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THE EFFECTS OF CIGARETTE SMOKE COMPARED TO 3-METHYLCHOLANTHRENE AND PHENOBARBITONE ON ALKOXYRESORUFIN METABOLISM BY LUNG AND LIVER MICROSOMES FROM RATS

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Abstract—The rates of metabolism of phenoxazone and a homologous series of its ethers (alkoxyresorufins) by liver and lung microsomes of rats exposed to cigarette smoke were compared with the metabolism in rats pretreated with 3-methylcholanthrene (3MC) or phenobarbitone (PB). The rate of resorufin production was dependent on the length of the ether side chain. Liver and lung microsomes from control rats differed in their activity profiles (rate of resorufin production plotted against side-chain length), showing highest activity with ethoxy- and benzyloxyresorufin respectively. 3MC and PB selectively induced hepatic microsomal resorufin production with only certain of the substrates and the two agents differed in their selectivity, inducing most greatly with ethoxy- and benzyloxyresorufin respectively. Pulmonary microsomal resorufin production was induced by 3MC with a substrate selectivity similar to that shown for liver, but PB suppressed pulmonary metabolism with all the substrates. A single, short exposure to cigarette smoke induced ethoxyresorufin O-deethylase activity transiently in liver and lung microsomes. Three consecutive daily short exposures to cigarette smoke caused a weak 3MC-like induction of liver microsomal alkoxyresorufin metabolism, but the effect on lung microsomes was like weak 3MC and PB inductions combined. It is concluded that cigarette smoke induces selected cytochrome P-450-linked alkoxyresorufin *O*-dealkylase activities to a similar extent in both lung and liver and that the effects of cigarette smoke are characteristic of both 3MC-type and non-3MC-type inducers.

Drugs and other xenobiotics, such as pesticides, food additives and industrial chemicals, are activated and detoxified by a group of cytochrome P-450-linked enzymes, the microsomal monooxygenases. Many different forms of constitutive and inducible cyt. P-450 are present in mammalian hepatic and extrahepatic tissues, with several of the forms showing distinctive, although often not unique, substrate and reaction specificities [1, 2]. It is now well established that pretreatment of rats with phenobarbitone (PB)¶ or 3-methylcholanthrene (3MC) respectively induces the synthesis of two different families of cytochrome P-450 [3].

One of the monooxygenase inducers that most commonly affects humans is cigarette smoke [4, 5]. The polycyclic aromatic hydrocarbons (PAH) in cigarette smoke are believed to make a major contribution to the inducing effect [5]. It is, therefore, not surprising that a form of cyt. P-450 analogous to a form induced in rats by 3MC, together with two monooxygenase activities that are selectively induced in rats by 3MC (aryl hydrocarbon hydroxylase, AHH, and ethoxyresorufin *O*-deethylase, EROD), are significantly higher in liver and placenta of smokers than of non-smokers [6–13]. Mechanistic studies of cigarette smoke induction require an animal model. Cigarette smoke induces pulmonary AHH and EROD in mice [14] and pulmonary AHH in rats [15, 16]. It is not certain, however, whether the changes induced in monooxygenase activities by cigarette smoke are the same as the changes induced by 3MC, nor are the relative extents of induction in lung and liver clear. Accordingly, we have studied the effects of cigarette smoke in rats on the pulmonary and hepatic microsomal metabolism of a homologous series of alkoxyresorufins, which act as probes for several different types of cyt. P-450 induction [17].

MATERIALS AND METHODS

Chemicals. The synthesis of phenoxazone, and of alkoxyresorufins from the sodium salt of resorufin (Molecular Probes Inc., 24750, Lawrence Road, Junction City, OR 97448, U.S.A.) and the appropriate alkyl iodide, were as described previously

‡ The mention of a proprietary product does not constitute an endorsement by the U.S. Department of Agriculture.

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¶ Abbreviations used: AHH, aryl hydrocarbon hydroxylase; DMSO, dimethylsulphoxide; EROD, ethoxyresorufin *O*-deethylase; 3MC, 3-methylcholanthrene; PAH, polycyclic aromatic hydrocarbon; PB, phenobarbitone; TLC, thin layer chromatography.

