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Retinoic Acid Inhibits Hydrocortisone-Stimulated Expression of Phenol Sulfotransferase in Bovine Bronchial Epithelial Cells

JOE D. BECKMANN,* ROBERT PALMATIER, AND BRIAN KLIEWER
Departments of Internal Medicine and Biochemistry, Section of Pulmonary and Critical Care, University of Nebraska Medical Center, Omaha, Nebraska 68198

The airway epithelium, which is commonly exposed to xenobiotics, contains the conjugative enzyme phenol sulfotransferase (PST). We have previously reported that hydrocortisone (HC) stimulates the expression of PST severalfold in cultured bovine bronchial epithelial cells (Beckmann et al., 1994, J. Cell. Physiol. *160*:603–610). Here we report that this stimulation is attenuated by retinoic acid (RA). Dose-response measurements of both enzyme activities and mRNA levels indicated a 50% inhibition of HC stimulated PST expression with 0.05 nM RA. Varied concentrations of RA had a general repressive effect on HC-stimulated PST expression, with no change in the half-maximal HC stimulatory concentration of 12.5 nM. Steady state kinetic measurements indicated no significant changes in apparent K_m values of 3–5 μM for the acceptor substrate, 2-naphthol; only HC- and RA-dependent changes in V_{max} were observed. These changes were likely due to altered enzyme expression, as evidenced by immunoblot and Northern blot hybridization analyses. Thus, the expression of PST within bronchial epithelial cells is not merely constitutive, but is subject to both positive and negative controls.

The rising incidence and death rates from lung cancer, especially among women, may be largely attributed to inhalation of potentially carcinogenic compounds such as found in cigarette smoke (Wingo et al., 1995). Airway epithelia are a primary source of chemically induced carcinomas (Auerbach et al., 1979; Cohen, 1982; Pfeifer et al., 1989), so xenobiotic metabolism at this site is of obvious importance. Metabolism of xenobiotics encountered during various exposures occurs by several enzymatic mechanisms. The cytochrome P-450s are well known, of which CYP1A1 induction and CYP2D6 polymorphism may play important roles in lung cancer (Gonzalez, 1995). In addition, conjugative reactions exist that usually perform a detoxification role. The glucuronosyl transferase and glutathione transferase enzyme families are inducible by xenobiotics (Bock et al., 1979; Bock and Lilienblum, 1979; Sims and Grover, 1974; Singh et al., 1987). However, human lungs exhibit little if any glucuronidation (Mehta and Cohen, 1979), and glutathione transferase is repressed in smokers (Gonzalez, 1995).

Sulfotransferases compose another class of diverse conjugative enzymes often involved in xenobiotic metabolism and detoxification (Falany and Wilborn, 1994). The phenol sulfotransferases (EC 2.8.2.1.) are a subfamily of conjugative enzymes that catalyze the sulfonation of hydroxylated aromatic compounds, using 3′-phosphoadenosine 5′-phosphosulfate (PAPS) as the reaction cosubstrate (Sekura and Jakoby, 1979). The observations that 44 and 24% of human and bovine bronchial tissue benzo(a)pyrene metabolites were sulfate esters (Autrup et al., 1980) indicates the probable importance of this pathway. Although sulfation of phenolic compounds is generally a detoxification process, esterification of certain molecules produces an ultimate carcinogen (Surh et al., 1990; Vaught et al., 1981). Therefore, regulation of phenol sulfotransferases and perhaps other members of this enzyme family is now an important factor to consider when describing the toxicology of many compounds.

