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AN INVESTIGATION OF THE FORMATION OF CYTOTOXIC, GENOTOXIC, PROTEIN-REACTIVE AND STABLE METABOLITES FROM NAPHTHALENE BY HUMAN LIVER MICROSOMES

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Abstract—Chemically reactive epoxide metabolites have been implicated in various forms of drug and chemical toxicity. Naphthalene, which is metabolized to a 1,2-epoxide, has been used as a model compound in this study in order to investigate the effects of perturbation of detoxication mechanisms on the *in vitro* toxicity of epoxides in the presence of human liver microsomes. Naphthalene (100 μM) was metabolized to cytotoxic, protein-reactive and stable, but not genotoxic, metabolites by human liver microsomes. The metabolism-dependent cytotoxicity and covalent binding to protein of naphthalene were significantly higher in the presence of phenobarbitone-induced mouse liver microsomes than with human liver microsomes. The ratio of *trans*-1,2-dihydrodiol to 1-naphthol was 8.6 and 0.4 with the human and the induced mouse microsomes, respectively. The metabolism-dependent toxicity of naphthalene toward human peripheral mononuclear leucocytes was not affected by the glutathione transferase μ status of the co-incubated cells. Trichloropropene oxide (TCPO; 30 μM), an epoxide hydrolase inhibitor, increased the human liver microsomal-dependent cytotoxicity (19.6 ± 0.9% vs 28.7 ± 1.0%; P = 0.02) and covalent binding to protein (1.4 ± 0.3% vs 2.8 ± 0.2%; P = 0.03) of naphthalene (100 μM), and reversed the 1,2-dihydrodiol to 1-naphthol ratio from 6.6 (without TCPO) to 2.6, 0.6 and 0.1 at TCPO concentrations of 30, 100 and 500 μM, respectively. Increasing the human liver microsomal protein concentration reduced the cytotoxicity of naphthalene, while increasing its covalent binding to protein and the formation of the 1,2-dihydrodiol metabolite. Co-incubation with glutathione (5 mM) reduced the cytotoxicity and covalent binding to protein of naphthalene by 68 and 64%, respectively. Covalent binding to protein was also inhibited by gestodene, while stable metabolite formation was reduced by gestodene (250 μM) and enoxacin (250 μM). The study demonstrates that human liver cytochrome P450 enzymes metabolize naphthalene to a cytotoxic and protein-reactive, but not genotoxic, metabolite which is probably an epoxide. This is rapidly detoxified by microsomal epoxide hydrolase, the efficiency of which can be readily determined by measurement of the ratio of the stable metabolites, naphthalene 1,2-dihydrodiol and 1-naphthol.

The cytochrome-P450 mediated metabolism of aromatic compounds can result in the formation of chemically reactive epoxide intermediates [1]. Such epoxides vary widely in their stability [2], and undergo either spontaneous isomerization to phenols [1] or further biotransformation [3], mediated by epoxide hydrolase (EH†) or glutathione-*S*-transferases (GST) to the corresponding diol or glutathione (GSH) conjugate, respectively. Alternatively, in some cases the reactive epoxides may bind covalently to cellular macromolecules and cause genotoxicity, cytotoxicity or hypersensitivity [4].

Epoxides derived from polycyclic aromatic hydrocarbons have been implicated in chemical carcinogenesis [2], while epoxides derived from clinically used drugs such as phenytoin [5] and carbamazepine [6, 7] have been implicated in hypersensitivity reactions. In general, the reasons for unique susceptibility to drug and chemical toxicity are poorly understood, although it has been postulated that an imbalance between bioactivation and detoxication may be responsible [8]. Thus, enhancement of bioactivation, i.e. by enzyme induction, has been shown to increase the toxicity associated with compounds such as bromobenzene [9]. Conversely, a recent study has indicated that a deficiency of GSH transferase μ is associated with a higher risk for lung cancer caused by cigarette smoking [10]. There is also indirect evidence which suggests that impaired EH activity is associated with anticonvulsant hypersensitivity [6, 11].

In order to investigate further the importance of the balance between bioactivation and detoxication in determining epoxide-mediated toxicity, naphthalene, a volatile aromatic hydrocarbon, which is metabolized in animals to a 1,2-epoxide [12], has

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† Abbreviations: ASC, ascorbic acid; EH, epoxide hydrolase; DMSO, dimethyl sulphoxide; GSH, glutathione; GST, glutathione-*S*-transferase; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulphonic acid; HSA, human serum albumin; MNL, mononuclear leucocytes; PB, phenobarbitone; PCR, polymerase chain reaction; SCE, sister chromatid exchange; TCPO, 1,1,1-trichloropropene-2,3-oxide.

been used as a model compound. Naphthalene-1,2-epoxide has been shown to be an obligatory intermediate in the conversion of naphthalene to 1-naphthol, 2-naphthol, *trans*-1,2-dihydroxy-1,2-dihydronaphthalene and three isomeric GSH conjugates [1]. Naphthalene is of direct toxicological interest since it causes selective toxicity affecting the Clara cells in the terminal airways of murine lung [13, 14]. In addition, naphthalene is a widespread environmental contaminant found in cigarette smoke [15] and various industrial manufacturing processes [15], and is a suspected carcinogen [16].

Previous studies investigating naphthalene toxicity have used animal microsomes prepared from various tissues to bioactivate the compound. The metabolism of naphthalene by human liver has not previously been investigated. Therefore, in this study naphthalene has been used as a model compound to investigate the effects of perturbation of detoxication mechanisms on its *in vitro* toxicity in the presence of human tissues. In addition, for comparison, we have used phenobarbitone (PB)-pretreated mice as a generating system. This has been previously used by others [6] and ourselves [11, 17] to investigate the mechanisms of anticonvulsant hypersensitivity.

