THE ADENYL CYCLASE SYSTEM AND CARCINOGENESIS: DECREASED RESPONSIVENESS OF MOUSE EPIDERMIS TO ISOPROTERENOL AFTER 3,4-BENZPYRENE TREATMENT

Andrew W. Murray and Ajit K. Verma

School of Biological Sciences, Flinders University of South Australia, Bedford Park, South Australia, 5042.

Received July 5, 1973

SUMMARY : The concentration of cyclic AMP in untreated and acetone-treated mouse epidermis was increased 9-11 fold 10 min. after the intraperitoneal injection of L-isoproterenol. The response to isoproterenol was unaffected 30 min. after a single application of 3,4-benzpyrene in acetone, but was reduced to a 2.7 fold increase when tested after 24 hours. The increase in epidermal cyclic AMP following isoproterenol injection varied between 2.1 and 2.5 fold with repeated applications of 3,4-benzpyrene over periods of up to 55 days. The response to isoproterenol was unaffected by 1,2-benzpyrene, a non-carcinogenic polycyclic hydrocarbon.

Recent studies with cultured cells have suggested that the adenyl cyclase system is an important regulator of normal cellular growth (1,2) and that alterations in this system accompany malignant transformation (3-6). Consequently it is of importance to determine whether changes in the production, metabolism and/or functions of cyclic nucleotides occur during the development of cancers in intact animals. It has been reported (7) that the responsiveness of hepatic adenyl cyclase to glucagon and epinephrine change in rats fed a diet supplemented with the carcinogen 2-acetylaminofluorene The changes occur before the morphological appearance of hepatomas. To our knowledge this is the only study of the properties of adenyl cyclase in pre-cancerous tissue.

In the present studies we have measured the isoproterenol-dependent accumulation of cyclic AMP in mouse epidermal cells following topical application of 3,4-benzpyrene, a carcinogenic polycyclic hydrocarbon. Previous work has shown that the cyclic AMP concentration of normal mouse

epidermis is increased about ten fold within ten minutes of a single injection of the β-receptor agonist, isoproterenol (8).

MATERIALS AND METHODS

The animals used were female Swiss albino mice and were three months old at the start of the experiments. The animals were kept in a room with an artificial rhythm of dark and light periods (dark from 5 a.m. to 5 p.m.). The back skin of the animals was shaved and only those animals were used which did not show a regrowth of hair over a 7 day period.

3,4-Benzpyrene (Calbiochem. Australia Ltd.) or 1,2-benzpyrene (Koch Light & Co. Ltd., Colnbrook, England) were dissolved as 1% solutions in acetone. The back areas of individual mice were treated with two drops of the benzpyrene solution (equivalent to 0.05 mg - 0.06 mg of hydrocarbon per mouse) twice a week.

At appropriate times after commencement of benzpyrene treatment the accumulation of epidermal cyclic AMP in response to an intraperitoneal injection of L-isoproterenol (Sigma Chemical Co.; 10 nmoles per g body weight) was measured. Except for the 30 min. and 24 hour treatments, this response was measured two days after the last application of benzpyrene. Six control (saline injected) and six test (isoproterenol injected) animals were used in each experiment. Ten minutes after injection the mice were killed by cervical dislocation and trichloroacetic acid extracts of the epidermis made as described before (8). All experiments were started at 10 a.m. and about 3 hours were required to obtain acid extracts from the 12 animals in a group; control and test animals were treated alternately during this period. After extraction of the trichloroacetic acid with ether, the aqueous samples were fractionated on Dowex 50 (H^+) columns, and the cyclic AMP contents measured by the binding-protein method of Gilman (9).

The DNA content of the acid insoluble material was measured by the

Table 1. Effect of isoproterenol on cyclic AMP levels in mouse epidermis.

Experiments were carried out as described in the Materials and Methods section.

3,4-BP; 3,4-benzpyrene. 1,2-BP; 1,2-benzpyrene.

