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Effect of cycloheximide on tryptophan binding to rat hepatic nuclei

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Summary. This study evaluated whether cycloheximide, an inhibitor of protein synthesis, would affect the binding of L-tryptophan to rat hepatic nuclei or nuclear envelopes. Previous reports have indicated that the binding of L-tryptophan to hepatic nuclear envelope protein was saturable, stereospecific, and of high affinity. Also, the administration of L-tryptophan rapidly stimulated hepatic protein synthesis. In this study, we determined that the addition of cycloheximide in vitro inhibited ^{3}H-tryptophan binding to hepatic nuclei or nuclear envelopes. Heat-treated cycloheximide failed to have this inhibitory binding effect. In vivo treatment of rats with cycloheximide diminished in vitro ^{3}H-tryptophan binding to hepatic nuclei of treated rats compared to controls. Puromycin, another inhibitor of hepatic protein synthesis, when added in vitro did not affect ^{3}H-tryptophan binding to hepatic nuclei but did diminish in vitro binding after in vivo treatment. Thus, cycloheximide added in vitro diminished ^{3}H-tryptophan binding to hepatic nuclei probably by its structural effect on the receptor while cycloheximide administered in vivo may also act in part by inhibiting protein synthesis.

Keywords: Amino acids – L-tryptophan – Hepatic nuclear binding – Cycloheximide – Rats

Introduction

Our laboratory has been concerned with the effects of various agents on L-tryptophan binding to rat hepatic nuclei. Earlier we reported that there exists a L-tryptophan receptor in hepatic nuclear envelopes and the binding of L-tryptophan to the receptor is saturable, stereospecific and of high affinity (Kurl et al., 1987, 1988). We have speculated that this specific binding of L-tryptophan to hepatic nuclei plays a vital role in the ability of L-tryptophan to rapidly stimulate hepatic protein synthesis (Sidransky and Verney, 1996, 1997; Sidransky et al., 1992). In consideration that other compounds may influence

(usually diminish) the hepatic nuclear binding of L-tryptophan, we have reported that a variety of agents (nutritional, hormonal and toxicological) have such an effect on in vitro ^{3}H-tryptophan binding to hepatic nuclei (Sidransky and Verney, 1996, 1997, 1999a,b; Sidransky et al., 1992). In the present study, we have investigated whether a potent inhibitor of protein synthesis, cycloheximide, would affect hepatic nuclear tryptophan binding.

Cycloheximide was selected mainly to determine whether its ability to inhibit protein synthesis (Ennis and Lubin, 1964; Korner, 1966) would be of importance in affecting in vitro ^{3}H-tryptophan binding to isolated hepatic nuclei or nuclear envelopes. Since cycloheximide acts at the level of the ribosomes in affecting protein synthesis, it was of interest to determine whether the minor degree of ribosomal contamination, which occurred in the preparation of isolated hepatic nuclei or nuclear envelopes, could possibly be of importance. Electron microscopic examination of our preparations of nuclei or nuclear envelopes revealed relatively pure preparations but some minor contamination with ribosomes or microsomes was detected (Kurl et al., 1987).

The results of this study revealed that cycloheximide and also four compounds structurally related to cycloheximide had an inhibitory effect on in vitro ^{3}H-tryptophan binding to hepatic nuclei and nuclear envelopes. This finding in in vitro experiments did not appear to be related to the inhibitory effect on protein synthesis due to cycloheximide. However, in vivo administration of cycloheximide to rats did suppress the subsequent in vitro ^{3}H-tryptophan binding (total and specific) to hepatic nuclei. This in vivo effect may be related in part to the inhibition of protein synthesis since in vivo inhibition of protein synthesis induced by puromycin acted likewise. Thus, our in vitro and in vivo results revealed that cycloheximide could inhibit tryptophan binding to hepatic nuclei, probably related to its structural effect on the receptor (in vitro) and also due in part to its inhibition of protein synthesis (in vivo).

Materials and methods

Animals

Female rats of the Sprague Dawley strain (Hilltop Lab Animals, Scottsdale, PA), weighing on the average 250 g, were used in all experiments. Rats, maintained in a temperature – controlled room with a 12-hour light/dark cycle, were fed a commercial diet (Purina Laboratory Chow #5001; Purina, St. Louis, MO). Rats were deprived of food overnight but had free access to water before they were used in experiments. In some experiments rats were treated intraperitoneally with test compounds one h before killing. Animals were killed by decapitation. These studies were approved by the institutional animal care and use committee.

Chemicals

The ^{3}H-tryptophan used in the experiments was L-(5-^{3}H)tryptophan, 1.15 TBq/nmol and L-(U-^{14}C)leucine, 12.9 GBq/nmol, obtained from Amersham/Searle (Arlington Heights, IL). L-tryptophan was obtained from US Biochemical (Cleveland, OH). Other chemicals were from Sigma (St. Louis, MO).

