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SHORT COMMUNICATION

Induction of Aryl Hydrocarbon Hydroxylase in Mouse 3T3 Cells: Relationship to the State of Cell Proliferation and Guanosine Cyclic 3′,5′-Monophosphate

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SUMMARY

RUDDON, RAYMOND W., LUNDEEN, DONNA E., AND RIKANS, LORA E.: Induction of aryl hydrocarbon hydroxylase in mouse 3T3 cells: relationship to the state of cell proliferation and guanosine cyclic 3′,5′-monophosphate. *Mol. Pharmacol.* **9**, 686-691 (1973).

The induction of aryl hydrocarbon hydroxylase was studied in a line of mouse 3T3 fibroblasts (Swiss) grown in continuous cell culture. The level of hydroxylase activtiy after induction by benz[*a*]anthracene appeared to be several times higher in this 3T3 line than in other continuously cultured cell lines previously studied. The induction of aryl hydrocarbon hydroxylase was linear for 24 hr after the addition of 13 μM benz[*a*]-anthracene to the culture medium. Induction of the enzyme by benz[*a*]anthracene was markedly greater in 3T3 cells grown in the presence than in the absence of serum and was higher in nonconfluent than in confluent cells. Moreover, the addition of 0.01 μM dibutyryl cyclic GMP (a putative signal for increased cell proliferation) to serumless medium containing 6 μM benz[*a*]anthracene markedly potentiated induction of aryl hydrocarbon hydroxylase by the hydrocarbon. These results suggest that the inducibility of the hydroxylase system by benz[*a*]anthracene is greater in proliferating than in nonproliferating cells.

Aryl hydrocarbon hydroxylase is a microsomal, NADPH-requiring, mixed-function oxidase involved in the metabolism of a number of carcinogenic polycyclic hydrocarbons. It is present in a wide variety of tissues from many species *in vivo* (1), in explants of rat lung in organ culture (2), and in a number of mammalian cells grown in cell culture (1, 3-5). The use of established cell culture lines to study microsomal enzyme induction offers several advantages over the intact animal. For example, the medium and concentrations of drugs bathing the cells can be more precisely controlled. In addition, cell culture systems provide the opportunity to study directly the malignant transformation of cells *in vitro* by exposure to chemical carcinogens and the role of metabolism of the carcinogenic chemicals in this process. Malignant transformation of 3T3 cells derived from BALB mice has been shown to occur after exposure of the cells to polycyclic hydrocarbons *in vitro* (6).

This report describes the characteristics of the induction of aryl hydrocarbon hydroxylase in a line of mouse 3T3 cells grown in

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continuous cell culture and the relationship of enzyme inducibility to the proliferative state of the cells. The level of the induced enzyme in this cell line appears to be significantly higher than in other continuously cultured cell lines (1, 3–5), and thus it may afford a unique opportunity to characterize further the control of aryl hydrocarbon hydroxylase activity and its role in carcinogenesis.

Benz[*a*]anthracene was obtained from Eastman Organic Chemicals. Benzo[*a*]pyrene, N^2,$O^{2\prime}$-dibutyryl cyclic GMP, N^6,$O^{2\prime}$-dibutyryl cyclic AMP, and NADPH were obtained from Sigma Chemical Company. *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid was purchased from Calbiochem. Dr. Harry Gelboin, Chief of the Chemistry Branch, National Cancer Institute, generously supplied the 3-hydroxybenzo[*a*]pyrene used as a standard for the aryl hydrocarbon hydroxylase assays.

