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Induction of aryl hydrocarbon hydroxylase and carbon monoxide-binding hemoproteins in mouse epidermis by tobacco carcinogens

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Elevated levels of the enzyme, aryl hydrocarbon hydroxylase (AHH), have been observed in various tissues of cigarette smokers [1–4] and in the lungs of mice [5] or rats [6] exposed to cigarette smoke. The most common method for measuring tumorigenicity of cigarette smoke condensate (CSC) and fractions derived from it consists of topical applications to mouse skin for extended periods. Recent evidence in this laboratory has indicated that a single application of tumorigenic CSC subfractions causes a 5- to 8-fold increase in epidermal AHH activity [7]. In the present study, the difference spectra of microsomal cytochromes and the level of AHH were measured in the epidermis of mouse skin 24 hr after application of the most tumorigenic neutral subfraction from CSC.

Preparation of CSC and its separation into neutral subfractions by column chromatography and solvent-partitioning have been described [8]. The dimethylsulfoxide-soluble fraction, designated 'fraction 20' has been demonstrated previously [9] and recently in our laboratory [7] to cause the greatest tumorigenic response on mouse skin of all the neutral subfractions. Fraction 20 was diluted with acetone to equal 60% of its concentration in crude CSC. 1,2-Benzanthracene (K & K Laboratories, New York, N.Y.)* was recrystallized from benzene and a solution (25 μmoles/ml) was prepared in acetone. A single application of 0·2 ml of fraction 20, benzanthracene or acetone was pipetted onto the shaved backs of 50- to 55-day-old ICR Swiss mice. The mice were killed by cervical dislocation 24 hr later and about 9 cm^2 of the treated section of skin was removed and washed in cold isotonic NaCl. The epidermis was scraped from the skin with a surgical scalpel and placed in ice-cold 0·25 M sucrose. All homogenization and centrifugation procedures were carried out at 4°.

Epidermal scrapings of ten mice/treatment or control group were pooled in 12 ml of 0·25 M sucrose and homogenized using a Polytron model PT-10-20 (Brinkman Instruments, Inc., Westbury, N.Y.). The homogenates were strained through gauze and centrifuged at 9000 *g* for 30 min. Supernatant was collected and centrifuged at 105,000 *g* for 1 hr. The microsomal pellet was resuspended in 2·5 ml of 1·15% KCl-0·05 M Tris buffer, pH 7·4, using a Potter–Elvehjem glass homogenizer with a Teflon pestle. Unless otherwise stated, this microsomal fraction was used for AHH assay and spectral studies. AHH activity was measured by the method of Wattenberg *et al.* [10] with minor modifications. The incubation mixture contained in a final volume of 1 ml: 0·1 ml microsomes; 0·5 ml of 0·1 M sodium phosphate buffer, pH 7·5; 3 μmoles $MgCl_2$; 0·56 μmole NADPH; 0·49 μmole NADH; and 80 nmoles benzo(a)pyrene (dissolved in a minimum of dimethylsulfoxide). The mixture was incubated at 37° for 20 min and the reaction stopped by addition of 1·0 ml of cold acetone. After extraction with 2·5 ml hexane, 2·0 ml of the organic phase was extracted with 2·5 ml of 1·0 N NaOH. The concentration of phenolic metabolites in the alkaline phase was analyzed with an Aminco–Bowman spectrophotofluorometer with activation at 396 nm and fluorescence at 522 nm. Duplicate samples were compared with standards containing 3-hydroxybenzo(a)pyrene and with appropriate blanks. The standard and blanks were carried through incubation and extraction procedures. Difference spectra of microsomes were determined with an Aminco DW-2 spectrophotometer by the method of Omura and Sato [11] and by a modification of this procedure as described by Zampaglione and Mannering [12]. Protein content was determined by the method of Lowry *et al.* [13] with bovine serum albumin as standard. Results were analyzed statistically using the paired *t*-test.

On a per mg protein basis, AHH activity in the epidermal scrapings was about 10–15 times greater than that in whole skin. The microsomal fraction had a 4-fold higher activity/mg of protein than the homogenized epidermis. Yields of microsomal protein were similar in control and treatment groups (Table 1). Application of 5·0 μmoles benzanthracene increased hydroxylation of benzopyrene 8-fold, and treatment with CSC fraction 20 increased activity of the enzyme 7-fold. This concentration of fraction 20 is both a tumor initiator [7] and promoter [9] on mouse skin.