MATERIALS AND METHODS

Chemicals. Naphthalene, human serum albumin (HSA, fraction V), PB, NADPH (tetrasodium salt), deoxynucleotide triphosphates, bromodeoxyuridine, phytohaemagglutinin, colcemid, aflatoxin B_1 and [^{14}C]naphthalene (sp. act. 370.3 MBq/mmol) were obtained from the Sigma Chemical Co. (Poole, U.K.). Trimethylpentane was obtained from the BDH Chemical Co. (Poole, U.K.). Naphthalene 1,2-dihydrodiol was synthesized according to the method of Platt and Oesch [18]. All solvents were of HPLC grade and were products of Fisons plc (Loughborough, U.K.). *Thermus aquaticus* (*Taq*) DNA polymerase was obtained from Perkin Elmer (Beaconsfield, U.K.). Oligonucleotide primers were purchased from ILS (London, U.K.).

Preparation of human and mouse liver microsomes. Human liver microsomes were prepared from histologically normal livers (N = 6; age range 21–56 years) obtained from kidney transplant donors as reported previously [19]. Ethical consent was obtained from the local ethical committee. Hepatic microsomes were also prepared from groups of six PB-pretreated mice (60 mg/kg body weight per day, i.p. in 0.9% w/v saline for 3 days) by the same centrifugation procedure used to obtain washed human liver microsomes. The microsomal pellets were stored at −80° until used. The microsomal protein content was determined by the method of Lowry *et al.* [20].

Isolation of peripheral blood mononuclear leucocytes (MNL). Peripheral blood MNLs were isolated from heparinised venous blood freshly drawn from healthy male volunteers (age range 22–38 years) by centrifugation through a density gradient as described previously [17].

Determination of the GST μ status using oligonucleotide-driven polymerase chain reaction (PCR) analysis. Genomic DNA was prepared from leucocytes of healthy volunteers by a standard method using Triton X-100 and proteinase K treatment.

PCR analysis was undertaken by the method of Brockmoller *et al.* [21] using oligonucleotide primers specific for the human GST μ gene. PCR was performed in a volume of 25 μL containing 50 mM KCl, 10 mM Tris–HCl pH 8.3, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, template DNA (100 ng), 0.2 mM of each deoxynucleotide triphosphate, 1 μM of each primer and 1.0 U of *Taq*-polymerase. Following denaturation of DNA at 94° for 4 min, amplification was carried out for 35 cycles as follows: denaturation for 1 min at 94°, annealing for 1.5 min at 53° and extension at 72° for 1.5 min using a Hybaid DNA thermal cycler (Teddington, U.K.). The PCR products were detected by electrophoresis on a 1.5% agarose gel. The primer sequences were as follows: 5′-CTGCCCTACTTGATTGATGGG-3′ for primer 1, 5′-CTGGATTGTAGCAGATCATGC-3′ for primer 2 and 5′-CTCCTGATTATGACAG-AAGCC-3′ for primer 3. The combination of primers 1 and 2 gave a fragment of 273 bp, while primers 2 and 3 gave a fragment of 650 bp.

Determination of the metabolism-dependent toxicity of naphthalene toward human peripheral MNL. Isolated MNLs (1×10^6) in *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulphonic acid (HEPES)-buffered balanced salt medium (1 mL) [22] were incubated with naphthalene (1–100 μM) and human or PB-induced mouse microsomes (0.5 mg protein/incubation) in the presence or absence of NADPH (1 mM) for 2 hr at 37°. Naphthalene was added in 10 μL of methanol/0.9% saline (1:1, v/v), which was non-toxic. For comparison with naphthalene, benzene (100 μM) which is also known to form an epoxide [1], was added to some incubations in the presence of human liver microsomes (0.5 mg). All incubations were carried out in quadruplicate.

With human liver microsomes, the effect of increasing the protein concentration on naphthalene cytotoxicity was assessed by increasing the concentration of microsomal protein (0.5–2 mg/incubation). In addition, in other experiments, the microsomal protein concentration was kept constant (0.5 mg) while the total protein concentration was increased by the addition of HSA (0.5 mg and 1.5 mg/incubation).

In order to characterize the cytotoxic metabolite, in some incubations, the cells were preincubated with the EH inhibitor, trichloropropene oxide (TCPO; 30 μM for 10 min). At this concentration, TCPO has previously been shown not to be directly cytotoxic [17]. In addition, in other experiments with human liver microsomes (0.5 mg/incubation), the effect of preincubation of cells with GSH (500 μM) and ascorbic acid (ASC; 0.01–2 mM), was assessed.

After 2 hr, the cells were sedimented and resuspended in drug-free medium (HEPES-buffered medium containing 5 mg/mL HSA). Incubations were continued for 16 hr at 37° and aliquots removed in order to determine cell viability by Trypan blue dye exclusion (0.2% Trypan blue) as reported previously [17].

Metabolism of naphthalene by human and mouse liver microsomes. [^{14}C]Naphthalene (100 μM,

3.7 kBq) was incubated with murine (0.5 mg protein) or human (0.5–2.0 mg protein) hepatic microsomes in HEPES-buffered medium (pH 7.4; final volume 1 mL). The reactions were initiated by the addition of NADPH (1 mM; omitted in control incubations) followed by incubation in a shaking water bath for 2 hr at 37°. Some incubations also contained TCPO (30 μM to 2 mM).

In order to determine whether metabolism of naphthalene by human liver was cytochrome P450-dependent, various compounds (25–250 μM) which are known to be cytochrome P450 enzyme inhibitors were incubated with naphthalene (100 μM) as described above except that the inhibitors were pre-incubated for 15 min with the microsomes prior to the addition of naphthalene. The compounds used were enoxacin, gestodene, sulphaphenazole and dimethyl sulphoxide (DMSO) which are known to be selective inhibitors of CYP1A [23, 24], CYP3A [25], CYP2C9 [26] and CYP2E1 [27], respectively.

