Vol. 131, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1985 Pages 103-108

PHENOBARBITAL AND SOME OTHER LIVER TUMOR PROMOTERS STIMULATE DNA SYNTHESIS IN CULTURED RAT HEPATOCYTES*

Anthony M. Edwards and Carolyn M. Lucas

Clinical Biochemistry, School of Medicine,
Flinders University of South Australia,
BEDFORD PARK, S.A., 5042, AUSTRALIA

Received June 26, 1985

Hepatocytes isolated from normal adult rats were maintained at sub-confluent density in a defined medium in primary culture. In control cultures with added epidermal growth factor and low concentrations of dexamethasone, the rate of DNA synthesis was initially low but increased after about 30 hours in culture. Addition of the xenobiotic liver tumor promoters phenobarbital, α-hexachlorocyclohexane or p,p'-dichlorodiphenyltrichloroethane to cultures after cell attachment caused concentration-dependent stimulation of DNA synthesis measured after 2 days in culture. While dexamethasone (30 nM) alone had little effect on hepatocyte DNA synthesis, the stimulatory effects of the xenobiotics required the permissive presence of the steroid.

Phenobarbital (PB) and some other xenobiotics including the environmental pollutants α-hexachlorocyclohexane (HCH) and p,p'-dichlorophenyltrichloroethane (DDT) are considered to be promoters of liver carcinogenesis (1,2). When administered after exposure to an initiating carcinogen, they increase the number and size of putative preneoplastic lesions in the liver (e.g. 3-5) and increase the subsequent appearance of liver tumors (e.g. 6-8). The mechanism(s) by which PB and other non-cytotoxic xenobiotics promote is unknown. Since xenobiotics cause at least transient stimulation of DNA synthesis in normal liver (e.g. 2,5,6,9) and stimulate growth of preneoplastic hepatocytes (5), enhancement of liver cell growth may be a least one component of promoter action (2,5) although alternate mechanisms have been suggested (e.g. 7,10,11). Based on

*This work was supported by grants from the Anticancer Foundation of the Universities of South Australia and the Flinders Medical Centre Research Foundation.

Abbreviations: DDT, p,p'-dichlorodiphenyltrichloroethane; EGF, epidermal growth factor; HCH, α-hexachlorocyclohexane; PB, sodium phenobarbital.

0006-291X/85 $1.50

previous work which established that significant levels of DNA replication and in some cases, mitosis, can be achieved in primary monolayer cultures of adult rat hepatocytes (e.g. 12-16), this study examined the effects of xenobiotic liver tumor promoters on hepatocyte DNA synthesis under the fully-defined and readily-manipulated conditions of cell culture.

MATERIALS AND METHODS

Animals and materials - Male wistar rats (Porton strain) weighing 200-300 g, given access to laboratory chow and water ad libitum were used for preparation of hepatocytes. The sources and formulations of media were as previously described (17). EGF was generously provided by Professor A.W. Murray, School of Biological Sciences, Flinders University of South Australia, or for some experiments purchased from Collarobative Research Inc. (Lexington, MA.). HCH and DDT were from Serva Feinbiochemica (Heidelberg, FRG) and phenobarbitone sodium (PB) from Prosanna Laboratories (Sydney, Australia). Other biochemicals were from Sigma (St. Louis, MO.).

Hepatocyte isolation and culture - Hepatocytes were isolated by a two-step collagenase perfusion procedure followed by low-speed centrifugations as in (18). Procedures for plating and maintaining hepatocytes in culture were as in (19) except that cells were dispensed into 90 mm collagen-coated culture dishes at a density of approx 4000 cells/cm^2. After 3 h for cell attachment the "standard culture medium" contained modified waymouth medium as in (17) without insulin but with added EGF (30 ng/ml) and dexamethasone (30 nM) unless otherwise specified. Test compounds were added with the post-attachment medium change and readded to cultures with subsequent daily medium changes. The vehicle for drug additions (dimethylsulphoxide, 0.2%) had no effect on hepatocyte DNA synthesis.

Measurements of DNA synthesis and DNA - For measurements of [^{3}H]thymidine incorporation into DNA the medium was changed to fresh "standard culture medium" containing 0.08 µCi/ml [methyl-^{3}H]thymidine (20 Ci/mmol, New England Nuclear, Boston MA.). Cultures were incubated for a further 6 h: over this period, [^{3}H]thymidine incorporation into DNA was shown to be approximately linear with respect to time. DNA was extracted from extensively-washed monolayers by a modification of the procedure in (12) as follows. Monolayers were incubated for 30 min at 37^0 with 3 M KOH (4 ml) then carefully mixed with 2 ml 15% trichloroacetic acid containing 6 M HCl. After standing at 4^0 (usually overnight) the mixture was centrifuged at 3000 g, the pellet resuspended in 1 ml 0.5 M perchloric acid and DNA extracted by heating at 80^0 for 15 min. After centrifugation for 10 min at 3000 g, [^{3}H]thymidine in an aliquot of supernatant was determined by scintillation counting and DNA content of the supernatant measured colorimetrically using Burton's diphenylamine reagent (20). In general the results shown in each Figure or Table were obtained with a single hepatocyte preparation. In every case, comparable results were obtained in replicate experiments with independent cell preparations.