Phenol sulfotransferases have been purified and studied from several tissues and species. In humans two distinct enzymes exist: a thermolabile enzyme (TL- or M-PST) which is reactive with micromolar concentrations of catecholamines (Whittemore et al., 1985, 1986), and a thermostable enzyme (TS- or P-PST) that is preferentially reactive with low concentrations of simple phenols (Campbell et al., 1987; Falany et al., 1990). Purification studies of bovine lung phenol sulfo-

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*To whom reprint requests/correspondence should be addressed at Department of Biochemistry, Section of Pulmonary and Critical Care, University of Nebraska Medical Center, Omaha, NE 68198.

transferase indicated thermostable activity (Baranczyk-Kuzma and Szymczyk, 1987) that was inactive with catecholamines (Baranczyk-Kuzma and Szymczyk, 1986), thereby being most comparable to the human P-PST enzyme. This comparison is supported by the molecular characterization of the bovine lung PST cDNA, which revealed a predicted amino acid sequence most similar to the human P-PST enzyme (Schauss et al., 1995). In this context, the bovine lung may be considered as less complex than human lung with respect to possible phenol sulfonation pathways.

As an initial model system with less potential complexity than human, we have been examining the regulation of phenol sulfotransferase expression using bovine bronchial epithelium. This tissue has a low basal level of phenol sulfotransferase expression as demonstrated both enzymologically and immunohistologically (Beckmann et al., 1993). Hydrocortisone and other corticosteroids, however, can stimulate the expression of this enzyme in cultured bovine bronchial epithelial cells by 3–5-fold (Beckmann et al., 1994). Here we report that the stimulatory effects of hydrocortisone can be attenuated with retinoic acid, which may have important implications for the dietary control of xenobiotic metabolism in the lung.

MATERIALS AND METHODS

Retinoic acid, hydrocortisone, and LHC-Basal medium were from Biofluids, Inc. (Rockville, MD). Insulin was from Sigma (St. Louis, MO) and bovine pituitary extract was prepared as previously described (Beckmann et al., 1992). RPMI 1640, penicillin/streptomycin, and fungizone were from GIBCO (Grand Island, NY). Unlabeled PAPS and [^{35}S]-PAPS (2 Ci/mmol) were from Pharmacia (Piscataway, NJ) and New England Nuclear (Boston, MA), respectively. 2-Naphthol was from Fluka (Ronkonkoma, NY). Other reagents were of the highest purities available.

Cell culture

Bovine bronchial epithelial cells were prepared and cultured under serum-free conditions as previously described (Beckmann et al., 1992). Total primary epithelial cells were suspended in a partially supplemented medium, LHC-D plus RPMI 1640 (1:1) containing pituitary extract (100 μg protein/ml) and insulin (5 μg/ml, LHC-D/RPMI + PI). LHC-D is LHC-Basal medium supplemented with calcium stock, stocks 4 and 11, trace elements, ethanolamine/phosphoethanolamine (Lechner and LaVeck 1985), penicillin (50 U/ml), streptomycin (50 μg/ml), and fungizone (1 μg/ml). From 2–4 × 10^6 cells were plated per 35 mm tissue culture well, which had been precoated with 30 μg type I bovine collagen (Vitrogen, Collagen Corp., Palo Alto, CA). After overnight attachment at 37°C in a 95% air/5% CO_2 humidified atmosphere, the wells were washed twice with LHC-D/RPMI to remove unattached cells and refed with LHC-D/RPMI + PI. After 2 days, the cultures received fresh LHC-D/RPMI + I, and were then fed with hormone-free LHC-D/RPMI for 24 h prior to hydrocortisone and retinoic acid exposures as indicated in the figure legends.

Enzyme extraction and analyses

Cell extracts were prepared by NP-40 detergent lysis method number two as previously described (Beckmann et al., 1994). Total protein concentrations were determined by a Coomassie Blue G-250 binding assay (Bio-Rad, Richmond, CA) using bovine serum albumin as the standard. Phenol sulfotransferase activities were measured in triplicate at 23°C using a modified (Beckmann, 1991; Beckmann et al., 1994) radioisotopic procedure (Ramaswamy and Jakoby, 1987; Sekura et al., 1979). Activities in the cultured cell extracts were normalized for total protein. Sigmoidal dose-response curves were analyzed using a general logistic function, which includes a "slope factor" and the IC_{50} or ED_{50} values as fitted parameters (DeLean et al., 1978).

