Inability of Vitamin A Deficiency to Alter Benzo(a)pyrene Metabolism in Syrian Hamsters¹

William A. Bornstein,² M. P. Lamden, A. H. L. Chuang, R. L. Gross, P. M. Newberne, and Edward Bresnick

Department of Biochemistry, The University of Vermont College of Medicine, Burlington, Vermont 05401 [W. A. B., M. P. L., A. H. L. C., E. B.], and Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 [R. L. G., P. M. N.]

ABSTRACT

Syrian golden hamsters were placed on a control or vitamin A-deficient diet. When their serum vitamin A content was significantly reduced, i.e., to less than 10% of controls, the hamsters were killed and lung aryl hydrocarbon hydroxylase activity and metabolism of benzo(a)pyrene were determined. The benzo(a)pyrene metabolite profile was similar with control and A-deficient systems, and only few quantitative differences were noted. Addition of β-retinyl acetate to the in vitro incubations did not substantially affect benzo(a)pyrene metabolism.

INTRODUCTION

Nutritional manipulation of vitamin A status exerts profound effects on tumorigenesis in a number of different test systems (reviewed in Ref. 41). One of the more extensively studied animal models in this regard is that of BP-induced respiratory tract carcinoma in the Syrian hamster (12, 30, 34, 38). With these rodent model systems, vitamin A deficiency clearly enhances tumorigenesis (6, 28–30, 41). However, supplementation with vitamin A or its analogs at pharmacological doses in excess of the quantity necessary to prevent frank deficiency results in more variable effects (21, 28, 38, 39). Such equivocal effects of pharmacological supplementation with vitamin A compounds have been hypothesized as being related to problems of distribution (41). Studies with recently synthesized analogs (i.e., retinoids) with superior pharmacological properties have tended to support this hypothesis (2, 10, 30, 41).

The mechanism by which vitamin A influences tumorigenesis is not known. BP as well as many but not all of the other chemical carcinogens known to be influenced by vitamin A status requires metabolic activation prior to initiating tumor formation (5, 7, 15, 17, 22, 24, 36, 37, 45). Activation is accomplished by an enzyme system that is present not only in microsomes but also in nuclei and leads to the formation of metabolites that bind to DNA and other macromolecules (1, 3, 4, 16, 19, 32, 44).

Vitamin A deficiency diminishes the level of rat hepatic microsomal cytochrome P-450 (13) and enhances the binding of BP to hamster tracheal epithelial DNA (8). In addition, Hill and Shih (14) have found that the formation by rodent hepatic microsomes of a metabolite that is strongly bound to reaction components was inhibited by the in vitro addition of any of several vitamin A compounds. On the other hand, they were unable to observe any effect of these compounds on the formation of 3-OH-BP by hepatic microsomes, although an inhibition of the further metabolism of 3-OH-BP was noted. Under the conditions of their experiments, only limited pulmonary metabolism of BP was seen.

HPLC has been recently applied to the analysis of BP metabolism; this technique permits quantitation of a rather wide spectrum of BP metabolites (35, 46). In this study we have applied this technique to a more definitive investigation of the possible effects of vitamin A deficiency on BP metabolism. We have manipulated the vitamin A status in vivo and have concentrated our attention on lung, a target organ for BP tumorigenesis (33).

MATERIALS AND METHODS

Chemicals. BP, NADPH, G-6-P, and G-6-P dehydrogenase were obtained from Sigma Chemical Company (St. Louis, Mo.). [3H]BP (5 Ci/mmol, generally labeled) was purchased from Amersham/Searle (Arlington Heights, Ill.) and was purified prior to use by alumina column chromatography (9). β-Retinyl acetate, which was the generous gift of Dr. Brooke Mossman, was stored under a N₂ atmosphere at −80°.

Animals. Weanling male Syrian golden hamsters (ARS/ Sprague-Dawley Division, The Mogul Corporation, Madison, Wis.) were individually housed in plastic cages with cellulose filter tops; one-eighth to one-quarter inch of ground corn cobs were used as bedding (Bed-O-Cobs; Anderson Cob Mills, Inc., Maumee, Ohio). Water and food were provided ad libitum. Both control and experimental groups received a semisynthetic diet formulated into agar gel. The control and experimental diets were complete and identical with respect to essential nutrients except that the former contained 30 IU of vitamin A per g of diet, whereas the latter contained no vitamin A.

Preparation of Tissue. Animals were sacrificed by decapitation, and blood was collected directly into plastic tubes. Lung and liver were rapidly excised and washed, and microsomes were isolated from tissue homogenates as previously described (5). The microsomes were used immediately. Serum and liver homogenates (in 0.25 M sucrose) were stored at −80° until utilized in the determination of their vitamin A contents. The concentration of protein was determined by the method of Lowry et al. (23) with bovine serum albumin as the reference standard.

