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Key terms: benzo(a)pyrene metabololism; enzymatic activity; induction; inhalation; methyl ethyl ketone; methylchloroform; microsomal cytochrome P-450; n-hexane; organic solvent; rat; rat liver; rat liver microsomal cytochrome P-450; steroid metabolism; xylene

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Changes in rat liver microsomal cytochrome P-450 and enzymatic activities after the inhalation of n-hexane, xylene, methyl ethyl ketone and methylchloroform for four weeks

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TOFTGARD R, NILSEN OG, GUSTAFSSON J-Å. Changes in rat liver microsomal cytochrome P-450 and enzymatic activities after the inhalation of n-hexane, xylene, methyl ethyl ketone and methylchloroform for four weeks. Scand j work environ health 7 (1981) 31—37. Groups of Sprague-Dawley rats were exposed, by inhalation, to n-hexane (900 ppm, 3,240 mg/m3), xylene (600 ppm, 2,625 mg/m3), methyl ethyl ketone (800 ppm, 2,345 mg/m3) and methylchloroform (800 ppm, 4,345 mg/m3) for four weeks. Increased liver weights and liver to body weight ratios were observed for all the solvents except n-hexane. An increased in vitro formation of certain metabolites of all the investigated substrates was found only in the rats exposed to xylene. The in vitro microsomal metabolism of biphenyl, benzo(a)pyrene, 4-androstene-3,17-dione and 5α-androstane-3α,17β-diol in combination with sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that n-hexane was without effect on rat liver microsomal cytochrome P-450 and that methyl ethyl ketone and methylchloroform depressed the formation of two metabolites of androstenedione but did not alter the concentration of cytochrome P-450 under the experimental conditions used. Xylene was shown to be a phenobarbital-like inducer of rat liver microsomal cytochrome P-450.

Key terms: benzo(a)pyrene metabolism, induction, organic solvents, steroid metabolism.

Organic solvents are of great interest in the field of occupational health because of their frequent use in different types of industries.

It is now evident that effects such as hepatotoxicity and carcinogenicity are the results of metabolic activation occurring in the body. This phenomenon has been shown for the hepatotoxicity and carcinogenicity of carbon tetrachloride, chloroform, and trichloroethylene (2, 18, 30), the carcinogenicity of dioxane (33), the hepatotoxicity of carbon disulfide (5, 6, 17), the leukemogenic effect of benzene (25), and the neurotoxicity of n-hexane and methyl n-butyl ketone (4, 27).

The enzyme system responsible for such activation through the formation of epoxides or other reactive metabolites is, in most cases, the one dependent on liver microsomal cytochrome P-450. The activity of this enzyme system, which consists of separate isoenzymes with different substrate specificities (12, 31), can be modulated by exogenous factors, including exposure to organic solvents. This modula-
tion may lead to an altered susceptibility to the toxic effects of the solvent itself or to other environmental contaminants. An inducing effect on liver microsomal cytochrome P-450 in rats has been shown after the inhalation of methylchloroform, benzene, carbon tetrachloride, and trichloroethylene (10, 20). On the other hand, both methylchloroform and carbon tetrachloride have been reported to decrease the liver microsomal content of cytochrome P-450 when administered intragastrically (28, 32), and this finding underlines the importance of using relevant administration routes in the evaluation of the biological effects of hydrocarbon solvents.

The present study was undertaken to investigate the effect of four commonly used hydrocarbon solvents with widely different chemical structures, n-hexane, xylene, methyl ethyl ketone and methylchloroform, on the liver microsomal cytochrome P-450 enzyme system in the rat after inhalation. Changes in the different forms of cytochrome P-450 and in the in vitro microsomal metabolism of biphenyl, benzo(a)pyrene, and the steroids 4-androstene-3,17-dione and 5α-androstane-3α,17β-diol were investigated.

Material and methods

Animals and experimental design

Male Sprague-Dawley rats weighing about 300 g were obtained from Anticimex (Sweden). The rats were kept in cages 5 d prior to treatment. They had free access to water and food and were kept in a room with controlled temperature and light (14 h light — 10 h dark).

Groups of four rats were exposed during the light period of the day to solvent vapors [n-hexane 910 ppm ± 240 (± SD) (3,276 ± 864 mg/m³) and xylene 630 ppm ± 170 (± SD) (2,756 ± 744 mg/m³) in one experiment and methyl ethyl ketone 760 ppm ± 200 (± SD) (2,229 ± 587 mg/m³) and methylchloroform 820 ppm ± 130 (± SD) (4,451 ± 706 mg/m³) in another experiment] 6 h each day, 5 d/week, during four weeks. Control groups were exposed to circulating air only. The animals were killed by decapitation on the morning of the day after the last exposure. No food or drinking water was offered during the exposures. Only water was allowed during the 24 h preceding sacrifice.

