0026-895X/92/020259-08$03.00/0

MOLECULAR PHARMACOLOGY, 41:259-266

Metabolic Oxidation and Toxification of *N*-Methylformamide Catalyzed by the Cytochrome P450 Isoenzyme CYP2E1

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Received June 17, 1991; Accepted October 30, 1991

SUMMARY

Alkylformamides, for example *N*-methylformamide, are hepatotoxic in rodents and humans. The mechanism by which *N*-methylformamide exerts its hepatotoxicity involves metabolic oxidation at the formyl moiety to yield a short-lived intermediate, perhaps methyl isocyanate, which reacts with glutathione to afford *S*-(*N*-methylcarbamoyl)glutathione. The hypothesis that the cytochrome P450 isozyme CYP2E1 catalyzes the metabolic toxification of *N*-methylformamide was tested. Hepatocytes obtained from mice that had received acetone, an inducer of CYP2E1, were incubated for up to 4 hr with *N*-methylformamide (5 and 10 mM). Whereas *N*-methylformamide caused cytotoxicity in these cells, as measured by release from the cells of lactate dehydrogenase, it was barely toxic, under these conditions, to cells from untreated mice. Coincubation of *N*-methylformamide with dimethylsulfoxide (10 mM), a CYP2E1 inhibitor, for 4 or 6 hr abolished the hepatocytotoxicity of *N*-methylformamide. Metabolism of *N*-methylformamide to *S*-(*N*-methylcarbamoyl) glutathione was measured in incubates with liver microsomes from rats, mice, or humans in the presence of glutathione. Pretreatment of rodents with acetone or ethanol induced the rate of metabolism of *N*-methylformamide and of *p*-nitrophenol, a known CYP2E1 substrate, but it did not increase aminopyrine *N*-demethylation. Metabolism of *N*-methylformamide and *p*-nitrophenol was elevated in microsomes from animals that had received acetone (1%) in their drinking water for 1 week to 230% and 200%, respectively, of control values in mouse microsomes and to 310% and 240%, respectively, of control values in rat microsomes. Pretreatment of animals with 4-methylpyrazole (200 mg/kg intraperitoneally, once daily for 3 days) increased metabolism of *N*-methylformamide to 410% of control values in rat liver microsomes but was without effect on murine microsomal metabolism of *N*-methylformamide. The metabolism of this compound was strongly inhibited by the CYP2E1 substrates or inhibitors dimethylsulfoxide (1–100 mM), *p*-nitrophenol (100 μM), and diethyldithiocarbamate (100 μM), which did not affect aminopyrine *N*-demethylation. A polyclonal antibody against rat CYP2E1 (10 mg of IgG/nmol of cytochrome P450) inhibited *N*-methylformamide metabolism in liver microsomes from rats and from a human by 75% and 80%, respectively. The rate of metabolism of *N*-methylformamide to *S*-(*N*-methylcarbamoyl) glutathione was determined in liver microsomes from six humans and correlated with extent of metabolic hydroxylation of chlorzoxazone, a CYP2E1 probe, and with amount of immunodetectable enzyme using an anti-rat CYP2E1 antibody (r=0.81 and 0.80, respectively). The results suggest that CYP2E1 is the predominant, if not sole, cytochrome P450 isozyme responsible for the metabolic toxification of hepatotoxic *N*-alkylformamides.

Formamides are important industrial chemicals. DMF, for example, is a solvent used widely in the manufacture of synthetic fibers, leathers, films, and surface coatings (2). Its production worldwide has been estimated to be 2×10^5 tons annually (3). NMF has solvent properties similar to those of DMF, and it also possesses antineoplastic activity against a variety of mouse tumors (4–6). The potential application of NMF as a chemotherapeutic agent in the treatment of cancer in humans was evaluated in several phase I and phase II clinical trials (7–11), but no beneficial therapeutic effect was achieved. In addition to nonspecific malaise and nausea, many patients suffered liver damage, which was occasionally so severe that it was the dose-limiting toxicity of the drug. Hepatotoxicity has also been reported in workers occupationally exposed to DMF under conditions of poor industrial hygiene (12). The hepatotoxicity of NMF can be reproduced in rodents (13–16). The mechanisms by which *N*-methylformamides cause liver damage in mice have been shown to be intrinsically linked to their metabolism (16). NMF is extensively metabolized in rodents

This work was supported by grants from the Wolfson Foundation, the Medical Research Council, and the Health and Safety Executive of Great Britain and by a Medical Research Council postgraduate studentship (to R.H.) and a research fellowship from the European Science Foundation (to J.M.).

ABBREVIATIONS: DMF, *N,N*-dimethylformamide; AMCC, *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine; CYP2E1, cytochrome P450 isozyme also known as IIE1, ac, j, or 3a (for latest nomenclature, see Ref. 1); DMSO, dimethylsulfoxide; GLC, gas-liquid chromatography; LDH, lactate dehydrogenase; NMF, *N*-methylformamide; PNP, 4-nitrophenol; SMG, *S*-(*N*-methylcarbamoyl)glutathione.