Preparation of nuclei and nuclear envelopes

Immediately after the rats were killed, the livers were removed and placed on ice until homogenization (within 15 minutes). Purified hepatic nuclei were prepared as described by Blobel and Potter (1966). The relative purity of the nuclei was determined previously by light and electron microscopy (Kurl et al., 1987). Nuclear envelopes of hepatic nuclei were isolated with the procedure of Harris and Milne (1974) as modified by Agutter and Gleed (1980) and routinely used in this laboratory (Kurl et al., 1987, 1988). Purified hepatic nuclei were treated with 0.001 M $NaHCO_3$, digested with Dnase I (10 mg/L), and centrifuged on a stepwise sucrose gradient ($\leq$2 M sucrose); the nuclear envelope band at interface (1.5 to 1.8 M sucrose) was then removed. The yield of isolated hepatic nuclei was 0.15 mg nuclear protein/g liver.

Binding of ^{3}H-tryptophan to nuclei or nuclear envelopes

Rat hepatic nuclei (0.15 mg protein) or nuclear envelopes (0.5 mg protein) were incubated with l-5-^{3}H-tryptophan (containing 278 kBq, 0.245 nmol tryptophan per assay, added last) in the absence or presence of a 2,000-fold excess of unlabeled tryptophan (10^{-4} M) or test compound ($\leq 10^{-4}$ M) in 5 ml at room temperature for 2 hours. These conditions were selected based on our previous finding (Kurl et al., 1987). The nuclei were incubated in and then washed three times with buffer (0.05 M Tris HCI, pH 7.5, 0.025 M KCI, 0.005 M $MgCl_2$, 0.0001 M phenylmethylsulfonyl fluoride, 0.0002 M dithiothreitol, and 0.25 M sucrose), and the nuclear envelopes were incubated in and then washed two times with buffer (0.05 M Tris HCl, pH 7.5, 0.002 M EDTA, 10% vol/vol glycerol, 0.001 M phenylethylsulfonyl fluoride, and 0.002 M β-mercaptoethanol). After the final wash, the nuclei or nuclear envelopes were suspended in the appropriate buffer, and radioactivity was measured after addition of a scintillation mixture (Opti Fluor; Packard Instruments, Downers Grove, IL). Binding of ^{3}H-tryptophan to hepatic nuclei or nuclear envelopes was expressed as cpm per unit of protein. This total binding (binding in the absence of unlabeled tryptophan or test compound) was then compared with binding in the presence of various concentrations of unlabeled tryptophan, unlabeled test compound, or both.

Preparation of microsomes

Postmitochondrial supernatants were prepared from liver homogenates of rats from the control and experimental groups and used to prepare microsomes (Sidransky et al., 1968).

In vitro protein synthesis

In all assays, liver microsomes of control and experimental rats and liver cytosol of control (water-treated) rats were used (Sidransky et al., 1968). The incubation mixture in a total volume of 1 ml consisted of 0.2 ml microsomes in TKM (0.05 M Tris HCl, pH 7.5, 0.025 M KCl, and 0.005 M $MgCl_2$), 0.3 ml cytosol prepared from the liver of control animals in 0.125 M sucrose in TKM, and 0.5 ml of a solution containing 0.7 μmol ATP, 0.28 μmol GTP, 7.0 μmol phosphenolpyruvic acid (tricyclohexyl ammonium salt), 1.5 μmol Tris, pH 7.4, 7.5 μmol NH_4Cl, 0.175 μmol $MgCl_2 \cdot 6H_2O$, 0.05 μmol L-[U-^{14}C]leucine (18.5 kBq), and 18 μg pyruvate kinase. Samples for in vitro incorporation were incubated for 60 minutes at 37°C in a water bath with mechanical shaking, and the reaction was stopped by addition of 0.1 ml KOH (1N) and kept on ice for 30 minutes. Aliquots (80 μl) were spotted on glass microfiber paper and washed with TCA (10%) three times (first wash contained 0.2% unlabeled L-leucine). The samples were dried, and after addition of a scintillation mixture (Opti Fluor) radioactivity was measured using a liquid scintillation spectrometer

(Beckman Instrument, Palo Alto, CA). The protein content was determined as described by Lowry et al. (1951).

Statistics

Data were analyzed by Student's paired t test (Snedecor and Cochran, 1980).

Results

In the first series of experiments we investigated whether the addition of cycloheximide (at concentrations varying from 10^{-4} to 10^{-18} M) would affect in vitro ^{3}H-tryptophan binding to hepatic nuclei. The results are summarized in Table 1. Cycloheximide is inhibitory to ^{3}H-tryptophan binding (16.7 to 37.2%) at all concentrations used, but maximally at 10^{-10} to 10^{-16} M. When various concentrations of cycloheximide were added to unlabeled L-tryptophan (10^{-4} M), cycloheximide diminished the inhibition of ^{3}H-tryptophan binding to hepatic nuclei by unlabeled L-tryptophan (10^{-4} M) alone with diminishing effects at concentrations from 10^{-4} to 10^{-16} M cycloheximide, but especially from 10^{-4} to 10^{-12} cycloheximide (Table 1). Upon adding the same varying concentrations of both unlabeled L-tryptophan and cycloheximide, the inhibitions were less at 10^{-4} and 10^{-6} M but similar or alike at 10^{-8} and 10^{-18} M when compared to unlabeled L-tryptophan alone. However, the combinations at 10^{-16} and 10^{-18} M induced appreciably less inhibition than that of cycloheximide alone at similar concentrations.