Mouse 3T3-M cells (Swiss) were obtained from Dr. Gail Wertz, Department of Human Genetics, University of Michigan Medical School. This line originally came from the laboratory of Basilico *et al.* (7) and contains a highly inducible aryl hydrocarbon hydroxylase system compared with the CCL-92 line of 3T3 fibroblasts (Swiss) obtained from the American Type Culture Collection.[1] For this reason, the 3T3-M line was selected for further study. Cells were grown as monolayer cultures in Blake bottles at 37° in Dulbecco's modified Eagle's medium (Grand Island Biological Company) supplemented with 10% calf serum (KC Biological, Inc., Lenexa, Kan.), 100 units/ml of penicillin G, and 100 μg/ml of streptomycin. The cultures revealed no uridine phosphorylase activity, which is presumptive evidence for absence of mycoplasmic contamination (8). Benzanthracene was dissolved in dimethyl sulfoxide and added to the culture medium to achieve a final concentration of 0.5% DMSO[2] in the medium. This concentration of DMSO did not appear to affect cell proliferation or morphology. Other drugs were added in sterile media directly to the cell cultures. Prior to homogenization the medium was removed, and the cells were rinsed three times in ice-cold HEPES-buffered saline (HBS) solution (pH 7.2) containing NaCl, 140 mM; KCl, 5.4 mM; $Na_2HPO_4 \cdot 7H_2O$, 0.7 mM; dextrose, 5.6 mM; and HEPES, 10 mM. After the final rinse the cell suspension was centrifuged at 1000 × *g* for 10 min at 0°, resuspended in HBS, and centrifuged again. The washed pellet (representing approximately 1 × 10^8 cells harvested from three Blake bottles for each experimental point) was then frozen at −80°. The freezing step facilitated the subsequent disruption of cells during homogenization, and cell pellets could be stored at −80° for several days without loss of aryl hydrocarbon hydroxylase activity. The pellets were thawed and suspended in 3–4 ml of a buffer (pH 7.4) containing Tris-HCl, 10 mM; sucrose, 250 mM; $MgCl_2$, 5 mM; and β-mercaptoethanol, 5 mM. Homogenization was performed in a Dounce ball type homogenizing tube by 10 strokes with a loose and 60–75 strokes with a tight pestle. Cell disruption was followed by phase contrast microscopy, and greater than 90% cell breakage was achieved with minimal breakage of nuclei by this method. Protein determinations were performed on the homogenates by a modification of the method of Lowry *et al.* (9).

The aryl hydrocarbon hydroxylase activity of the 3T3 cell homogenates was determined essentially by the method of Nebert and Gelboin (3), with the following modifications. The standard assay mixture contained, in a volume of 1.05 ml, 30 μmoles of Tris-HCl (pH 7.5), 0.18 μmole of NADPH, 4 μmoles of $MgCl_2$, 0.5–1.0 mg of homogenate protein, and 80 nmoles of benzo[*a*]pyrene (added in 0.05 ml of methanol just prior to incubation). The reaction mixtures were shaken at 37° for 30 min in air. The incubation and all subsequent steps were carried out in a darkened room without fluorescent lighting, since the reaction product, 3-hydroxybenzo[*a*]pyrene, is extremely light-sensitive. The formation of product was linear for at least 30 min under these conditions. The reaction was stopped by the addition of 1.0 ml of cold acetone, followed by 5.0 ml of hexane. The samples were then

[1] L. E. Rikans, and R. W. Ruddon, unpublished observations.

[2] The abbreviations used are: DMSO, dimethyl sulfoxide; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; cGMP, guanosine cyclic 3′,5′-monophosphate; cAMP, adenosine cyclic 3′,5′-monophosphate.

shaken for 10 min at 37°, transferred to 50-ml shaker bottles, and shaken for an additional 10 min at room temperature. They were then centrifuged at 2500 rpm in an International model K centrifuge for 10 min, and a 3.0-ml aliquot of the hexane phase was removed and placed in another 50-ml shaker bottle containing 4.0 ml of 1 N NaOH. The mixtures were then shaken for 20 min and centrifuged for 10 min at 2500 rpm. Following centrifugation, a 3-ml aliquot of the alkaline aqueous phase was removed, an additional 4.0 ml of 1 N NaOH were added, and the samples were again shaken for 20 min and centrifuged for 10 min as above. After the second extraction, a 4.0-ml aliquot of the alkaline aqueous phase was removed and pooled with the first 3-ml aliquot. This second NaOH extraction was found to improve the extraction efficiency and to make the checks from sample to sample more reproducible. Variation between identical samples by this method was routinely less than ±5%. The concentration of 3-hydroxylated benzo[*a*]pyrene in the alkaline phase was then determined spectrophotofluorometrically with activation at 396 nm and fluorescence at 522 nm in an Aminco-Bowman spectrophotofluorometer. The aryl hydrocarbon hydroxylase activity of each cell sample was determined in triplicate, and tissue blanks, incubated without substrate, were subtracted from each of the experimental values.