[18, 19]. Their structures were confirmed by NMR and mass spectroscopy. Resorufin, phenoxazone and the alkoxyresorufins were dissolved in dimethylsulphoxide (DMSO) to provide 1 mM stock solutions, which were stored in the dark for up to 1 year without degradation. Before performing the studies described below the purity of the alkoxyresorufins was checked by thin-layer chromatography (TLC) on silica gel plates (Merck; 20 cm × 20 cm × 0.25 mm; aluminium-backed) developed with benzene–acetone (3/1; v/v). The purity of resorufin was checked by TLC as described and by gas chromatography (2 m × 1.5 mm column packed with 3% UCON HB2000 and 6% KOH on Chromosorb W-AW, 80–100 mesh) using helium as carrier gas (30 ml/min), an oven temperature of 150° and an NPD detector (Packard, model 427). PB was purchased from BDH Chemicals Ltd. (Poole, Dorset, U.K.) and 3MC, NADPH and bovine serum albumin were obtained from the Sigma Chemical Co. (Poole, Dorset, U.K.). All chemicals were of the highest purity available. Standard commercial cigarettes were made up of a rod of flue-cured tobacco and a ventilated cellulose acetate filter; the cigarettes were designed to yield 10 mg total particulate matter in 9 puffs.

Animals. Male Wistar rats, weighing either 250–300 g (Study 1) or 350–400 g (Study 2) were supplied by Charles River Laboratories (Margate, Kent, U.K.). Food (CRM[X] pellets, Labsure, Poole, U.K.) and water were available *ad libitum*, except during periods of cigarette smoke exposure or sham exposure. For the exposure of rats to cigarette smoke a BAT-Mason inhalation system was used, which releases a precisely-calibrated dilution of cigarette smoke into a nose-only inhalation chamber [16, 20]. The smoke was generated by drawing 35 ml puffs of 2-sec duration into a dilution chamber, where it was diluted with air to give a chamber concentration of 6 mg total particulate matter per litre. The rats were exposed to diluted fresh smoke for 9 min per day. Control rats were kept in identical conditions and exposed for comparable time periods to air only, to simulate the stress experienced under restraint. In Study 1, rats were given either a single cigarette smoke exposure or a corresponding sham (air) exposure (tube controls). In Study 2, rats were given, once daily, i.p., for 3 days, 3MC (20 mg/kg in corn oil) or PB (80 mg/kg in 0.9% sodium chloride) or corresponding corn oil or saline vehicle control injections; a fifth group of rats was exposed to tobacco smoke once a day for three consecutive days.

Preparation of microsomes. Animals were killed by cervical dislocation at selected times after smoke exposure (Study 1) or 24 hr after the last treatment (Study 2). Livers and lungs were excised, washed, weighed and placed in beakers containing ice-cold 1.15% KCl (w/v). The liver microsomes were prepared essentially as described by Ioannides and Parke [21], except that the microsomal pellets were resuspended in 1.15% KCl (w/v) and centrifuged a second time at 105,000 *g* for 60 min. The microsomal pellets were then resuspended in glycerol–EDTA–phosphate buffer (20% glycerol, 1 mM EDTA, 50 mM potassium phosphate, pH 7.4) to give a protein concentration of 10–12 mg protein/ml and were kept at −80°. Lung has a tendency to float and its high connective tissue content impairs homogenisation with a Potter-Elvejhem homogenizer. This problem was overcome by subjecting the scissor-minced lung to an Ultra-Turax homogenizer (International Laboratory Appliances, Type X1020) for 3–5 sec at approximately one third of maximum speed, prior to homogenization with a Potter-Elvejhem homogenizer (4–5 slow up and down strokes). Microsomes were prepared as described for liver, resuspended in the glycerol–EDTA–phosphate buffer at 4–6 mg protein/ml and stored at −80° without detriment to the activities measured (storage effect data not shown).