AHH Assay. AHH activity was measured essentially according to the method of Nebert and Gelboin (26). The incubation mixture contained, in a final volume of 0.5 ml,
Tris chloride buffer (pH 7.5), 40 µmol; MgCl₂, 1.5 µmol; NADPH, 0.13 µmol; BP, 0.04 µmol; G-6-P, 0.33 µmol; G-6-P dehydrogenase, 0.08 unit; and microsomal protein, 70 to 250 µg. The assay mixtures were incubated at 37° for 10 min with gentle shaking. The reaction was stopped by immersing the tubes in ice water, and 0.5 ml of cold acetone and 1.5 ml of hexane were added immediately. The mixture was vigorously blended on a Vortex mixer for 1 min and then centrifuged. The hydroxylated BP in the organic (upper) layer was extracted in 1.5 ml of 1 N NaOH solution, and the fluorescence of the alkaline solution was measured at 522 nm with the excitation wavelength set at 396 nm. AHH activity was measured in duplicate samples with 3 levels of enzyme protein. The assays were performed under conditions of linearity with respect to both time and protein.

**Microsomal Metabolism of [³H]BP.** Incubations were performed in 0.5-ml volumes containing NADPH, 0.33 mM; G-6-P, 0.26 mM; Tris chloride (pH 7.5), 50 mM; MgCl₂, 3 mM; G-6-P dehydrogenase, 0.07 unit; lung microsomes equivalent to 0.7 to 0.9 mg of protein; and [³H]BP (100 µCi/µmol), 10 µCi/assay in 10 µl of DMSO. β-Retinyl acetate, when present, was at a concentration of 5 µM and was added in 5 µl of DMSO. Each assay tube contained DMSO at a final concentration of 3% (v/v). DMSO at this concentration had no effect upon enzyme activity. Preincubation was carried out for 5 min at 37° in the absence of substrate; the complete system was then incubated for an additional 15 min. The reaction was terminated by the addition of 0.5 ml of acetone, and the metabolites were extracted into 1.0 ml of ethyl acetate. The aqueous phase was washed with 0.5 ml of ethyl acetate, and the organic phases were combined, dried over anhydrous sodium sulfate, passed through 0.45-µm Millipore filters, and reduced to a residue under a stream of dry N₂. The residues were stored under N₂ at -20° until HPLC analyses were performed.

The chromatography was performed on a 3.9-mm x 30-cm µBondapak C₁₈ column (Waters Associates, Milford, Mass.) with a reverse-phase linear gradient of 60 to 80% methanol in water over the course of 40 min with a flow rate of 1.0 ml/min. The sample was dissolved in 80 µl of 100% methanol, from which a 40-µl aliquot was taken for injection onto the column. The eluent was monitored continuously for absorbance at 254 nm, while fractions were collected directly into scintillation vials every 0.5 min. Quantitation was by liquid scintillation counting techniques with the use of a Beckman LS-3133T spectrometer and a Triton X-100-based counting fluor. The counting efficiency for ³H was greater than 30%. A mixture of nonradioactive metabolite standards (a generous gift from Dr. Harry V. Gelboin of the National Cancer Institute) was periodically cochromatographed with authentic 9-hydroxybenzo(a)pyrene and 3-OH-β-retinyl acetate. We have recently observed a metabolite eluting with this retention that was formed by mouse liver microsomes in substantial quantity even in the absence of an epoxide hydrase inhibitor. The mass spectral pattern and UV spectrum of this metabolite in their studies was identical with those obtained with the authentic 4,5-oxide of BP, i.e., the K-region oxide.

**RESULTS**

Serum and hepatic vitamin A levels were severely depressed in the experimental group (Table 1). The reduction in vitamin A content of serum and liver was 92% in each case. However, the difference in body weights between the control and experimental groups was not statistically significant. Rats with similarly depressed serum and hepatic vitamin A levels but normal body weights were found by Hauswirth and Brizuela (13) to have decreased hepatic cytochrome P-450 and altered activity of certain drug-metabolizing enzymes. Our experiments were performed with hamsters that were not in the debilitating stages of vitamin A deficiency, i.e., at the time of large weight loss.