In three experiments, we investigated the effects of length of incubation (30, 60, 90 or 120 min) on the cycloheximide (10^{-10} M) effect on in vitro ^{3}H-

Table 1. Effect of varying levels of unlabeled L-tryptophan (Trp), of cycloheximide (CHEX) singly or combined on inhibition of ^{3}H-trp binding to rat hepatic nuclei

Concentration M	% inhibition due to test compounds singly or combined			
	Singly		Combined	
	Trp	CHEX	Constant Trp (10^{-4} M) + Variable CHEX	Both Similar Variables
10^{-4}	(32) 68.7 ± 0.65[a]	(25) 18.6 ± 1.63	(16) 27.2 ± 3.36[b]	(16) 27.2 ± 3.36[b]
10^{-6}	(1) 56.6	(11) 23.1 ± 2.60	(10) 27.9 ± 4.80[b]	(6) 26.1 ± 5.18
10^{-8}	(1) 49.3	(11) 24.5 ± 2.13	(12) 44.1 ± 5.01[b,c]	(6) 46.5 ± 4.48
10^{-10}	(2) 27.2 ± 6.15	(19) 33.6 ± 2.42[d]	(13) 48.4 ± 5.10[b,e]	(6) 44.1 ± 3.48
10^{-12}	(2) 32.4 ± 0.28	(12) 29.4 ± 1.77[d]	(9) 43.1 ± 2.59[b,e]	(5) 34.9 ± 2.42
10^{-14}	(2) 19.8 ± 1.77	(16) 37.2 ± 2.39[d]	(9) 51.7 ± 3.39[b,e]	(5) 26.2 ± 1.39[f]
10^{-16}	(2) 7.7 ± 0.42	(10) 35.2 ± 4.79[d]	(4) 58.8 ± 3.80[e,g]	(3) 11.8 ± 1.44[f]
10^{-18}	(3) 8.0 ± 3.93	(4) 16.7 ± 4.48	(3) 58.9 ± 0.57[b,e]	(3) 8.2 ± 4.65

[a] Number of experiments in parenthesis. Mean ± SEM. [b] $P < 0.01$, compared with Trp (10^{-4} M) alone group. [c] $0.05 > P > 0.01$, compared with Combined Trp (10^{-4} M) + Variable CHEX (10^{-4} M) groups. [d] $P < 0.01$, compared with CHEX (10^{-4} M) group. [e] $P < 0.01$, compared with Combined Trp (10^{-4} M) + Variable CHEX (10^{-4} M) groups. [f] $P < 0.01$, compared with CHEX singly of same concentration groups. [g] $0.05 > P > 0.01$, compared with Trp (10^{-4} M) alone group.

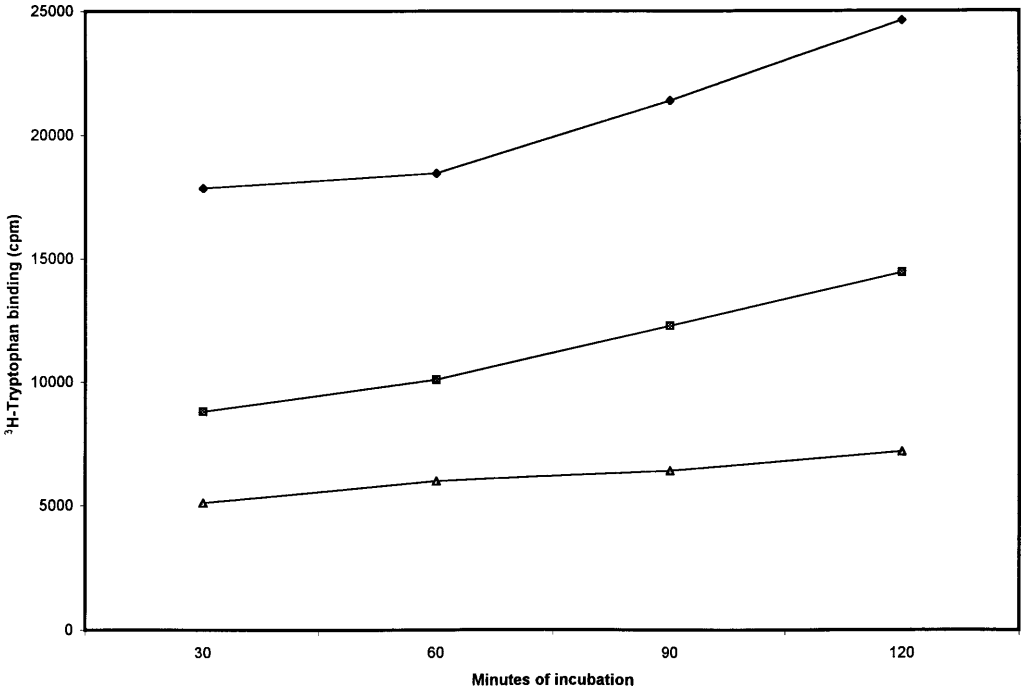

Fig. 1. Time course of ^{3}H-tryptophan binding to hepatic nuclei without (water, ◆——◆) or with addition of cycloheximide (10^{-10}M) (■——■) or of unlabeled L-tryptophan (10^{-4}M) (▲——▲). Values are means of two experiments

