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MECHANISM OF ACTION OF BENZO(a)PYRENE AND NICOTINE ON HORMONE PRODUCTION BY RAT PITUITARY TUMOR CELLS

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SUMMARY: The effects of the cyclic aromatic hydrocarbon, benzo(a)pyrene (BaP) and that of the tobacco alkaloid, nicotine, on prolactin (PRL) and growth hormone (GH) synthesis by rat pituitary tumor cells in culture (GH cells) have been studied. Treatment of GH cells with nicotine (0.1-300 µg/ml) neither affected the growth, nor significantly altered the general pattern of hormone production in these cells. BaP at concentrations greater than 5 µg/ml irreversively inhibited the growth of these cells. The sublethal concentrations of BaP, which did not affect either 1) cell growth, or 2) amino acid transport or 3) total protein synthesis or degradation, did however inhibit specifically, hormone synthesis by these cells. More interestingly concentrations of nicotine which did not affect either cell growth or hormone synthesis, modulated both of these cellular processes in the presence of BaP. A concentration dependent stimulation of microsomal BaP monooxygenase activity was observed in nicotine or BaP treated cells. The effects of these drugs on stimulation of BaP monooxygenase activity seems to be additive. Nicotine also enhanced the association of radioactivity (presumably [^{3}H] BaP metabolites) with DNA in [^{3}H] BaP treated cells. It is concluded that nicotine by itself did not demonstrate any cytotoxic effect nor influence hormone synthesis in GH cells. However, this constituent of tobacco smoke stimulated BaP monooxygenase activity and the interaction of [^{3}H] BaP metabolites with cellular DNA and also modulated BaP induced inhibition of hormone synthesis in GH cells.

INTRODUCTION: The cyclic aromatic hydrocarbon, benzo(a)pyrene (BaP) is an established potent carcinogen (1). Its abundance in the environment and its intake from other sources such as tobacco smoke, qualifies it as one of the most prevalent chemical carcinogens to which living systems are currently exposed. Ectopic hormone production by lung tumors obtained from heavy smokers suggest that certain constituents of cigarette smoke are responsible for such cellular differentiation (2.3). Hormones have been associated with induction and progression of tumors in many experimental systems; but the role of hormones

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in the process of initiation and progression of carcinogenesis is not clearly defined as yet.

It is of significant interest to investigate the effect of these chemical constituents of cigarette smoke on hormone production by specific cell types which normally produce hormones. Specific hormone production, which is a differentiated function of a specific cell type in the normal pituitary gland, can be studied in cultured rat pituitary tumor cells (GH cells). GH cells also respond to physiological and pharmacological agents in a fashion similar to the normal pituitary gland (4). Different subclones of GH cells synthesize and secrete different amounts of prolactin (PRL) and growth hormone (GH). To understand the mechanism of action of BaP and that of the tobacco alkaloid nicotine, the effect of these chemical agents on hormone production by GH cells and also by human lung tumor cells, has been examined. In this report, the results of our studies on PRL and GH synthesis by control and drug treated GH cells are presented.

Methods: Cells used in this investigation were multihormone producing clonal strains of GH cells. Isolation and characterization of these GH cell strains were described previously (4). Growth of these cells was measured by determination of cell protein (5).

Assay for PRL and GH Production, Synthesis and Degradation: The production of PRL and GH by control and drug treated cells was measured in the culture media and in sonic extracts of cells by complement fixation assay (6). PRL synthesis in intact cells was quantitated by indirect antibody precipitation of [^{3}H] PRL in culture medium and in cell extracts after pulse labelling of the cells with [^{3}H] leucine. To determine the transport of [^{3}H] leucine in control and drug treated cells, intracellular levels of trichloroacetic acid-soluble radioactivity at different time periods after pulse were measured in those cells. Degradation of newly synthesized [^{3}H] PRL was studied by kinetic measurements of rate of disappearance of PRL-antibody precipitable radioactivity during the chase period after a short pulse.