Steady state kinetic analyses of phenol sulfotransferase activities in cell extracts were conducted using the same assay method, except 2-naphthol concentrations were varied from 0.75–10 μM (Beckmann et al., 1994). Measured rates of sulfonation were used to determine K_m, V_{max}, and their standard errors using the weighted procedure of Wilkinson (Wilkinson, 1961), these being calculated on a Macintosh computer by the use of a configured graphical spreadsheet program (Cricket Graph III, Computer Associates, Islandia, NY).

Antibodies and immunoblot analyses

Mouse anti-bovine phenol sulfotransferase was as previously described (Beckmann et al., 1993). Cell extracts, prepared as described above, were subjected to polyacrylamide gel electrophoresis in the presence of dodecyl sulfate (Laemmli, 1970) and electrophoretic transfer (Towbin et al., 1979) to Immobilon membranes (Millipore, Bedford, MA). Membranes were allowed to air dry, rewet in methanol followed by water, and then probed as previously described (Beckmann et al., 1994) using a chemiluminescence detection kit (ECL, Amersham, Arlington Heights, IL). Relative band intensities were determined by Macintosh computer-facilitated integrative analysis after transmittance scanning densitometry (Hoeffer, model GS-300, San Francisco, CA).

RNA extraction and analysis

Total cell RNA was extracted and purified as previously described (Chomczynski and Sacchi, 1987). Samples of 10–15 μg were electrophoretically separated on 1% agarose gels (MOPS/formaldehyde system, Maniatis et al., 1982) and subsequently transferred by capillary blotting to nitrocellulose membranes using 20× SSPE. After UV crosslinking fixation (Stratalinker, Stratagene, La Jolla, CA), the Northern blots were blocked with 5× SSPE/1% SDS/5× Denhardt's solution, and then probed using a 459 bp 5′ portion of the bovine PST cDNA (Schauss et al., 1995) labeled with ^{32}P by random hexamer priming (Feinberg and Vogelstein, 1984). Hybridization was at 42°C in 2× SSPE/1 × Denhardt's/0.1% SDS/5% dextran sulfate/50% formamide. Probed blots were washed 4–5 times at room temperature in 0.1× SSPE/0.1% SDS prior to exposure to DuPont Cronex film using one intensifying screen at −80°C. As a loading control hybridization probe, a mouse β5 tubulin cDNA was used (Sullivan and Cleveland, 1986), which detects two transcripts for

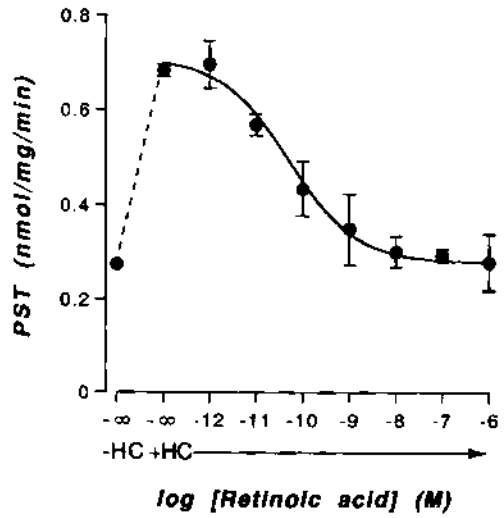

Fig. 1. Effects of increasing retinoic acid supplementation on the stimulation of expression of phenol sulfotransferase (PST) activity by hydrocortisone in cultured bronchial epithelial cells. Primary cells were initially cultured for 4 days in the presence of insulin, followed by one day of culture in the absence of hormonal supplementation (see Materials and Methods). Cultures were then exposed to the indicated combinations of ± hydrocortisone (HC, 20 nM) and retinoic acid at the indicated concentrations for 48 h prior to extraction (see Materials and Methods). Values are averages of triplicate measurements ± SD, being normalized per mg protein being assayed. The curve was derived from the logistical equation (see Materials and Methods) using a "slope factor" of −0.6 and an IC_{50} value of 0.05 nM.