and humans *in vivo* (6, 16–20) and in suspensions of mouse hepatocytes *in vitro* (21). The metabolic pathway of NMF that is associated with the generation of the hepatotoxic lesion is initiated by oxidation at the formyl moiety, a novel reaction in xenobiochemistry, to yield an intermediate, probably the highly poisonous methyl isocyanate (CH_3NCO) (16, 19). This intermediate reacts with GSH and yields SMG (19) (see Fig. 1). SMG is metabolized further to the mercapturate AMCC, which is a major urinary excretion product of NMF (17, 22). AMCC is also a urinary metabolite of DMF in humans and rodents (22, 23). Unlike the parent formamides, which are devoid of direct cytotoxic properties, *N*-alkylcarbamic acid thioesters, such as SMG and AMCC, are directly cytotoxic and not innocuous detoxification products of the reactive intermediate (24). Details of the enzymes that catalyze the metabolic oxidation of *N*-alkylformamides are virtually unknown, except that the hepatic microsomal cytochrome P450 system seems to be involved (25). The *N*-formyl moiety is a functional group present in several endogenous substances, for example chemotactic *N*-formylmethionine peptides (26), and many exogenous agents (27, 28), yet little is known about its metabolic toxification. In light of this dearth of knowledge, the hypothesis was tested here that *N*-formyl oxidation and formamide toxification are catalyzed by the cytochrome P450 isozyme CYP2E1, which catalyzes the metabolism of a number of xenobiotics of low molecular weight, such as ethanol, acetone, pyridine, and benzene (29). In order to test this hypothesis, the hepatocellular toxicity of NMF and its metabolism were studied *in vitro* in preparations of rodent and human liver in which CYP2E1 activity was specifically modulated using suitable enzyme inducers or inhibitors.

Materials and Methods

Chemicals. Diethyldithiocarbamate, 4-methylpyrazole, NMF, and PNP were bought from Aldrich Chemical Co. (Poole, UK). NMF was purified by distillation to >99% purity (GLC) before use. Chloroxazone, collagenase, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, GSH, streptozotocin, NADP, and chemicals used for Western blot analysis were obtained from Sigma Chemical Co. Ltd. (Poole, UK). SMG was synthesized by Drs. D. H. Han and P. G. Pearson, University of Washington, (Seattle, WA), as described previously (24). 5-Fluoro-2(3*H*)-benzoxazolone and 6-hydroxychlorozoxazone were provided by Dr. R. Peter, Universität Basel (Basel, Switzerland). All other chemicals and reagents were in the laboratory and had been commercially available.

Animals and pretreatments. Male BALB/c mice (18–23 g) that had been bred at Aston University, from Charles Rivers breeding stock, were used. Male Sprague-Dawley rats (90–130 g) were purchased either from Charles Rivers Laboratories (Wilmington, MA), for use in the purification of CYP2E1, or from Bantin and Kingmans Ltd. (Hull, UK), for use in the experiments described below. New Zealand white rabbits were purchased from R&R Rabbitry (Stanwood, WA). Animals were pretreated as follows. Rats received one injection of streptozotocin (65 mg/kg, via the tail vein) 4 weeks before the preparation of microsomes, to induce diabetes (30). Acetone and ethanol were given orally, acetone either as a 1% solution in drinking water for 7 days (mice) or as a 50% solution in saline (5 ml/kg) by single oral gavage 24 hr before preparation of microsomes (rats) (31, 32) and ethanol as a 10% solution in drinking water for 14 days (32). 4-Methylpyrazole was injected once daily (200 mg/kg in saline, intraperitoneally) for 3 days before preparation of microsomes (33). Control animals received water or saline via the appropriate route.

Cytotoxicity of NMF towards mouse hepatocytes. Isolation of mouse hepatocytes by collagenase perfusion of the liver, incubation of cells with NMF (5 and 10 mM) with or without DMSO (10 mM), and determination of cytotoxicity by measurement of release into the cellular supernatant of LDH were as described before (21). Hepatocytes were used only when initial viability was >85%, as determined by trypan blue exclusion.

Preparation of liver microsomes. Excess samples of healthy human liver tissue were obtained after graft reduction of donor liver from the Liver Transplant Unit at the Queen Elizabeth Hospital (Birmingham, UK). Tissues originated from six organ donors, three females (age 10, 28, and 34 years) and three males (age 12, 45, and 55 years). Homogenate (25%) of human or rodent liver was prepared in Tris-buffered (50 mM, pH 7.4) KCl solution (0.154 M). Human liver samples were stored at −70° for 3 weeks to 8 months before use. Microsomes were obtained in the usual way by differential centrifugation of homogenate, first at 9000 × *g* for 20 min and then at 100,000 × *g* for 1 hr, in a Beckman L8-60M ultracentrifuge. The microsomal pellet was suspended in Tris buffer (50 mM, pH 7.4), recentrifuged at 100,000 × *g* for 1 hr, and resuspended in phosphate buffer (50 mM, pH 7.4). In control incubates, microsomes were used after inactivation by immersion in boiling water for 10 min.

Microsomal incubations. All incubations were carried out, with duplicate samples, in 5-ml glass vials under shaking at 37°. Incubates contained substrate, microsomes (2–4 mg of protein/ml), an NADPH-generating system (20 mM glucose-6-phosphate, 10 mM NADP, and 4 IU glucose-6-phosphate dehydrogenase) or NADPH (10 mM), and, in the case of incubations with NMF, also GSH (10 mM), in potassium phosphate buffer (0.05 M, pH 7.4). Substrate concentration was 400 μM chlorzoxazone, 100 μM PNP, 5 mM aminopyrine, and 5 or 10 mM NMF; in the experiments in which enzyme kinetics were investigated, NMF concentration was 0.4–10 mM. The final volume was 1 ml (incubates with chlorzoxazone or PNP) or 2 ml (incubates with NMF or aminopyrine). In some experiments, DMSO (0.1–100 mM), PNP (30–100 μM), or diethyldithiocarbamate (100 μM) was added as alternative substrate or inhibitor of CYP2E1. Incubations were initiated by addition of NADPH or the NADPH-generating system after a 3-min preincubation period, and they were terminated after 15 min (PNP oxidation), 20 min (chlorzoxazone metabolism), or 30 min (metabolism of NMF or aminopyrine). During these time periods, rates of metabolism were linear with time. Linearity with time was not checked in the case of PNP oxidation in microsomes from acetone-pretreated rats, in which a third of the available substrate was metabolized during the overall incubation.