Assay of BaP Monooxygenase: BaP monooxygenase was measured in microsomal preparations from control and drug treated cells according to the method of DePierre et al (7).

Measurement of Radioactivity Associated with Purified DNA Isolated from [^{3}H] BaP Treated Cells: Cells were grown in 60mm dishes and treated with different concentrations of [G-^{3}H] BaP (Amersham 17.4Ci/nmole). DNA from cells grown in the presence of different concentrations of the radioactive drugs was then isolated according to the method of Gross-Bellard, et al. (8). The DNA preparation was made free of protein and RNA by extensive treatment with Proteinase K and RNase I (8). Radioactivity associated with such DNA preparation was measured by precipitation with cold trichloroacetic acid and subsequently by counting the radioactive precipitate by collecting on a GFC filter. Ninety to ninety five percent the TCA precipitable radioactivity associated with such DNA preparation could be solubilized by DNase I treatment.

RESULTS AND DISCUSSIONS: Results presented in Fig. 1, show the growth of GH cells in the presence of BaP alone, nicotine alone and in the presence of both drugs together. Nicotine concentrations ranging from 0.1-50 µg/ml (Fig. IA) and 100-300 µg/ml (Fig. 1 B) did not affect the growth of these cells. Treatment of GH cells with BaP at concentrations higher than 5 µg/ml irreversibly inhibited growth of these cells (Fig. 1 C). BaP concentrations which were not inhibitory did however drastically affect the growth of cells when added in combination with sublethal concentrations of nicotine (Fig. 1 C & D). These results demonstrate that nicotine enhanced the toxic effect of BaP on GH cells. A concentration dependent inhibition of PRL (Fig. 2 A) and GH (Fig. 2 C) production was observed in BaP treated cells. Although treatment of the cells with BaP (5 µg/ml) for 5 days, did not affect the growth of

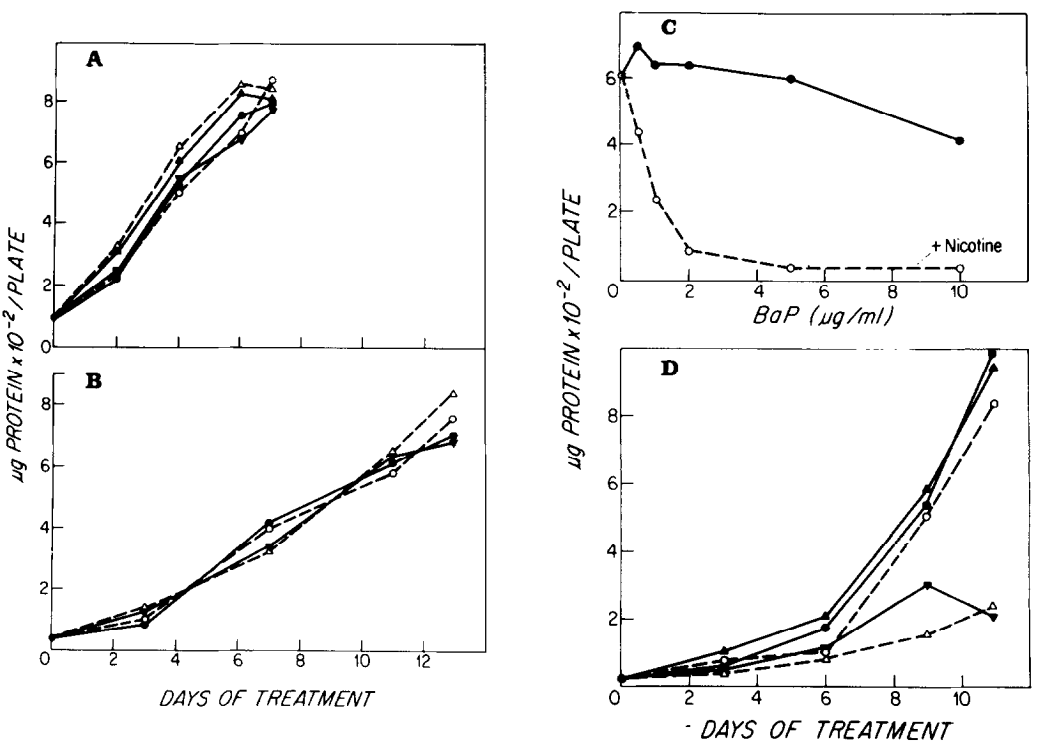


Figure 1 Effect of Nicotine and BaP on Growth of GH Cells:

Panel A: -●- = control; -▲- = 0.1 µg/ml; -o- = 0.5 µg/ml; -Δ- = 10.0 µg/ml; -▼- = 50 µg/ml. Panel B: -●- = control; -▼- = 100 µg/ml; -o- = 300 µg/ml; -Δ- = 300 µg/ml. Panel C: -●- = BaP; -o- = BaP + Nicotine (300) µg/ml Panel D: -●- control, ▲ = 5 µg/ml BaP; -o- = 5 µg/ml BaP + 100 µg/ml nicotine; -▼- = 5 µg/ml BaP + 200 µg/ml nicotine -Δ- = 5 µg/ml BaP + 300 µg/ml nicotine.

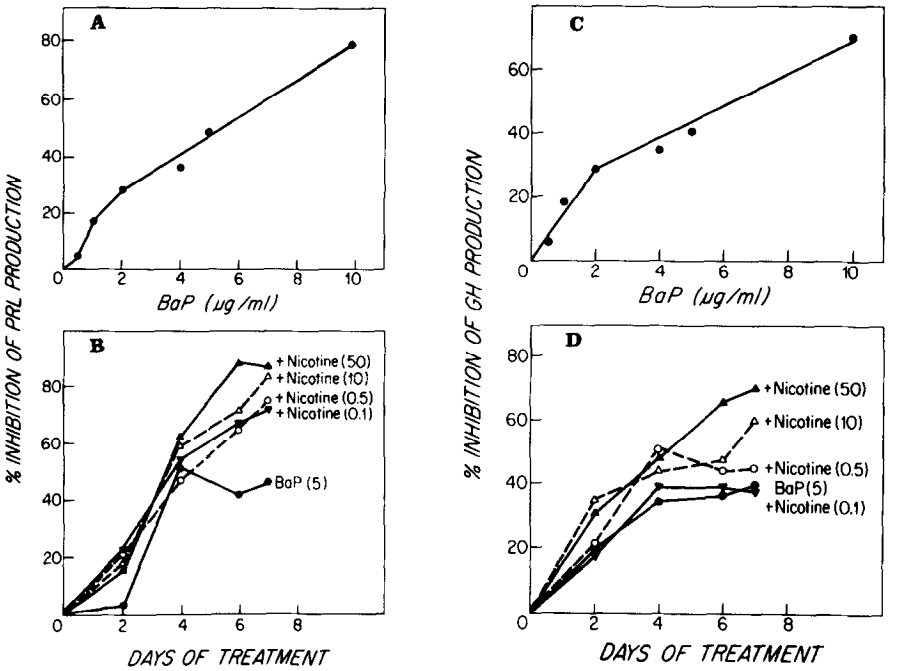


Figure 2 Effect of BaP and Nicotine on Prolactin (PRL) and Growth Hormone Production (GH): PRL and GH production in control and treated cells were determined by measurement of the hormone in the medium by microcomplement fixation assay. (7) Panel A shows the effect of different concentrations of BaP on PRL productions by GH_3 cells treated with the drug for five days. Percent inhibition of PRL production (µg/ml protein/day) by different concentrations of BaP was calculated from PRL determinations in the medium of GH_3 cells grown in the absence of the drug under identical conditions. Panel B shows the effect of different concentrations of nicotine on the BaP induced inhibition of PRL production by GH_3 cells. GH_3 cells were grown in the presence of BaP alone or BaP and nicotine together at the concentrations (µg/ml) of the drugs indicated in parenthesis, treated for the specified period of time. Panel C shows the percent inhibition of GH production as observed after treatment of the cells with indicated concentrations of BaP for five days. Panel D shows the effect of different concentrations (µg/ml) of nicotine as indicated by the numerals in parenthesis; on the BaP induced inhibition of GH production.