Inhalation exposure

The rats were exposed in a glass desiccator fitted with inlet and outlet tubing. The volume of the desiccator was 21 l, and an air flow of 8 l·min⁻¹ was maintained during the exposure. The desired composition of the exposure atmosphere was obtained by the mixing of measured portions of air and saturated solvent vapor. Every 2 h the exposure level was monitored from 0.2-ml air samples taken in the animal's breathing zone with a prewarmed gas-tight syringe. These samples were injected into a gas chromatograph (Varian Aerograph Series 1400). A calibration curve was constructed with the use of gas standards containing known concentrations of the solvent.

Preparation of liver microsomes

Rat liver microsomes were prepared as previously described (31). The microsomes were suspended and diluted in a 0.05 M potassium buffer, pH 7.4, containing 10⁻⁴ M EDTA (ethylenediaminetetraacetate) to a final concentration of about 30 mg of microsomal protein per milliliter. The protein concentration was determined by the method of Lowry (16), bovine serum albumin being used as the standard.

Microsomal enzyme assays

All enzyme assays were performed with freshly prepared microsomes. The total concentration of cytochrome P-450 was determined from the reduced carbon monoxide difference spectrum (21).

The assays of the biphenyl and the benzo(a)pyrene hydroxylase activities were performed as described elsewhere (31). In the high-pressure liquid chromatographic
separation of benzo(a)pyrene metabolites, phenol fraction I and phenol fraction II were eluted with the same retention times as 9-hydroxy- and 3-hydroxy-benzo(a)pyrene. Incubations with 4- (4-14C)androstene-3,17-dione and 5a(4-14C)androstane-3a,17β-diol were performed as described earlier (3, 7).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The liver microsomes were diluted to a concentration of 1 mg of protein per milliliter in 0.05 M sodium phosphate buffer, pH 7.4, containing 10⁻⁴ M EDTA. The microsomal suspensions were treated with sodium dodecyl sulfate (SDS) [150 mg/mg of protein (0.5 mmol/mg of protein)] and β-mercaptoethanol [75 mg/mg of protein (1.0 mmol/mg of protein)]. Electrophoresis was performed on slab gels (model 220 Dual Vertical Slab Gel Electrophoresis Cell, BIO-RAD Laboratories, USA) with the use of the discontinuous buffer system of Laemmli (14). The slab gels contained 7.5 % acrylamide in the separating gel and had the dimensions 140 mm (migration distance) × 100 mm × 0.75 mm (thickness). Twenty sample wells were loaded with 5 µg of protein each; 5 mA was applied per gel during stacking and 10 mA per gel during separation. As judged from the mobilities of the standard proteins phosphorylase B (mol wt 94,000), bovine serum albumin (mol wt 68,000), ovalbumin (mol wt 45,000), and carbonic anhydrase (mol wt 30,000), the resolution obtained during electrophoresis was sufficient to distinguish differences of 500 in the apparent molecular weights of the different types of liver microsomal cytochrome P-450. The gels were stained for protein at 60°C with 0.2% Coomassie brilliant blue R-250 in water:acetic acid (5:1) for 20 min and destained overnight at the same temperature in acetic acid:ethanol:water (1.5:1:17.5).

The qualitative identification, based on heme staining, of four different protein bands, induced by either phenobarbital or 3-methylcholanthrene, as cytochrome P-450s (RLvMc P-450₉ₑ, RLvMc P-450₅ₒ, RLvMc P-450₅₃, and RLvMc P-450₉₃) has been described elsewhere (31). Relative quantitation of the amount of protein in the different bands was performed by densitometric scanning on a Beckman scanning densitometer model R-112 at 500 nm after the gels had been stained with Coomassie brilliant blue R-250. A linear relationship between peak area and the amount of protein applied has been reported earlier (19).

Statistics

Student’s t-test was used, and p-values of less than 0.05 were considered significant.

