), dithiothreitol (1 mM), and protease inhibitors aprotonin (1 \(\mu\)g/ml), phenylmethylsulfonyl fluoride (100 \(\mu\)M), and pepstatin (0.75 \(\mu\)M). The suspension was lysed by sonication, and the suspension was subjected to centrifugation at 15,000g for 20 min at 4°C. Supernatant solutions were aliquoted and stored at \(-80°C\) until use.

#### Preparation of Rat Liver Cytosols
F344 and WKY rats were sacrificed by decapitation following carbon dioxide anesthesia. Livers were removed rapidly, minced, and homogenized (25% w/v) in the homogenization buffer described above and centrifuged at 100,000g for 1 h at 4°C. The supernatant (cytosol) was aliquoted and stored at \(-80°C\) until use.

#### MDA N-Acetyltransferase Activity Assay
MDA N-acetyltransferase assays were measured using modifications of high-performance liquid chromatography assays as described previously (Leff et al., 1999). Suitably diluted liver cytosol or bacterial lysate, acetyl coenzyme A (1 mM), and MDA (0.1 mM) were incubated at 37°C in a total volume of 300 \(\mu\)l. MDA was dissolved in 1% dimethyl sulfoxide, and the percentage of dimethyl sulfoxide in the reaction mixture was 0.1%. Controls for the recombinant rat Nats substituted bacterial lysates from E. coli transfected with plasmid without a rat Nat1 or Nat2 insert. Controls for liver cytosol substituted water for acetyl coenzyme A. The reaction was stopped by the addition of 1 M perchloric acid. Following centrifugation of precipitated protein, reaction supernatants were injected (40 \(\mu\)l) onto a 125 × 4 mm LiChroCART (EMD Chemicals, Inc., Darmstadt, Germany) C18 column (5 \(\mu\)m) fitted with a similar LiChroCART guard column (4 × 4 mm). Reactants and products were eluted from the column with a 10-min linear gradient (2 ml/min) from 100% sodium perchlorate (pH 2.5) to 100% acetonitrile. Under the conditions of this assay, MDA was eluted at 13.1 min, N-acetyl-MDA was eluted at 14.4 min, and \(N,N'\)-diacetyl-MDA was eluted at 15.1 min. Protein concentrations were determined by methods described previously (Bradford, 1976). Because the amount of \(N,N'\)-diacetyl-MDA formed was minimal compared with N-acetyl-MDA, only the latter was quantitated and N-acetyltransferase activities were calculated as nanomoles of monoaethylated MDA product per minute per milligram of bacterial lysate or liver cytosolic protein.

#### MDA Treatment
Female F344 (rapid acetylator), WKY (slow acetylator), or F344/WKY hybrid rats (200–250 g each) were fasted for 24 h (to enhance gastric emptying of MDA) and administered MDA (18.8, 37.5, 75, or 150 mg/kg) or vehicle (corn oil) by intragastric gavage. Following MDA administration, rats were again provided unlimited access to LabDiet 5001 rodent diet (PMI Nutrition International, Richmond, IN). Twenty-four hours after dosing, the rats were anesthetized with xylene/ketamine (1 mg/kg) and blood was collected from the dorsal vena cava before sacrifice by exsanguination. Following centrifugation at 14,000g for 10 min, plasma was collected and stored at \(-80°C\) until later analysis. Livers were harvested with some portions snap-frozen, and other sections were formalin fixed, embedded in paraffin, and mounted on microscope slides for histological assessment.

#### Clinical Analysis and Histological Examination
Alanine transaminase (ALT) is highly concentrated in the liver, and an increase in plasma ALT levels is indicative of hepatic damage. Plasma ALT levels were measured using standard kits obtained from Thermotrace (Melbourne, Australia). For histological analysis, paraffin-embedded liver sections (5 \(\mu\)m) were cut and stained with hematoxylin and eosin.

#### Statistical Analyses
Results are reported as mean ± S.E. One-way analysis of variance followed by Student-Neumann-Keuls multiple comparisons test was used to test the significance of dose-dependent increases in ALT levels. Student’s \(t\) test was used to analyze differences between rapid and slow acetylators.
Results

N-Acetylation of MDA by Rat N-Acetyltransferases. The N-acetylation of MDA to N-acetyl-MDA was catalyzed by recombinant rat Nat1 and Nat2 (Fig. 1). Catalytic rates by rat Nat2 20 were approximately 100-fold higher than Nat2 21A (Fig. 1). The N-acetylation of MDA to N-acetyl-MDA was significantly (2–3-fold) higher in F344 (rapid acetylator) than in WKY (slow acetylator) liver cytosol (Fig. 1).