The kinetics of induction of aryl hydrocarbon hydroxylase in 3T3-M cells by 13 μM benz[*a*]anthracene in the presence of calf serum is illustrated in Fig. 1. In all similar experiments the range of induction by benz[*a*]anthracene (13 μM) after 24 hr of treatment of 3T3 cells in serum was 6–9-fold. This induced hydroxylase activity is about one-half the level of enzyme activity in uninduced rat liver cells *in vivo* and is several times higher than the induced activities in other 3T3 lines previously studied (1, 5, 10). Induced levels similar to this have been observed, however, in 3T3-L cell (5) and 3T3-HTC cell (10) hybrids.

Since aryl hydrocarbon hydroxylase was so rapidly inducible in the 3T3-M cells (approximately twice control by 2 hr after exposure to benz[*a*]anthracene), it was of interest to determine how rapidly gene transcription might be stimulated by the inducing agent in these cells. When actinomycin D, 1 μg/ml (a dose which inhibits RNA synthesis 95% in 3T3-M cells), was administered along with 13 μM benz[*a*]anthracene at zero time, induction of the enzyme was completely blocked (Fig. 2). This is consistent with results from other cell lines, suggesting that the initial phase of stimulation of aryl hydrocarbon hydroxylase by polycyclic hydrocarbons requires the synthesis of induction-specific RNA (11). However, in 3T3-M cells the accumulation of the RNA required for translation of components of the aryl hydrocarbon hydroxylase system must be extremely rapid. By 1 hr after exposure to benz[*a*]anthracene, all the essential RNA had apparently been synthesized, since there was no blockade of induction by actinomycin D added 60 min after the hydrocarbon.

The dose-response relationship between benz[*a*]anthracene and aryl hydrocarbon hydroxylase activity in 3T3-M cells which had been grown with or without serum during the 24 hr of exposure to benz[*a*]anthracene prior to harvesting is illustrated in Fig. 3. In the presence of serum there was a 6-fold increase in enzyme activity 24 hr after exposure to 12 μM benz[*a*]anthracene. How-

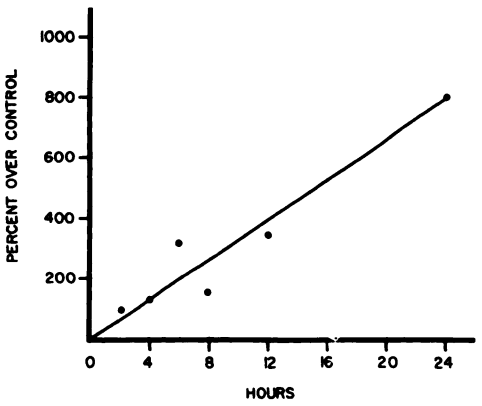

FIG. 1. *Kinetics of induction of aryl hydrocarbon hydroxylase by benz[a]anthracene (13 μM) in 3T3-M cells grown in medium containing serum*

Benz[*a*]anthracene was dissolved in DMSO and added to the culture medium. The cells were harvested 2, 4, 6, 8, 12, and 24 hr after addition of the hydrocarbon. The results are normalized from two separate experiments (triplicate assays at each time point).