CYP2E1 was purified from livers of streptozotocin-treated rats (30). A small amount of the purified enzyme that was not used for rabbit immunization (see below) was used in a preliminary metabolism experiment. Incubations were conducted with 0.25 nmol of purified enzyme, 1 nmol of cytochrome P450 reductase, 0.25 nmol of cytochrome b_5, 10 mM GSH, and 10 mM NMF, for 1 hr.

Quantitation of metabolites. Sample preparation and quantitation of SMG, 4-nitrocatechol, and formaldehyde generated by metabolism of NMF, PNP, and aminopyrine, respectively, were conducted as previously described, 4-nitrocatechol (32) and formaldehyde (34) spec-

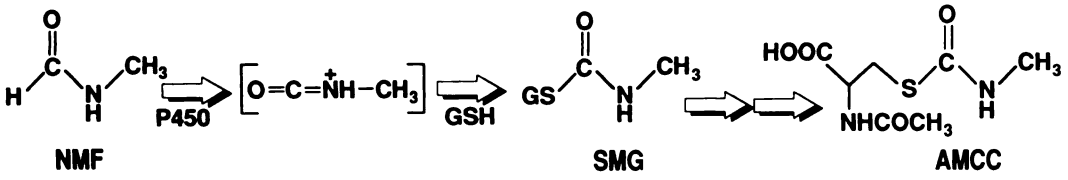

Fig. 1. Metabolism of NMF.

trophotometrically using a Cecil CE 594 instrument and SMG by GLC after derivatization of the thioester with ethanol in alkali to furnish ethyl *N*-methylcarbamate (25). Primarily a Pye Unicam series 204 gas chromatograph was used, as specified before (25). For the determination of apparent K_m and V_{max} values for SMG formation, GLC analysis was performed on a HP-5890A gas chromatograph fitted with a nitrogen-selective detector and a HP-3396 integrator. A fused silica capillary HP-20M column (25 m × 0.32 mm i.d., 0.32-μm film thickness) was used at 130°, with helium as carrier gas. Apparent K_m and V_{max} values were derived from Eadie-Hofstee plots, in which v is plotted against v/a (v = rate of metabolite formation and a = substrate concentration). At the NMF concentrations used (0.4–1 mM), this relationship was linear.

Metabolism of chlorzoxazone to 6-hydroxychlorzoxazone was determined principally as described by Peter *et al.* (35), with some modifications.[1] Incubations with chlorzoxazone were terminated by addition of phosphoric acid (43%, w/v) (50 μl) and internal standard [5-fluoro-2(3*H*)-benzoxazolone] (5 μg). Incubates were extracted twice with dichloromethane (1 ml). Organic and aqueous layers were separated by centrifugation (6000 × *g*) for 10 min. The combined organic layers were evaporated to dryness (at room temperature). Residues were dissolved in an acetonitrile/water mixture (4:6, v/v) (100 μl). Aliquots (20 μl) were analyzed by high performance liquid chromatography. The system consisted of Waters 510 pumps, WISP 710 autoinjector, Shimadzu SPD-6A UV detector (287 nm), and Waters Maxima 820 work station. Separation was achieved on a 50- × 4.6-mm Supelcosil LC-8-DB (5 μm) column in series with a 20- × 2-mm guard column filled with LiChrosorb RP-8 (5 μm) packing material, using a mixture of acetonitrile/phosphoric acid (0.5%) (26:74, v/v) as mobile phase, at a flow rate of 1.8 ml/min. Retention times of 6-hydroxychlorzoxazone, 5-fluoro-2(3*H*)-benzoxazolone, and chlorzoxazone were 0.9, 1.5, and 2.6 min, respectively. Calibration curves were established using authentic 6-hydroxychlorzoxazone.

Cytochrome P450 levels were measured spectrophotometrically as described by Gibson and Skett (36). Protein content of microsomal suspensions was determined according to the method of Lowry *et al.* (37).

Preparation of antibody. A female rabbit was immunized by once weekly injection (subcutaneously) of CYP2E1 antigen (100 μg of protein) for 3 weeks, followed by a 100-μg boost after 12 weeks. Total IgG was isolated from blood collected after the last injection (38). This polyclonal antibody pool recognized selectively a single rat liver microsomal protein band (apparent molecular weight, 53,000), which co-migrated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with purified rat CYP2E1. Slight cross-reactivity to a protein of lower apparent molecular weight was removed by immunoabsorbtive techniques (30). Briefly, the total IgG pool was passed over a column of Sepharose 4B gel that had a mixture of microsomal proteins (isolated during CYP2E1 purification; CM-Sepharose/30 mM plus 45 mM NaP_i fractions) covalently linked to it. These protein fractions contained very little CYP2E1 protein (by Western blot). The final antibody fraction recognized a single protein band in human liver microsomes, which was of slightly higher apparent molecular weight (54,000) than rat CYP2E1. This protein was assumed to be human CYP2E1, based on its relative electrophoretic mobility and high affinity towards the antibody.

Western blot analysis. Proteins in human liver microsomal samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on gels containing 7.5% (w/v) acrylamide, by the method of Laemmli (39), in a Mini-Protean apparatus (Bio-Rad). Proteins were electroblotted onto nitrocellulose as described by Towbin *et al.* (40), using a Transblot apparatus (Bio-Rad). The nitrocellulose was incubated at room temperature for 1 hr in 10 mM Tris-buffered saline (pH 7.4) with 5% (w/v) skim milk and 0.2% Triton X-100, to block nonspecific antibody binding. The nitrocellulose was then incubated for 1 hr at room temperature and for 16 hr at 4° with the antibody (1 μg/ml) in Tris-buffered saline/milk. The blots were washed three times with Tris-buffered saline and incubated for 2 hr (at room temperature) in Tris-buffered saline/milk with 1/1000 Protein A (from *Staphylococcus aureus*) conjugated to horseradish peroxidase. The antigenic components were visualized with 0.0025% (w/v) 4-chloronaphth-1-ol in 10 mM Tris·HCl (pH 7.4), with 0.01% (v/v) hydrogen peroxide. Band intensity was measured with a LKB 2202 Ultrascan laser densitometer.