tryptophan binding to hepatic nuclei. Also, the effect of addition of unlabeled L-tryptophan (10^{-4}M) was determined. The results are revealed in Fig. 1. It is apparent that cycloheximide had an inhibitory effect on binding at all four intervals of incubation and that the inhibitory effect was less than due to unlabeled L-tryptophan.

Next, we investigated whether the time of addition of cycloheximide (10^{-10}M) to the in vitro ^{3}H-tryptophan binding to hepatic nuclei was influential on the effects observed in Table 1. In the first series of experiments, water or unlabeled L-tryptophan (10^{-4}M) was added at 0 time. Then after $^1/_2$h water, unlabeled L-tryptophan (10^{-4}M) or cycloheximide (10^{-10}M) was added. After 1 h, ^{3}H-tryptophan was added to all groups and incubations were stopped at 2 h. The total bindings of each group revealed diminished binding (−60.1 to −63.7%) with pretreatment with unlabeled L-tryptophan. Addition of cycloheximide diminished binding of the water group by 45.5% and of the unlabeled L-tryptophan group by 43.5% compared to water control group but its effect on the unlabeled L-tryptophan group was less diminished (−43.5%) than due to unlabeled L-tryptophan alone (−63.7%). In the second series of experiments, water or unlabeled L-tryptophan (10^{-4}M) was added at 0 time and incubated with hepatic nuclei and ^{3}H-tryptophan for 2 h (usual incubation procedure as for Table 1). Incubation was stopped, nuclei were spun down, washed X 2, and then water, unlabeled L-tryptophan (10^{-4}M) or cyclohex-

Table 2. Effect of cycloheximide (CHEX) addition before or after ^{3}H-tryptophan (trp) binding to rat hepatic nuclei

Effects before binding					
Incubation conditions[a]				^{3}H-tryptophan binding to hepatic nuclei	
0	$^1/_2$ h	1 h	2 h	cpm/mg protein	% change
Water	Water	^{3}H-trp	Stop	(4) 19,342 ± 3,189[b]	
Water	Trp	^{3}H-trp	Stop	(3) 7,725 ± 2,669[c]	−60.1
Water	CHEX	^{3}H-trp	Stop	(4) 10,535 ± 1,066[d]	−45.5
Trp	Water	^{3}H-trp	Stop	(4) 7,030 ± 1,589[d]	−63.7
Trp	CHEX	^{3}H-trp	Stop	(3) 10,922 ± 1,507[e]	−43.5

Effects after binding					
Incubation conditions[a]			^{3}H-tryptophan binding to hepatic nuclei		
0	2 h[f]	$2^1/_4$ h	Total	Non-Specific	Specific
			cpm/mg protein		
Water	Water	Stop	(4) 24,103 ± 1,772		
Trp	Water	Stop		(4) 8,268 ± 865	(4) 15,835 ± 1,146
Water	Trp	Stop	(3) 7,495 ± 1,116[g]		
Trp	Trp	Stop		(3) 7,479 ± 1,574	(3) 16 ± 422[h]
Water	CHEX	Stop	(4) 14,120 ± 1,739[d]		
Trp	CHEX	Stop		(4) 8,827 ± 1,244	(4) 5,293 ± 1,670[h]

[a] Tryptophan (Trp) was added at 10^{-4} M and cycloheximide (CHEX) was added at 10^{-10} M. [b] Number of experiments in parenthesis. Means ± SEM. [c] $P < 0.01$, compared to Water, Water group. [d] $0.05 > P > 0.01$, compared to Water, Water group. [e] $P < 0.01$, compared with Trp Water group. [f] Binding reaction was stopped after 2 h incubation. Isolated nuclei to were then incubated further with added compound for $^1/_4$ h. [g] $P < 0.01$, compared to Water, Water Group. [h] $P < 0.01$, compared to control specific binding group.

imide (10^{-10} M) were added for an additional 15 min incubation. The results are summarized in Table 2 and reveal that the control groups retained binding similar to the usual 2 h incubation conditions whereby there was 66% specific binding. Addition of unlabeled L-tryptophan caused marked diminution of total binding (−69%) and of specific binding (−99%) and addition of cycloheximide caused appreciable diminution of total binding (−41%) and of specific binding (−67%), but less than with unlabeled L-tryptophan. Thus, it appears that cycloheximide also increases breakdown of total and of specific binding.