cells, such treatment did reduce PRL and GH production by 50% and 60% respectively. Nicotine, at concentrations ranging from 0.1-300 µg/ml did not affect the PRL and GH production by these cells (data not shown). However, it is clear from the results presented in Fig. 2 B & D that BaP-induced inhibition of hormone production by GH cells was further enhanced by nicotine concentrations as low as 0.1 µg/ml. The 40-50% inhibition of PRL and GH production which was observed at BaP concentration of 5 µg/ml, could be enhanced to the extent of 80-90% with simultaneous addition of nicotine (0.1-50 µg/ml) in the growth medium along with the same concentration of BaP (Figs. 2 B & D).

EFFECT OF BaP ON PRL SYNTHESIS & DEGRADATION: Assay of hormones by micro-complement fixation determines the immunoreactive material present in cells at a given time. To have an estimate of the rate of PRL synthesis, cells were prelabelled with [^{3}H] leucine for 4, 6, 12, and 24 hours, and the relative amounts of [^{3}H] PRL synthesized during these periods in control and BaP treated cells were determined. No significant difference in the appearance of [^{3}H] leucine in intracellular TCA soluble radioactivity (data not shown) and its incorporation into total TCA precipitable radioactivity between control and BaP treated (5 days) cells were observed (Fig. 3 A, -O-). These results suggest that BaP treatment of the cells (5 µg/ml) did not affect either the transport of the specific amino acid or total protein synthesis. Results presented in Fig. 3 A show the amounts of [^{3}H] PRL that could be precipitated with anti-PRL antibody from total cellular [^{3}H] protein synthesized during 4, 6, 12 or 24 hours labelling period in control and in cells treated with increasing concentrations of BaP. The total TCA precipitable radioactivity varied depending on the labelling period, but percentage of [^{3}H] PRL in total [^{3}H] protein synthesized remained approximately the same. However, the results presented in Fig. 3 A demonstrate a concentration dependent inhibition, specifically, of PRL synthesis in BaP treated cells (5 µg/ml for 5 days). The degree of inhibition of [^{3}H] PRL synthesis by a given concentration of BaP was approximately the same regardless of the duration of the labelling period.

The observed inhibition of [^{3}H] PRL synthesis in GH cells by BaP may be due to comparatively higher turnover rate of newly synthesized [^{3}H] PRL in drug treated cells. This possibility was examined by studying the rate of degradation of [^{3}H] PRL in control and drug treated cells. Results presented in Fig. 3 B show that the degradation rate of such newly synthesized [^{3}H] PRL in control and BaP treated cells was unaffected by drug treatment of the cells. The relative increase of [^{3}H] PRL in both control and BaP treated cells (Inset, Fig. 3) reflects the higher rate of degradation of a fraction of cellular proteins in comparison to PRL. These results in addition to those presented in Fig. 2 demonstrate that BaP specifically inhibited hormone synthesis in GH cells. Nico-