The reactions were terminated by the addition of 2 mL of ice-cold methanol and the protein precipitated overnight at −20°. After centrifugation, the supernatant was removed and the polar metabolites were assayed in the methanol-water phase by radiometric HPLC. Covalent binding to the precipitated protein was measured as described below [28].

Analysis of stable metabolites by radiometric HPLC. Unchanged naphthalene was extracted from the methanol-water phase with trimethyl pentane [28] and the remaining aqueous phase analysed for the 1,2-dihydrodiol and 1-naphthol essentially as described by Buckpitt *et al.* [28]. The methanol–water phase was concentrated under a stream of nitrogen and 50-μL aliquots injected onto a C_{18} Spherisorb 5ODS column (25 cm × 0.46 cm; HPLC Technology, Macclesfield, U.K.). The metabolites were eluted with a mobile phase of 40% methanol–water for 10 min followed by a 10 min linear gradient to 70% of methanol–water at 1 mL/min using a HPLC (Spectra Physics) linked to a radiometric detector (Flo-one Beta, Canberra, Packard). Naphthalene 1,2-dihydrodiol and 1-naphthol eluted at 7 and 21 min, respectively. Radioactivity was monitored throughout the run and peaks were integrated and expressed as percentage radioactivity eluting from the column. The metabolites were identified by comparison of their retention times with those of co-injected authentic compounds.

For mass spectrometric analysis of the metabolites, microsomal incubations were set up as described above. Approximately 300 nmol of the metabolites (approximately 80% 1,2-dihydrodiol and 20% 1-naphthol) in ethanol were injected onto a silica column (75 mm × 6 mm i.d.), the metabolites being separated using petroleum ether:ethyl acetate 2:1 (v/v). The peak eluates were combined, evaporated to dryness under nitrogen and redissolved in 250 μL of ethanol, which was then analysed by EI mass spectrometry, and gave an identical spectrum to that of authentic materials (1,2-dihydrodiol: M/Z 162 ($[M]^+$; 26%), 144 (29%), 131 (15%) and 116 (86%)).

Irreversible binding of radiolabelled naphthalene to protein. Radiolabelled material irreversibly bound to precipitated protein was determined by exhaustive solvent extraction. The protein was extracted successively with methanol (5 mL × 2) and 70% (v/v) aqueous methanol (5 mL × 1), and then dissolved in 1 M NaOH (2 mL). Aliquots were taken for quantification of radioactivity and for protein estimation.

Determination of genotoxicity of naphthalene. Whole blood (0.5 mL) freshly drawn from healthy male volunteers was added to RPMI 1640 (4.5 mL) supplemented with 10% heat inactivated foetal calf serum, 2 mM L-glutamine and 50 μg/mL gentamicin. Cultures were stimulated with phytohaemagglutinin (PHA-P; 10 μg/mL) and bromodeoxyuridine (50 μM). After 48 hr, the cells were resuspended in unsupplemented RPMI, and the cultures exposed to naphthalene (100 μM) in the presence of a drug metabolizing system consisting of human liver microsomes (0.5 mg) and NADPH (0.2 mM, omitted from control). Aflatoxin B_1 (30 μM) was used as a positive genotoxin under identical conditions. After 2 hr, the cells were washed once in RPMI and then resuspended in supplemented RPMI for the remainder of the 72 hr culture. Colcemid (0.2 μg/mL) was added to the cultures 3 hr prior to the cell harvest. Methanol in acetic acid fixed metaphases were prepared by the method of Dean and Danford [29]. After ageing in the dark for 24 hr, the slides were washed, stained with Hoechst 33258 (5 μg/mL) for 20 min, rinsed and then exposed to long wavelength UV irradiation for 12 min. The slides were then incubated in 2 × sodium chloride/sodium citrate buffer (65°, 25 min), washed in deionized water and stained with 4% Giemsa for sister chromatid exchange (SCE) determination. A minimum of 2000 metaphases were scored for mitotic index analysis, 100 metaphases for proliferative index and 30 metaphases for SCE analysis from each culture.

Statistical analysis. Results of quadruplicate incubations are expressed as mean ± SEM. Statistical analysis was performed using the Kruskal–Wallis test, accepting $P < 0.05$ as significant.

RESULTS

Comparison of the metabolism of naphthalene to cytotoxic, protein-reactive and stable metabolites by human and mouse liver microsomes

Naphthalene was bioactivated to cytotoxic and protein-reactive metabolites by both human and mouse liver microsomes (0.5 mg), the cytotoxicity and covalent binding to protein being significantly higher with the PB-mouse liver microsomes than with the human liver microsomes (Fig. 1A and B).

With human liver microsomes, the major stable metabolite was the 1,2-dihydrodiol, while with PB-mouse liver microsomes, 1-naphthol was the major metabolite. The ratio of 1,2-dihydrodiol to 1-naphthol was 8.6 and 0.4 with the human and mouse microsomes, respectively (Fig. 1C).

In contrast to naphthalene, benzene (100 μM) was not bioactivated by human liver microsomes, the cell death being 7.9 ± 1.4% and 5.8 ± 0.8% in the absence and presence of NADPH, respectively.