Addition of NADH to oxidized skin microsomes (Fig. 1a) produced a cytochrome b_5 difference spectrum with peaks at 423 and 557 nm and a trough at 413 nm. Relative magnitude of the 423–413 nm absorbance was not altered by pretreatment of the mice with either benzanthracene or fraction 20. Further reduction of NADH-reduced microsomes by the addition of sodium dithionite (Fig. 1b) revealed the presence of other hemoprotein species with a Soret maximum at 421 nm. Treatment with fraction 20 increased the magnitude of this peak, while treatment with benzanthracene increased the magnitude and shifted the peak to 430 nm. Bubbling carbon monoxide into dithionite-reduced microsomes (Fig. 1c) produced a difference spectrum with a peak at 420 nm and a trough at 433 nm. The 420 nm peak increased in response to treatment with both benzanthracene and fraction 20. A modified method of studying carbon monoxide-binding hemoproteins used by Zampaglione and Mannering [12] and by Bickers *et al.* [14] involves bubbling carbon monoxide into both reference and sample cuvettes, recording baseline, and then adding dithionite to the sample cuvette, thus producing a carbon monoxide-reduced microsomes minus carbon

Table 1. Induction of skin microsomal aryl hydrocarbon hydroxylase by application of benzanthracene or CSC fraction 20.*

Treatment	Epidermal microsomal protein (mg/g skin, wet wt)	Benzo(a)pyrene hydroxylation (pmoles product formed/mg protein/min)
Acetone	1·70 ± 0·68	42 ± 11
1,2-Benzanthracene	1·34 ± 0·35	323 ± 29†
Fraction 20	1·43 ± 0·22	281 ± 11†

* Protein content and benzo(a)pyrene hydroxylation were determined in resuspended microsomes obtained from pooled epidermal scrapings of ten mice/group as described earlier. Each value represents the mean ± S. E.M. from three experiments.

† Significantly different from acetone-treated control groups ($P < 0·01$).

* Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

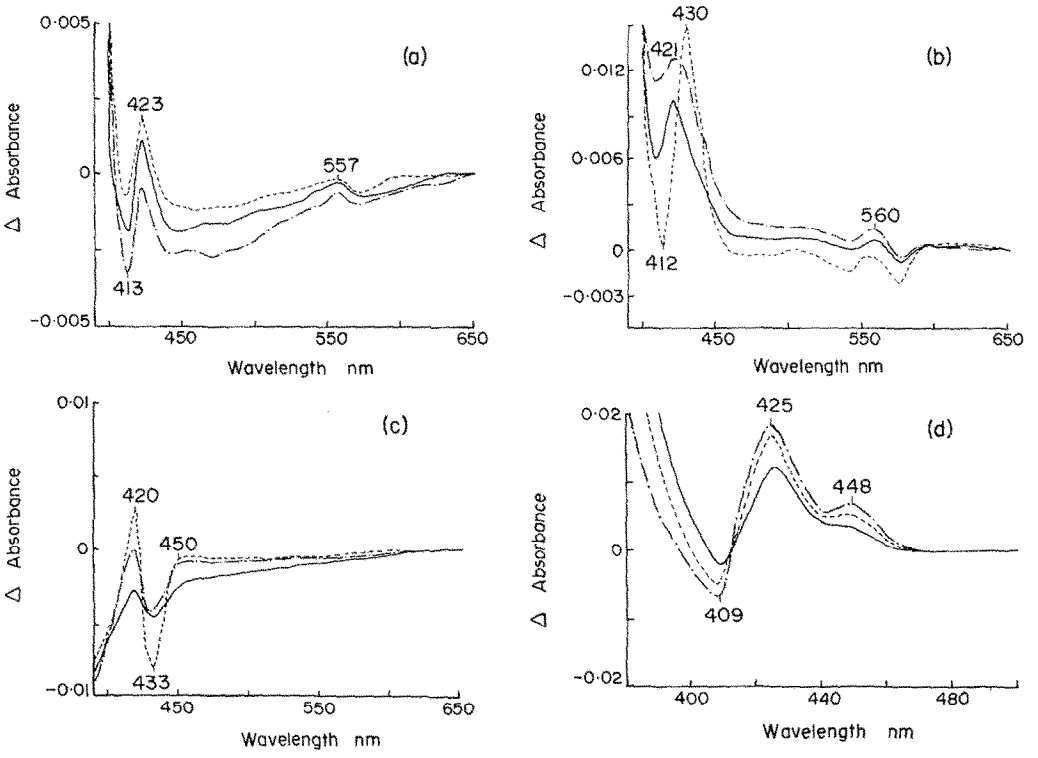

Fig. 1. Difference spectra of mouse epidermal microsomes. Key: (——) acetone-pretreated; (– – – –) 1,2-benzanthracene-pretreated; (— · · —) CSC fraction 20-pretreated. The curves represented are averages drawn from data points obtained in three separate experiments. (a) NADH-reduced minus oxidized microsomes, protein concentration = 0·5 mg/ml. (b) Dithionite-reduced minus NADH-reduced microsomes, protein concentration = 0·5 mg/ml. (c) Carbon monoxide bubbled-dithionite-reduced minus dithionite-reduced microsomes, protein concentration = 0·5 mg/ml. (d) Carbon monoxide bubbled-dithionite-reduced minus carbon monoxide-bubbled microsomes, protein concentration = 1·0 mg/ml.

monoxide-oxidized microsomes difference spectrum. Using this modification, a spectrum with peaks at 425 and 448 nm was obtained (Fig. 1d), and the magnitude of both peaks was increased by treatment with fraction 20 or benzanthracene.

Aryl hydrocarbon hydroxylase is an enzyme of the mixed function oxidase type, requiring NADPH and O_2 [15,16], and is inducible in lung tissue after exposure of rats to cigarette smoke [6]. Our results indicate that this enzyme system is inducible in mouse skin after treatment with a fraction of cigarette smoke condensate containing a high concentration of polycyclic hydrocarbons. That this same fraction of CSC is one of the most tumorigenic might be related to its ability to increase metabolism of the polycyclic hydrocarbons, resulting in high concentrations of proximal carcinogens in contact with epidermal cells.

The involvement of cytochrome P-450 in skin AHH activity has been suggested previously by inhibition of benzo(a)pyrene hydroxylation by human foreskin in a carbon monoxide atmosphere [17]. Furthermore, Bickers *et al.* [14] demonstrated the appearance of a CO-binding component in rat skin microsomes after induction of AHH with polychlorinated biphenyls, and Poland *et al.* [18] have found induction of cytochrome P-448 in skin of mice after administration of the potent AHH inducer, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Our results indicate that induction of skin AHH by application of benzanthracene or CSC fraction 20 is accompanied by an increase in content of CO-binding hemoprotein. Apparently a relatively small increase in this component is associated with a large increase in AHH activity.

The nature of the cytochrome(s) induced in mouse skin by fraction 20 or benzanthracene is as yet unclear. Other researchers [19–21] have demonstrated the appearance in liver microsomes of a new cytochrome, termed P-448 or P_1-450, after treatment of rabbits, rats or genetically responsive mice with polycyclic hydrocarbons. Our results in skin, however, support the finding of Pohl *et al.* [22] that the major cytochrome in mouse skin appears to be cytochrome P-420, as indicated in Fig. 1c. Homogenization using a Potter–Elvehjem Teflon-glass homogenizer, instead of a Polytron, in separate experiments in our lab, and other techniques to protect cytochromes during homogenization by Pohl *et al.* [22] did not alter the quantity of P-420 present. Furthermore, Pohl *et al.* [22] demonstrated the presence of P-420 in unhomogenized mouse skin. Poland *et al.* [18], however, have demonstrated a peak at 450 nm in CO-treated dithionite-reduced mouse skin microsomes, although a sizable 420 nm peak was seen in one of the control animals. These workers resuspended microsomes in 30% glycerol, which has a protective effect on the integrity of cytochrome P-450 [23] and may explain the failure of Pohl *et al.* and us to observe a peak at 450 nm.

The failure to observe a definite peak at 450 nm in the carbon monoxide difference spectra (Fig. 1c), and the presence of a peak at 448 nm in the dithionite difference spectra of carbon monoxide-bubbled microsomes (Fig. 1d) indicate that conclusions concerning the particular carbon monoxide-binding pigment present in an enzyme system must be made carefully when the 'modified procedure' described by Zampaglione and Mannering [12] and by Bickers *et al.* [14] is used.

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