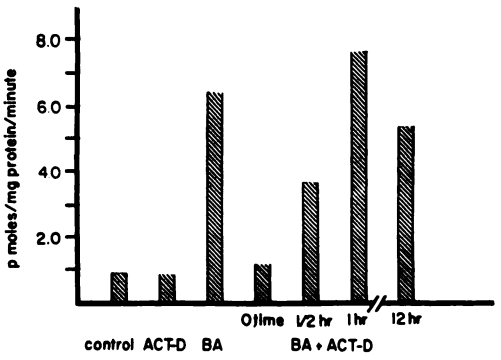

FIG. 2. *Time course of actinomycin D blockade of aryl hydrocarbon hydroxylase induction by benz[a]anthracene (1.5 μM)*

Benz[a]anthracene (BA) was added at zero time to all the cultures except the control and the cultures containing actinomycin D (ACT-D) alone. Actinomycin D (1 μg/ml) was added to the cultures labeled "BA + ACT-D" at various times after the addition of benz[a]anthracene. All cultures were incubated for 24 hr and then assayed for hydroxylase activity.

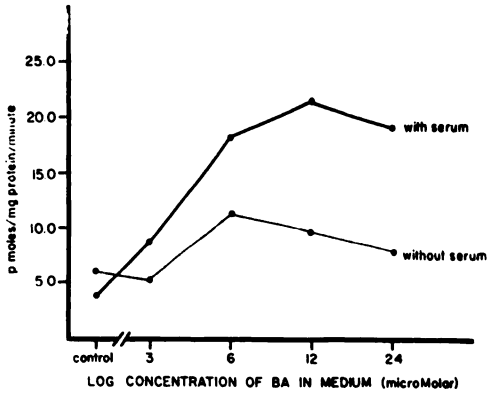

FIG. 3. *Relationship between concentration of benz[a]anthracene added to culture medium and specific activity of aryl hydrocarbon hydroxylase*

Various concentrations of benz[a]anthracene (BA) were added to cultures of nonconfluent cells in a medium with or without serum. Enzyme activity was determined 24 hr after addition of drug. The concentration of benz[a]anthracene is plotted on a logarithmic scale.

ever, in the absence of serum the peak response was attained at 6 μM benz[a]anthracene, and the induced level was only about twice the control. A similar dependence on the presence of serum for the induction of aryl hydrocarbon hydroxylase by polycyclic hydrocarbons has been shown to exist in primary cultures of fetal rat liver cells by Gielen and Nebert (12). These authors postulated that the serum effect is related to the presence of hormones, phospholipids, or other factors which may act synergistically with the polycyclic hydrocarbon in the induction of this enzyme. However, another possibility is that the rate of cell proliferation is an important factor in the inducibility of aryl hydrocarbon hydroxylase activity by various chemicals. For example, it has been reported that the enzyme activity is much more inducible by polycyclic hydrocarbons in human lymphocytes which have been stimulated to proliferate by the plant lectins phytohemagglutinin and pokeweed mitogen than in unstimulated, nonproliferating cells (13, 14). The kinetics of this potentiation of the induction effect suggests that the peak potentiation occurs 72 hr after exposure to the lectins. This corresponds to the time of maximal DNA synthesis in lectin-stimulated human lymphocytes (15).

In our studies the removal of calf serum from the growth medium diminished the rate of proliferation of 3T3-M cells. Thus it is possible that the role of serum in the induction of aryl hydrocarbon hydroxylase by benz[a]anthracene is related to its growth-stimulatory properties. The possible relationship between cell proliferation rate and enzyme inducibility is further strengthened by the data presented in Table 1. When grown to confluence, untransformed 3T3 cells exhibit contact inhibition, and the rate of cell proliferation markedly diminishes (16). Density-dependent inhibition of proliferation rate has also been observed in 3T3-M cells.[1] When the activity of aryl hydrocarbon hydroxylase was compared in contact-inhibited cells and in nonconfluent cells (which continue to proliferate for several hours even after the removal of serum) treated with benz[a]anthracene for 24 hr, the induced levels were several times higher in nonconfluent cells. Another observation of these experiments was that dibutyryl cGMP appeared to have a slight stimulatory

TABLE 1

Induction of aryl hydrocarbon hydroxylase by various agents in confluent and nonconfluent 3T3 cells in serum-free medium

In these experiments the cells were grown to the appropriate density in medium containing 10% calf serum. The serum-containing medium was then removed and replaced with medium without serum but containing drug. The cell cultures were then incubated for an additional 24 hr before harvesting. Calculation of percentage increase over control is based on the DMSO control for benz[*a*]anthracene induction and the non-DMSO control for dibutyryl cGMP. In experiments 3 and 4 benz[*a*]anthracene dibutyryl cGMP, and dibutyryl cAMP were dissolved in DMSO prior to addition to the medium.

