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EFFECT OF INHIBITORS OF MICROSOMAL ENZYMES ON AFLATOXIN B_1-INDUCED CYTOTOXICITY AND INHIBITION OF RNA SYNTHESIS IN ISOLATED RAT HEPATOCYTES

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Previous studies conducted in this laboratory demonstrated that AFB_1 activation and deactivation was effectively inhibited by metyrapone and TCPO in isolated hepatocytes. The present study was undertaken to study the toxic effect of AFB_1 on hepatocyte and RNA synthesis, and to assess the influence of the inhibitors on AFB_1-induced cytotoxicity and AFB_1-inhibited RNA synthesis. AFB_1 at 50 μM was toxic and inhibited macromolecular synthesis by greater than 70% at 180 min of incubation whereas at lower concentrations of AFB_1 (0.05-10 μM) dose-and time-dependent decreases in cell viability, protein and RNA synthesis were observed. Using [^{3}H]-AFB_1 (0-1.5 μM), the uptake and covalent binding of the toxin were also dose-and time-dependent. Initial rates of these processes to reach half-maximum was found to be 0.25 μM AFB_1. In cells treated with AFB_1 (5 μM) and metyrapone (1.0 mM) or SKF-525A (10 μM), the cell viability was similar to the control and [^{3}H]-uridine incorporation was significantly higher than AFB_1 treated cells. AFB_1 and TCPO (0.5 mM) treated cells exhibited further decreases in cell viability and RNA synthesis. Results suggest that the binding of AFB_1 to DNA and impairment of transcriptional activity may lead to cell death.

It has been recognized that Aflatoxin B_1 (AFB_1) produced by strains of aspergillus flavus, is hepatotoxic and carcinogenic in laboratory and domestic animals (1-3). Microsomal activation of AFB_1 into a reactive epoxide, AFB_1-2,3-epoxide, and the subsequent interation of the epoxide with cellular DNA, RNA and protein has been implicated in the mechanism of acute toxicity and carcinogenesis (4-7). Although AFB_1 is known to bind cellular macromolecules covalently (8-13) and to inhibit RNA (14-17) and protein synthesis (14,18) preceeding histopathological alterations, direct correlation between the AFB_1-induced toxicity, DNA binding, and inhibition of macromolecular synthesis in a given system is still lacking. Recent studies conducted with isolated rat hepatocytes demonstrate that activation of AFB_1 and deactivation of AFB_1-2,3-epoxide can be inhibited by metyrapone and 1,2-

Abbreviations: AFB_1, Aflatoxin B_1; TCA, trichloroacetic acid; TCPO, 1,2-epoxy-3,3,3-trichloropropane.

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epoxy-3,3,3-trichloropropane (TCPO) respectively (19). The present study was undertaken to investigate the effect of these microsomal enzyme inhibitors on AFB_1-induced toxicity and AFB_1-inhibited transcriptional activity employing hepatocytes in vitro.

Materials and Methods

Materials: AFB_1 and reagent kits for lactate dehydrogenase (LDH) were obtained from Calbiochem-Behring Corp. L-[4,5-^{3}H]-leucine (75 Ci/mmole), [5-^{3}H]-uridine (29.7 Ci/mmole), [Methyl-^{3}H]-thymidine (45 Ci/mmole) and NCS solubilizer were supplied from Amersham Radiochemicals. Moravek Biochemical, Inc. was the source of [^{3}H]-Aflatoxin B_1 (13-14 Ci/mmole). SKF-525A was a gift from Smith-Kline & Beckman Corp. Other chemicals and biochemicals were obtained from sources given previously (19).

Preparation and Incubation of Hepatocytes: Hepatocytes were prepared from male Wistar rats of 250-300g body weight and incubated under conditions described previously (19). In over one hundred isolations, the yield of hepatocytes was 121 ± 87 x 10^6 cells per liver with 94 ± 2% were viable. The cellular content of DNA, RNA and protein was 2.1 ± 0.2, 8.5 ± 0.4 and 179 ± 9 mg/10^8 cells respectively. At the end of 180 min incubation, the viability in controls was 89 ± 3% and showed no significant changes in cellular content of macromolecules. The LDH activity in the medium was 5.7U and 6.7U/10^6 cells at zero and 180 min respectively. The cells were capable of incorporating radioactive labeled precursors into DNA, RNA and protein in a linear fashion. Cells were pre-incubated for 15 min before the addition of unlabeled or [^{3}H]-labeled AFB_1 and the various inhibitors of microsomal enzymes. At times indicated, aliquots (100 µl) of cell suspension were removed for determination of cell viability and LDH released into the medium. The intracellular concentration (pmole/10^6 cell) of free and bound [^{3}H]-AFB_1 were determined by methods described previously (19). AFB_1 bound to DNA or protein were expressed as dpm/mg of macromolecule.