[1] R. Peter, personal communication.

Results

Effect of pretreatment with acetone or coincubation with DMSO on cytotoxicity of NMF in mouse hepatocytes. The toxicity of monoalkylformamides such as NMF has been conclusively linked to the cytochrome P450-mediated oxidation of the *N*-formyl moiety, which yields eventually *S*-(*N*-alkylcarbamoyl)glutathione (16, 19) (see Fig. 1). Initially, the role that CYP2E1 plays in the metabolic toxification of NMF was investigated. Mice received acetone in their drinking water according to a treatment schedule that doubled microsomal PNP oxidation, compared with values obtained with microsomes from control mice (see below). Hepatocytes were isolated and incubated in suspension with NMF. Fig. 2 shows that hepatocytes from acetone-pretreated mice were dramatically more susceptible to NMF-induced cytotoxicity than liver cells from naive animals. When hepatocytes were incubated with NMF for 6 hr in the presence of 10 mM DMSO, an inhibitor of CYP2E1 (41), NMF toxicity was significantly diminished (Fig. 3).

Microsomal oxidation of NMF and effect of induction and inhibition of CYP2E1. In order to characterize the microsomal oxidation of NMF to SMG, we determined the apparent K_m and V_{max} values for this metabolic route catalyzed by rat liver microsomes. They were 3.9 ± 0.9 mM and 0.38 ± 0.11 nmol/min/nmol of cytochrome P450 (mean ± standard deviation, three to five experiments), respectively. The hypothesis that this biotransformation is catalyzed by CYP2E1 was tested. Mice and rats were pretreated with the CYP2E1 inducers acetone, ethanol, or 4-methylpyrazole (29, 31–33, 42–44). Hepatic microsomes were prepared and incubated with NMF in the presence of GSH. Metabolically generated SMG was measured, and the amount produced was compared with the ability of the different microsomal preparations to catalyze the oxidation of PNP or aminopyrine. Whereas the oxidative metabolism of PNP is mediated preferentially, perhaps exclusively, via CYP2E1 (32), the P450 isozymes that contribute to the catalysis of the metabolic *N*-demethylation of aminopyrine have not been clearly identified, but CYP2E1 does not seem to be involved (45). Fig. 4 shows that aminopyrine *N*-demethylation was not affected significantly by any of the pretreatments. In contrast, treatment of rodents with acetone caused a marked increase in the rate at which NMF or PNP was metabolized in microsomes from either rodent species. Pretreatment with ethanol also elevated the rate of microsomal NMF and PNP metabolism, although not as consistently as did acetone administration. In the case of animals that had received 4-methylpyrazole, the rate at which NMF or PNP was metabolized was elevated in microsomes from rats but not in microsomes from mice (Fig. 4).

PNP is a CYP2E1 substrate (46), and DMSO (41) and diethyldithiocarbamate (47) are relatively specific inhibitors of CYP2E1. Their presence impeded metabolism of NMF to SMG in rat hepatic microsomes at concentrations that did not affect

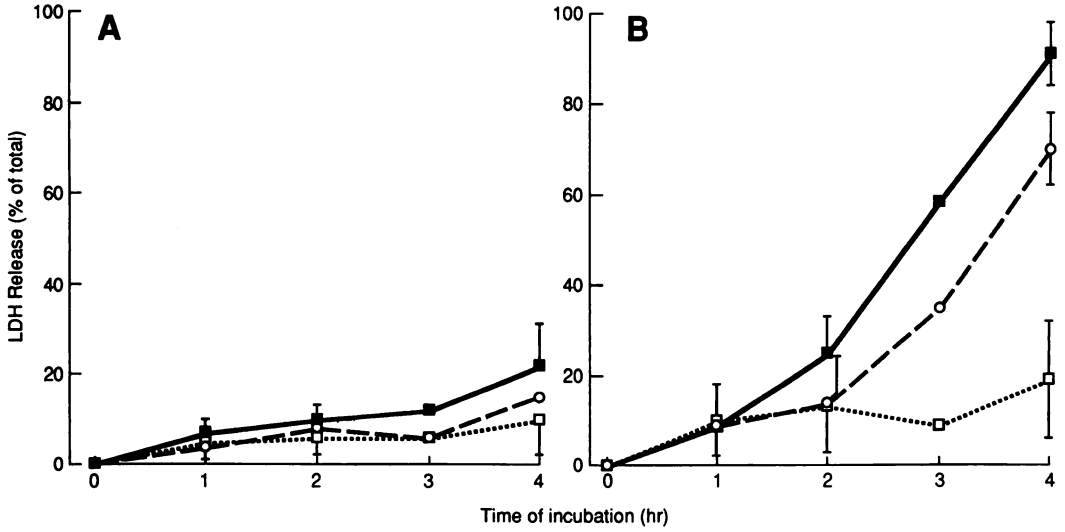

Fig. 2. Cytotoxicity in hepatocytes from control mice (A) and mice that had received acetone (B), incubated without (□) or with NMF at 5 mM (○) or 10 mM (■). Hepatocytes were incubated for up to 4 hr, and LDH released from cells was measured in the supernatant. Values are the mean of two (3-hr point) or the mean ± standard deviation of three experiments (1-, 2-, and 4-hr points), each performed with duplicate incubates, and are expressed as percentage of total LDH released by addition of Triton. For the sake of clarity of presentation, error bars have been omitted from several 5 mM NMF data points, where the standard deviation was not greater than that shown for the respective 10 mM NMF data points. For details of pretreatment schedules, see Materials and Methods; incubations and LDH analysis were performed as described before (21).