Treatment (No. applications in parentheses)	pMoles cyclic AMP/μg DNA		
	Saline	Isoproterenol	Isoproterenol/Saline
Control (untreated)	0.52 ± 0.09	5.35 ± 0.92	10.3
		†4.74 ± 0.57	9.1
Control (5 acetone treatments)	0.71 ± 0.27	7.82 ± 0.71	10.9
30 min. 3,4-BP (1)	0.45 ± 0.07	4.91 ± 0.55	10.9
24 hr. 3,4-BP (1)	0.88 ± 0.25	2.36 ± 0.25	2.7
9 days 3,4-BP (3)	0.86 ± 0.11	1.88 ± 0.36	2.2
†9 days 3,4-BP (3)	0.81 ± 0.10	1.82 ± 0.38	2.2
23 days 3,4-BP (7)	1.12 ± 0.14	2.40 ± 0.42	2.1
‡23 days 3,4-BP (7)	0.32 ± 0.04	3.17 ± 0.42	10.0
*55 days 3,4-BP (16)	0.81 ± 0.18	2.03 ± 0.33	2.5
9 days 1,2-BP (3)	0.63 ± 0.05	5.65 ± 1.04	9.0

†Animals in these groups were injected i.p. with dibenamine (10 mg per Kg body weight) 30 min. before injection of isoproterenol.

‡Treated with 3,4-benzpyrene for 23 days; response to isoproterenol tested after a further 14 days with no 3,4-benzpyrene treatments.

*The animals were tested 3 days after the last application of 3,4-benzpyrene.

method of Burton (10) after washing as described by previous workers (8).

To estimate the metabolism of isoproterenol, trichloroacetic acid extracts of epidermis and heart were made as described above, 10 min. or 1 hour after an intraperitoneal injection of ^{3}H-isoproterenol (10 nmoles per g body weight; 10 μCi). The samples were lyophilized and chromatographed on Whatman 3 MM paper in n-butanol-acetic acid-H_2O (4:1:1, by vol; see Ref. 11). Radioactivity associated with unchanged isoproterenol (R_f 0.65 -

0.7), 3-O-methylisoproterenol (0.75 - 0.8) and conjugates of these two compounds (R_f 0.20 - 0.3) were measured by liquid scintillation counting after elution from the paper with water.

No papillomas or malignant carcinomas were observed over the test period (up to 55 days after the initial treatment with 3,4-benzpyrene).

RESULTS AND DISCUSSION

Table 1 shows the effect of the intraperitoneal injection of isoproterenol on cyclic AMP levels of mouse epidermis after 10 min. A 9-11 fold increase was observed in control animals and in animals tested 30 min. after 3,4-benzpyrene treatment. However the degree of stimulation was significantly reduced 24 hours after a single treatment with 3,4-benzpyrene and remained low after further exposure to the carcinogen. The effects of 3,4-benzpyrene were reversible; animals treated for 23 days showed a normal response to isoproterenol after a further 14 days with no carcinogen applications. The response to isoproterenol was unaffected by treatment with 1,2-benzpyrene, a non-carcinogenic polycyclic hydrocarbon.

One possibility for the decreased response to isoproterenol in 3,4-benzpyrene-treated mice is that the hydrocarbon induces the formation of enzymes which inactivate catecholamines. The major pathways for isoproterenol metabolism are methylation by catechol O-methyltransferase (12), and the formation of conjugated derivatives (13). The O-methyltransferase is intracellular and widely distributed (14), consequently inactivation should be most extensive in those non-neuronal tissues which accumulate isoproterenol by the $Uptake_2$ mechanism (15). In Table 1 it is shown that prior injection of dibenamine, an inhibitor of $Uptake_2$ (16), did not increase the response of 3,4-benzpyrene-treated mice to isoproterenol.

Direct measurement of metabolites in extracts of skin and heart after injection of ^{3}H-isoproterenol indicated that there were no gross changes

in the metabolism of this compound in 3,4-benzpyrene-treated mice. Test animals were used 24 hours after a single application of 3,4-benzpyrene; control animals were treated with acetone. Ten minutes after ^{3}H-isoproterenol injection the percentage of radioactivity extracted from benzpyrene-treated mouse skin associated with isoproterenol, 3-0-methylisoproterenol and conjugated derivatives was 51, 29 and 20 respectively; in control animals the percentages were 53, 25 and 22 respectively. After 1 hour a much higher proportion of the radioactivity was associated with conjugated derivatives; values of 50% and 48% of the extracted radioactivity were obtained for test and control animals respectively. Similar patterns of metabolism were found in extracts of mouse heart 10 min. and 1 hour after injection of ^{3}H-isoproterenol In all experiments the extracts from three mice were pooled for chromatographic analysis. The pattern of ^{3}H-isoproterenol metabolism was similar in the skin of mice which had been treated with 3,4-benzpyrene for 55 days.

