Induction of Increased Benzpyrene Hydroxylase Activity in Pulmonary Tissue *in Vitro** (32716)

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In the present report a technique for inducing an increased benzpyrene (BP) hydroxylase activity in rat pulmonary tissue *in vitro* will be described. BP hydroxylase converts the potent carcinogen BP to weakly carcinogenic or noncarcinogenic hydroxy and quinone derivatives (1). This enzyme system was first found in liver where it occurs as one of a group of closely related microsomal enzyme systems which metabolize many polycyclic hydrocarbons and also a wide range of other compounds which are foreign to the organism (2–4). BP hydroxylase has subsequently been shown to be present in other tissues including those of the major portals of entry, i.e, the lung and the gastrointestinal tract (5–7).

One of the properties of BP hydroxylase as well as related microsomal enzyme systems is that an increase in their activities can be induced *in vivo* by a variety of organic compounds (1–8). Any mechanism which offers the possibility of increasing the detoxification of BP in the respiratory system would appear to be of particular importance because of the widespread occurrence of this carcinogen in a form in which it is inhaled. Since a technique for studying induction of BP hydroxylase in organ culture might have potential value, investigations were undertaken to determine if this could be accomplished. The rat lung was chosen for the initial experimental work because a considerable amount of data has been obtained on induction of increased BP hydroxylase activity in the lung of this species, and also because preliminary work indicated that it could be cultured successfully under conditions that would be satisfactory for carrying out induction studies.

Methods and Materials. The organ culture technique which was employed is a modification of the method of Trowell (9). Cubes of pulmonary tissue of approximately 1.5 mm were prepared from the lungs of female Sprague-Dawley rats 48 days of age. These cubes were placed on squares of hardened ashless Whatman filter paper supported by a stainless steel grid which had been put into a fused-silica crucible containing the culture medium. The crucible was within a small Petri plate. The volume of the medium was such that the filter paper remained moist but the medium did not cover the tissue. The atmosphere contained 95% oxygen and 5% carbon dioxide. The medium consisted of 10% chick embryo extract and 22% bovine serum in Hanks' balanced salt solution. Water-soluble inducers were added directly to the medium whereas inducers soluble in organic solvents were first dissolved in dimethylsulfoxide (DMSO) and then added to the medium with the final concentration of DMSO being 1% by volume. This concentration has no demonstrable effect on the BP hydroxylase activity of the lung tissue. All compounds tested were at a concentration of 0.03 μmoles/ml except where otherwise indicated. Incubation was carried out at 37°C for 46 hours.

For determining BP hydroxylase activity in the lung culture experiments, a modification of a previously described technique was employed (8). A 0.1% homogenate of the tissue cubes which had previously been frozen at —40°C was made in cold (0–2°C) isotonic KCl with a microhomogenizer of the Potter-Elvehjem type attached to a mechanical stirrer. The reaction mixture consisted of 100 μl homogenate; 95 μl of a solution containin 100 μg NADPH, 100 μg of NADH, 4 μmoles nicotinamide, 3 μmoles KCl, and 300 μmoles $NaH_2PO_4 \cdot Na_2HPO_4$ buffer pH 7.4; and 5 μl of acetone containing 1 μg of BP. The reaction was started by the addition of the BP solution. Incubation was carried out for 10 min at 37°C. The re-

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TABLE I. Effects of Phenothiazines and Certain Other Compounds on the *in Vitro* and *in Vivo* Induction of Benzpyrene Hydroxylase Activity in Pulmonary Tissue.

Compound tested	Lung culture experiments		*in Vivo* experiments	
	Benzpyrene hydroxylase activity (units /mg protein)[a]	Ratio: test (Control)	Benzpyrene hydroxylase activity (units /mg protein)[b]	Ratio: test (Control)
Control (nonincubated)	7.9 ± 2.9	—	8.1 ± 1.7	—
Control (incubated)[c]	9.5 ± 1.9	—	—	—
Phenothiazine	36.4 ± 7.4	3.8	67.0 ± 6.7	8.3
10-Acetylphenothiazine	34.4 ± 4.5	3.6	53.5 ± 1.1	6.6
Chlorpromazine sulfoxide HCl	27.8 ± 3.7	2.9	53.5 ± 4.3	6.6
Chlorpromazine HCl	29.9 ± 4.5	3.1	44.0 ± 6.2	5.4
Methyl (10-phenothiazine) propionate	9.8 ± 3.2	1.0	11.6 ± 2.2	1.4
3-Methylcholanthrene	25.1 ± 1.1	2.6	64.3 ± 8.4	7.9
Benzo(a)pyrene	20.6 ± 2.1	2.2	41.8 ±10.0	5.2
Pyrene	11.1 ± 1.6	1.2	22.4 ± 3.0	2.8
beta-Naphthoflavone	34.9 ± 3.2	3.7	32.4 ± 3.8	4.1
2,5-Diphenyltriazole	23.8 ± 1.9	2.5	70.3 ±11.6	8.5

[a] Each value is obtained from duplicate determinations on separate lung cultures from three rats except for the controls which are derived from nine rats. Mean ± SD.