RESULTS AND DISCUSSION

Fig. 1 shows the results of measuring the rate of [^{3}H]thymidine incorporation into hepatocyte DNA after various times in culture. In

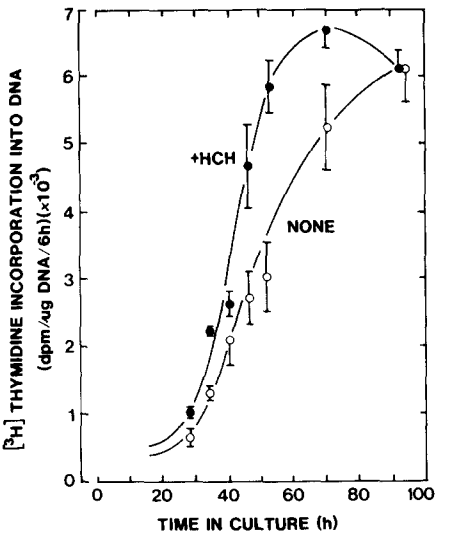

Fig. 1. Effect of HCH on DNA synthesis in hepatocyte monolayers after various times in culture. Hepatocytes were isolated, allowed to attach to dishes, then maintained with or without HCH, 30 μM as in 'METHODS'. [^{3}H]Thymidine was added at the times shown and incorporation into DNA measured after a further 6 h incubation. Values are means±S.D from 4 dishes.

control cultures (with EGF and dexamethasone) the rate was initially low but after about 30 h in culture, rose progressively during the period studied (up to 4 d in culture). HCH significantly stimulated the rate of [^{3}H]thymidine incorporation at most time points between 1 and 3 days in culture ($p < 0.001$ for all times except 40 h) although after 4 days, the rate of DNA synthesis was similar in cultures with or without HCH. [^{3}H]Thymidine incorporation after 2 or 3 days in culture in the presence or absence of HCH was inhibited by > 85% if 10 mM hydroxyurea was added to cultures 2 h prior to, and during exposure to [^{3}H]thymidine, suggesting that observed incorporation represented predominantly replicative DNA synthesis. Autoradiographic studies (not shown) established that in the period 46-70 h in culture, nuclei of 30-50% of the cells in monolayers were labelled with [^{3}H]thymidine. In general, the labelled cells had typical hepatocyte morphology.

Thus under culture conditions (relatively low cell density, with EGF) where significant levels of DNA synthesis were seen in controls, HCH

enhanced the initial rise in synthetic rate. The transience of stimulation may reflect at least in part, limitations of the culture system. Under the relatively simple conditions used (collagen substratum, serum-free medium with limited hormone supplements) there may have been little completed cell division as discussed by others (14,16).

Fig. 2 shows the concentration-dependence of effects of HCH, PB and DDT on DNA synthesis in hepatocytes measured after 46 h in culture. DNA synthesis was increase up to 85%, 50% and 30% above the relevant control values with 30 μM HCH, 30 μM DDT and 2 mM PB respectively. Armato et al. (23) recently reported that a variety of tumor promoters including PB, DDT and γ-HCH at 10^{-10} M, stimulated DNA synthesis in neonatal hepatocyte cultures. In the present study, much higher drug concentrations were required, consistent with levels required for induction of γ-glutamyltranspeptidase (19,24) or monooxygenase activity (24) in adult hepatocyte cultures.

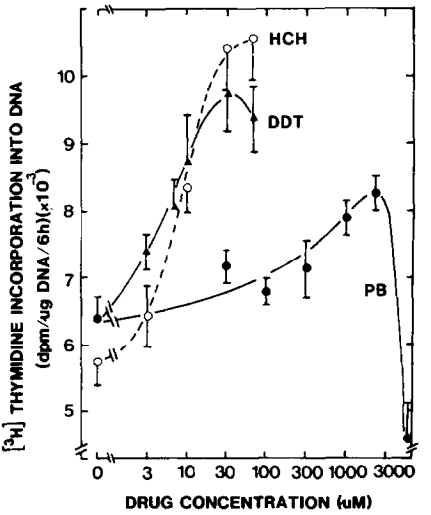

Fig. 2. Concentration-dependence of the stimulation of hepatocyte DNA synthesis by xenobiotics. After attachment, hepatocytes were maintained with daily additions of xenobiotics to the concentration shown. After a total 46 h in culture, fresh medium (without xenobiotics) containing [^{3}H]thymidine was added and incorporation into DNA measured as in 'METHODS'. Hepatocytes from 2 separate preparations were used (dashed and solid lines). Higher levels of incorporation in these cultures reflect lowere cell density than in Fig. 1. Values are means±S.D from 4 dishes.