A typical HPLC pattern of BP metabolites is shown in Chart 1. Cochromatography with BP metabolite standards revealed that hamster pulmonary microsomes can form in vitro the 3 known BP trans-diols, 4,5-, 7,8-, and 9,10-dihydriodiol; the 3 quinones, 1,6-, 3,6-, and 6,12-quinones; and 2 groups of phenols. The phenol peaks cochromatographed with authentic 9-hydroxybenzo(a)pyrene and 3-OH-β-retinyl acetate, respectively. These regions have been found by Selkirk et al. (35) using rat liver microsomes to contain these metabolites predominantly. An additional peak in the quinone region eluted with a retention time approximating that of the 4,5-oxide of BP. Lam and Wattenberg (20) have recently observed a metabolite eluting with this retention that was formed by mouse liver microsomes in substantial quantity even in the absence of an epoxide hydrase inhibitor. The mass spectral pattern and UV spectrum of this metabolite in their studies was identical with those obtained with the authentic 4,5-oxide of BP, i.e., the K-region oxide.

**Table 1** 

<table>
<thead>
<tr>
<th>Vitamin A levels in control and deficient hamsters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutritional status</td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td>Control (2)</td>
</tr>
<tr>
<td>A-deficient (5)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, number of groups; each group consisted of 2 animals.

* p < 0.0001.

* p > 0.05.
However, the definitive identity of this peak as well as the assertion that benzo(a)pyrene 4,5-oxide may accumulate in the absence of an inhibitor of epoxide hydrase remain controversial.

Three additional radioactive peaks, designated UKI, UKII, and UKIII, were observed in the regions of high, moderate, and low relative polarities, respectively. The structures and importance of these species are unknown. Radioactivity associated with these peaks was not quantitatively affected by whether microsomes were obtained from vitamin A-depleted or control animals (data not shown).

Lung microsomal metabolism of BP is quantitatively presented in Table 2. Radioactivity of each metabolite is expressed as a percentage of that appearing in all peaks cochromatographing with defined authentic metabolites. Very few significant differences resulted from either the in vivo or in vitro manipulation of the vitamin A status. The formation of benzo(a)pyrene 3,6-quinone by pulmonary microsomes from control animals was increased slightly but significantly by the in vitro addition of β-retinyl acetate. Under the same conditions the formation of the second group of phenols (largely 3-OH-BP) was decreased significantly. However, of greater interest is the observation that none of the metabolite peaks was quantitatively altered by the nutritional status of the animals from which the microsomes were obtained.

Levels of AHH activity were determined with the use of hepatic microsomes from the same animals in which the pulmonary metabolism had been assessed (Table 3). The former assay measures the BP phenols, predominantly 3-OH-BP. Although a slightly greater AHH activity was observed with microsomes from vitamin A-deficient animals, this difference was not statistically significant. We have, however, found this increase to be quite reproducible both in this species and in other rodent species (data not shown).

**DISCUSSION**

Vitamin A may exert its inhibitory effect upon chemical carcinogenesis by numerous possible mechanisms. Of these various possibilities an alteration in the metabolism of chemical carcinogens is attractive and is supported by the finding that, in vitamin A deficiency, reduced levels of cytochrome P-450 (13) were observed. Furthermore, evidence has been obtained suggesting gross alterations in BP metabolism by hepatic microsomes obtained from animals fed a normal diet when incubated in the presence of vitamin A analogs (14).

In this study we have been unable to demonstrate any substantial quantitative differences in the metabolic profiles obtained when BP is incubated with pulmonary microsomes from vitamin A-deficient or control hamsters. Furthermore,
the addition of a vitamin A analog in vitro exerted little effect on the metabolism of BP by these microsomes. The addition of a vitamin A analog in vitro exerted little activity remaining at the origin after development in a paper chromatographic system. One cannot conclusively infer altered metabolism of BP from their results.

Even prior to the present study, evidence had accumulated that suggested that metabolic alterations would be unlikely as the sole mechanism by which vitamin A compounds influence tumorigenesis. For example, vitamin A manipulations could be carried out as long as 10 weeks after exposure of rats to another polycyclic aromatic hydrocarbon, 3-methylcholanthrene, and still drastic reductions in the formation of metaplastic nodules were observed (27). Furthermore, retinoids have been convincingly demonstrated to alter tumor induction by direct-acting carcinogens, which do not require metabolic activation (25). These and other data imply that vitamin A compounds act during the postinitiation phase of tumorigenesis. Effects during this phase may be mediated through the hormone-like action of vitamin A compounds, e.g., changes in DNA synthesis and/or DNA repair (40). These aspects of vitamin A interaction with target tissues for chemical carcinogenesis are currently under investigation in our laboratory.

REFERENCES


Inability of Vitamin A Deficiency to Alter Benzo(a)pyrene Metabolism in Syrian Hamsters


Updated version: Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/38/6/1497

E-mail alerts: Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions: To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions: To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.