Chemicals

The following chemicals were purchased: 2-hydroxybiphenyl, 4-hydroxybiphenyl, benzo(a)pyrene, and 5a-androstane-3a,17β-diol from the Sigma Chemical Co, St Louis, LA, USA; ethyl methyl ketone analytical grade (ag) and n-hexane (ag) from E Merck, Darmstadt, Federal Republic of Germany; xylene from Aristar, BDH, Chemicals Ltd, Poole, England; 1,1,1-trichloroethane from Fisher Scientific Co, Fair Lawn, NJ, USA; bovine serum albumin (Fr, V) from Miles Laboratories Ltd, Stokes Poges, England; biphenyl from Merck-Schuchardt, München, Federal Republic of Germany; 3-hydroxybiphenyl from ICN Pharmaceuticals Inc, Plainview, NY, USA; [G-3H]benzo(a)pyrene (specific activity 26 Ci per mmol) and 4-(4-14C)androstene-3,17-dione (specific activity 0.06 Ci per mmol) from Radiochemical Centre, Amersham, England; and reference benzo(a)pyrene metabolites from IIT Research Institute, Chicago, IL, USA. The purity of the organic solvents was determined by gas chromatography. Xylene contained 23 % ethylbenzene, 2 % o-xylene, 64.5 % m-xylene, 10 % p-xylene, and 0.5 % toluene. The level of benzene contamination was 4 ppm; 11 ppm of 1,1,2-trichloroethane was found in the methylchloroform.

Results

Of the organic solvents investigated, only xylene significantly impaired the growth of the rats during the four weeks’ exposure [35 ± 3 g (± SD) increase against 61 ± 2 g for the control group].

The effects on liver weight and the total concentration of liver microsomal cyto-
chrome P-450, while there was no increase for the other solvents.

The SDS-polyacrylamide gel electrophoresis of liver microsomes revealed no significant changes in the different forms of cytochrome P-450 after exposure to n-hexane, methyl ethyl ketone or methyl-

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**Table 1. Effects of n-hexane, xylene, methyl ethyl ketone, and methylchloroform inhalation on rat liver — Mean ± SD.**

| Substance | Liver weight (g) | Liver weight nmoles P-450 | Body weigh mg microsomal protein |
|-----------|-----------------|--------------------------|---------------------------------|
| n-Hexane  | 11.3 ± 0.8      | 0.028 ± 0.0007           | 0.57 ± 0.13                     |
| Xylene    | 12.2 ± 0.4      | 0.031 ± 0.0005           | 0.70 ± 0.05                     |
| Control   | 11.2 ± 0.1      | 0.027 ± 0.0004           | 0.59 ± 0.12                     |
| Methyl ethyl ketone | 11.5 ± 0.5 | 0.029 ± 0.0008 | 0.63 ± 0.05                     |
| Methylchloroform | 11.7 ± 0.7 | 0.029 ± 0.0014 | 0.56 ± 0.07                     |
| Control   | 10.2 ± 0.5      | 0.025 ± 0.0005           | 0.59 ± 0.04                     |

* The experimental rats were exposed to 900 ppm of n-hexane (3,240 mg/m³), 600 ppm of xylene (2,675 mg/m³), 800 ppm of methyl ethyl ketone (2,345 mg/m³) or 800 ppm of methylchloroform (4,345 mg/m³) for 6 h a day, 5 d a week, during four weeks. The controls were exposed to circulating air only.

**Table 2. Hydroxylation of biphenyl and benzo(a)pyrene in rat liver microsomes after exposure to n-hexane, xylene, methyl ethyl ketone and methylchloroform — Mean ± SD.**

| Substance | Biphenyl (pmol meta-bolite·mg protein⁻¹·min⁻¹) | Benzo(a)pyrene a (pmol metabolite·mg protein⁻¹·min⁻¹) | 9,10-Diol | 4,5-Diol | 7,8-Diol | Phenol I | Phenol II | Quinones |
|-----------|-----------------------------------------------|---------------------------------------------------|-----------|----------|----------|----------|----------|----------|
| n-Hexane  | 66 ± 7                                        | < 30                                              | 1,210± 210 | 25± 4    | 18± 8    | 25± 11   | 335± 59  | 30± 6    |
| Xylene    | 96 ± 6                                        | < 30                                              | 1,380± 50* | 25± 4    | 18± 8    | 25± 11   | 335± 59  | 30± 6    |
| Control   | 53 ± 10                                       | < 30                                              | 1,050± 260 | 25± 4    | 18± 8    | 25± 11   | 335± 59  | 30± 6    |
| Methyl ethyl ketone | 30 ± 5  | < 30                              | 1,030± 165 | 25± 4    | 18± 8    | 25± 11   | 335± 59  | 30± 6    |
| Methylchloroform | 32 ± 6 | < 30                             | 760± 155 * | 25± 4    | 18± 8    | 25± 11   | 335± 59  | 30± 6    |
| Control   | 32 ± 6                                        | < 30                                              | 760± 155  | 25± 4    | 18± 8    | 25± 11   | 335± 59  | 30± 6    |

* The experimental rats were exposed to 900 ppm of n-hexane (3,240 mg/m³), 600 ppm of xylene (2,675 mg/m³), 800 ppm of methyl ethyl ketone (2,345 mg/m³) or 800 ppm of methylchloroform (4,345 mg/m³) for 6 h a day, 5 d a week, during four weeks. The controls were exposed to circulating air only.

