Vol. 160, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1989 Pages 217-221

ALTERED HEPATIC MICROSOMAL FUNCTION AND ELEVATED PROTOONCOGENE EXPRESSION AS RESIDUAL EFFECTS IN RATS EXPOSED TO DELTA-9-TETRAHYDROCANNABINOL

Hullahalli R. Prasanna, Kenji D. Nakamura, Syed F. Ali,
Ming H. Lu, William Slikker, Jr., and Ronald W. Hart

Food and Drug Administration
National Center for Toxicological Research
Jefferson, AR 72079

Received March 6, 1989

The microsomal activation of the potent hepatocarcinogen aflatoxin B_1 (AFB_1) and the expression of selected protooncogenes were investigated in the livers of rats exposed to Δ^9-tetrahydrocannabinol (THC). At equimolar levels of cytochrome P-450, the microsome-mediated binding of AFB_1 to DNA was significantly lower (56% of the controls) in preparations from drug exposed rats. Hepatic expression of the *c-k-ras* protooncogene was 3-fold higher in THC exposed animals. These results suggest the possible occurrence of long lasting residual effects in the rats exposed to THC.

Δ^9-tetrahydrocannabinol (THC), the principal psychoactive constituent of marijuana and hashish, is known to be biologically harmful to many species (1,2). Much of the information available on the effects of THC suggest that it causes alterations in various metabolic pathways including those for xenobiotics (3,4). There is, however, a paucity of information with regard to the possible residual effects in animals exposed to this drug. Since the liver has been recognized to be the principal site for the metabolism of various xenobiotics including THC (5), we have chosen this tissue to examine the residual effect of THC on (a) the microsome-mediated biotransformation of the potent hepatocarcinogen, Aflatoxin B_1 (AFB_1) (6), including the glutathione S-transferase (GST) mediated modulation of AFB_1 binding to DNA (7) and (b) the expression of the *c-k-ras* and *c-myc* protooncogenes which are implicated in tumorigenesis (8).

MATERIALS AND METHODS

Chemicals were obtained from the following sources: NADPH, bovine serum albumin, glutathione (GSH), and calf thymus DNA (highly polymerized) from Sigma Chemical Co., St. Louis, MO.; 1-chloro-2,4- dinitrobenzene (CDNB) from Fisher Scientific Co.; [^{3}H] AFB_1 (specific activity 24 Ci/mmol) and AFB_1 were obtained from Moravek Biochemical Inc., Brea, CA. HPLC of AFB_1 and [^{3}H] AFB_1 indicated high purity for these compounds (>98%), hence they were used without further purification. Highly pure (>98%) Δ^9-THC was obtained from the National Institute on Drug Abuse (NIDA), USA.

Treatment of Rats

Male Sprague Dawley rats, 2 months of age, were obtained from NCTR's breeding colony and were divided into control and THC-treated groups and maintained individually in cages. THC (dissolved in a solution containing 89% saline, 1% Triton X-100 and 10% ethanol) was administered by gavage at a dose of 20 mg/kg body weight (1 ml/kg body weight), 5 days a week for 90 days. Control rats received the vehicle solvent only. Drug treatment was terminated and the animals were maintained for a further period of 60 days

0006-291X/89 $1.50

before sacrifice. The animals were maintained on a Purina rat chow diet and the control rats were pair fed to the treated rats throughout the study. The rats were maintained under a 12-hr light/dark cycle with temperature and relative humidity of 25 ± 2°C and 50 ± 4%, respectively.

Preparation of Hepatic microsomes
Microsomes were prepared from the livers of control and THC-treated rats by differential centrifugation. Briefly, the livers were minced and mixed with 1:8 (w/v) 0.25 M sucrose containing 10 mM Tris pH 7.4. All subsequent operations were conducted at 4°C. The tissue suspensions were homogenized using a Potter-Elvejhem homogenizer. Homogenates were centrifuged at 10,000 x g for 30 minutes to remove mitochondria, nuclei and cellular debris. The microsomal pellet was obtained by centrifuging the post mitochondrial supernatant at 105,000 x g for 60 minutes. The microsomal pellet was suspended in 20 ml of 0.15 M KCl and recentrifuged at 105,000 x g for 60 minutes. The resulting pellet was suspended in 0.25 M sucrose containing 20% glycerol and stored at -80°C until used. The cytochrome P-450 content was determined according to Omura and Sato (9). Protein and DNA content of homogenates were estimated by the methods of Lowry, *et al* (10) and Burton (11), respectively. The activity of cytosolic glutathione transferase involved in the modulation of binding of AFB_1 to DNA (7) was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate, as previously described (12).

Microsome-Mediated Binding of AFB1 to DNA
Binding assays were conducted with 2 mM NADPH, 2 μM [^{3}H]AFB_1, 1 mg calf thymus DNA, and microsomal protein equivalent to 1 nmol cytochrome P-450 in 100 mM potassium phosphate buffer (pH 7.4) with or without 5 mM GSH and with or without hepatic cytosol equivalent to 20 mg liver tissue and with or without THC (in ethanol). After incubating the mixture for 60 min at 37°C, DNA from the mixture was isolated and the binding of AFB_1 was determined as described (13).