Effect of TCPO on the metabolism of naphthalene

Previous studies with anticonvulsants [5, 17] have

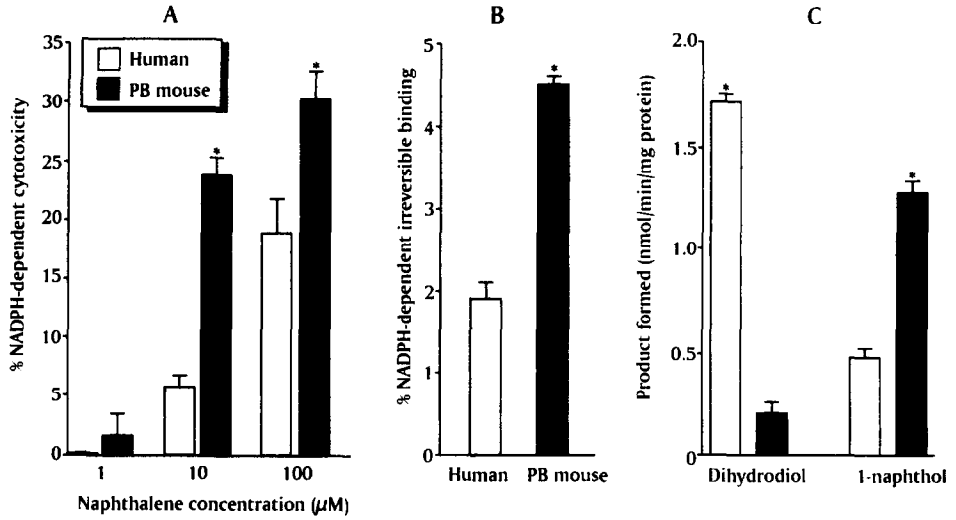

Fig. 1. The metabolism of naphthalene (100 μM) to (A) cytotoxic (towards peripheral MNLs), (B) protein-reactive and (C) stable metabolites in the presence of either pooled human liver microsomes (0.5 mg) or pooled PB-induced mouse liver microsomes (0.5 mg). The results represent the mean ± SEM of quadruplicate incubations. Statistical analysis performed by comparing the human and mouse microsomal incubations: *$P < 0.05$.

used TCPO as an inhibitor of EH. In the present study, co-incubation of MNL with TCPO at a concentration of 30 μM, which has previously been shown not to alter cell viability [17], increased the cytotoxicity of naphthalene (Fig. 2) in the presence of human liver microsomes.

To investigate further the effects of TCPO on covalent binding to protein and stable metabolite formation, several concentrations of TCPO were used. Concentrations of TCPO higher than 30 μM inhibited ($P < 0.05$) the *in vitro* metabolism of naphthalene (Fig. 3A). TCPO has previously been shown to inhibit the cytochrome P450 enzymes at high concentrations [30]. Thus, it was found that while concentrations of TCPO up to 500 μM increased ($P < 0.05$) covalent binding to protein of naphthalene (100 μM), higher concentrations reduced covalent binding to protein (Fig. 3B). TCPO reduced the amount of 1,2-dihydrodiol formed while resulting in an increase in 1-naphthol formation (Fig. 3C), such that the ratio of 1,2-dihydrodiol to 1-naphthol changed from 6.6 in the absence of TCPO to 2.6, 0.6 and 0.1 at TCPO concentrations of 30, 100 and all concentrations above 500 μM, respectively.

Effect of protein concentration on the metabolism of naphthalene

Bioactivation of naphthalene (100 μM) to a cytotoxic metabolite decreased with increasing human liver microsomal protein concentration (Table 1). The effect of preincubating cells with TCPO (30 μM) was also diminished by increasing the microsomal protein (Table 1).

To investigate this further, parallel experiments were undertaken where incubations containing microsomal protein in excess of 0.5 mg were replaced

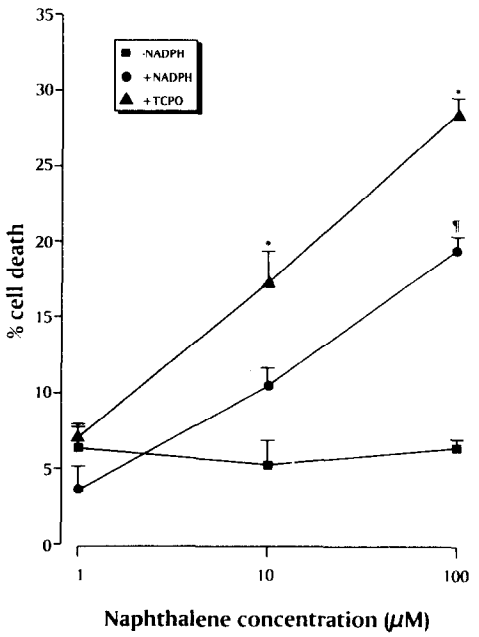

Fig. 2. The concentration-dependent toxicity of naphthalene toward peripheral MNLs in the absence or presence of NADPH (1 mM), and after incubation of the MNLs with TCPO (30 μM) in the presence of NADPH. The results represent the mean ± SEM of quadruplicate incubations. Statistical analysis performed by comparing incubations with and without NADPH: ¶$P = 0.02$, and incubations with and without TCPO in the presence of NADPH: *$P = 0.02$.