Assays. The 7-*O*-dealkylation of the alkoxyresorufins and the 7-hydroxylation of phenoxazone were measured fluorimetrically by the production of resorufin, according to the method of Burke and Mayer [19], using a Perkin-Elmer spectrofluorimeter (model MPF-3) with excitation and emission wavelengths at 510 nm and 586 nm respectively and excitation and emission slit widths both 6 nm. The reaction was carried out at 37° in a 10 mm pathlength fluorimeter cuvette containing 2 ml of 100 mM potassium–phosphate buffer (pH 7.6), 0.1–0.2 mg of microsomal protein, 10 μl of phenoxazone or alkoxyresorufin (1 mM in DMSO) and 10 μl of NADPH (50 mM). Reaction conditions were always chosen to ensure initial reaction rates of at least 2 min duration and a linear proportionality between reaction rate and microsomal protein concentration over the range used. The spectrofluorimeter was calibrated intermittently by adding 10 μl of authentic resorufin (10 μM in DMSO) to 2.03 ml of 10 mM potassium phosphate buffer (pH 7.6) plus 0.1 mg of microsomal protein. Microsomal protein content was measured by the method of Lowry *et al.* [22], using bovine serum albumin as standard.

Statistics. Results for control and treated rats were compared by Student's *t*-test and differences were treated as significant when P was less than 0.05.

RESULTS

In all the enzyme assays 100 mM potassium phosphate buffer was used. This produced consistently higher activities with both liver and lung microsomes from both control and 3MC-treated rats than did Tris–HCl buffer (10–100 mM) or concentrations of potassium phosphate buffer higher or lower than 100 mM (data not shown). All specific activities are nmol resorufin formed/min/mg protein and given as mean ± SEM for groups of 4–5 individual rats.

Study 1: *time-course of the induction of EROD activity by a single exposure to cigarette smoke*

Lung microsomes from tube-control rats showed a very low but accurately measurable EROD activity of 0.015 ± 0.001 nmol/min/mg protein. Following a single 9-min exposure to cigarette smoke there was a significant 3.9-fold increase in lung EROD activity after 4 hr, followed by a slow decrease to basal activity by 24 hr after exposure (Fig. 1). Liver microsomes from tube-control rats had a much higher EROD activity (0.295 ± 0.015 nmol/min/mg protein) than control lung microsomes. A single

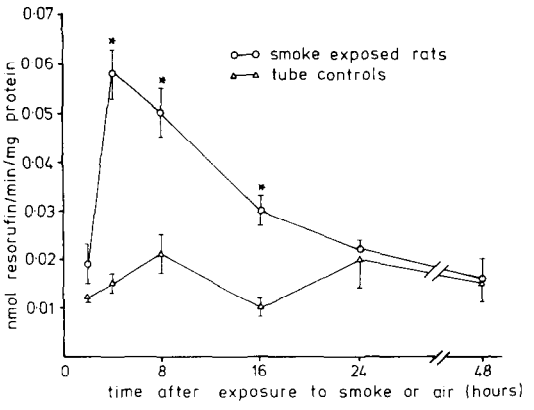

Fig. 1. Time-course of the induction of ethoxyresorufin *O*-deethylase activity in rat lung microsomes by cigarette smoke. Rats were exposed to cigarette smoke or air once (at time zero) as described in the Methods. Results are means ± SEM for 4–6 rats per time point, measured individually. * Test significantly different from the same time control, $P < 0.05$.

exposure to cigarette smoke caused an increase in liver EROD activity that was smaller (2.2-fold) and which peaked later (8 hr after exposure) than that seen with lung microsomes (Fig. 2). Although the smoke-induced increase in liver microsomal EROD at 8 hr was not statistically significant, this was probably due to the large standard error of the treated group, since the smaller increase at 4 hr was significant. Smoke treatment did not cause any change in liver or lung microsomal protein concentrations.