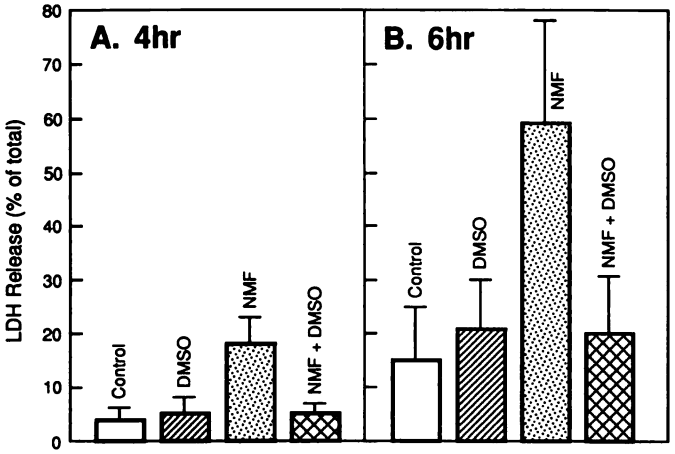

Fig. 3. Effect of DMSO (10 mM) on cytotoxicity of NMF (10 mM) in hepatocytes. Cells were incubated for 4 hr (A) or 6 hr (B) without (□) or with either DMSO (▨), NMF (▩), or NMF and DMSO (▩). Results are the mean ± standard deviation of three experiments, each performed with duplicate incubates, and are expressed as percentage of total LDH released by addition of Triton. The difference between cytotoxicity induced by NMF alone and that seen with NMF and DMSO together is significant ($p < 0.05$, Students t test). Incubations of LDH analysis were performed as described before (21).

aminopyrine *N*-demethylation (Fig. 5). Diethyldithiocarbamate at 100 μM, which did not reduce aminopyrine metabolism, inhibited metabolic generation of SMG from NMF in rat liver microsomes by 88 ± 7% (three experiments), compared with incubations without inhibitor.

A monospecific antibody against rat CYP2E1 was added to incubations of NMF with microsomes from rat or human liver. Fig. 6 shows that the antibody inhibited NMF metabolism substantially, in rat liver microsomes maximally by 75% (Fig. 6A) and in liver microsomes from a human maximally by 82% (Fig. 6B). Microsomal *N*-demethylation of aminopyrine was not altered by the presence of this antibody.

In a preliminary experiment, SMG was also detected in incubates of NMF (10 mM) and GSH with purified rat liver CYP2E1 after reconstitution with cytochrome P450 reductase and cytochrome b_5. In order to maximize metabolite formation, the mixture was incubated for 1 hr, a time during which NMF oxidation was probably not linear with time. The amount of SMG generated (27 nmol/nmol of cytochrome P450) was 5 times that found under identical incubation conditions in microsomes from livers of streptozotocin-treated rats, which served as the source of the purified enzyme. The difference in SMG production between purified enzyme and microsomes mirrors the contribution of CYP2E1 to the overall microsomal cytochrome P450, as determined by PNP oxidation (result not shown).

Correlation of NMF metabolism with metabolism of chlorzoxazone and immunodetectable CYP2E1. There should be a linear correlation between the rates of two reactions that are catalyzed by the same microsomal enzyme when microsomal preparations with varying enzyme activities are compared (48). In view of this fact, the rates at which microsomes from six different human livers catalyzed the metabolism of NMF and of chlorzoxazone were measured. The metabolic hydroxylation of chlorzoxazone, a muscle relaxant, is catalyzed selectively by CYP2E1; thus, it is a specific probe for this enzyme (35). The rates of both catalytic reactions in the human microsomes were indeed correlated, because standard linear regression analysis afforded a correlation coefficient of $r = 0.81$, corresponding to a significance level of $p < 0.05$ (Fig. 7A). The rate of NMF metabolism was also compared with the amount of CYP2E1 measured in the human microsomes by immunoblot analysis using an antibody against rat CYP2E1. Enzyme was detected in all six microsomal samples (Fig. 8), and the plot of rate of NMF metabolism versus amount of immunostainable enzyme is shown in Fig. 7B. Linear regression analysis yielded a correlation coefficient of $r = 0.80$.