The simplest explanation of these results is that application of 3,4-benzpyrene to mouse skin causes a rapid change in the responsiveness of epidermal adenyl cyclase to isoproterenol. The effects are, however, not immediate and it is possible that a metabolite of 3,4-benzpyrene is responsible. Previous studies have shown marked increases in the activity of aryl hydrocarbon hydroxylase in mouse skin within 16 hours of the topical application of polycyclic hydrocarbons (17). 3,4-Benzpyrene and/or its metabolites have been shown to bind to skin proteins (18) and covalently to DNA after incubation with a microsomal hydroxylating system (19), but it is not known if either reaction is involved in the carcinogenic activity of the hydrocarbon. Presumably assays of adenyl cyclase activity in cell-free preparations will be needed to conclusively decide if the properties of this enzyme are altered by 3,4-benzpyrene treatment. Unfortunately the response of isolated epidermal adenyl cyclase to isoproterenol is much less than that observed *in vivo* (20). Possible effects on a cyclic AMP phosphodiesterase are unlikely because of the similar or higher basal

levels of epidermal cyclic AMP in 3,4-benzpyrene-treated animals (Table 1).

The evidence is good that fluctuations in cyclic AMP levels are involved in the natural regulation of the growth of epidermal cells (8, 21-23), although the nature of the physiological modulators of epidermal adenyl cyclase is not certain (8). Consequently the early growth stimulatory effects of 3,4-benzpyrene on epidermal cells (24) may result from an inability of adenyl cyclase to respond to hormonal stimulation. A similar mechanism has been proposed to explain the increased cellular proliferation of psoriatic epidermis (25). In current experiments we are attempting to determine whether there is a causal link between changes in the adenyl cyclase system and carcinogenesis.

ACKNOWLEDGEMENTS

This work was supported by grants from the University of Adelaide Anti-Cancer Foundation, and the Australian Research Grants Committee.

REFERENCES

1. Burger, M.M., Bombick, B.M., Breckenridge, B.M., and Sheppard, J.R., Nature, New Biology, 239, 161 (1972).
2. Otten, J., Johnson, G.S., and Pastan, I., J. Biol. Chem., 247, 7092 (1972).
3. Johnson, G.S., Friedman, R.M., and Pastan, I., Proc. Nat. Acad. Sci. U.S.A., 68, 425 (1971).
4. Butcher, F.R., Scott, D.F., Potter, V.R., and Morris, H.P., Cancer Res., 32, 2135 (1972).
5. Otten, J., Bader, J., Johnson, G.S., and Pastan, I., J. Biol. Chem., 247, 1632 (1972).
6. Sheppard, J.R., Nature, New Biology, 236, 14 (1972).
7. Christofferson, T., Morland, J., Osnes, J., and Elgjo, K., Biochim. Biophys. Acta, 279, 363 (1972).
8. Marks, F., and Grimm, W., Nature, New Biology, 240, 178 (1972).
9. Gilman, A.G., Proc. Nat. Acad. Sci. U.S.A., 67, 305 (1970).
10. Burton, K.A., Biochem. J., 62, 315 (1956).
11. Hertting, G., Biochem. Pharm., 13, 1119 (1964).
12. Conolly, M.E., Davies, D.S., Dollery, C.T., Morgan, C.D., Paterson, J.W., and Sandler, M., Br. J. Pharmac., 46, 458 (1972).
13. Axelrod, J., and Tomchick, R., J. Biol. Chem., 233, 702 (1958).
14. Inscoe, J.K., Daly, J., and Axelrod, J., Biochem. Pharmacol., 14, 1257 (1965)
15. Iversen, L.L., Brit. J. Pharmacol., 41, 571 (1971).
16. Iversen, L.L., Salt, P.J., and Wilson, H.A., Brit. J. Pharmacol., 46, 647 (1972).

17. Wiebel, F.J., Leutz, J.C., and Gelboin, H.V., Arch. Biochem. Biophys., 154, 292 (1973).
18. Heidelberger, C., and Modenhauer, M.G., Cancer Res., 16, 442 (1956).
19. Gelboin, H.V., Cancer Res., 29, 1272 (1969).
20. Duell, E.A., Voorhees, J.J., Keisery, W.H., and Hayes, E., Arch. Derm., 104, 601 (1971).
21. Powell, J.A., Duell, E.A., and Voorhees, J.J., Arch. Derm., 104, 359 (1971).
22. Marks, F., and Rebien, W., Naturwissenschaften, 59, 41 (1972).
23. Voorhees, J.J., Duell, E.A., and Kelsey, W.H., Arch. Derm., 105, 384 (1972).
24. Glucksmann, A., Cancer Res., 5, 385 (1945).
25. Voorhees, J.J., and Duell, E.A., Arch. Derm., 104, 352 (1971).