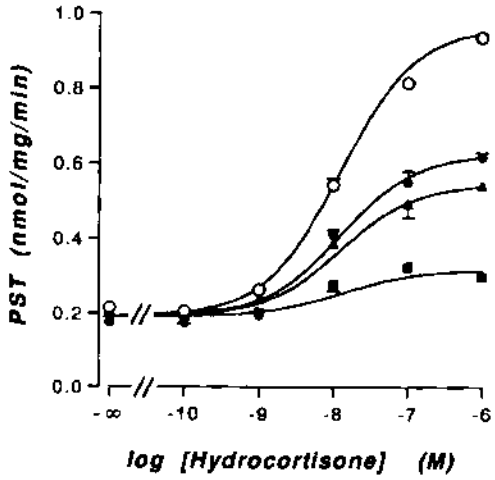

Fig. 2. Retinoic acid (RA) attenuates stimulation of phenol sulfotransferase (PST) expression even at elevated hydrocortisone concentrations. Primary cells were cultured as described in the legend of Figure 1, and finally incubated for 90 h at the indicated concentrations of hydrocortisone with varied amounts of RA. Open circles, no RA; closed circles, 0.1 nM RA; closed triangles, 10 nM RA; closed squares, 1 μM RA. Symbols cover the ±SD of triplicate measurements unless the error bars indicate otherwise. The fitted curves were derived from the logistic function (see Materials and Methods) using a "slope factor" of 0.85 and an ED_{50} of 12.5 nM.

double verification of load equivalencies. Quantitation was achieved by densitometry as described above.

RESULTS

Effect of retinoic acid on phenol sulfotransferase expression

We previously reported that nanomolar concentrations of hydrocortisone stimulated bovine bronchial epithelial cell phenol sulfotransferase expression (Beckmann et al., 1994). Inclusion of additional supplements, which included 3.3 nM retinoic acid, decreased the hydrocortisone response (Beckmann et al., 1994). To precisely evaluate the role of retinoic acid in the control of phenol sulfotransferase expression, bronchial epithelial cells were exposed for 48 h to hydrocortisone in the presence of increasing concentrations of retinoic acid (Fig. 1). Complete attenuation of the hydrocortisone stimulatory effect was accomplished by 10–1,000 nM retinoic acid, with a calculated IC_{50} value of 0.05 nM. These effects were not due to direct inhibition of the phenol sulfotransferase per se, because inclusion of up to 1 μM retinoic acid in control extract enzyme assays had no effects (not shown).

To examine the ability of retinoic acid to attenuate the stimulation of phenol sulfotransferase activity by hydrocortisone in greater detail, hydrocortisone dose-response measurements were made at several fixed retinoic acid concentrations (Fig. 2). Nanomolar retinoic acid concentrations caused a general repressive effect even at elevated hydrocortisone concentrations (Figure 2); there was no apparent change in the calculated ED_{50} value of 12.5 nM for hydrocortisone stimulation in the presence of incomplete retinoic acid blockade. Approximately 50% attenuation was achieved by 0.1 nM retinoic acid, which is consistent with the initial observation (Fig. 1). These results also demonstrate that retinoic acid alone (−∞ controls) has little if any effect on the basal level of phenol sulfotransferase expression.

The effects of varied retinoic acid concentrations on the time-courses for hydrocortisone induction of phenol sulfotransferase activities were also measured (not shown). Rates of enzyme induction during the first 4–5 days after exposure to 20 nM hydrocortisone plus varied retinoic acid concentrations were less than that observed with hydrocortisone alone.

Immunoblot analyses of phenol sulfotransferase protein levels

To determine if the changes in phenol sulfotransferase activities (Figs. 1 and 2) could be ascribed to simple changes in protein level, immunoblot analyses were performed. Exposure to hydrocortisone (20 nM, 72 h) increased immunodetectable protein by approximately 10-fold (Fig. 3). Coexposure with 0.1 nM retinoic acid reduced this stimulation by about 50%, which is very comparable to the degree of attenuation observed upon measurement of enzyme activities (Fig. 2). Retinoic acid alone had little if any effect on immunodetectable protein level, once again compatible with enzyme measurements (Fig. 2).