The results in Table 3 compare the effects of cycloheximide (10^{-4} M to 10^{-16} M) on in vitro ^{3}H-tryptophan binding to hepatic nuclei and hepatic nuclear envelopes. In each of 2 experiments the hepatic nuclei and nuclear envelopes were prepared from the same rat livers. The findings indicate that the inhibitory effects of cycloheximide on ^{3}H-tryptophan binding were similar in hepatic nuclei and nuclear envelopes.

Table 3. Effect of varying levels of cycloheximide (CHEX) alone or with unlabeled L-tryptophan (Trp) on inhibition of ^{3}H-Trp binding to hepatic nuclei

Test compounds		Inhibition of ^{3}H-Trp binding to hepatic nuclei or nuclear envelopes[a]	
		Nuclei	Nuclear envelopes
Trp	10^{-4} M	64.4	67.6
CHEX	10^{-4} M	19.6	28.4
"	10^{-6} M	32.3	28.3
"	10^{-8} M	20.9	21.9
"	10^{-10} M	31.7	27.4
"	10^{-12} M	30.4	31.3
"	10^{-14} M	48.9	54.1
"	10^{-16} M	46.6	53.9
Trp 10^{-4} M + CHEX 10^{-8} M		37.8	44.8
" + CHEX 10^{-10} M		46.5	45.2
" + CHEX 10^{-12} M		54.4	50.8

[a] Values are the means of 2 experiments where nuclei and nuclear envelopes were prepared from same rat livers.

Table 4. Effect of cycloheximide and related compounds on inhibition of ^{3}H-tryptophan binding to hepatic nuclei

Concentration	Inhibition of ^{3}H-tryptophan binding to hepatic nuclei (%) by the following compounds			
	4-phenacylpyridino-N-oxide monohydrate	4-(2-phenylethyl)-pyridine	4-chlorophenyl-4-phenethyl-piperidino ketone	2-(3,4-dimethoxy-phenyl)-3-oxo-3-pyridin-4-YL-propionitrile
10^{-4} M Trp (4) 69.8 ± 2.3[a]				
10^{-4} M	(4) 35.2 ± 6.6	(4) 38.5 ± 5.2	(4) 50.0 ± 4.8	(4) 51.4 ± 3.0
10^{-6} M	(1) 39.6	(1) 30.1	(1) 27.5	(1) 43.4
10^{-8} M	(3) 31.9 ± 2.3	(3) 29.7 ± 1.1	(3) 29.9 ± 9.1	(3) 34.5 ± 2.3
10^{-10} M	(1) 4.3	(1) 1.7	(1) +3.0	(1) 38.0
10^{-12} M	(2) 24.4 ± 12.3	(2) 18.0 ± 1.4	(2) 13.1 ± 12.8	(2) 18.6 ± 0.3
10^{-14} M	(1) +12.4	(1) +12.8	(1) +13.0	(1) 24.3

[a] Number of experiments in parenthesis. Mean ± SEM.

Table 4 summarizes the data obtained using four cycloheximide-related compounds, 4-phenacylpyridino-N-oxide monohydrate, 4-(2-phenylethyl)-pyridine, 4-chlorophenyl-4-phenethyl)-piperidino ketone and 2-3,4-dimethoxy-phenyl)-3-oxo-3-pyridin-4-YL-propionitrile. In general, each of these compounds had an inhibitory effect on in vitro ^{3}H-tryptophan binding to hepatic nuclei. The effects were, in general, similar but in a few cases even more marked than those induced by cycloheximide alone.

Table 5. Effect of in vivo cycloheximide (CHEX) treatment on in vitro ^{3}H-tryptophan binding to hepatic nuclei

Treatment[a]		^{3}H-tryptophan binding to hepatic nuclei %	
0	1 h	Total[b]	Specific[c]
Water	Kill	(6) 100	(6) 67.2 ± 0.2[d]
L-tryptophan (5 mg/100 gBW)	Kill	(3) 54.4 ± 2.0[e]	(3) 31.9 ± 4.4[e]
CHEX (150 μg/100 gBW)	Kill	(5) 49.6 ± 8.9[e]	(5) 29.6 ± 6.0[e]
CHEX (500 μg/100 gBW)	Kill	(1) 61.6	(1) 24.1
CHEX + L-tryptophan	Kill	(1) 52.9	(1) 30.0

[a] Rats were fasted overnight and in the morning were treated as indicated. Water and L-tryptophan were tube-fed while CHEX was administered intraperitoneally. [b] 100% represents 27,527 cpm per mg nuclear protein of 6 experiments. [c] Specific binding was total binding minus non-specific binding (binding in presence of excess unlabeled tryptophan (10^{-4} M)). [d] Number of experiments in parenthesis. Mean ± SEM. [e] $P < 0.01$.