Measurement of protooncogene expression
Methods for the isolation and determination of the levels of mRNA for a specific protooncogene have been previously described (14). Briefly, rat livers were homogenized in 4 M guanidinium thiocyanate solution, mRNA was isolated by ethanol precipitation and purified by affinity chromatography and the levels of transcript determined by blot hybridization. The following plasmids were used as probes: *v-myc*, pMyc3Pst and *v-K-ras*, HiHi3 were gifts of J.M. Bishop and R.W. Ellis, respectively. The β-actin probe, pHFβA-1, was obtained from L. Kedes.

Statistical Analysis
Student's t-test was used to evaluate the statistical significance of the results.

RESULTS

No significant difference was found between the two groups in either total liver weight, liver protein, microsomal and cytosolic protein, cytochrome P-450 and DNA contents and cytosolic glutathione S-transferase activities (data not shown). Data presented in Table I shows that the microsome mediated binding of AFB_1 to DNA is significantly reduced (56% of controls) in preparations from THC exposed rats. Inhibition of the binding of AFB_1 to DNA is also caused by the addition of THC *in vitro*, but the degree of inhibition was greater when THC was added to control microsomes (30% vs 24%). Although the efficacy of the modulation of the binding of AFB_1 to DNA by cytosolic glutathione S-transferases did not change between the two groups (Table II), the potential of THC to inhibit the activity of glutathione S-transferases is indicated (Table III; 20% inhibition). A three-fold increase in the expression of *c-k-ras* protooncogene in the livers of THC exposed rats is demonstrated in Table IV. The specificity of this effect by THC is revealed by its lack of effect on the expression of either β-actin or *c-myc* protooncogene.

DISCUSSION

Although THC affects biotransformation of various xenobiotics (4), this report, to our knowledge, is the first to identify possible perturbations which continue as long lasting residual effects in animals withdrawn from the drug. Reduced binding of AFB_1 to DNA in

TABLE I

EFFECT OF Δ^9-THC *IN VIVO* AND *IN VITRO* ON THE MICROSOME MEDIATED BINDING OF AFLATOXIN B_1 TO DNA

	pmols AFB_1 bound/mg DNA/nmol cytochrome P450/60 min	
	Control	THC-Treated
Microsome	156 ± 18	68 ± 17[1]
Microsomes + THC	109 ± 7[2]	52 ± 9

The results are presented as the mean ± SD. Analyses were conducted on duplicate samples from individual animals and each group consisted of 5 animals. The final concentration of THC was 6.4 μM, and ethanol was 2%.

[1]$p < 0.001$ when compared with control microsomes.

[2]$p < 0.01$ when compared with control microsomes.

microsomal preparations from THC exposed rats is an indication of the lasting effect of THC on the efficacy of microsomal function. This could be due to (a) an irreversible binding of THC or its metabolites with microsomes or (b) alterations caused at the genetic level which could influence factors involved in microsomal function. Inhibition of binding of AFB_1 to DNA by THC *in vitro* is not surprising because of its strong interaction with microsomes (15) particularly with cytochrome P-450 (16). Inhibition of the activity of glutathione S-transferases which is involved in the modulation of binding of AFB_1 to DNA (7) by THC *in vitro*, suggests that the detoxification of AFB_1 by conjugation with GSH, could be decreased *in vivo* when THC is present in the system. However, this effect does not remain as a residual effect as is borne out by the data presented in Table II. Inhibition of binding of AFB_1 to DNA by microsomal preparations from THC treated rats does indicate a potential for a slow metabolic activation of AFB_1 *in vivo* in THC exposed rats. Unless AFB_1 and its metabolites are effectively scavenged and removed from circulation,

TABLE II

EFFECT OF HEPATIC CYTOSOLIC GLUTATHIONE TRANSFERASE FROM THC EXPOSED RATS ON THE MICROSOME MEDIATED BINDING OF AFB_1 TO DNA

Cytosol	pmols AFB1 Bound/mg DNA/nmol of cytochrome P-450/60 min	Percent Inhibition
—	136 ± 15	0
Control	77 ± 10	43
THC-treated	65 ± 8	52

Aliquots of cytosol containing 1500 units of glutathione transferases (assayed with CDNB as substrate) was used. GSH at a final concentration of 5 mM was included in these experiments. The values are mean ± SD. Analyses were conducted in duplicate for each cytosol sample. Cytosols from 5 animals in each group were used. The source of the microsomes for each experiment was from the control animals.