Steady state kinetic properties of expressed phenol sulfotransferase

Previous studies with bronchial epithelial cell phenol sulfotransferase activities across a very broad range

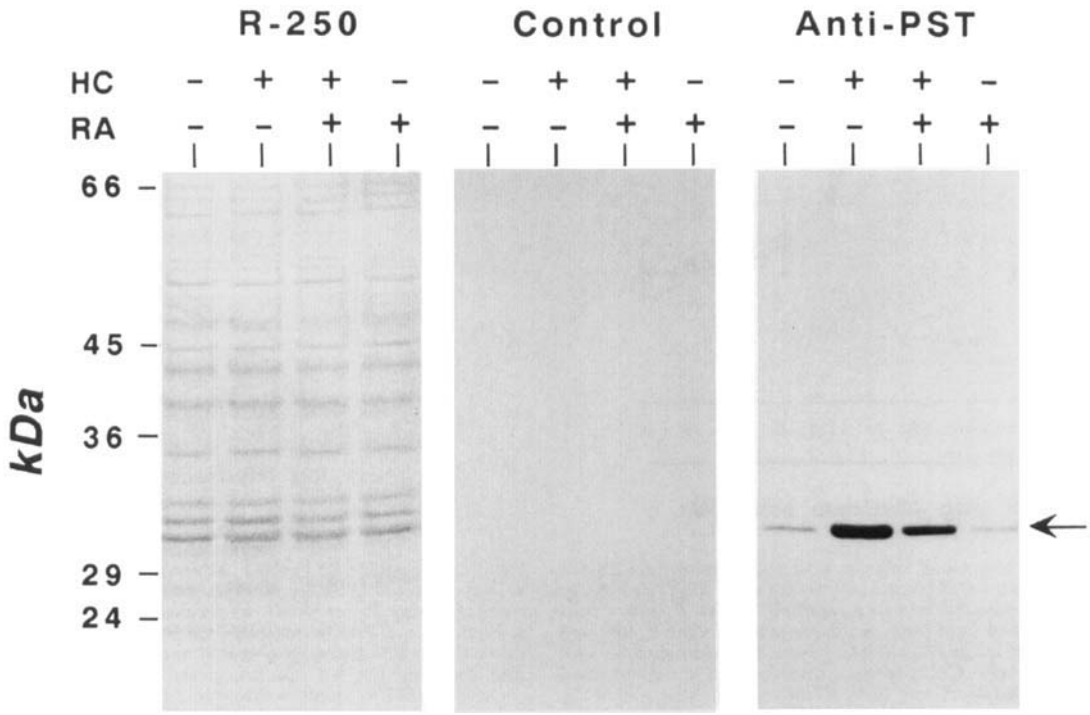

Fig. 3. Immunoblot analysis indicates changes in expression of phenol sulfotransferase protein in cultured bovine bronchial epithelial cells. Cells were initially cultured as described in the legend of Figure 1, and subsequently exposed to ± hydrocortisone (± HC, 20 nM) and ± retinoic acid (± RA, 0.1 nM) for 72 h. Cell extracts were prepared, electrophoresed, blotted and probed as described in Materials and Methods. Each lane contained 40 μg protein. The left panel (R-250) is a gel stained for total protein to demonstrate overall loading and blotting equivalence. Replicate blots were probed with mouse anti-bovine phenol sulfotransferase (Anti-PST) and normal mouse serum (control). The arrow shows the 32 kDa PST protein detected by chemiluminescence.

(3.8 nM–1 mM) of 2-naphthol concentration have shown a single "peak" of activity (Beckmann et al., 1994). The apparent K_m value for 2-naphthol was not changed by hydrocortisone stimulation. To test the possibility that retinoic acid might change this kinetic parameter, assays were conducted at numerous substrate concentrations (Fig. 4). The simple converging linear patterns observed regardless of hydrocortisone and retinoid exposures indicated no overt alteration in apparent K_m values for 2-naphthol. Weighted analyses of such data indicated expression of a "low K_m" PST (Table 1), with apparent K_m values of 3–5 μM for 2-naphthol; these values are within error of our previously reported measurements (Beckmann et al., 1994). The determined V_{max} values are consistent with the enzymatic activities measured at 20 μM 2-naphthol (Figs. 1 and 2), which is a "saturating" substrate concentration.