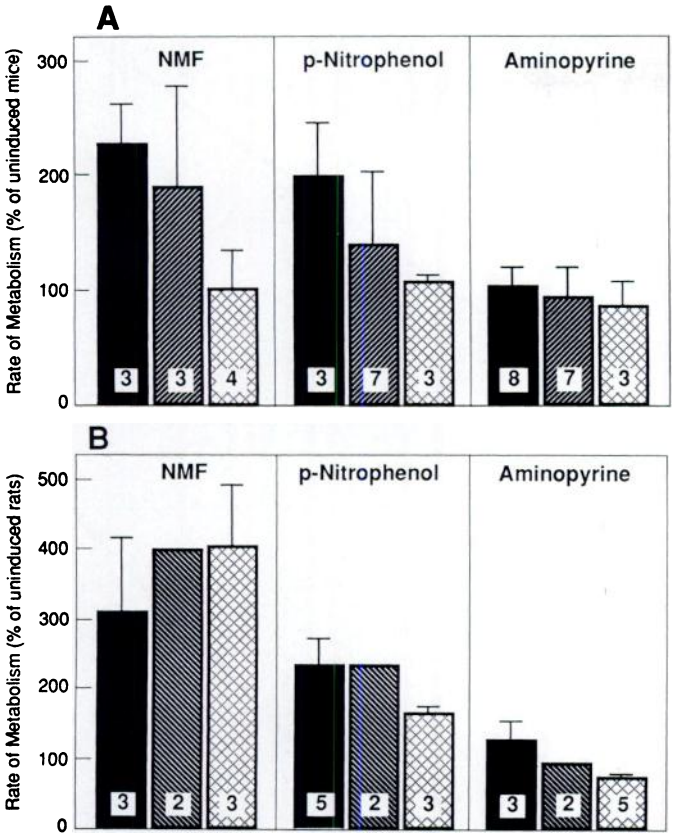

Fig. 4. Influence of pretreatment of mice (A) or rats (B) with acetone (■), ethanol (▨), or 4-methylpyrazole (▩) on the microsomal metabolism of NMF, PNP, and aminopyrine. Rates of metabolism of NMF, PNP, and aminopyrine were, respectively, 0.48±0.14, 0.89±0.29, and 4.80±1.24 nmol/nmol of cytochrome P450/min in microsomes from control mice (n=9-3) and 0.44±0.10, 0.87±0.13, and 4.70±1.59 nmol/nmol of cytochrome P450/min in microsomes from control rats (n=6). Values are the mean ± standard deviation, numbers of experiments are given at the *bottom of bars*. Rate of metabolism of NMF in microsomes from mice treated with ethanol was significantly different from that observed in control microsomes (p<0.05), but the difference was not significant in the case of PNP oxidation. Details of pretreatment schedule, incubation conditions, and metabolite analysis are described in Materials and Methods.

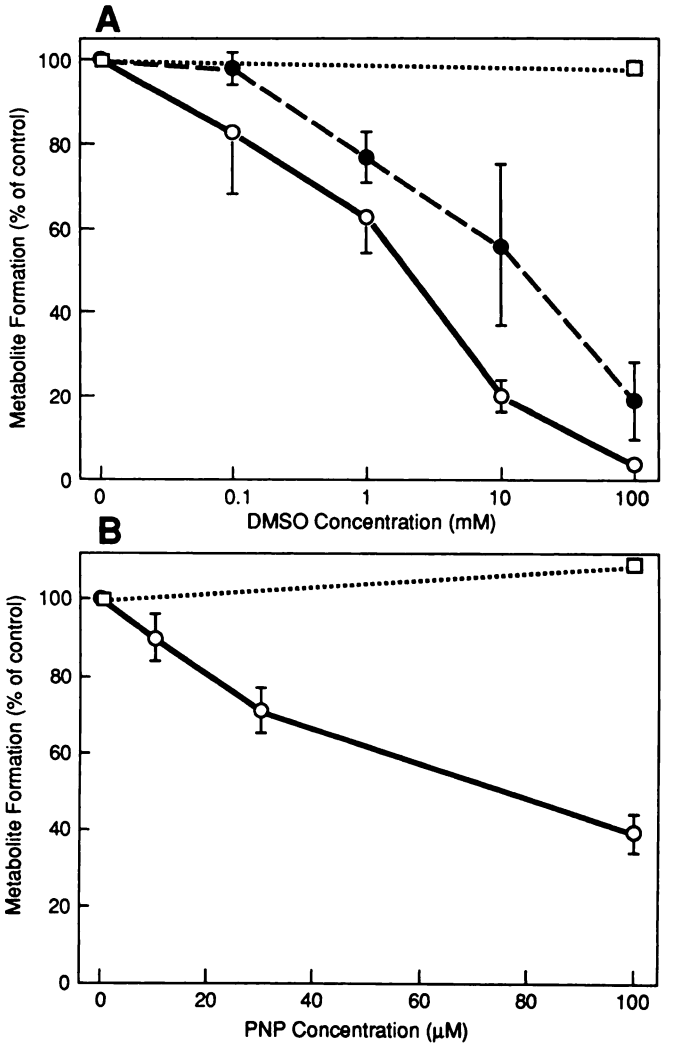

Fig. 5. Effect of DMSO (A) and PNP (B) on metabolism of NMF (○), PNP (●), or aminopyrine (□) in rat liver microsomes. Values, which are the mean of two or the mean ± standard deviation of three or four experiments, are expressed as percentage of rate of metabolic generation of SMG from NMF, *p*-nitrocatechol from PNP, or formaldehyde from aminopyrine in microsomal incubates without inhibitor. In each experiment, incubations were conducted in triplicate. Details of incubation conditions and metabolite analysis are described in Materials and Methods.

Discussion

The activation and detoxification of many toxic chemicals can be rationalized in terms of oxidation by relatively few cytochrome P450 enzymes (47). In the case of a considerable number of toxic molecules of low molecular weight, such as dialkylnitrosamines (49), benzene, styrene, halomethanes, vinyl halides, ethyl carbamate (47), or acetonitrile (33), the cytochrome P450 isozyme that is predominantly responsible for their activation has been identified as CYP2E1. The results of the work described here provide, for the first time, compelling evidence for the contention that CYP2E1 is also crucially involved with the metabolic toxification of hepatotoxic *N*-alkylformamides. This evidence can be summarized as follows. (i) Microsomal oxidation of NMF to a product that *N*-methylcarbamoylates GSH was induced by pretreatment of rodents with selective inducers of CYP2E1. (ii) Cytotoxicity of NMF towards hepatocytes was reduced in the presence of DMSO, and it was exacerbated in cells obtained from mice that had received acetone, compared with cells from control mice. (iii) Metabolic generation of SMG from NMF in rodent microsomes was effectively suppressed by the CYP2E1 inhibitors or alternative substrates DMSO, PNP, and diethyldithiocarbamate. (iv) NMF oxidation was inhibited dramatically in hepatic microsomes from rats or humans upon incubation with an antibody against rat CYP2E1. (v) Metabolism of NMF to SMG was catalyzed by purified rat liver CYP2E1. (vi) A correlation was found in liver microsomes from six humans between extent of metabolism of NMF and both rate of metabolism of the CYP2E1 probe chlorzoxazone (35) and amount of immunostainable CYP2E1.