Incorporation of Radioactively Labeled Precursors: To measure the synthesis of nucleic acid, [^{3}H]-uridine (20 µCi/ml) or [^{3}H]-thymidine (10 µCi/ml) was added to control and AFB_1-treated cells. To measure protein synthesis, [^{3}H]-leucine (20 µCi/ml) was used. Incubations were routinely conducted for 180 min. In experiments where both AFB_1 and inhibitors of microsomal enzymes were used, [^{3}H]-uridine (2 µCi/ml) was added after 15 min pre-treatment and incubation was conducted for 120 min. At zero and indicated times, duplicate samples (25 µl) were precipitated with cold 10% trichloroacetic acid (TCA), collected on a glass fiber disc (Whatman GF/C), washed with 5% TCA, and radioactivity determined by scintillation counting (20). The radioactivity was expressed as dpm/10^6 cell.

Other Methods: The cell viability was measured by trypan blue exclusion (0.27% final trypan blue concentration) and LDH activity was determined by the disappearence of NADH at 340 nM (21). DNA and RNA were determined by the diphenylamine and orcinol reactions respectively (22). Protein was assayed by the method of Lowry et al. (23).

Results and Discussion

As depicted in Table 1, at the end of 3 h incubation of hepatocytes with varying concentrations of AFB_1 (0-50 µM), cell viability decreased with increasing amounts of AFB_1 added. The decreases were accompanied by increase

Table 1. Effect of AFB_1 on Hepatocytes and Synthesis of Macromolecules

AFB_1 added (μM)	0	0.05	0.10	1.0	5.0	10.0	50.0
				(Percent Change)			
Cell Viability	-5	-6	-7	-12	-15	-19	-20
LDH released into medium	+17	+17	+11	+13	+62	+86	+120
				Mean + S.E.M. (10^5 dpm/10^6 cells)			
Incorporation of precursors							
[^{3}H]-uridine (N=10)	19 ± 1	19 ± 1	17 ± 1	15 ± 2	12 ± 1*	6 ± 0.5*	5 ± 0.5*
[^{3}H]-leucine (N=8)	28 ± 1	29 ± 2	27 ± 4	23 ± 2	19 ± 2*	13 ± 2*	7 ± 0.7*
[^{3}H]-thymidine (N=4)	5 ± 0.5	5 ± 0.5	5 ± 0.3	5 ± 0.3	5 ± 0.3	4 ± 1.0	3 ± 1.0

Hepatocytes were incubated with AFB_1 and labeled precursors for 180 min. Changes in cell viability and LDH released into the medium were compared with samples taken at zero time.
*Indicates p value <0.05 compared to untreated cells.

of LDH released into the medium. At 50 μM of AFB_1, a toxic dose, the ability of hepatocytes to incorporate [^{3}H]-uridine and [^{3}H]-leucine was inhibited by 74% and 73% respectively. Since the DNA of resting liver cells was not replicating, significant changes in [^{3}H]-thymidine incorporation were not observed. Although lower concentrations of AFB_1 (0.05-10 μM) caused some changes in cell viability and LDH release into the medium, significant inhibition of RNA and protein synthesis was noted only in cells treated with 5 μM and 10 μM of AFB_1. The inhibition of RNA synthesis observed *in vitro* with isolated hepatocytes is in agrement with studies conducted with liver slices(14), nuclei isolated from rats treated with AFB_1 *in vivo* (15) and in intact animals (16,17). The inhibition of [^{3}H]-leucine incorporation was similar to liver slices prepared from rats receiving AFB_1 at the LD_{50} dose and liver slices incubated with 30 μM and 60 μM AFB_1 (14). In studies conducted with liver slices [^{14}C]-leucine incorporation into liver proteins was inhibited by 51% and 76% at 30 μM (similar to an *in vivo* LD_{50} dose) and 60 μM AFB_1 respectively (14) whereas in the present study 50 μM AFB_1 inhibited protein synthesis by 73% (Table 1). At AFB_1 concentrations lower than 50 μM, concentration and time dependent inhibitions of RNA and protein synthesis were observed suggesting that isolated hepatocytes can be a useful system to investigate the effect of AFB_1 on macromolecular synthesis.