**Table 3. Metabolism of 4-androstene-3,17-dione and 5α-androstane-3α,17β-diol in rat liver microsomes after exposure to n-hexane, xylene, methyl ethyl ketone and methylchloroform — Mean ± SD.**

| Substance | 4-androstene-3,17-dione (nmol metabolite·mg protein⁻¹·min⁻¹) | 5α-androstane-3α,17β-diol (nmol metabolite·mg protein⁻¹·min⁻¹) |
|-----------|-------------------------------------------------------------|-------------------------------------------------------------|
| n-Hexane  | 0.22±0.03, 1.40±0.10, 0.60±0.11                            | 0.71±0.05, 0.26±0.05, 0.25±0.04, 0.12±0.08, 0.30±0.01 |
| Xylene    | 0.26±0.07, 1.42±0.19, 1.33±0.18                              | 0.54±0.03, 0.22±0.05, 0.24±0.02, 0.13±0.01, 0.35±0.01 |
| Control   | 0.20±0.03, 1.22±0.18, 0.84±0.07                              | 0.83±0.11, 0.22±0.04, 0.27±0.03, 0.11±0.01, 0.30±0.02 |
| Methyl ethyl ketone | 0.24±0.01, 1.08±0.06, 0.68±0.03 | 0.90±0.18, 0.45±0.09, 0.36±0.06, 0.14±0.02, 0.38±0.07 |
| Methylchloroform | 0.18±0.01, 0.82±0.13, 0.45±0.03 | 0.75±0.03, 0.28±0.06, 0.30±0.02, 0.10±0.02, 0.29±0.06 |
| Control   | 0.17±0.03, 1.32±0.19, 0.85±0.10                              | 0.78±0.12, 0.28±0.06, 0.36±0.03, 0.14±0.02, 0.34±0.01 |

* The experimental rats were exposed to 900 ppm of n-hexane (3,240 mg/m³), 600 ppm of xylene (2,675 mg/m³), 800 ppm of methyl ethyl ketone (2,345 mg/m³) or 800 ppm of methylchloroform (4,345 mg/m³) for 6 h a day, 5 d a week, during four weeks. The controls were exposed to circulating air only.
chloroform, while increases in two forms were noted after exposure to xylene. Relative quantitation of these forms by densitometric scanning revealed a 100% increase in RLvMc P-450 and a 35% increase in RLvMc P-450 ($\alpha$).

Following exposure to xylene, significant increases were noted in the in vitro liver microsomal metabolism of biphenyl to 2- and 4-hydroxybiphenyl (table 2). n-Hexane and methyl ethyl ketone had no significant effects, while methylchloroform reduced the formation of 2- and 4-hydroxybiphenyl. The formation of 4,5-dihydroxy-4,5-dihydrobenzo(a)pyrene from benzo(a)pyrene was selectively increased five times after exposure to xylene, and the formation of 9,10-dihydroxy-9,10-dihydrobenzo(a)pyrene was increased by 50% (table 2). Exposure to methyl ethyl ketone and methyl chloroform significantly decreased the formation of both 9,10-dihydroxy-9,10-dihydrobenzo(a)pyrene and phenols in phenol fraction II. Exposure to n-hexane did not affect the liver microsomal metabolism of benzo(a)pyrene.

The effects of solvent exposure on the liver microsomal metabolism of 4-androstene-3,17-dione and 5a-androstane-3a, 17β-diol are shown in table 3. The formation of 16-hydroxylated androstenedione metabolites increased 60% following exposure to xylene. This phenomenon was mainly due to an increased formation of 16-ketotestosterone, as demonstrated by gas chromatography/mass spectrometry. Exposure to methyl ethyl ketone increased the formation of 7α-hydroxyandrostenedione but decreased the formation of both 6β-hydroxyandrostenedione and 16-hydroxyandrostenedione. Exposure to methyl chloroform also decreased the formation of the two latter metabolites. No effects were observed following the exposure to n-hexane.