Hydrocortisone and retinoic acid regulate PST mRNA levels

The most common mechanism whereby steroids and retinoids modulate protein expression is via gene regulation by their corresponding receptor proteins (Evans, 1988; Yamamoto, 1985). To determine if this might also apply to the positive and negative regulation of phenol sulfotransferase expression by hydrocortisone and retinoic acid, respectively, Northern blot hybridization analyses were performed (Fig. 5). Hybridization with the bovine PST cDNA demonstrated a visually obvious

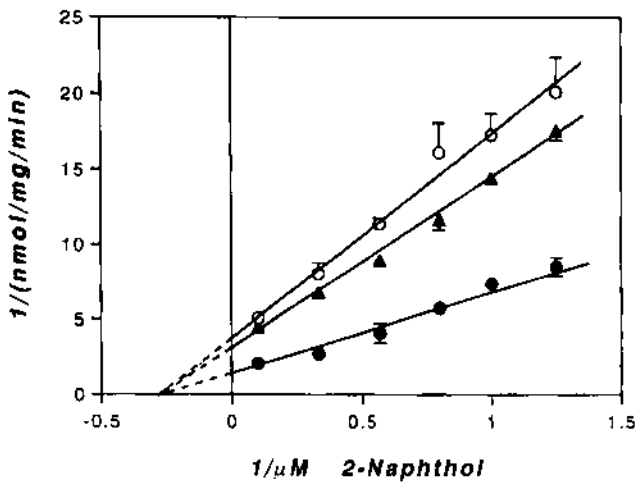

Fig. 4. Retinoic acid and hydrocortisone do not alter the phenol sulfotransferase K_m values for the acceptor substrate. Extracts from control cultures (open circles), cultures exposed for 4 days to 20 nM hydrocortisone (closed circles), or 4 day exposure to hydrocortisone plus 100 nM retinoic acid (closed triangles) were assayed at the indicated concentrations of 2-naphthol. Symbols encompass ± SD of averaged triplicate measurements unless error bars show otherwise. Results of weighted analyses from additional experiments are presented in Table 1.

TABLE 1. Steady-state kinetic parameters for 2-naphthol sulfonation by bovine bronchial epithelial cell culture extracts[1]

HC (nM)	RA (nM)	K_m ± SE (μM)[2]	V_{max} ± SE (nmol/mg/min)[2]
0	0	3.68 ± 0.36	0.272 ± 0.013
20	0	2.95 ± 0.37	0.642 ± 0.041
20	0.001	4.15 ± 0.49	0.628 ± 0.037
20	0.010	4.17 ± 0.37	0.521 ± 0.025
20	0.10	4.56 ± 0.46	0.506 ± 0.033
20	1.0	3.22 ± 0.50	0.441 ± 0.044
20	10	5.26 ± 0.57	0.571 ± 0.036
20	100	3.08 ± 0.29	0.295 ± 0.013
20	1000	3.01 ± 0.16	0.292 ± 0.007

[1]Primary cells (see Materials and Methods) were exposed to the indicated concentrations of hydrocortisone (HC) and retinoic acid (RA) for 4 days.
[2]Steady state kinetic constants and their standard errors were calculated by the weighted analysis of Wilkinson (1961). 2-Naphthol concentrations were varied as shown in Figure 4.

increase in cognate transcript levels in response to hydrocortisone, which was attenuated by simultaneous exposures to increasing concentrations of retinoic acid. No obvious effect of retinoic acid alone was observed. Using tubulin transcript levels as internal controls for slight variations in loading, densitometric analyses revealed a 10-fold increase in PST mRNA levels in response to hydrocortisone (not shown). The repression of this stimulation by retinoic acid displayed a dose-response very similar to that observed by analysis of enzyme activities, with half-maximal inhibition at approximately 0.1 nM.