Study 2: comparative effects of smoke, 3MC and PB on phenoxazone and alkoxyresorufin metabolism

With either liver or lung microsomes the homologous series of alkoxyresorufins (methoxy to octoxy, plus benzyloxy) were each 7-*O*-dealkylated to a common product, resorufin, while the unsubstituted parent compound, phenoxazone, was correspondingly 7-hydroxylated also to form resorufin. The rate of resorufin production was, however, greatly dependent on the length of the alkoxy side-chain, resulting in a distinctive activity profile (reaction rate plotted against side-chain length). Previous studies have shown that resorufin is the sole metabolite from phenoxazone and each of the alkoxyresorufins and that an increase in side-chain length does not cause side-chain hydroxylation to take precedence over *O*-dealkylation [19, 23]. No statistically significant differences in enzymic activity were observed between the microsomes prepared from tube-control rats and the microsomes obtained from corn oil- or saline-injected animals.

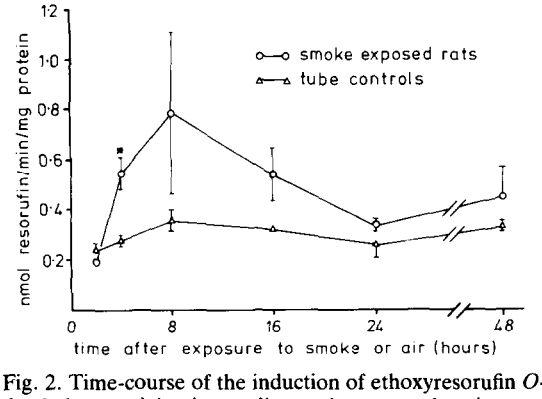

Fig. 2. Time-course of the induction of ethoxyresorufin *O*-deethylase activity in rat liver microsomes by cigarette smoke. Rats were exposed to cigarette smoke or air once (at time zero) as described in the Methods. Results are means ± SEM for 4–6 rats per time point, measured individually. * Test significantly different from the same time control, $P < 0.05$.

Lung microsomes

With lung microsomes from control rats the highest resorufin production activities were with benzyloxy- and pentoxyresorufin (0.265 ± 0.041 and 0.076 ± 0.006 nmol/min/mg protein respectively) (Fig. 3A). The activity profiles were similar for con-

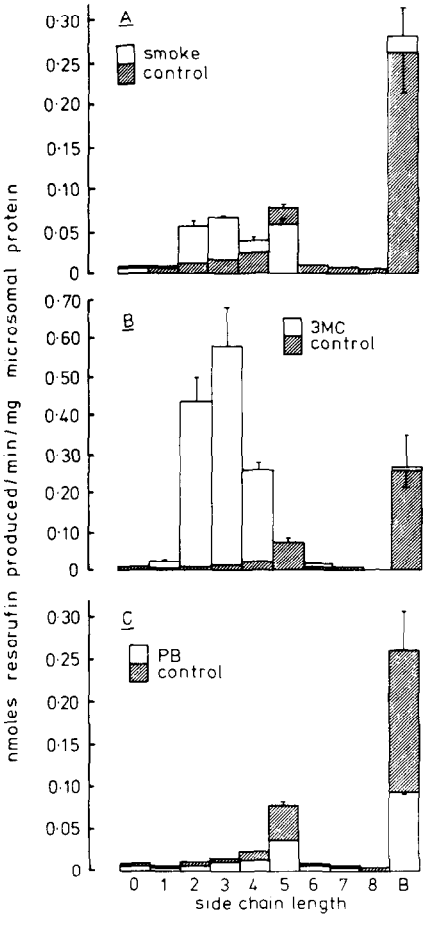

Fig. 3. Effects of exposure to cigarette smoke (A) or treatment with 3-methylcholanthrene (B) or phenobarbitone (C) on the metabolism of phenoxazone and a homologous series of alkoxyresorufins in rat lung microsomes. Rats were exposed to cigarette smoke or treated with 3MC or PB once daily for 3 days and killed 24 hr after the last exposure or treatment, as described in the Methods. Results are means ± SEM for 4–6 rats per treatment, measured individually. Each bar originates at the abscissa. Note the differences in ordinate scales. The numbers below the abscissa identify the substrate by the length of its side chain (O = phenoxazone, the unsubstituted parent compound; B = benzyloxyresorufin).