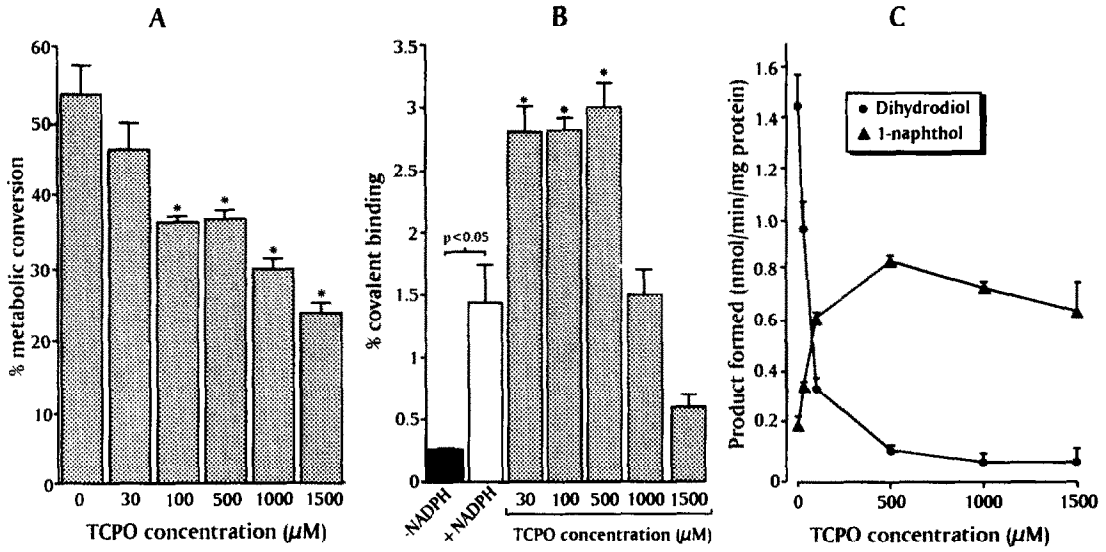

Fig. 3. The concentration-dependent effect of TCPO on (A) the metabolic turnover of naphthalene, (B) the *in vitro* covalent binding to protein of radiolabelled compound (3.7 kBq) and (C) the metabolism of naphthalene to stable metabolites. In graph B, the first two sets of incubations were performed in the absence of TCPO, while incubations with TCPO also contained NADPH (1 mM). The results represent the mean ± SEM of quadruplicate incubations. Statistical analysis performed by comparing incubations with and without TCPO (at the different concentrations) in the presence of NADPH: $^*P < 0.05$.

Table 1. The effect of increasing the human liver microsomal protein concentration on the cytotoxicity of naphthalene (100 μM) towards peripheral blood MNLs in the absence and presence of NADPH (1 mM), and after preincubation of the cells with TCPO (30 μM)

Incubations conditions	Microsomal protein concentration 0.5 mg	1.0 mg	2.0 mg
	% MNL cell death		
−NADPH	4.2 ± 1.2	4.6 ± 1.6	4.4 ± 1.1
NADPH	20.7 ± 2.2†	15.9 ± 2.0†	13.6 ± 2.8*
+TCPO	26.5 ± 2.0	19.4 ± 3.6	14.4 ± 3.6

The results represent the mean ± SEM of five separate experiments performed in quadruplicate. Statistical analysis performed by the Kruskal–Wallis test comparing (a) incubations with and without NADPH: $^*P < 0.01$, $^\dagger P < 0.005$; (b) with and without TCPO in the presence of NADPH: no significance detected; and (c) in the presence of NADPH with or without TCPO at different protein concentrations: no significant difference detected.

by an equivalent amount of HSA. In keeping with the effect of increasing microsomal protein concentration, HSA also reduced the metabolism-dependent cytotoxicity of naphthalene as well as the effect of preincubation of the cells with TCPO (Fig. 4). In contrast, the total covalent binding to protein increased from 2.34 ± 0.2% at a microsomal protein concentration of 0.5 mg to 2.97 ± 0.2% (N.S.) and 4.3 ± 0.2% ($P < 0.05$) at microsomal protein concentrations of 1.0 and 2.0 mg, respectively, while addition of equivalent amounts of HSA had little effect (data not shown). Similarly, HSA had a minor effect on 1,2-dihydrodiol formation, while increasing the microsomal protein increased the amount of 1,2-dihydrodiol formed (Fig. 5). Both the increase in microsomal protein and HSA decreased 1-naphthol formation (Fig. 5).

Effect of GSH and ASC on the metabolism of naphthalene

Preincubation of MNLs with GSH (5 mM) reduced the cytotoxicity of naphthalene (100 μM) from 11.9 ± 1.4% (mean ± SEM) to 3.8 ± 0.5% ($P < 0.05$). Covalent binding of naphthalene to microsomal protein was also reduced from 1.4 ± 0.04% to 0.5 ± 0.04% ($P < 0.05$) by GSH (5 mM). ASC had no effect on the metabolism-dependent cytotoxicity of naphthalene (9.6 ± 1.8, 14.0 ± 2.0, 14.4 ± 2.3, 12.5 ± 2.1 and 14.3 ± 0.9% at 0 μM, 1 μM, 100 μM, 1 mM and 2 mM of ASC, respectively).

Effect of cytochrome P450 *inhibitors on the metabolism of naphthalene*

Of the cytochrome P450 inhibitors used, gestodene and enoxacin inhibited stable metabolite formation (Table 2). However, only gestodene significantly inhibited the covalent binding to protein (Table 2).

Effect of naphthalene on SCE frequency in the presence of human liver microsomes

Metabolism of naphthalene by human liver enzymes had no effect on mitotic and proliferative indices, nor did it result in an increased SCE frequency in human peripheral lymphocytes in