Treatment	Specific activity	
	Confluent	Nonconfluent
	pmoles/min/mg protein	
Experiment 1		
Control	3.64	3.16
Control − NADPH[a]	0.00	
Control + DMSO (0.5%)	3.73	2.39
Benz[*a*]anthracene (13 μM)	5.77 (+54%)	9.41 (+293%)
Dibutyryl cGMP (0.1 μM)	5.15 (+38%)	4.96 (+56%)
Experiment 2		
Control		3.15
Control + DMSO (0.5%)		3.90
Benz[*a*]anthracene (13 μM)		9.15 (+134%)
Experiment 3		
Control (+DMSO)	4.78	
Benz[*a*]anthracene (12 μM)	6.92 (+45%)	
Dibutyryl cGMP (0.1 μM)	5.02	
Benz[*a*]anthracene (12 μM) + dibutyryl cGMP (0.1 μM)	11.31 (+136%)	
Experiment 4		
Control (+DMSO)	4.27	
Benz[*a*]anthracene (6 μM)	7.38 (+73%)	
Dibutyryl cGMP (0.01 μM)	3.28	
Benz[*a*]anthracene (6 μM) + dibutyryl cGMP (0.01 μM)	12.75 (+198%)	
Dibutyryl cAMP (0.01 μM)	4.06	
Benz[*a*]anthracene (6 μM) + dibutyryl cAMP (0.01 μM)	4.14	

[a] In this control, NADPH was omitted from the incubation mixture employed to determine enzyme activity. In one other additional controls, DMSO was added to the culture medium.

effect on hydroxylase activity. cGMP has been reported to be elevated in lymphocytes which have been stimulated to shift from a nonproliferative to a proliferative state by phytohemagglutinin or concanavalin A (17). Thus it was considered that elevated cGMP concentrations may have a permissive effect on the induction of aryl hydrocarbon hydroxylase. This appears to be the case, as shown in experiments 3 and 4 of Table 1. In these experiments exogenously added dibutyryl cGMP (0.1 or 0.01 μM) produced a marked potentiation of the induction of hydroxylase activity by benz[*a*]anthracene (12 or 6 μM). Dibutyryl cAMP at a concentration of 0.01 μM had no effect alone and inhibited the induction of hydroxylase by the hydrocarbon.

Three lines of evidence suggest that the inducibility of aryl hydrocarbon hydroxylase by benz[*a*]anthracene is related to the state of proliferation of 3T3-M cells. The enzyme is much more inducible in the presence than in the absence of serum, in nonconfluent than in confluent cells, and in the presence of exogenously added dibutyryl cGMP. The role of cGMP in regulating the levels of aryl hydrocarbon hydroxylase in these cells is unclear. Its direct effect appears to be minimal, and it has been reported that it

does not induce the hydroxylase activity in fetal rat liver cells (12). Thus it may act in some way like the addition of serum, i.e., as one of the signals involved in the stimulation of cell proliferation, as proposed by Hadden *et al.* (17).

Recently Whitlock and Gelboin (18) reported that temporary inhibition of protein synthesis in cultured cells by cycloheximide or puromycin potentiates the induction of aryl hydrocarbon hydroxylase by benz[*a*]anthracene after the inhibitor has been removed from the medium. They postulated the presence of a labile protein which reversibly inhibits the induction of hydroxylase activity at a post-transcriptional level. It may be that the level of post-transcriptional inhibitor is higher in nonproliferating than in proliferating cells. At any rate, the relationship between cell proliferation and enzyme inducibility is an intriguing one and warrants further examination.

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