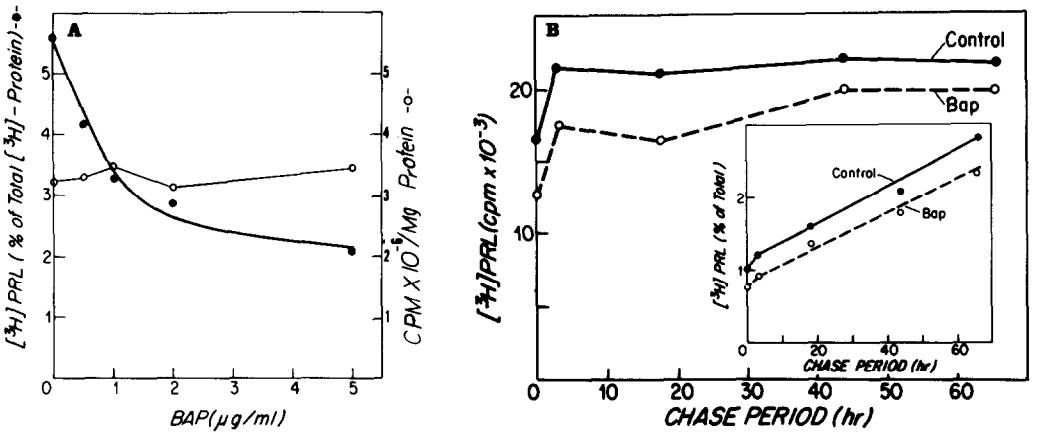
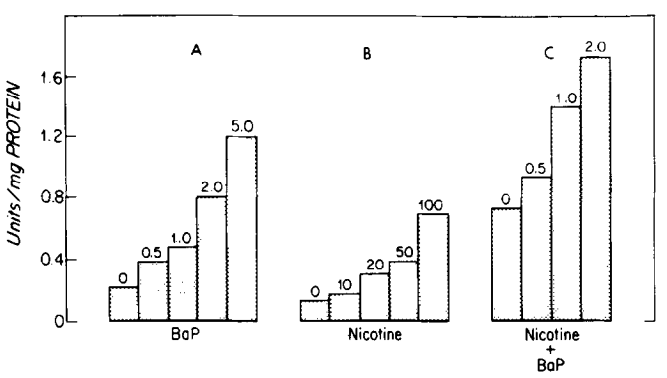


Figure 3 Effect of BaP on Prolactin (PRL) Synthesis and Degradation: GH cells were grown in 25cm^2 tissue culture flask in the absence (-o-) or the presence (-●-) of the different concentrations of BaP under the conditions described in legend to Fig. 1. PRL synthesis was quantitated by the indirect antibody precipitation of the PRL in the culture media and in the cells after pulsing for six hours with [^{3}H] leucine (10µCi/ml, in a leucine free medium (MEM minus leucine, Gibco). [^{3}H] PRL in the sonic extract of the cells and in the culture medium was precipitated with the anti-PRL-antibody under the conditions described previously (4). The [^{3}H] PRL-antibody complex was dissolved in SDS-buffer and TCA precipitable radioactivity was determined in an aliquot.

For degradation studies cells were grown in the absence or the presence of BaP (5 µg/ml; for five days) and pulse labelled with [^{3}H] leucine under the conditions described above. After pulse labelling with [^{3}H] leucine, the cells were washed several times with the preequilibriated medium (F10) and fresh medium (F10) was then added and incubated at 37°. At the onset of the chase period, a set of flasks (in duplicate) containing the prelabelled cells were withdrawn and [^{3}H] PRL and total [^{3}H] protein in the cell extract and in medium were quantitated. Such [^{3}H] PRL and [^{3}H] total protein determination were carried out in the cell extracts and the culture medium of the control (-●-) and the BaP treated (-o-) cells withdrawn at indicated times during the chase period. In the inset of Fig. 3, these results are expressed as [^{3}H] PRL as the percentage of the total [^{3}H] protein in control (-●-) and BaP treated (-o-) cells withdrawn at the indicated time periods during the chase period.

tine by itself, did not affect cell growth or hormone production. Its modulation of BaP induced effects, suggests that the tobacco alkaloid may influence cellular mechanism/s which is/are involved in the metabolism of, and thus, the biological action of the cyclic aromatic hydrocarbon. To explore this possibility the enzyme BaP monooxygenase which is responsible for the conversion of cyclic aromatic hydrocarbon into the terminal biologically active compound was assayed in control, BaP and nicotine treated GH cells.