Next, we investigated whether in vivo treatment of rats with cycloheximide (150 or 500 µg/100 g body weight) would affect subsequent in vitro ^{3}H-tryptophan binding to hepatic nuclei. Table 5 summarizes the results of 3–6 experiments. Hepatic nuclei of cycloheximide-treated rats revealed much (38.4 to 50.4%) inhibition of total binding. Specific binding was determined in each case by subtracting non-specific binding (^{3}H-tryptophan binding in the presence of excess (10^{-4} M) unlabeled L-tryptophan) from total binding. Here too, cycloheximide inhibited specific binding (from 67.2% for controls to 24.1 to 29.6%). Rats that were treated in vivo with unlabeled L-tryptophan alone or with cyclohexmide revealed diminished total and specific binding of hepatic nuclei. In 3 of the above experiments, in vitro ^{14}C-leucine incorporation into protein using microsomes of liver of control (water) and of cycloheximide-treated rats were assayed and revealed that the experimental group compared to the control group had 65.6% incorporation into protein. On the other hand, the results revealed similar binding effects with cycloheximide as with L-tryptophan in in vivo treatment on subsequent in vitro ^{3}H-tryptophan binding to hepatic nuclei. Even though the in vivo findings were consistent with the earlier in vitro findings, the mechanisms of action were probably different for cycloheximide than for L-tryptophan under these experimental conditions.

In consideration that the in vivo effect of cycloheximide on subsequent in vitro ^{3}H-tryptophan binding to hepatic nuclei may be related partly to induced inhibition of protein synthesis, we investigated the effects of other inhibitors of protein synthesis. We selected DL-ethionine and puromycin, two potent inhibitors of hepatic protein synthesis (Simpson et al., 1950; Darken, 1964), that are structurally unrelated and act by different mechanisms. The results are summarized in Table 6. In vitro assay of ^{3}H-tryptophan binding to hepatic nuclei in the presence of unlabeled L-tryptophan, DL-ethionine, or puromycin (all at 10^{-4} M) revealed little or no binding inhibition by DL-ethionine or

Table 6. Effect of treatment with L-tryptophan, DL-ethionine or puromycin in vitro or in vivo on ^{3}H-tryptophan binding to hepatic nuclei and ^{14}C-leucine incorporation into proteins by hepatic microsomes

Groups	In vitro experiments	In vivo experiments[a]		
	% inhibition of ^{3}H-tryptophan binding to hepatic nuclei	In vitro ^{3}H-tryptophan binding to hepatic nuclei		In vitro ^{14}C-leucine incorporation into protein by microsomes
		Total[b]	Specific[c]	
		%		%
Water	0	(5) 100	(5) 67.2 ± 0.2	(4) 100[d]
Tryptophan 10^{-4} M	(11) 67.9 ± 1.0[e,f]	(3) 54.4 ± 2.0[f]	(3) 31.9 ± 4.4[f]	(4) 160.6 ± 11.4[g]
DL-ethionine 10^{-4} M	(9) +12.5 ± 3.7	(4) 81.6 ± 14.6	(4) 55.6 ± 10.5	(4) 84.7 ± 14.1
Puromycin 10^{-4} M	(4) 10.3 ± 4.6	(4) 58.9 ± 16.6	(4) 28.4 ± 6.0[f]	(4) 68.1 ± 15.4

[a] Rats were fasted overnight and in the morning were treated for 1 h before killing. Compounds were administered intraperitoneally in doses per 100 g body weight: L-tryptophan, 5 mg; DL-ethionine, 100 mg; and puromycin, 4 mg. [b] 100% represents 26,578 ± 1,978 cpm per mg nuclear protein of 6 experiments. [c] Specific binding was total binding minus non-specific binding (binding in presence of excess unlabeled L-tryptophan (10^{-4} M)). [d] 100% represents 10,846 ± 1,900 cpm per mg microsomal RNA of 4 experiments. [e] Number of experiments in parenthesis. Mean ± SEM. [f] $P < 0.01$, compared with water group. [g] $P\ 0.05 > P > 0.01$, compared with water group.

by puromycin. In vivo intraperitoneal administration of L-tryptophan, DL-ethionine, or puromycin for 1 h and subsequent in vitro assay of ^{3}H-tryptophan binding to hepatic nuclei revealed decreased total and specific (total minus non-specific in presence of unlabeled L-tryptophan (10^{-4}M)) binding (much with L-tryptophan or puromycin but little with DL-ethionine), while in vitro ^{14}C-leucine incorporation into protein by hepatic microsomes of L-tryptophan-treated rats showed increased (+60.6%) while those of DL-ethionine or puromycin-treated rats show decreased (15.3 and 31.9%, respectively) incorporation into protein.

Earlier, we reported that L-leucine alone had little or no inhibitory effect on in vitro ^{3}H-tryptophan binding to rat hepatic nuclei (Sidransky and Verney, 1997). Yet, when L-leucine was added with unlabeled L-tryptophan, it negated the effect of the latter alone on in vitro ^{3}H-tryptophan binding to hepatic nuclei (Sidransky and Verney, 1997). Thus, we investigated whether L-leucine would influence the effect of cycloheximide on in vitro ^{3}H-tryptophan binding to hepatic nuclei. Table 7 summarizes the data. L-leucine addition negated the inhibitory binding effect of cycloheximide, similar to that reported earlier with unlabeled L-tryptophan (Sidransky and Verney, 1997).