MDA Dose-Response Study. Acute MDA caused a dose-dependent increase in plasma ALT levels in F344/WKY hybrid rats with a threshold MDA dose of 37.5 mg/kg (Fig. 2). Analysis of hematoxylin- and eosin-stained slides showed portal edema, inflammation, bile duct necrosis, and hemorrhage that was more severe as the MDA dose increased (Fig. 3). The lowest MDA dose that significantly showed these increases was 37.5 mg/kg, which was utilized for subsequent comparison of rapid and slow acetylator rats (see below).

Comparison of MDA Toxicity in Rapid versus Slow Acetylator Rats. Following MDA (37.5 mg/kg) administration, plasma ALT levels were 5- to 6-fold higher in F344 than in WKY rats (Fig. 4). No evidence of liver damage was observed in F344 or WKY rats administered vehicle (Fig. 5). MDA caused more severe liver damage in F344 compared with WKY rats as determined by pathologic assessment. Livers from F344 rats administered MDA had exacerbated bile duct necrosis, inflammation, hemorrhage, and portal expansion relative to WKY rats (Fig. 5).

Discussion

Our study showed that the Nat2 acetylation polymorphism modified MDA hepatotoxicity in a rat model. Kanz et al. (1992, 1995, 1998) previously showed that MDA is toxic to bile duct epithelial cells, leading to hepatocyte damage in rats. Other studies have found that metabolic activation is required and that oxidation plays an important and complex role in MDA toxicity (Bailie et al., 1993; Kautiainen et al., 1998). Glutathione depletion exacerbates MDA toxicity in rat biliary epithelial cells and hepatocytes, which suggest that glutathione might play an important cytoprotection role in MDA hepatotoxicity (Kanz et al., 2003). It has been suggested (Kautiainen et al., 1998) that, in analogy with oxidation of other amines such as benzidine, a reactive intermediate [(4-imino-2,5-cyclohexadien-1-ylidene)-methyl]-4-amino-benzene for hemoglobin adducts is formed through peroxidative oxidation. In humans exposed to MDI, highly variable levels of N-acetyl-MDA were detected in the urine and hemoglobin adducts derived from N-acetyl-MDA were identified (Sepai et al., 1995). Nevertheless, the metabolites directly responsible for the bile injury and/or hepatotoxicity remain unknown.

A major urinary metabolite of MDI and MDA in both humans (Robert et al., 1995; Schutze et al., 1995; Dalene et al., 1996) and rats (Gledhill et al., 2005) is the N-acetyl-MDA metabolite. Thus, we hypothesized that the NAT2 acetylation polymorphism would modify MDA-induced hepatotoxicity. One previous study in humans found that plasma MDA levels averaged 1.48 μg/l in 11 human pipe-layers with slow acety-
lator genotype, whereas it was undetectable (<0.05 μg/l) in 13 pipe-layers with intermediate acetylator genotype and six pipe-layers with rapid acetylator genotype (Dalene et al., 1996). Nevertheless, when adjusted for estimated cumulative exposure during the preceding 3 months, in a multiple regression analysis, no significant association between NAT2 genotype and plasma MDA level remained (Dalene et al., 1996). Because human exposures to MDI or MDA are variable and administration of MDA to humans is not ethical, we tested this hypothesis in a rapid and slow acetylator rat model. The rat model was chosen, because previous studies have shown that MDA hepatotoxicity has been studied extensively in the rat and that the mechanism for the Nat2 polymorphism is similar to humans. One previous study that examined the role of Nat2 polymorphism toward MDA hepatotoxicity reported that hepatic damage following MDA administration was significantly greater in C57BL/6J (rapid) versus A/J (slow) acetylator male but not female mice (Weber et al., 1984).

Because humans and rats express both hepatic NAT1 and NAT2, it was important to assess the capacity of these NAT isozymes to catalyze the N-acetylation reaction. We investigated this through recombinant expression of individual rat Nat2 in bacteria. Although the absolute levels MDA N-acetyltransferase activities expressed in the recombinant system are not relevant to those observed in vivo, comparisons of rapid versus slow acetylator rat Nat2 are valid to assess the role of the rat Nat2 polymorphism in the N-acetylation of MDA. We found that MDA was readily N-acetylated by both rat Nat1 and Nat2, which mimics the N-acetylation of monoamines (Hein et al., 1993) and other diamines (Zenser et al., 1996) by both human NAT1 and NAT2. Furthermore, N-acetylation by Nat2 20 (the form expressed in F344 rapid acetylators) was approximately 100-fold higher than Nat2 21A (the form expressed in WKY slow acetylators), consistent with results from other aromatic amines (Doll and Hein, 1995). Although the N-acetylation of MDA is catalyzed by both human NAT1 and NAT2, recent studies in our laboratory have shown that recombinant human NAT2 allozymes