Results presented in Fig. 4 (left panel) show the microsomal BaP monooxygenase activity measured in BaP (Fig. 4 A) or nicotine (Fig. 4 B) treated GH cells. A concentration dependent increase of the enzyme activities in microsomes of nicotine or BaP treated cells were observed. The enzyme activity



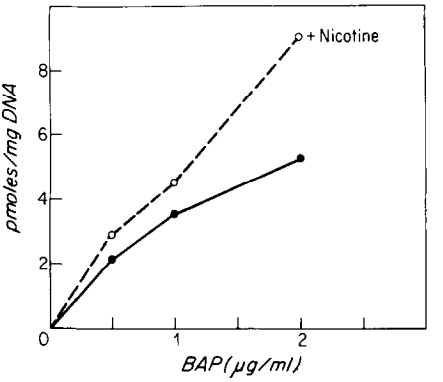


Figure 4 Left panel: Effects of BaP and Nicotine on BaP Monooxygenase Activity: GH_3 cells were grown as suspension culture in 1 liter spinner flasks (Belco), to a density of 5-8x10^5 cells/1 in the absence or in the presence of specified drugs. Cells were then harvested, washed and microsomes were prepared according to the method of DePierre et al (4). Assay conditions for BaP monooxygenase activity is described in "Materials and Methods" section.
Figure 4 Right panel: Effects of Nicotine on the Association of Radioactivity with DNA in [^{3}H] BaP Treated Cells: GH_3 cells were grown as monolayer in 75cm tissue culture flasks in the absence or presence of nicotine. [^{3}H] BaP solution (20μCi/ml) was mixed with non-radioactive BaP and an aliquot of this mixture was then added to each of the flasks to achieve the indicated fianl concentrations. After the drug treatment period (four days), the medium was removed and the cells were washed and harvested and DNA from these cells were isolated according to the method of Gross-Bellard et al (5). DNA content in these preparations were determined by spectroflourometric method (9). The [^{3}H] BaP-metabolite (pmoles) associated with DNA was calculated from the specific activity of the [^{3}H] BaP at each concentrations of the drug. Nicotine concentration was 100 μg/ml in [^{3}H] BaP and Nicotine (-o-) treated cells.

measured in the microsomal preparation of cells treated simultaneously with both drugs showed cumulative effect on this stimulation of BaP monooxygenase activity (Fig. 4 C). These results demonstrate that the low basal BaP monooxygenase activity can be stimulated further by treatment of GH cells with either of the cigarette smoke constituents, i.e., nicotine or BaP.

EFFECT OF NICOTINE ON THE ASSOCIATION OF RADIOACTIVITY WITH DNA IN [^{3}H] BaP TREATED CELLS: Metabolites of cyclic aromatic hydrocarbons interact with DNA leading to the formation of DNA-adducts. Results presented in the previous section demonstrated that nicotine enhanced the effects of BaP on a) growth of cells, b) hormone production by GH cells and c) also, modulates microsomal BaP monooxygenase activity in these cells. Results presented in Fig. 4 (right panel) demonstrate the interaction of [^{3}H] BaP-metabolites with DNA of GH cells grown in the presence or absence of nicotine. Cold TCA precipitable radioactivity associated with purified DNA isolated from [^{3}H] BaP treated cells in-

creased with increasing concentrations of the drug. The association of the radioactive compound, presumably the biologically active metabolite of [^{3}H] BaP, was further enhanced in the presence of nicotine (100 µg/ml). Most of the TCA precipitable radioactivity (90-95%) could be solubilized after treatment with DNase 1, suggesting that, the measured radioactivity was indeed associated with DNA. These results suggest that nicotine stimulates the binding of [^{3}H] BaP metabolites to cellular DNA presumably via stimulation of enzymatic conversion of [^{3}H] BaP into the terminal compound. Such parallel effects of nicotine on the binding of [^{3}H] BaP metabolites to cellular DNA and on the cellular differentiated function of hormone synthesis, suggest that these two events are correlated.

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