[b] Each value is obtained from duplicate determinations on the lungs from four animals. Mean ± SD.

[c] Tissue from control rats incubated in the organ culture system for 46 hours.

action was stopped by the rapid addition of 400 μl of 3:1 acetone-distilled water. The mixture was shaken with 900 μl of petroleum ether (Skelly-Solve B, bp 66–68°C). An aliquot of the 1 ml organic-solvent phase was extracted with 200 μl of 1 *N* NaOH. The fluorescense of the hydroxy derivatives of BP in the aqueous extract was determined in a 3 × 83-mm Pyrex cell adapted for Farrand photoelectric fluorometer model A-3. The instrumental standardization procedure and calculation of units have been described previously (8). Protein concentrations were determined by the method of Lowry *et al.* (10) using bovine serum albumin as a standard.

In the *in vivo* induction studies, 1 ml of solution containing 0.03 mmole of the compound under investigation was administered by oral intubation to 48-day old Sprague-Dawley rats 46 hours prior to sacrificing. Water or DMSO were used as the vehicles. Neither of these in the volume employed alters BP hydroxylase activity. For determining BP hydroxylase activity, a 1.25% homogenate of rat lung was made in cold (0–2°C) isotonic KCl with a Virtis-45 homogenizer. Each specimen was homogenized for 90 sec with a blade-rotor speed of approximately 15,000–25,000 rpm. The reaction mixture consisted of 2 ml of homogenate; 1 ml of a solution containing 1 mg NADPH, 0.5 mg NADH, 60 μmoles nicotinamide, 50 μmoles KCl, and 5,000 μmoles of $NaH_2PO_4 \cdot Na_2HPO_4$ buffer at pH 7.4; and 0.1 ml acetone containing 25 μg of BP. Incubation was carried out for 20 min at 37°C. The remainder of the procedure was performed as described previously (8).

Results. Induction of increased BP hydroxylase activity was produced in the lung culture system by the addition to the medium of compounds which induce increased BP hydroxylase activity *in vivo*, Table I. The magnitude of the induction was less in the lung culture system than in the intact animal. Many of the compounds in Table I cannot be studied at a higher concentration in the *in vitro* system because of either lack of solubility in the medium or toxicity as manifested by visible evidence of cell damage. However, phenothiazine and chlorpromazine

sulfoxide HCl can be studied at a concentration of 0.1 μmole/ml culture media. This added amount of compound does not result in any significantly different level of BP hydroxylase activity than that obtained with 0.03 μmole/ml as employed in the studies presented in Table I. These data indicate that for at least these two compounds a maximum induction effect *in vitro* has been obtained.

Examination by light microscopy of sections of the cubes of lung tissue cultured for 46 hours showed some alterations from the appearance of uncultured control specimens. A loss of volume of the alveolar air spaces was generally observed and also an increase in the number of alveolar macrophages. In the alveolar septa changes occurred which resulted in the appearance of cells with large hyperchromatic nuclei. No morphologic distinctions could be made between tissue incubated in the presence of compounds being tested for inducing capacity and the corresponding controls.

Discussion. With the technique employed, induction of increased BP hydroxylase activity in pulmonary tissue has been obtained *in vitro*. In comparing the relative effectiveness of various inducers *in vitro* and *in vivo* it will be noted that 3-methylcholanthrene and 2,5-diphenyltriazole have less activity in the lung culture system than 10-acetylphenothiazine and phenothiazine although *in vivo* they have an equivalent or greater inducing capacity than the two phenothiazines. This suggests that reliance on the *in vitro* technique to predict *in vivo* activity may result in underestimation of the inducing capacity of a compound in the intact animal.

In addition to the *in vitro* technique described in this report, induction of increased BP hydroxylase activity in a tissue culture of embryonic cells has recently been reported (11). There are several possible advantages to these *in vitro* techniques for studying BP hydroxylase induction. They provide a method for a more direct investigation of factors regulating the induction process than studies in which the entire animal is employed. Factors such as systemic toxicity or hormonal changes due to the compound administered can be eliminated as well as metabolic modifications of the inducer by tissues other than the one under study. A further use of this type of technique would be in the study of induction in certain tissues such as the human lung where it is almost impossible to obtain sequential specimens. Depending upon the purpose for which it is used, these advantages would have to be balanced against the artifacts which may be introduced by a culture technique.

Summary. A technique has been developed for obtaining induction of increased benzpyrene hydroxylase activity in short-term cultures of rat pulmonary tissue. Several phenothiazines, polycyclic hydrocarbons, and other compounds which induce increased benzpyrene hydroxylase activity *in vivo* when administered to rats will likewise induce an increased activity of this enzyme system *in vitro* when added to the culture system.

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