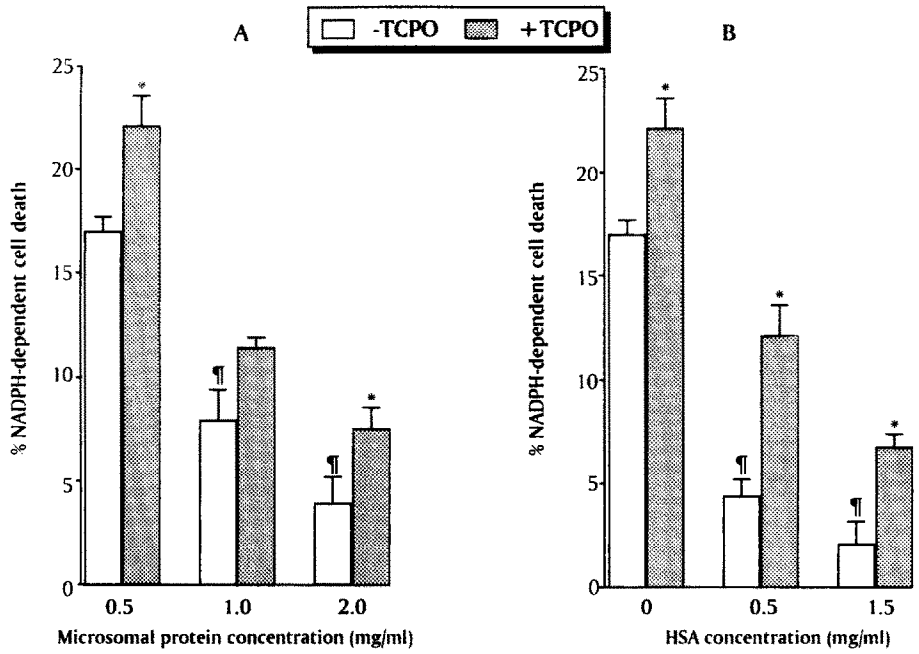

Fig. 4. The effect of protein concentration on the metabolism dependent toxicity of naphthalene (100 μM) toward peripheral MNLs with and without preincubation of the cells with TCPO (30 μM). Graph A represents the effect of increasing human liver microsomal protein on cytotoxicity. Graph B represents the effect of increasing non-microsomal protein (i.e. HSA) while keeping the microsomal protein constant at 0.5 mg/incubation. The results represent the mean ± SEM of quadruplicate incubations. Statistical analysis performed by comparing incubations with and without TCPO (30 μM): ¶$P < 0.05$, and for incubations carried out without TCPO, incubations with 0.5 mg of microsomal protein have been compared to incubations with the higher concentrations of (microsomal and non-microsomal) protein: *$P < 0.05$.

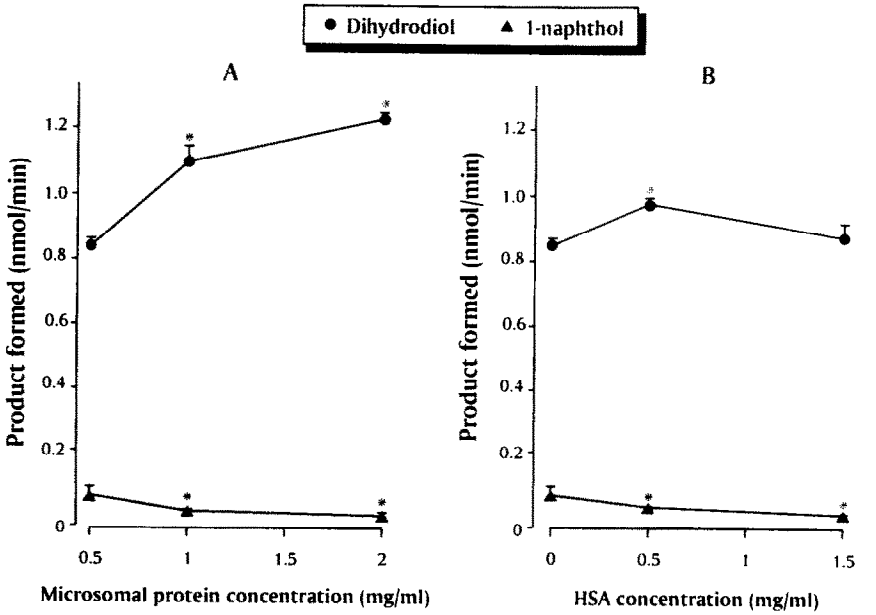

Fig. 5. The effect of increasing microsomal (A) and non-microsomal, i.e. HSA (B) protein on the metabolism of naphthalene (100 μM) to the 1,2-dihydrodiol and 1-naphthol. For experiments with HSA, the microsomal protein concentration was kept constant at 0.5 mg/incubation. The results represent the mean ± SEM of quadruplicate incubations. Statistical analysis performed by comparing the amounts of 1,2-dihydrodiol and 1-naphthol formed at the lowest protein concentration, i.e. 0.5 mg, with that formed at the higher protein concentrations: *$P < 0.05$.

Table 2. The effect of co-incubation of naphthalene (100 μM) with various cytochrome P450 inhibitors including enoxacin, gestodene, sulphaphenazole and DMSO on its *in vitro* metabolic conversion and its bioactivation to a protein-reactive metabolite in the presence of pooled human liver microsomes (0.5 mg)

Cytochrome P450 inhibitor	Concentration (μM)	% of control covalent binding to protein	% Metabolic turnover
Enoxacin	25	87 ± 6.9	63 ± 2.3
	100	76 ± 5.7	55 ± 2.1
	250	64 ± 7.8	51 ± 2.2
Gestodene	25	58 ± 3.4	55 ± 2.1
	100	66 ± 2.8	54 ± 3.3
	250	51 ± 2.0	46 ± 1.2
Sulphaphenazole	25	75 ± 4.8	62 ± 1.0
	100	76 ± 3.6	56 ± 0.8
	250	85 ± 11.3	65 ± 1.3
DMSO	25	75 ± 2.9	60 ± 1.9
	100	81 ± 4.6	63 ± 1.0
	250	75 ± 4.7	62 ± 2.4

The results represent the mean ± SEM of quadruplicate incubations. The covalent binding to protein and percentage metabolic turnover in the absence of any cytochrome P450 inhibitors (i.e. control incubations) was 3.5 ± 0.6% and 60 ± 1.5%, respectively. Statistical analysis performed by the Kruskal–Wallis test comparing incubations with and without cytochrome P450 inhibitors: *P < 0.05.