The argument in favor of a substantial involvement of CYP2E1 in NMF oxidation is further strengthened by the observed difference between species in inducibility of NMF metabolism. Whereas acetone and ethanol have been shown to induce CYP2E1 in all species investigated thus far, pyrazole derivatives induces this enzyme in the rat and rabbit but not in certain mouse strains, including BALB/c (50), the strain used in the present investigation. In these mice, pyrazoles

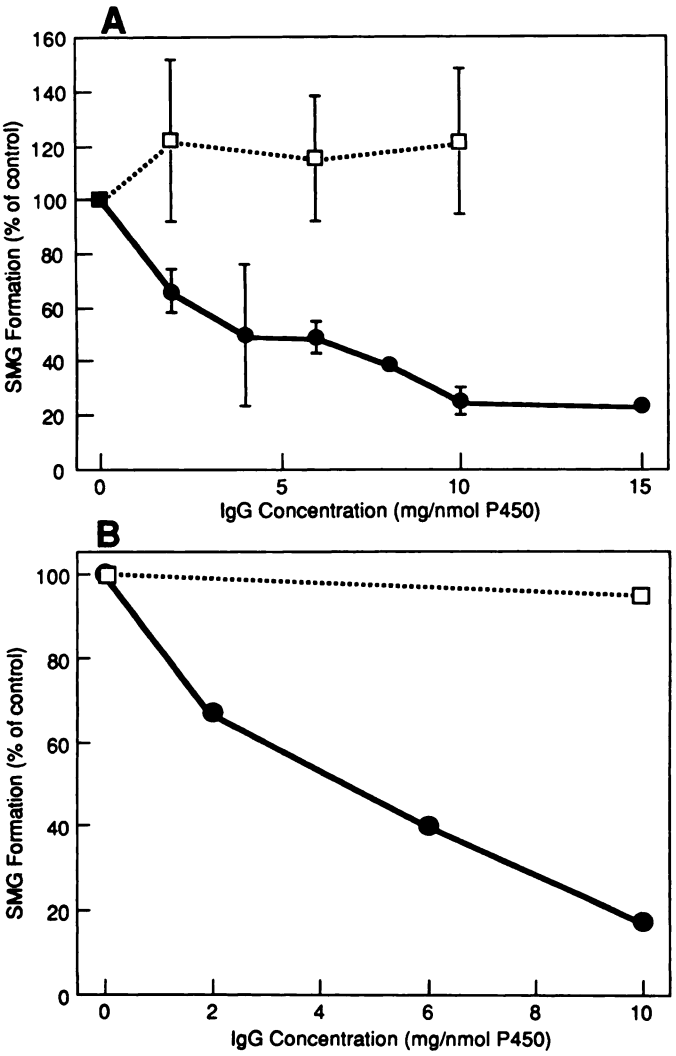

Fig. 6. Effect of anti-rat CYP2E1 IgG (●) or of preimmune IgG (□) on the metabolism of NMF in rat (A) or human (B) liver microsomes. Values, which are expressed as percentage of the rate of metabolic production of SMG from NMF in control microsomes, are the mean of two or the mean ± standard deviation of three experiments. Microsomes in A were from three rats and microsomes in B were from an individual human; in each experiment, incubations were conducted in triplicate. The rate of uninhibited metabolism of NMF by rat or human microsomes was, respectively, 0.53±0.12 and 0.42 nmol of SMG/nmol of P450/min. In a preliminary experiment, metabolism of aminopyrine was not inhibited by the anti-rat CYP2E1 IgG. Details of incubation conditions and metabolite analysis are described in Materials and Methods.

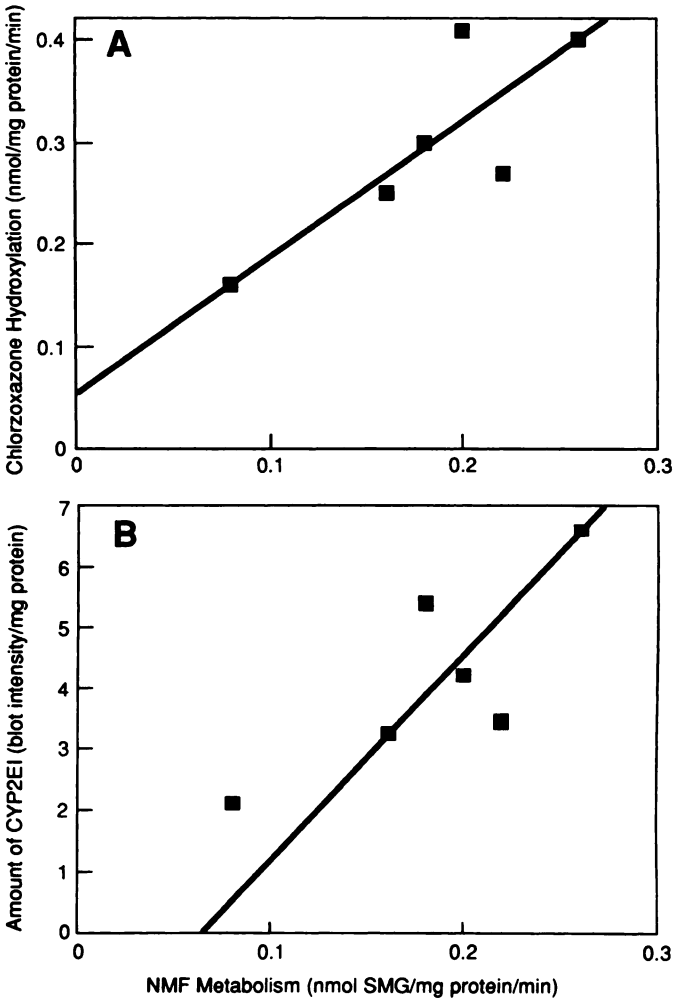

Fig. 7. Correlation of metabolism of NMF with hydroxylation of chlorzoxazone (A) and immunodetectable CYP2E1 (B) in microsomes from six human livers. Metabolic activities are the mean of three and protein levels the mean of two determinations. Standard linear regression analysis gave correlation coefficients of r=0.81 (A) and r=0.80 (B) (p<0.05). Details of incubation conditions and metabolite and immunoblot analyses are described in Materials and Methods.