trol and PB-induced lung microsomes but were very different with 3MC-induced lung microsomes. 3MC treatment caused large, significant inductions of pulmonary *O*-dealkylase activity with ethoxy- and propoxyresorufin (44- and 41-fold induction respectively) and lesser induction with butoxy- and methoxyresorufin (11- and 4-fold respectively) (Fig. 3B). PB treatment, in contrast, caused a decrease in lung microsomal resorufin production activity with all the substrates (Fig. 3C). The decreases were particularly large and statistically significant with pentoxy- and benzyloxyresorufin (51% and 42% decrease respectively). Three daily exposures to cigarette smoke caused small but significant increases in lung microsomal *O*-dealkylase activity with ethoxy- and propoxyresorufin (6- and 5-fold increase respectively) and a significant 24% decrease in activity with pentoxyresorufin (Fig. 3A).

Liver microsomes

Control liver and lung microsomes had very different activity profiles, with liver showing its greatest resorufin production activity with ethoxy- and propoxyresorufin. Control liver microsomes were more active than control lung microsomes with all but two of the substrates, but were significantly less active than lung microsomes with pentoxy- and benzyloxyresorufin (64% and 46% less active respectively) (Fig. 4A). Control, PB- and 3MC-induced liver microsomes had very different activity profiles. 3MC induced very large increases in hepatic microsomal *O*-dealkylase activity with the C1 to C4 ethers (methoxy-, ethoxy-, propoxy- and butoxyresorufin), the largest increase (145-fold) being with ethoxyresorufin (Fig. 4B). PB treatment caused a significant increase in resorufin production activity with every substrate except phenoxazone, but for the C1 to C4 ethers the increase was much less than that caused by 3MC. PB induced ethoxy- and propoxyresorufin *O*-dealkylase activities by 8- and 5-fold respectively, compared to 145- and 136-fold induction by 3MC. The largest PB-inductions were with benzyloxy- and pentoxyresorufin (144- and 87-fold induction respectively) and for these activities the increase was much more by PB than by 3MC (12- and 11-fold induction respectively by 3MC) (Fig. 4C). Three daily 9-min exposures to cigarette smoke caused small but significant increases in hepatic microsomal *O*-dealkylase activity with the C1 to C4 ethers, primarily with ethoxy- and propoxyresorufin (2.5- and 2-fold increase respectively), but did not induce activity with pentoxy- or benzyloxyresorufin (Fig. 4A).

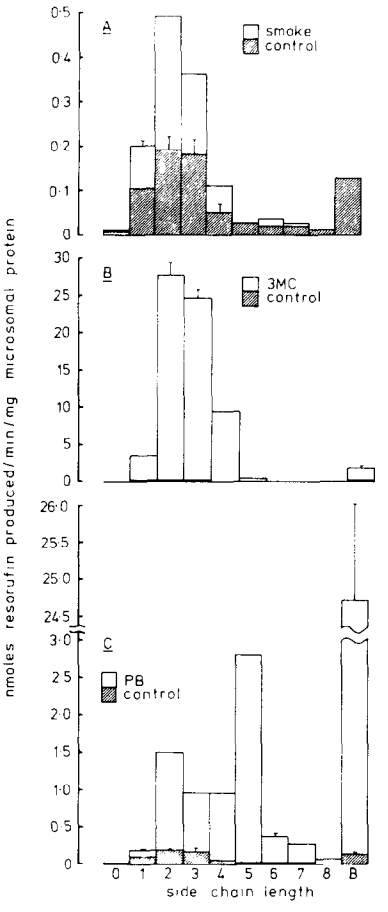

Fig. 4. Effects of exposure to cigarette smoke (A) or treatment with 3-methylcholanthrene (B) or phenobarbitone (C) on the metabolism of phenoxazone and a homologous series of alkoxyresorufins in rat liver microsomes. Rats were exposed to cigarette smoke or treated with 3MC or PB once daily for 3 days and killed 24 hr after the last exposure or treatment, as described in the Methods. Results are means ± SEM for 4–6 rats per treatment, measured individually. Each bar originates at the abscissa. Note the differences in ordinate scales. The numbers below the abscissa identify the substrate by the length of its side chain (O = phenoxazone, the unsubstituted parent compound; B = benzyloxyresorufin).