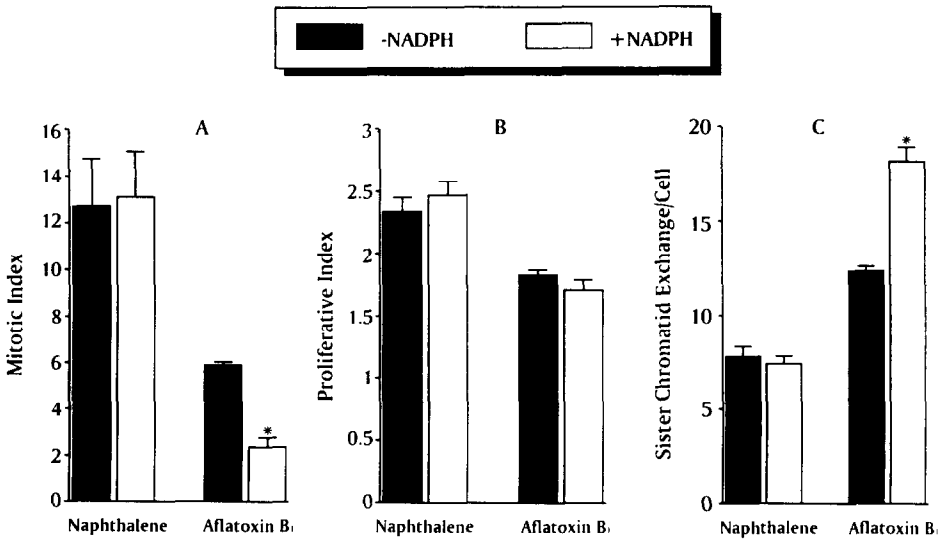

Fig. 6. The effect of naphthalene (100 μM) and aflatoxin B_1 (30 μM) on (A) mitotic index, (B) proliferative index and (C) SCE frequency in human peripheral lymphocytes in the presence of pooled human liver microsomes (0.5 mg). The values are the mean ± SEM from experiments performed with blood from five individuals. Statistical analysis performed by comparing incubations with and without NADPH: *P < 0.002.

contrast to the potent hepatocarcinogen aflatoxin B_1 (Fig. 6). Aflatoxin B_1 also resulted in a significant (P < 0.05) increase in SCE frequency (12.3 ± 0.3) compared with solvent (DMSO : methanol) controls (8.5 ± 0.5) in the absence of human liver microsomes.

GSTμ status and the metabolism-dependent cytotoxicity of naphthalene

To determine the effect of GSTμ status on the cytotoxicity of naphthalene (100 μM), individuals were typed for their GSTμ status using a genotyping assay [21]. MNLs were then isolated from six individuals who were GSTμ positive and six individuals who were negative. In both groups, the cytotoxicity of naphthalene increased with the addition of NADPH and after preincubation of cells with TCPO (30 μM; Fig. 7). However, there was no difference in either the metabolism-dependent

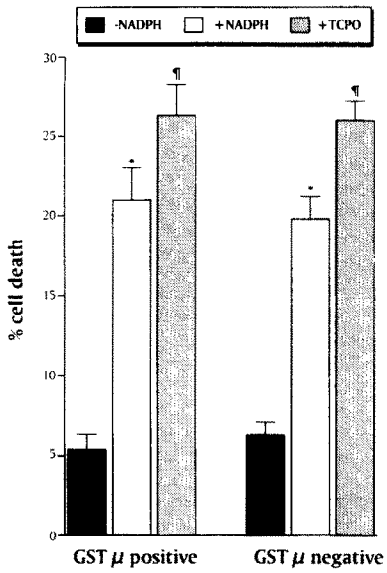

Fig. 7. A comparison of naphthalene toxicity toward peripheral MNLs in the presence and absence of NADPH (1 mM), and after preincubation with TCPO (30 μM) in the presence of pooled human liver microsomes (0.5 mg) and MNLs isolated from individuals genotyped as being either GST μ positive (N = 6) or GST μ negative (N = 6). Statistical analysis performed by comparing incubations with and without NADPH: *$P < 0.005$, and with and without TCPO in the presence of NADPH: ¶$P < 0.05$.

cytotoxicity of naphthalene or the effect of TCPO between individuals who were GSTμ positive and those who were negative.

DISCUSSION

The results of this study clearly indicate that human liver is capable of metabolizing naphthalene rapidly and efficiently to stable, protein-reactive and cytotoxic but not genotoxic metabolites. Two stable metabolites were detected, the 1,2-dihydrodiol and 1-naphthol. The former accounted for 80% of the metabolism, being a product of sequential 1,2-epoxidation and hydrolysis [12, 31], while the latter is formed by spontaneous rearrangement of the 1,2-epoxide [12, 31], which is known to have a half-life of only 3.6 min in an aqueous environment [3]. In keeping with animal studies [31–33], the metabolism of naphthalene by human liver seems to be mediated by more than one cytochrome P450 enzyme as demonstrated by the lack of complete inhibition by a series of selective P450 inhibitors.

The balance between bioactivation of naphthalene to the 1,2-epoxide and its detoxication by microsomal EH was important in determining the degree of bioactivation, as measured in our study by the two end-points, covalent binding to protein and cytotoxicity to peripheral blood mononuclear cells. Furthermore, this balance was also important in determining the ratio of the stable metabolites (1,2-dihydrodiol : 1-naphthol). Thus, addition of TCPO, an inhibitor of microsomal EH [34], reversed the metabolic profile of naphthalene in human liver microsomes (Fig. 3), in a concentration-dependent manner, indicating that >90% of the hydrolysis of the epoxide was enzyme mediated, as well as increasing the covalent binding to microsomal protein (Fig. 3B) and cytotoxicity (Fig. 2).