induce microsomal coumarin hydroxylase P450Coh (50). This species difference is faithfully mirrored by the inducibility of NMF metabolism; pretreatment with 4-methylpyrazole affected NMF oxidation in rat microsomes but not in microsomes from BALB/c mice, whereas ethanol or acetone caused an increase in the rate of *N*-formyl oxidation equally in microsomes from BALB/c mice and rats.

Previously we have been able to demonstrate (25) that the microsomal oxidation of NMF was inhibited in the presence of carbon monoxide and abolished by the absence of NADPH, suggesting the involvement of cytochrome P450, but was unaffected by pretreatment of mice with the cytochrome P450 inducers phenobarbital or β-naphthoflavone. Furthermore, microsomal NMF biotransformation was not inhibited by coincubation with proadifen (25), which is an inhibitor of a broad spectrum of cytochromes P450 but not of CYP2E1 (51). Pretreatment of mice with phenobarbital failed to exacerbate NMF-induced hepatotoxicity *in vivo* in mice, and treatment with proadifen did not reduce the severity of liver damage caused by NMF (52). All these findings are consistent with a crucial role of CYP2E1 in the metabolic toxification of *N*-alkylformamides, and they indicate that NMF, like chlorzoxazone and PNP, may well be a specific probe for CYP2E1 activity. The apparent K_m reported here for the metabolic conversion of NMF to SMG in rat liver microsomes, 3.9 mM, is well within the range of drug concentrations that were measured in blood and tissues of rodents that had received hepatotoxic doses of NMF. For example, in CBA CA mice administration of 6.8 mmol/kg NMF via the intraperitoneal route yielded a peak plasma concentration of 8.5 mM, and plasma drug levels did not decrease below values equivalent to the K_m reported here until >12 hr after drug administration (53).

The major pathway of disposition of DMF in humans and animals is metabolic hydroxylation at the *N*-methyl moiety to

Fig. 8. Western blot of six human liver microsomal samples probed with a rabbit anti-rat CYP2E1 antibody. Gels were loaded with approximately 30 μg of microsomal protein per lane. Details of immunoblot analysis are described in Materials and Methods.

generate *N*-(hydroxymethyl)-*N*-methylformamide (54–56). DMF is also metabolized to AMCC *in vitro* (22, 23), even though this biotransformation route has hitherto not been observed in microsomal preparations *in vivo* (25),[2] so that mechanistic details of the steps leading from DMF to AMCC, probably via *N*-(hydroxymethyl)-*N*-methylformamide and SMG, have hitherto not been verified. Knowledge of the metabolic generation of *N*-methylcarbamic acid thioesters, such as SMG, and their toxic precursor(s) from DMF in the liver of humans is relevant for the assessment of the potential hazard associated with occupational exposure to DMF. Such knowledge is especially felicitous in the light of a recent report on liver damage in humans after occupational overexposure to DMF (12). Results of preliminary experiments suggest that the microsomal hydroxylation of DMF to *N*-(hydroxymethyl)-*N*-methylformamide is markedly increased in microsomes from rodents that have received the CYP2E1 inducer acetone.[2] Therefore, it seems probable that the metabolism of DMF, like that of NMF, is at least in part catalyzed by CYP2E1.

The occupational corollary of the findings presented here is that humans exposed to formamides run an increased risk of experiencing liver damage under conditions that may cause induction of CYP2E1, such as fasting or consumption of alcohol. When present together with alcohol, formamides can cause symptoms comparable to those precipitated by alcoholic drinks in combination with disulfiram, such as facial flushing. Alcohol incompatibility reactions have been reported in humans exposed to NMF in clinical trials (8–11) or to DMF in the work environment (57–59). It is unlikely that formamides act like disulfiram, because they do not inhibit alcohol or aldehyde dehydrogenase enzymes *in vitro* (60, 61). Instead, a formamide metabolite generated via catalysis by CYP2E1, e.g., alkyl isocyanate, might inhibit alcohol or aldehyde dehydrogenases. Alternatively, the interaction responsible for the formamide-alcohol incompatibility reaction might occur directly at the level of hepatic CYP2E1, an enzyme that contributes to the metabolic clearance of ethanol from the body. These hypotheses require experimental verification.

Acknowledgments

We wish to thank Dr. A. Strain and colleagues at the Liver Unit, Queen Elizabeth Hospital, and Dr. J. K. Chipman, School of Biochemistry (both University of Birmingham, UK), for access to human liver samples, Dr. R. Peter (Department of Pharmacology, Universität Basel, Switzerland) for samples of 5-fluoro-2(3*H*)-benzoxazolone and 6-hydroxychlorzoxazone, Dr. T. A. Baillie (School of Pharmacy, University of Washington) for stimulating discussions, Dr. A. Boobis (Royal Postgraduate Medical School, London, UK) for the initial suggestion that CYP2E1 might be involved in formamide metabolism, and Mr. G. Smith for help with the figures.

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