DISCUSSION

The current data reveal that nanomolar concentrations of retinoic acid can prevent the hydrocortisone-stimulated expression of phenol sulfotransferase activity, protein levels, and message levels within cultured bovine bronchial epithelial cells. These results likely explain our prior observation (Beckmann et al., 1994) that inclusion of all hormonal supplements used to formulate the LHC-9 culture medium (Lechner and LaVeck, 1985) antagonized the stimulation of PST expression in these cells by hydrocortisone. Thus, the expression of PST in this system is not simply constitutive, but it is subject to both positive and negative controls.

Retinoic acid displayed a general repressive effect on phenol sulfotransferase expression, even in the presence of excess hydrocortisone (Fig. 2). This is in contrast to a second possible result of a "rightward" shift in the hydrocortisone dose-response curve. This strongly suggests that the two hormones are acting via distinct receptors, which is consistent with our current understanding of steroid and retinoid receptors. The retinoid receptor(s) appears to exert a dominant effect over the glucocorticoid receptor in the control of phenol sulfotransferase expression.

Retinoic acid also attenuates expression of cholesterol sulfotransferase expression in rabbit and human tracheobronchial epithelial cells during "squamous differentiation" (Rearick et al., 1987a,b). While incompletely characterized in these systems, current evidence indicates sulfoconjugation of cholesterol by dehydroepiandrosterone sulfotransferase (Aksoy et al., 1993). This steroid sulfotransferase is biochemically and genetically distinct from phenol sulfotransferases (Aksoy and Weinshilboum, 1995; Otterness et al., 1992; Wilborn et al., 1993). Also, our cell culture extracts and purified bovine phenol sulfotransferase enzyme display non-detectable cholesterol sulfotransferase activities (data not shown). Therefore, our observations are not due to assays of a steroid sulfotransferase. However, the previously reported assays of cholesterol sulfotransferase were conducted in the presence of Triton X-100, because the detergent was found to "activate" the enzyme activity (Rearick et al., 1987a). Triton X-100 is a substrate of phenol sulfotransferase (Spolter and Rice, 1980), so it is possible that previous measurements may be a mixture of steroid and phenol sulfotransferase activities.

Retinoic acid is a reported morphogen for the normal differentiation of tracheobronchial epithelium to a pseudostratified ciliated histology (Jetten et al., 1987). In the absence of this dietary hormone, airway epithelial squamous metaplasia occurs (Clark et al., 1980). It is therefore interesting that phenol sulfotransferase expression in human nasal polyp epithelium appears restricted to regions of basal cell hyperplasia with squamous cell metaplasia, whereas little expression is observed in adjacent epithelia of normal histology (Beckmann et al., 1995). These results provide further evidence for dynamic regulation of phenol sulfotransferase expression in airway epithelial cells in vivo, as indicated by the current results in vitro.

The control of gene expression by steroids and retinoids is complex. Although these hormones are frequently positive modulators, repression of gene expression has also been observed (Felli et al., 1991; Yang-Yen et al., 1990). Retinoic acid antagonizes estrogen stimulation of TGF-alpha and pS2 gene expression in MCF-7 cells, although the mechanism is unknown (Fontana et al., 1992). Retinoic acid also inhibits tyrosine aminotransferase gene induction by dexamethasone in a rat hepatocyte line (Pan et al., 1992). One possible mechanism for such counter-regulation would be mutually exclusive binding to specific gene regulatory DNA sequences. Alternatively, there is evidence for inhibitory protein-protein interactions between steroid receptors and other transcription factors (Yang-Yen et al., 1990), so perhaps such binding occurs with the retinoic acid receptor in our cell culture system. A more precise molecular mechanism for the repressive effect of retinoic acid on phenol sulfotransferase gene expression is not yet clear. We are currently characterizing the bovine phenol sulfotransferase gene in order to explain these observations. The presence of response elements for the steroid receptor(s) seems likely, based on reports of consensus estrogen and glucocorticoid response sequences upstream of an estrogen sulfotransferase gene (Komatsu et al., 1993).