It has been reported that cycloheximide boiled in aqueous solution at pH 7 for 1 h loses its activity (Merck Index, 1996). We, therefore, compared the in

Table 7. Effect of L-leucine on the inhibition of ^{3}H-tryptophan binding to hepatic nuclei due to cycloheximide

Groups	Inhibition of ^{3}H-tryptophan binding to hepatic nuclei %
Water	0
L-leucine 10^{-4}M	(4) 6.1 ± 2.4[a]
L-tryptophan 10^{-4}M	(4) 67.5 ± 0.4
Cycloheximide 10^{-10}M	(4) 41.2 ± 8.5
Cycloheximide 10^{-10}M + L-leucine 10^{-4}M	(4) 1.8 ± 1.2[b]
Cycloheximide 10^{-14}M	(1) 37.0
Cycloheximide 10^{-14}M + L-leucine 10^{-4}M	(2) 7.6 ± 6.3

[a] Number of experiments in parenthesis. Mean ± S.E.M. [b] $P < 0.01$, compared with cycloheximide 10^{-4}M group.

Table 8. Effects of cycloheximide and of heat inactivated cycloheximide on in vitro ^{3}H-tryptophan binding to hepatic nuclei and on in vitro ^{14}C-leucine incorporation into hepatic nuclear protein

Group Tryptophan 10^{-4}M (3) 65.9 ± 1.3[a]		Inhibition of ^{3}H-tryptophan binding to hepatic nuclei %	
		Cycloheximide	Boiled cycloheximide
Cycloheximide	10^{-8}M	(3) 33.0 ± 4.4	(3) 9.3 ± 7.8[b]
"	10^{-10}M	(3) 23.9 ± 7.0	(3) 2.5 ± 6.0
"	10^{-12}M	(2) 32.8 ± 9.6	(2) 14.0 ± 3.1
"	10^{-14}M	(2) 31.7 ± 9.7	(2) 14.3 ± 11.1
"	10^{-16}M	(2) 37.1 ± 4.2	(2) 14.2 ± 18.0
"	10^{-18}M	(2) 32.7 ± 4.1	(2) 10.4 ± 14.7

[a] Number of experiments in parenthesis. Mean ± SEM. [b] $0.05 > P > 0.01$, compared with cycloheximide.

vitro ^{3}H-tryptophan binding to hepatic nuclei using regular cycloheximide and heat boiled cycloheximide. Table 8 reveals that the effects of cycloheximide on in vitro ^{3}H-tryptophan binding to hepatic nuclei did not occur when using boiled (inactivated cycloheximide). In one experiment, we investigated whether the effect of cycloheximide on in vitro ^{3}H-tryptophan binding to hepatic nuclei could possibly be directly affected by contamination of the nuclei by microsomes or ribosomes. Using a routine preparation of hepatic nuclei, we measured whether ^{14}C-leucine incorporation into protein occurred in the presence of control cell cytosol. The results revealed that only a small degree (15% of that in Table 6) of incorporation occurred under these conditions and this was not appreciably changed by 10^{-10}M cycloheximide (regular, (+8%) or boiled (−1.4%). The results were similar with added unlabeled tryptophan (10^{-4}M)).

Discussion

Our present study on the effect of cycloheximide on in vitro ^{3}H-tryptophan binding to hepatic nuclei or nuclear envelopes indicates that the effect is probably due to its structural arrangement which affects and alters the nuclear receptor so that it diminishes ^{3}H-tryptophan binding and that the in vitro effect is not due to inhibition of hepatic protein synthesis. The inhibitory binding effect appears to be on binding and also on breakdown of binding. Although we have earlier determined some of the structural components of the L-tryptophan molecule which are vital for binding to the receptor protein in hepatic nuclei and nuclear envelopes (Sidransky, et al.), such an investigation dealing with the structural components of the complex molecule of cycloheximide or of the complex molecules of four related compounds, 4-phenacylpyridino-N-oxide monohydrate, 4-(2-phenylethyl)-pyridine, 4-chlorophenyl-4-phenethyl-piperidino ketone and 2-(3,4-dimethoxy-phenyl)-3-oxo-3-pyridin-4-YL-propionitrile, has not been conducted. Yet review of the molecular structures of these compounds suggests that they contain common sites which conceivably could act upon the receptor protein to block binding with L-tryptophan. Boiling of a cycloheximide solution for 1 hour, which inactivates the biological activity of cycloheximide, seemed to alter its structure such that it no longer had an inhibitory effect on in vitro ^{3}H-tryptophan binding to hepatic nuclei (Table 8).

The in vivo administration of cycloheximide to rats 1 h before killing affected (diminished) the subsequent in vitro ^{3}H-tryptophan binding to their isolated hepatic nuclei in comparison to that with isolated hepatic nuclei of control rats (Table 5). The decrease in total and specific ^{3}H-tryptophan binding of such experiments was more (48.3%) than the inhibition of in vitro ^{3}H-tryptophan binding when cycloheximide was added in vitro (32–36%) (Table 1). Although this difference may be related to different levels of cycloheximide affecting hepatic nuclei in vivo and in vitro, it may also be related in part due to the inhibition of protein synthesis induced in vivo by administering the cycloheximide 1 h before killing. The results with puromycin, another inhibitor of protein synthesis, suggests that in vivo inhibition of protein synthesis affected (inhibited) the subsequent in vitro ^{3}H-tryptophan binding to hepatic nuclei. On the other hand in vitro addition of puromycin did not essentially affect ^{3}H-tryptophan binding to hepatic nuclei (Table 6).