Table I Effect of dexamethasone on stimulation of DNA synthesis in hepatocytes by xenobiotics. The procedures for hepatocyte culture were as in 'METHODS' except that the medium contained 1 mM ornithine in place of arginine; and dexamethasone was omitted except as shown. After 46 h in culture (±xenobiotics after attachment), DNA synthesis was measured as in 'METHODS'. Values are means±S.D from 4 dishes.

	[^{3}H]Thymidine incorporated (dpm/μg DNA/6 h)(x 10^{-3})	
Additions	None	+ Dexamethasone 30 nM
None	2.25±0.17	2.36±0.18
DDT, 30 μM	2.60±0.55	4.35±0.17[a]
HCH, 30 μM	2.29±0.26	3.89±0.41[a]

[a]Significantly greater than dexamethasone alone, ($p < 0.001$).

Table 1 shows effects of dexamethasone on DNA synthesis. For these studies a selective modification of the "standard culture medium" was used to minimize out-growth of fibroblasts (21) which could occur more readily in the absence of dexamethasone (22). The rate of DNA synthesis after 46 h in culture was similar in monolayers maintained with or without dexamethasone but stimulation by HCH or DDT required the permissive presence of the steroid. This resembles the glucocorticoid requirement for induction of hepatocyte γ-glutamyltranspeptidase (19,24) and monooxygenases (25) by xenobiotics in culture.

The ability to observe a clear and reproducible stimulation of DNA synthesis by xenobiotics *in vitro* suggests that hepatocyte primary cultures will be useful in studying the nature and mechanism of the effects of xenobiotic tumor promoters on at least some aspects of hepatocyte growth.

REFERENCES

1. Pitot, H.C. and Sirica, A.E. (1980) Biochim. Biophys. Acta 605, 191-215.
2. Schulte-Hermann, R., Schuppler, J., Ohde, G., Bursch, W. and Timmermann-Trosiener, I. (1982) Chemical Carcinogenesis, pp. 231-260, Plenum Press, New York.

3. Goldsworthy, T., Campbell, H.A. and Pitot, H.C. (1984) Carcinogenesis 5, 67-71.
4. Ito, N. Tatematsu, M., Nakanishi, K., Hasegawa, R., Takano, T., Imaida, K. and Ogiso, T. (1980) Gann 71, 832-842.
5. Schulte-Hermann, R., Ohde, G., Schuppler, J. and Timmermann-Trosiner, I. (1981) Cancer Res. 41, 2556-2562.
6. Peraino, C., Fry, R.J.M., Staffeldt, E. and Christopher, J.P. (1975) Cancer Res. 35, 2884-2890.
7. Peraino, C., Staffeldt, E.F., Haugen, D.A., Lombard, L.S., Stevens, F.J. and Fry, R.J.M. (1980) Cancer Res. 40, 3268-3273.
8. Schulte-Hermann, R. and Parzefall, W. (1981) Cancer Res. 41, 4140-4146.
9. Schulte-Hermann, R. (1974) CRC Crit. Rev. Toxicol. 3, 97-158.
10. Abanobi, S.E., Lombardi, B. and Shinozuka, H. (1982) Cancer Res. 42, 412-415.
11. Bursch, W., Lauer, B., Timmermann-Trosiener, I., Barthel, G., Schuppler, J. and Schulte-Hermann, R. (1984) Carcinogenesis 5, 453-458.
12. McGowan, J.A., Strain, A.J. and Bucher, N.L.R. (1981) J. Cell. Physiol. 108, 353-363.
13. Russell, W.E., McGowan, J.A. and Bucher, N.L.R. (1984) J. Cell. Physiol. 119, 183-192.
14. Michalopoulos, G., Cianciulli, H.D., Novotny, A.R., Kligerman, A.D., Strom, S.C. and Jirtle, R.L. (1982) Cancer Res. 42, 4673-4682.
15. Nakamura, T., Tomita, Y. and Ichihara, A. (1983) J. Biochem. 94, 1029-1035.
16. Enat, R., Jefferson, D.M., Ruiz-Opazo, N., Gatmaitan, Z., Leinwand, L.A. and Reid, L.M. (1984) Proc. Natl. Acad. Sci. USA. 81, 1411-1415.
17. Edwards, A.M. (1982) Cancer Res. 42, 1107-1115.
18. Whiting, M.J. and Edwards, A.M. (1979) J. Lipid Res. 20, 914-918.
19. Edwards, A.M. and Lucas, C.M. (1985) Carcinogenesis 6, in press.
20. Burton, K.A. (1956) Biochem. J. 62, 315-322.
21. Leffert, H.L. and Paul, D. (1972) J. Cell. Biol. 52, 559-568.
22. Edwards, A.M. and Lucas, C.M. (1982) Epithelial Cell Differentiation and Neoplasia pp. 173-191. Australian Cancer Soc., Sydney.
23. Armato, U., Andreis, P.G. and Romano, F. (1984) Carcinogenesis 5, 1547-1555.
24. Edwards, A.M. (1984) Biochem. Pharmac. 33, 3839-3845.
25. Edwards, A.M., Glistak, M.L., Lucas, C.M. and Wilson, P.A. (1984) Biochem. Pharmac. 33, 1537-1546.