DISCUSSION

The rate of resorufin production from phenoxazone and a homologous series of alkoxyresorufins was highly dependent on the presence and the length of an alkoxy side-chain, but the influences of chain length on reaction rate were very different for liver and lung microsomes and for control, 3MC-induced and PB-induced microsomes. The results presented here for control, PB- and 3MC-induced liver microsomes from male Wistar rats are in close agreement with results reported previously for male Sprague–Dawley rats [17]. The remarkable difference in substrate selectivity (i.e. in activity profile) between control lung and liver microsomes is similar to results reported for female BALB/c mice [24], but the substrate preference of lung microsomes for pentoxy- and benzyloxyresorufin was much more pronounced in the rat than in the mouse. With rat lung microsomes pentoxyresorufin was the substrate with the second highest activity (after benzyloxyresorufin) and had 8-fold higher activity than ethoxyresorufin, whereas in mouse lung microsomes the second highest activity (after benzyloxyresorufin) was shown by ethoxyresorufin and pentoxyresorufin had only 37% of the activity of ethoxyresorufin. Whilst benzyloxyresorufin had the highest activity in both rat and mouse lung microsomes, it had 26-fold higher activity than ethoxyresorufin in rat but only 6-fold

higher activity than ethoxyresorufin in mouse. In rats the ratios of *O*-dealkylation specific activities between lung and liver microsomes (lung/liver) were 0.05, 2.8 and 1.8 for ethoxy-, pentoxy- and benzyloxyresorufin respectively, whereas in mice these ratios were 0.1, 0.7 and 1.8 respectively. The results for rats show that, whereas liver was 13- to 19-fold more active than lung in the *O*-dealkylation of methoxy-, ethoxy- and propoxyresorufin, lung was 2- to 3-fold more active than liver in the *O*-dealkylation of pentoxy- and benzyloxyresorufin. Thus, although rat liver microsomes have a higher microsomal concentration of total cyt. P-450 and a higher specific activity for several monooxygenation reactions than have lung microsomes [25], it cannot be assumed that all compounds will be metabolized with a higher microsomal specific activity by liver than by lung of rats. In rabbits, however, in contrast to rats, several monooxygenase reactions have similar or higher microsomal specific activities in lung than in liver [25, 26]. Normal rabbit lung microsomal cyt. P-450 is almost entirely comprised of two forms of cyt. P-450 analogous to PB-induced hepatic forms and this is the probable explanation for the high pulmonary activities of certain microsomal monooxygenase reactions in this species [27–29]. Similarly, lung microsomes of untreated rats contain a form of cyt. P-450 that is immunochemically indistinguishable from the major form induced by PB in rat liver, cyt. P-450b [3], and which is primarily responsible for the regio-selective hydroxylations of *n*-hexane and xylene by lung microsomes of untreated rats [30, 31]. Since PB-induced forms of rat liver microsomal cyt. P-450 show high substrate selectivity for pentoxy- and benzyloxyresorufin relative to ethoxyresorufin [17, 32], the selectivity of rat lung microsomes for pentoxy- and benzyloxy- rather than ethoxyresorufin further suggests a functional preponderence of "hepatic PB-induced" forms of cyt. P-450 in normal rat lung microsomes. The results reported here for rat are in accordance with reports that rat lung cyt. P-450 is highly inducible by 3MC and other PAH, but is refractory to induction by PB [3, 25, 30, 33]. The effect of 3MC induction on the substrate selectivity of alkoxyresorufin metabolism, causing an increase in the *O*-dealkylations primarily of the C1 to C4 ethers, was similar in both rat liver and lung microsomes, suggesting that 3MC induced functionally similar forms of cyt. P-450 in lung and liver. There is a report that 3MC induces similar but not identical forms of cyt. P-450 in rat lung and liver respectively [34], while immunoanalysis shows that 3MC induces both cyt. P-450 forms P-450c and P-450d in rat liver but only the form P-450c in rat lung [3]. Purified cyt. P-450c has a high substrate selectivity for ethoxy- compared to methoxyresorufin, whereas purified cyt. P-450d shows the converse selectivity (M. D. Burke and C. R. Wolf, unpublished observations). In 3MC-induced rats, lung microsomes showed a high ratio (27:1) between ethoxy- and methoxyresorufin dealkylation rates, whereas this ratio was much lower (8:1) for liver microsomes (Figs. 3 and 4). This suggests that the comparative inductions of immunoreactive P-450c and P-450d in lung and liver are reflected at the functional level also. However, since microsomes contain considerably more immunoreactive cyt. P-450 than spectrally active (and, by inference, functionally active) cyt. P-450 [35, 36], yet it is not known whether this discrepancy applies uniformly to all forms of cyt. P-450, it cannot be assumed that changes induced in the immunoquantified pattern of individual forms of cyt. P-450 in a tissue correctly reflect changes induced in the overall pattern of cyt. P-450-catalysed reactions in that tissue.