By utilizing other inhibitors of protein synthesis, such as puromycin and ethionine, as well as cycloheximide, we were able to determine whether inhibitors of protein synthesis that act by different mechanisms would influence in vitro ^{3}H-tryptophan binding to hepatic nuclei. Puromycin and ethionine, unlike cycloheximide, did not inhibit in vitro ^{3}H-tryptophan binding of hepatic nuclei. However, in vivo administration of puromycin did appreciably affect the subsequent in vitro ^{3}H-tryptophan binding to hepatic nuclei (Table 6). On the other hand, in vivo administration of ethionine had only a small effect on in vitro ^{3}H-tryptophan binding to hepatic nuclei. This difference may be related to the lesser degree of inhibition of hepatic protein synthesis due to ethionine (−15.3%) than due to puromycin (−31.9%) in our

experiments (Table 6). In vivo administration of cycloheximide under similar conditions caused a 34.4% decrease in hepatic protein synthesis, as measured by in vitro ^{14}C-leucine incorporation into protein. Thus, we conclude that the effect of cycloheximide in vitro on ^{3}H-tryptophan binding to hepatic nuclei appears to rest specifically with structural aspects of the molecule. Boiling of cycloheximide for 1 h alters the structure such that it no longer has the inhibitory binding effect. On the other hand, the in vivo effect of cycloheximide, may be related to both the effect of cycloheximide itself as well as due to its effect to inhibit protein synthesis. Support for the latter interpretation comes from our findings that puromycin, which appears to inhibit hepatic protein synthesis (as measured in vitro) to a similar degree as that of cycloheximide, affects hepatic nuclear tryptophan binding in vivo but not in vitro. Our results with ethionine are not clear.

In earlier studies, we reported that some amino acids, such as L-alanine, L-phenylalanine and L-tyrosine, competed with L-tryptophan for in vitro ^{3}H-tryptophan binding to rat hepatic nuclei (Sidransky and Verney, 1996; Sidransky et al., 1992) while others, such as L-leucine, L-isoleucine, and L-valine, did not compete with L-tryptophan for in vitro ^{3}H-tryptophan binding to rat hepatic nuclei (Sidransky and Verney, 1997). Nonetheless, L-leucine together with unlabeled L-tryptophan (excess 10^{-4} M) caused significantly less inhibition of ^{3}H-tryptophan binding to hepatic nuclei than did unlabeled L-tryptophan alone (Sidransky and Verney, 1997). The mechanism by which L-leucine acts under the above conditions is not known, yet speculation has been offered (Sidransky and Verney, 1997). In our present study we checked whether L-leucine, which abrogated the inhibition of ^{3}H-tryptophan binding to hepatic nuclei due to unlabeled L-tryptophan alone, would affect the in vitro hepatic nuclei tryptophan binding effect of cycloheximide. L-leucine did prevent the in vitro inhibitory binding effect due to cycloheximide. It is of much interest that L-leucine has a similar effect with cycloheximide as has been shown earlier with L-tryptophan.

In reviewing other studies on the effect of cycloheximide on receptor binding affinity, DeGroot and Rue (1983) reported that the administration of cycloheximide caused a marked decrease in the binding capacity of nuclear triiodothyronine (T_3) which was interpreted as being due to diminished synthesis and availability of receptor protein rather than due to alterations in binding receptor to chromatin. Thomas et al. (1992) reported that cycloheximide inhibited the number of tadpole erythrocyte nuclear specific T_3 binding sites during spontaneous or T_3 induced metamorphosis which was related to inhibition of protein synthesis. Belcheva et al. (1966) reported that cycloheximide treatment of NG108-15 neurohybrid cells inhibited opioid binding to nuclear membrane fractions attributed to inhibition of receptor synthesis. Thus, the effect of cycloheximide on receptor binding is in many cases related to its effect on protein synthesis which diminishes receptor synthesis. Such may also be the case in vivo with hepatic nuclear tryptophan receptor.

In an earlier study by Moja et al. (1991), they investigated the effect of cycloheximide on the plasma and tissue tryptophan levels in rats. They reported that cycloheximide treatment rapidly increased free tryptophan levels

in plasma, liver and brain. Also, rats treated with a tryptophan-free amino acid mixture revealed rapidly diminished tryptophan levels in plasma, liver and brain, while prior treatment with cycloheximide prevented this decrease. The results supported the concept that the levels of tryptophan in blood and tissues are rapidly influenced by body protein synthesis. Also, the dietary administration of tryptophan to fasted (Sidransky et al., 1968) or fed (Sarma et al., 1971) animals raises the circulating tryptophan levels and stimulates hepatic protein synthesis.

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