TABLE III

INHIBITION OF GLUTATHIONE S-TRANSFERASE ACTIVITY *IN VITRO* BY THC

	GST Activity/ml cytosol	
	Without THC added *in vitro*	With THC added *in vitro*
Control	6.6 ± 0.4	5.3 ± 0.3[1]
THC-treated	6.1 ± 0.7	5.0 ± 0.7

Final concentration of THC was 4.8 μM and ethanol was 2%. GSH transferase activity was determined as μmol of CDNB conjugated per minute at 25°C. Analyses were conducted on duplicate samples. Each group consisted of 5 animals.

[1]$p < 0.001$ when compared with controls.

the potential exists for an enhanced toxic effect by AFB_1 in THC exposed rats. The biologcial significance of the increased expression of *c-k-ras* oncogene in THC-exposed rats remains to be determined. Elevated levels of this protooncogene have been observed during tissue regeneration (17) as well as in liver tumors (18). However, since it has been shown that an elevated level of *k-ras* expression in concert with an elevated *myc* oncogene expression can cause transformation of primary cells (19), it would not be unreasonable to suggest that an elevated basal level of expression of *c-k-ras* might be detrimental. An establishment of a pattern of elevated expression of *k-ras* protooncogenes in a time dependent fashion might clarify the significance. The absence of a significant effect of THC exposure on the expression of β-actin gene and *c-myc* protooncogene suggests a specific effect on gene expression. This hypothesis is further supported by the effects of THC on the formation of inducible hepatic enzymes such as tyrosine aminotransferase and tryptophan oxygenase (20). Further work is needed to understand the implications of the various biological manifestations of THC exposure. The significance of the present study is that the THC induced perturbation in microsomal activation system for AFB_1 and the expression of *c-K-ras* protooncogene in rats are long lasting residual effects which are observed many weeks after drug cessation. One possible explanation for the results of our assays is the lasting toxic effects of THC in rats exposed to the drug.

TABLE IV

ACTIN AND PROTOONCOGENE EXPRESSION IN LIVERS OF THC-TREATED RATS

	Relative Expression		
	C-K-ras	β-Actin	*C-myc*
Control	0.5 ± 0.2	1.1 ± 0.3	1.1 ± 0.5
THC-treated	1.6 ± 0.5[1]	0.9 ± 0.3	0.9 ± 0.5

Duplicate assays were performed for each sample. Each group consisted of 6-10 animals. Values presented are mean ± SD.

[1]$p < 0.001$ when compared with controls.

REFERENCES

1. Braude, M.C. (1976) *In Pharmacology of marijuana* (M.C. Braude and S. Szara, eds.). Raven Press, New York, 21-26.
2. Hollister, L.E. (1986) *Pharmacol. Rev. 38*, 1-20.
3. Martin, B.R. (1986) *Pharmacol. Rev. 38*, 45-74.
4. Dingell, J.V., Miller, K.W., Heath, E.C. and Klausner, H.A. (1974) *Biochem. Pharmac. 22*, 949-958.
5. Christensen, H.D., Freudenthal, R.I., Gidley, J.T., Rosenfeld, R., Goegle, G., Testino, L., Brine, D.R., Pitt, C.G. and Wall, M.E. (1971) *Science 172*, 165-167.
6. Wogan, G.N. (1973) *Meth. Cancer Res. 7*, 309-344.
7. Lotlikar, P.D., Insetta, S., Lyons, P.R. and Jhee, E.C. (1980) *Cancer Lett. 9*, 143-149.
8. Chang, E.H., Further, M.E., Scolnick, E.M. and Lowy, D.R. (1982) *Nature 297*, 479-483.
9. Omura, T. and Sato, R. (1964) *J. Biol. Chem. 239*, 2370-2378.
10. Lowry, O.H., Rosebrough, N.M., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem. 193*, 265-275.
11. Burton, K. (1956) *Biochem. J. 62*, 315-322.
12. Habig, H.W., Pabst, M.J. and Jakoby, W. (1974) *J. Biol. Chem. 249*, 7130-7139.
13. Prasanna, H.R., Lotlikar, P.D., Hacobian, N. and Magee, P.N. (1986) *Cancer Lett. 33*, 259-267.
14. Nakamura, K.D. and Hart, R.W. (1987) *Mech. Age. Dev. 39*, 177-187.
15. Burstein, S.H. and Kupfer, D. (1971) *Ann. N.Y. Acad. Sci. 191*, 61-67.
16. Kupfer, D., Jansson, I. and Orrenius, S. (1972) *Chem. Biol. Interac. 5*, 201-206.
17. Goyette, M., Petropoulos, C.J., Shank, P.R. and Fausto, N. (1983) *Science 219*, 510-512.
18. Yaswen, Y, Goyette, M., Shank, P.R. and Fausto, N. (1985) *Mol. Cell. Biol. 5*, 780-786.
19. Land, H., Prada, L.F. and Weinberg, R.A. (1983) *Nature 304*, 596-602.
20. Friedman, M.A. and Wrenn, J.M. (1977) *Toxicol. Appl. Pharmacol. 41*, 345-352.