The time course of the induction of EROD activity following a single exposure to cigarette smoke was similar to that found for induction of pulmonary AHH in rats [15, 37] and mouse [14]. The greater extent of induction in the lung than the liver, which is also seen for AHH [14–16, 37], might be expected, since the lung is exposed directly to the smoke, whereas the liver may encounter diminished concentrations of the putative inducing agents therein, due to their systemic distribution and metabolism. Rat liver microsomal AHH is also induced, about 100%, by oral administration of tobacco leaf homogenates, but the induction takes much longer (several days) than does induction by inhalation of cigarette smoke [38]. Exposure of the rats to cigarette smoke on three successive days induced hepatic and pulmonary microsomal *O*-dealkylation with primarily ethoxy- and propoxyresorufin: this selectivity of induction is similar to the effect of 3MC and suggests that cigarette smoke acted as a weak 3MC-type inducer on both liver and lung. Cigarette smoke also caused a selective decrease in pulmonary microsomal pentoxyresorufin *O*-dealkylation, however, which was similar to the effect of PB: this suggests that cigarette smoke also acted on the lung as an inducer of a different type from 3MC, although there was no such effect on the liver. Rats that were exposed to cigarette smoke on three successive days were killed 24 hr after the final exposure, but greater changes in alkoxyresorufin dealkylation activities might have been seen had the rats been killed sooner after the third smoke exposure, in the light of the results for a single exposure to cigarette smoke.

O-Dealkylation activities with methoxy- and ethoxyresorufin, but not with pentoxy- and benzyloxyresorufin, are approximately 3- to 7-fold higher in liver microsomes from human cigarette smokers (more than 30 cigarettes per day) than in non-smokers [10, 13]. Thus, the effect of cigarette smoke on rat liver alkoxyresorufin metabolism was similar to the effect reported for humans. However, it has proved difficult to establish that the increased hepatic microsomal EROD activity in human smokers is really due to an induction of "PAH-induced" forms of cyt. P-450 and not merely a fortuitous result of the large interindividual variation that is seen in this activity [10, 13]. Although human liver microsomal EROD activity was inhibited by a monoclonal antibody specific for 3MC-induced forms of rat liver cyt. P-450 (P-450c and P-450d), there was not a significantly greater antibody inhibition in liver microsomes from smokers compared to non-smokers [13]. These results for rat suggest that cigarette smoke does, in fact, have a 3MC-like inducing effect on hepatic and pulmonary microsomal alkoxyresorufin metabolism, plus a different type of inducing effect on the lung.

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