**Fig. 3.** Representative photomicrographs (original magnification 200×) of liver histopathology changes (hematoxylin and eosin staining) 24 h after MDA dose to F344/WKY hybrid rats. MDA doses were as follows: 0 mg/kg (A), 18.8 mg/kg (B), 37.5 mg/kg (C), 75 mg/kg (D), and 150 mg/kg (E). Interpretation of the changes is as follows: normal liver morphology (A); slight expansion and inflammation of perportal area (B); inflammation and hepatocyte damage extended into midzonal area of liver (C); moderate damage and inflammation (D); and large area of portal expansion, inflammation, hemorrhage, and necrosis (E).

**Fig. 4.** Changes in plasma ALT levels 24 h after MDA treatment (37.5 mg/kg) in F344 (rapid) and WKY (slow) acetylator rats. Data points represent mean ± S.E.M. for six rats. a, plasma ALT levels were significantly higher (p < 0.05) in F344 rats treated with MDA versus vehicle. b, plasma ALT levels were significantly lower in WKY than in F344 rats following MDA administration.

**Fig. 5.** Histopathology changes (hematoxylin and eosin staining) in rat liver 24 h after vehicle or MDA (37.5 mg/kg) treatment in F344 (rapid) and WKY (slow) acetylator rats. A and B are from rapid and slow acetylator rats treated with vehicle. C and D are from rats treated with MDA. No damage was observed in vehicle-treated rats (A and B). Necrotizing hepatitis with portal damage consisting of necrosis, portal expansion, hemorrhage, and inflammation was more prominent in rapid (C) than in slow (D) acetylator rats. Representative photomicrographs (original magnification 200×) are shown.
encoded by rapid acetylator alleles catalyze MDA N-acetylation at rates substantially higher than recombinant human NAT2 allozymes encoded by slow acetylator alleles (unpublished data). Differences in MDA N-acetylation rates between F344 and WKY rat liver cytosols were 2- to 3-fold, reflecting the contribution of both Nat2 and Nat1, the latter of which does not differ between F344 and WKY inbred rat strains (Doll and Hein, 1995). Nevertheless, the magnitude of difference in MDA N-acetylation rates between F344 and WKY rat liver cytosol is consistent with other aromatic amine substrates (Hein et al., 1991b) and with MDA N-acetylation rates in cytosols derived from hepatocytes of human rapid versus slow NAT2 acetylators (unpublished data).

Previous studies showed a dose-response relationship for MDA-induced liver injury in the rat with a threshold dose between 25 and 75 mg/kg and the maximal response at around 100 to 150 mg/kg (Bailie et al., 1993). To minimize the possibility of saturating metabolic pathways, particularly N-acetylation, we selected a threshold dose (37.5 mg/kg) of MDA to conduct our comparisons between rapid and slow acetylators. Our conclusions from this study would be strengthened by comparisons of hepatotoxicity at additional doses or alternative methods of MDA administration.

N-Acetylation has been shown to both increase and decrease toxicities related to various xenobiotics and drugs (Hein, 2002). Because MDA possesses two amino groups, N-acetylation of one amino group does not necessarily prevent and in fact may enhance metabolic activation of the other amino group. A good example is the well documented capacity of many aromatic amines to induce urinary bladder cancer. For monoarylamines present in tobacco smoke, N-acetylation competes with N-oxidation and, consequently, human NAT2 slow acetylators are at increased risk for urinary bladder cancer (Garcia-Closas et al., 2005). In contrast, for diamines such as benzidine, N-acetylation does not compete and in fact most likely enhances oxidation of the second amine, thereby increasing risk in human NAT2 rapid acetylators (Carreon et al., 2006). Workers exposed to benzidine show relatively high levels of N-acetylbenzidine in the urine but virtually no N,N'-diacytethylbenzidine (Hsu et al., 1996; Rothman et al., 1996). DNA adducts derive from N-acetylbenzidine in the rat (Kennelly et al., 1984) and humans (Rothman et al., 1996), suggesting that N-acetylation is an activation pathway for benzidine. Nevertheless, the effects of N-acetylation on benzidine metabolism do not necessarily generalize to MDA and further studies are needed.

In summary, we found that the N-acetyltransferase 2 genetic polymorphism modifies MDA hepatotoxicity in the rat. Because both the frequency of the NAT2 acetylation polymorphism in human populations and potential exposures to MDA or MDI are relatively high, gene-environmental interactions for these compounds should be investigated further in both the rat model and in human populations.

References
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