The role of the balance between bioactivation and detoxication in determining naphthalene toxicity was further confirmed by the use of hepatic microsomes prepared from mice pretreated with PB (Fig. 1). It is known that mouse liver contains less EH than human liver [34], while PB-induction has a greater effect on drug oxidation than epoxide hydrolysis [35]. In accordance with this, the ratio of stable metabolites (1,2-dihydrodiol : 1-naphthol) was 20-fold higher with human liver microsomes than with PB mouse microsomes (Fig. 1C), whilst cytotoxicity (Fig. 1A) and covalent binding to protein (Fig. 1B) of naphthalene were significantly higher with the mouse than with the human liver microsomes. It also provides an explanation for why the PB-induced mouse microsomes are more efficient than human microsomes in the generation of cytotoxic metabolites from the aromatic anticonvulsants [11], a group of drugs whose idiosyncratic toxicity has also been postulated to be due to the formation of reactive arene oxide metabolites [6, 11].

The protection afforded to the MNLs with the higher microsomal and non-microsomal protein concentrations (Fig. 4) can be attributed to a reduction in the amount of the epoxide reaching the target cell. However, two other factors were also important with respect to the formation of the protein-reactive and stable metabolites from naphthalene in the presence of higher protein concentrations. First, raising the microsomal protein concentration, but not HSA, led to an increase in the total amount of microsomal EH, as reflected by the increase in dihydrodiol formation (Fig. 5). Secondly, total cytochrome P450 content also increased with higher microsomal protein concentrations which was reflected in an enhanced degree of covalent binding to protein.

Using the isolated perfused mouse lung it has been demonstrated that naphthalene toxicity is due to epoxides rather than phenolic or quinone metabolites [36]. This is supported in our study by the lack of effect of ASC on cytotoxicity. Two naphthalene epoxide enantiomers can be formed by the cytochrome P450-mediated metabolism of naphthalene [37], the 1*R*,2*S*-epoxide which has been postulated to be responsible for the Clara cell toxicity in mouse lung [37], and the 1*S*,2*R*-epoxide which is the poorer substrate for EH both in the mouse [3] and the rat [31], and therefore, represents the major source of 1-naphthol formed [31]. An alternative explanation for the difference in the bioactivation of naphthalene and the ratio of stable metabolites (1,2-dihydrodiol : 1-naphthol) in the human and mouse microsomes may be due to the difference in the ratios of enantiomeric epoxides formed by the respective microsomes. However, this seems unlikely as it has been shown that mouse liver microsomes exhibit no stereoselectivity in the epoxidation of naphthalene [37]. Given that murine lung microsomes

metabolize naphthalene stereoselectively to the 1*R*,2*S*-epoxide [37], it is likely that the tissue-selective toxicity of naphthalene may be partly the result of inadequate detoxication, which is supported by the finding of Buckpitt *et al.* [28] that the 1,2-dihydrodiol:1-naphthol ratio in mouse liver was 17-fold higher than in mouse lung. With human liver microsomes, the rapid turnover of epoxide to diol precluded direct examination, by measurement of diastereoisomeric thiol conjugates, of the stereoselectivity of epoxidation, a problem which has also been encountered where primate liver has been used as human surrogate [14].

Apart from epoxide hydrolysis, GSH provides a major detoxication process for naphthalene epoxides [3]. In the present work, addition of GSH reduced covalent binding to protein and cytotoxicity. Therefore, apart from EH, the GSH transferases may be important in the detoxication of naphthalene epoxides. GSTμ is an enzyme which is polymorphically expressed in humans being absent in 50% of the population [38]. Since GSTμ is known to metabolize certain epoxides [39], we examined the functional importance of this enzyme in the detoxication of naphthalene epoxide by using cells, in the lymphocyte toxicity assay, from individuals who had been genotyped for GSTμ. However, our results indicate the lack of importance of GSTμ in the detoxication of naphthalene epoxide with no difference being observed either in the absence or presence of the EH inhibitor TCPO (Fig. 7).

The nature and severity of the toxicity mediated by epoxides is in part dependent on its biochemical and chemical stability [1]. Thus, naphthalene epoxide which is significantly more stable than benzene oxide [1, 40], was able to diffuse away from its site of formation in the microsomes and cause selective damage to lymphocytes, while benzene was not cytotoxic. In contrast to the cytotoxicity, however, naphthalene was not genotoxic as assessed by SCE induction (Fig. 6), under conditions in which aflatoxin B_1 [41], a known hepatocarcinogen was bioactivated, both in the absence and presence of a hepatic drug metabolizing system (Fig. 6). To the best of our knowledge, this is the first study to use both human liver microsomes and human peripheral lymphocytes to assess the genotoxicity of naphthalene. Thus, our results indicate that naphthalene is unlikely to be a genotoxin in humans. This is further strengthened by the fact that under very similar conditions, we were able to demonstrate bioactivation of naphthalene to cytotoxic and protein-reactive metabolites.

In conclusion, our studies indicate that human liver is capable of metabolizing naphthalene to cytotoxic and protein-reactive, but not genotoxic, metabolites which we believe to be an epoxide(s). If this is not rapidly detoxified by microsomal EH, it can bind to microsomal proteins and/or damage cell membranes. Naphthalene epoxide represents a useful substrate for investigating the functional and toxicological properties of EH since it is neither too unstable to prevent detection nor so stable as to be non-toxic. An added advantage is that its detoxication can be monitored by measurement of the ratio of the stable metabolites, naphthalene 1,2-dihydrodiol and the product of spontaneous rearrangement, 1-naphthol.

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