In order to correlate the cellular uptake of AFB_1 with inhibition of the transcriptional activity, the kinetics of AFB_1 uptake and binding were first

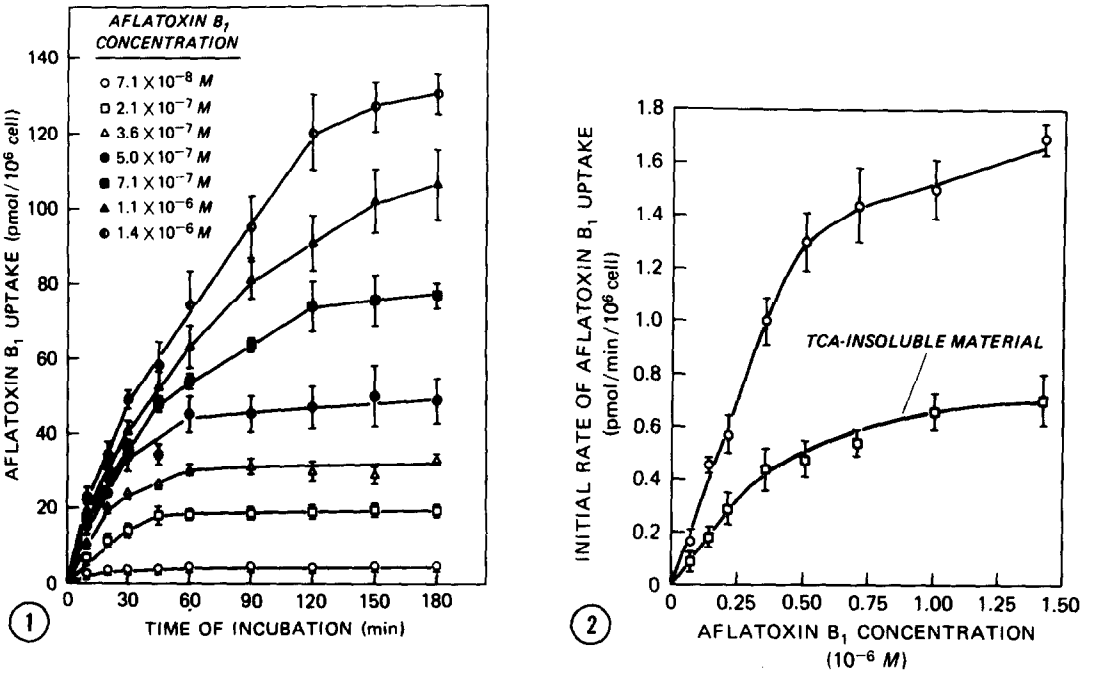

Figure 1. The time course of Aflatoxin B_1 uptake. Incubation of hepatocytes and the uptake of [^{3}H]-AFB_1 were conducted as described under "Methods". Final concentrations of AFB_1 are indicated in the Figure. Data are given as mean ± S.E. for duplicate determination from 6-8 experiments.

Figure 2. The kinetics of Aflatoxin B_1 uptake. Incubation of hepatocytes and the uptake of [^{3}H]-AFB_1 were similar to Figure 1. Rates were calculated from samples taken at 5, 10, 15 and 30 min after the addition of [^{3}H]-AFB_1. (○), total AFB_1; (□), bound AFB_1

investigated. As shown in Fig. 1, the time required to reach maximum intracellular concentration of AFB_1 varied. At low concentrations (0.071-0.5 μM), 45-60 min incubations were needed for AFB_1 to reach the plateau whereas at higher concentrations (up to 1.5 μM), the maximum intracellular AFB_1 concentration (pmole/10^6 cell) was reached by 120-180 min. When the initial rates of AFB_1 uptake and total binding (pmole/min/10^6 cell) were calculated the concentration for half-maximum activity was 0.25 μM (Fig. 2). Therefore, in subsequent studies of the effect of microsomal enzyme inhibitors on AFB_1-induced changes, the AFB_1 was added at 5 μM and incubation was terminated at the end of 2 h.