The most obvious function of the phenol sulfotransferase within the airway epithelium would be to provide a route of xenobiotic detoxification (Mulder, 1981). Sulfonation of certain compounds such as N-hydroxylarylamines (Gilissen et al., 1994), however, results in their bioactivation to the likely ultimate carcinogens. Therefore, modulation of phenol sulfotransferase activity may be an important determinant of detoxification vs. carcinogenesis. The ability to regulate airway epithelial cell phenol sulfotransferase activity in vitro will allow

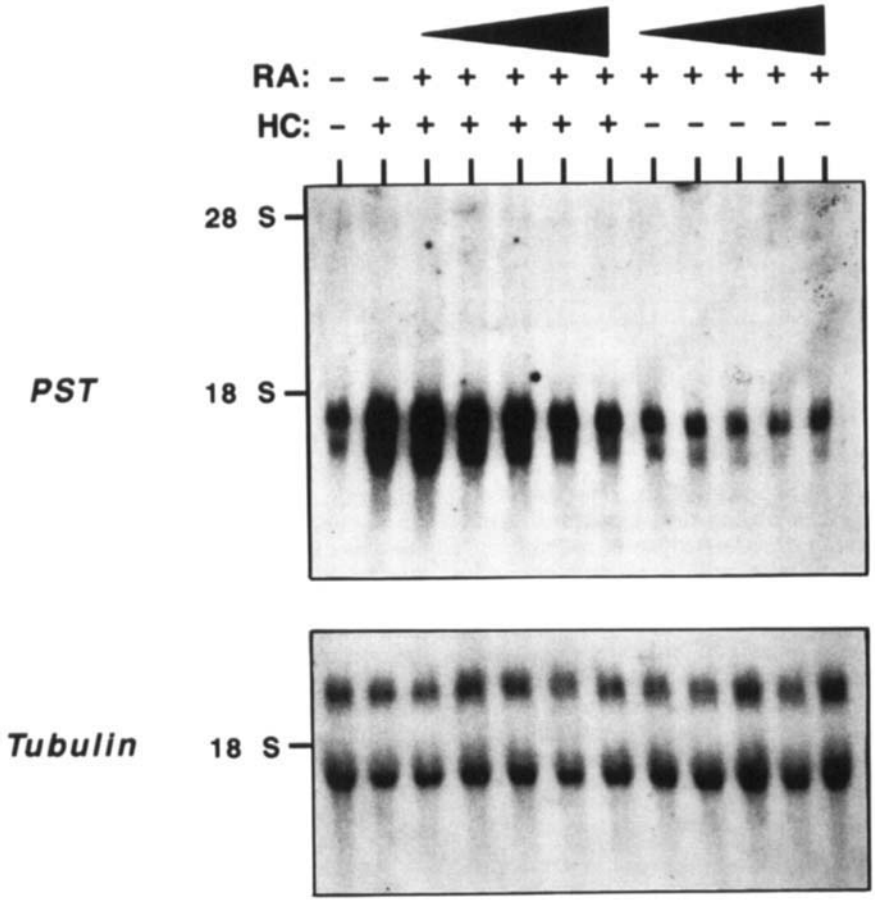

Fig. 5. Phenol sulfotransferase (PST) mRNA levels are regulated by hydrocortisone and retinoic acid. Primary cells were incubated in the absence of hydrocortisone (HC) and retinoic acid (RA) for 5 days before 50 h exposures to ± 20 nM HC in the absence or presence of increasing concentrations of RA (6 pM, 0.125 nM, 2.5 nM, 50 nM, 1 μM). Duplicate gels (10 μg RNA/lane) were blotted to nitrocellulose and hybridized to either the bovine PST cDNA or the mouse β5 tubulin cDNA probes followed by autoradiography as shown.

us to determine if any phenotypic endpoints can be observed, such as varied resistance to cytotoxic phenolic compounds. Such chemicals are of considerable interest, being abundant pollutants within organic combustion products such as cigarette smoke (Wynder and Hoffmann 1979). Whether our current results can explain the increased incidence of lung cancer in male smokers with elevated dietary β-carotene (Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group, 1994) will be an interesting question for further investigation.

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