Exposure to xylene increased the in vitro liver microsomal formation of 5a-androstane-3a,7β,17β-triol and 5α-androstane-3a,17β,18-triol and decreased the formation of 5α-androstane-2α,3a,17β-triol from 5α-androstane-3a,17β-diol. Methyl ethyl ketone increased the formation of 5α-androstane-2β,3α,17β-triol, while methyl chloroform reduced the formation of 5α-androstane-3a,7β,17β-triol. No effects were observed following the exposure to n-hexane.

Discussion

The results of this study and another recent study by Savolainen et al (26) imply that aromatic hydrocarbon solvents such as xylene are potential inducing agents for cytochrome P-450 and cytochrome P-450-dependent reactions in the liver. This assumption is also supported by the similar effects of toluene on cytochrome P-450 (results to be published). Methyl ethyl ketone and methyl chloroform do not seem to influence the total amount of liver microsomal cytochrome P-450 significantly, but they tend to depress some cytochrome P-450 dependent reactions. Although n-hexane did not influence either the liver microsomal cytochrome P-450 or any of the cytochrome P-450-mediated reactions investigated, an induction of cytochrome P-450 has been reported after the exposure of mice to a higher dose during a short period of time (13). Similar results have also been reported with respect to the effects of methyl chloroform on rats (10). These findings may suggest a dose-dependent influence of n-hexane and methyl chloroform on cytochrome P-450, and they indicate the importance of the length of exposure. The inducing capacity of xylene may partly be due to the large uptake of xylene in comparison to the uptake of methylchloroform and aliphatic solvents (1, 29).

SDS-polyacrylamide gel electrophoresis revealed that xylene induced RLvMc P-450 and RLvMc P-450($\alpha$), which are the same cytochrome P-450 forms that are induced to the greatest extent by phenobarbital (31). Furthermore, both xylene and phenobarbital increased the formation of 2- and 4-hydroxybiphenyl. Phenobarbital increased the formation of 4,5-dihydroxy-4,5-dihydrobenzo(a)pyrene more than tenfold (31), while xylene increased the formation of this metabolite fivefold. The metabolism of androstenedione and androstanediol was also affected in a similar manner following exposure to xylene or phenobarbital. These data strongly indicate that xylene is a phenobarbital-like inducer of liver microsomal cyto-
chrome P-450 in the rat. Methyl ethyl ketone, methylchloroform, and n-hexane did not cause any detectable induction either in the total amount of liver microsomal cytochrome P-450 or in the multiple forms, as demonstrated by SDS-polyacrylamide gel electrophoresis. No increases were detected in the formation of the different metabolites of biphenyl or benzo(a)pyrene. However, methyl ethyl ketone and methylchloroform reduced the metabolism of the two mentioned substrates to some extent.

Since cytochrome P-450 metabolizes endogenous substrates such as steroids, an induction of liver microsomal cytochrome P-450 may cause endocrine disturbances (22, 24). Whether the increased in vitro formation of 16-hydroxy-androstenedione after xylene exposure or the decreased formation of the same metabolite after methyl ethyl ketone and methylchloroform exposure are relevant for the in vivo situation remains to be established. It may be noted that reproduction disturbances have been reported in men exposed to organic solvents (8).

The induction of hepatic cytochrome P-450 by xylene has several toxicological implications. An increased metabolism of xylene itself with an increased formation of p-tolualdehyde from p-xylene can lead to the destruction of lung microsomal cytochrome P-450 after transport of the metabolite to the lung, as proposed by Patel et al (23). The increased formation of 4,5-dihydroxy-4,5-dihydrobenzo(a)pyrene implies an accelerated formation of the mutagenic 4,5-epoxy-4,5-dihydrobenzo(a)pyrene (15). The toxicological importance of this metabolite may be questionable, however, in view of recent reports on the efficient deactivation of this compound by epoxide hydrase and glutathione-S-transferase (11, 34). In contrast further metabolism of 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene can lead to the formation of the very potent carcinogen and mutagen 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (15). When animals given phenobarbital are exposed to n-hexane, an increased formation of 2-hexanol occurs (9) and probably leads to an accelerated production of the neurotoxic metabolite 2,5-hexanediene.

The hepatotoxicity of trichloroethylene and the covalent binding of trichloroethylene metabolites to DNA (deoxyribonucleic acid) increase after pretreatment with phenobarbital (2). Since xylene is shown to be a phenobarbital-like inducer, it is reasonable to assume that synergistic toxic effects may occur upon simultaneous exposure to xylene and other solvents.

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