Metyrapone is known to inhibit cytochrome P-450 linked mixed function oxidases and to reduce AFB_1 uptake and binding in isolated hepatocytes (19); SKF-525A, a cytochrome P-450 inhibitor, is known to prevent toxicity caused

Table 2. The Effect of SKF-525A and Metyrapone on [^{3}H]-AFB_1 Uptake and Binding

Incubation Condition	Intracellular [^{3}H]-AFB_1		Specific Radioactivity of [^{3}H]-AFB_1	
	Total	Bound	DNA-Bound	Protein-Bound
	(pmoles/10^6 cell)		(pmole/mg)	
Control	55.1 ± 8.5	25.9 ± 5.8	127 ± 3	7.4 ± 0.8
+SKF-525A (10 μM)	26.3 ± 5.6*	9.9 ± 1.6*	37 ± 5*	2.4 ± 0.7*
+Metyrapone (10 mM)	10.4 ± 0.7*	4.2 ± 0.2*	16 ± 4*	0.3 ± 0.1*

Hepatocytes incubated with various inhibitors and [^{3}H]-AFB_1 for 60 min. Data presented as Mean ± S.E.M of 4-6 experiment. *Indicates p value <0.05 compared to control.

by bromobenzene in primary hepatocyte culture (24). The effects of SKF-525A and metyrapone on AFB_1 uptake and binding to DNA and protein were compared. Results presented in Table 2 demonstrate that SKF-525A at 10 μM, a non-toxic dose, inhibited the biotransformation of AFB_1 as also observed with 1 mM metyrapone. The ability of SKF-525A and metyrapone in preventing activation of AFB_1 and its subsequent binding to DNA and protein (Table 2) and TCPO to enhance AFB_1 binding to DNA and protein (19) offers the possibility to study the influence of these inhibitors on AFB_1-inhibited RNA synthesis and AFB_1-induced toxicity. Data shown in Table 3 indicates that both SKF-525A and metyrapone prevented AFB_1-inhibited RNA synthesis and AFB_1-induced toxicity whereas TCPO enhanced these effects. These results further support the notion that activated AFB_1 binds DNA resulting in the impairments of DNA template activity (14-17) leading to cell death. The molecular basis of inhibition of

Table 3. The Influence of Microsomal Enzyme Inhibitors on AFB_1-Induced Cell Toxicity and AFB_1-Inhibited RNA Synthesis

Incubation Condition	Untreated Cells	Cells treated with AFB_1 (5 μM)			
		+None	+Metyrapone (1 mM)	+SKF-525A (10 μM)	+TCPO (0.5 mM)
		Mean + S.D.			
Number of Experiments	11	11	5	4	6
Cell Viability (%)	89 ± 4	83 ± 5	87 ± 5	85 ± 10	75 ± 5*
[^{3}H]-Uridine Incorporated (10^2 dpm/10^6 cell)	1.3 ± 0.2	0.8 ± 0.1*	1.0 ± 0.1	1.1 ± 0.1	0.6 ± 0.1*

Hepatocytes were pre-incubated with AFB_1 and various inhibitors for 15 min before the addition of [^{3}H]-uridine (2 μCi/ml) and incubated for 120 min. *Indicates p value <0.05.

RNA synthesis and its relationship to cell death still remain as a gap in our knowledge if indeed the two are truly casually related. Nevertheless, this multi-faceted insult of AFB_1 on cellular macromolecules may be related to the progressive decrease of cell viability observed.

In intact animals, efforts to correlate impairments of cellular functions, cytotoxicity, mutagenesis, teratogenesis, and carcinogenes, is caused by AFB_1 have not been very successful since these changes are known to be modulated by a number of factors (25-27). Employing isolated hepatocytes as described here, the systemic effects associated with the whole animal and cellular dedifferentiation inherent in growing cells can be avoided, and also pretreatment of rats with inducers of cytochrome P-450 (28) and culture medium supplemented with large quantities of hormones (29) are unnecessary. The relationship between AFB_1 binding to macromolecules and impairments of transcription activity has been established *in vitro*. Investigations are currently in progress to assess the intracellular translocations of AFB_1. Future experiments employing hepatocytes suspensions should yield information concerning the mechanism of initiation of carcinogenesis.

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