Biochemical Pharmacology, Vol. 39, No. 7, pp. 1261–1263, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

SHORT COMMUNICATIONS

Depressant effect of very low levels of aflatoxin B_1 on mouse glyoxylase-I activity and methylglyoxal disposal*

(*Received* 15 *September* 1989; *accepted* 30 *November* 1989)

The glyoxylase system (GLO-I and GLO-II, the former in particular) functions in preventing the accumulation of methylglyoxal in tissues, a product of glucose metabolism via the non-phosphorylated pathway and known to express general anti-bacterial, viral and tumor effects [1, 2]. Unregulated accumulation of methylglyoxal in tissues is cytotoxic. Notably, it inhibits protein synthesis, mediates hypoglycemia and causes damage to deoxyribonucleic acid [2–4]. Similar cytotoxic effects stemming from significant exposure to aflatoxin B_1 (AFB_1) have been reported [5–7]. This is of major interest since the AFB_1 is found in several foods due to contamination with moulds belonging to the *Aspergillus* strain [7]. When whole mouse liver homogenate was incubated at 37° with 5, 25 and 125 ng AFB_1/mL, a slight (12, 11 and 5%, respectively) diminution of GLO-I activity occurred [8] whereas at 1500 ng/mL, the activity of the enzyme increased by 17%. Although these changes were insignificant, they persisted. This raised the question of whether the *in vitro* observation is valid *in vivo* and if the AFB_1 could interfere with the metabolic role of GLO-I in methylglyoxal disposal. The present investigation therefore examines the effect of dietary AFB_1, at levels likely to be attained in human food, on colon (since unabsorbed AFB_1 is expected to interact with gut microflora) and liver GLO-I activity and sensitivity to methylglyoxal in the mouse. The mouse was chosen because it metabolizes AFB_1 in a manner similar to humans [7].

Materials and methods

Chemicals. Aflatoxin B_1, reduced glutathione, methylglyoxal, NaCl, KH_2PO_4 were purchased from the Sigma Chemical Co. (St Louis, MO).

Preparation of the AFB_1 diet. Powdered normal mouse chow was obtained from the Tema Food Complex, Tema, Ghana (TLC analysis of the chow was negative for AFB_1 and presumed to be $<4\ \mu g$/kg [9]). A minute amount of aflatoxin B_1 was dissolved in water and added to the powdered chow to give 0.0166 ng AFB_1/10 g of food. The AFB_1 chow mixture was pelleted into into usual form and oven dried. Control chow was similarly pelleted without addition of AFB_1.

Animals. Male and female ddy mice (8–10 weeks old) were obtained from the Laboratory Animal Unit of the Institute and divided into test and control groups. The test animals were placed on the AFB_1-chow and the controls on the normal chow. Both groups of mice were allowed free access to their respective diets and water. The male offspring of these animals (test and controls), after weaning, continued on the respective diets received by the parent stock until finally being used for the study.

Tissue preparation. The test and control animals were killed by cervical dislocation. The liver as well as 1.0 cm of the proximal (right) colon were removed and washed thoroughly with excess ice-cold normal saline. The entire liver was hand homogenized and 10 mg of the resulting pulp was suspended in 0.5 mL deionized water and allowed to stand at ambient temperature for 45 min. The lysate was clarified by centrifugation at 15,000 *g* for 20 min. The resulting supernatant was used within 6 hr for the determination of GLO-I activity (see below) and protein by a modification of the Lowry procedure [10]. The colon was treated in a similar manner.

GLO-I assay. The activity of GLO-I was determined essentially as described by Jellum and Elgjo [11]. Briefly, 0.02 mL of the 15,000 *g* supernatant was mixed with 1.0 mL of 50 mM phosphate buffered saline, pH 6.7, containing 1.25 mM reduced glutathione and 1.13 mM methylglyoxal.

After 15 min at ambient temperature, the absorbance of lactoylglutathione formed was read at 240 nm ($\varepsilon = 3.37\ mM^{-1}\,cm^{-1}$ [12]). The appropriate blank for each assay was also performed. P values were obtained with the Student's *t*-test.

Results and discussion

GLO-I activity. Offspring of mice fed food to which very low levels of AFB_1 (0.0166 ng AFB_1/10 g feed) was added showed a significant decrease in the activity of colon glyoxylase-I (GLO-I) by a mechanism which is not clear at present. The fall in activity of 39 and 50%, respectively, at 3 and 6 months of age was significant. A similar observation was made in the liver at the age of 6 months (Fig. 1). Jellum and Elgjo [11] reported a similar effect on GLO-I activity with *S*-hexyl reduced glutathione. Other studies [1, 2] have also shown that the activity of glyoxylase fell in the presence of pancreatic and kidney extracts.

Methylglyoxal sensitivity. To find out if the fall in GLO-I activity in the AFB_1 fed mice resulted in decreased disposal of methylglyoxal, groups of test and control mice (aged 6 months) were injected by intraperitoneal route with methylglyoxal in a standard dose of 150 mg/kg body weight in normal saline; once daily for 3 days. Before the treatment was carried out each mouse (both test and control animals) ingested 11–13 g of its respective diet daily (data not

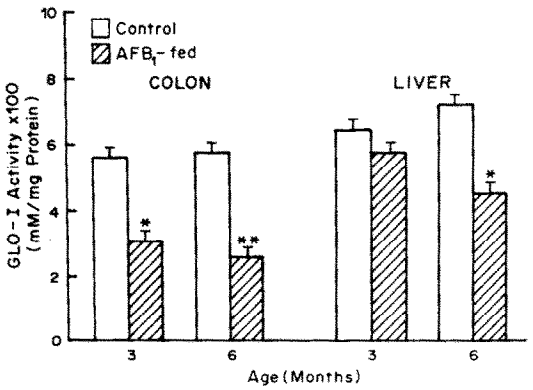

Fig. 1. Effect of AFB_1 on mice colon and liver glyoxylase-I activity. The assay mixture is as described in Materials and methods. At the end of a 15 min incubation at ambient temperature, absorbance changes were measured at 240 nm. Bars represent mean ± SE of 8–12 mice. Asterisks indicate values significantly different (*$P < 0.01$; **$P < 0.001$) from controls using the Student's *t*-test.

* Part of this paper was presented at the Ghana–Japan joint scientific conference by the NMIMR, University of Ghana, Legon, held on 7–9 August, 1989.

Table 1. Serum glucose and protein levels in AFB_1-fed and control mice before and after intraperitoneal injection of methylglyoxal (MG)*

	Glucose (mg/L)			Protein (g/L)		
	Before MG	After MG	% Difference	Before MG	After MG	% Difference
Control mice						
1	971	961	−1.0	59	55	−6.8
2	1271	1058	−16.8	51	50	−2.0
3	1358	1286	−5.3	QNS	QNS	—
4	1168	1156	−1.0	58	58	0
5	1183	1186	+0.3	56	57	+1.8
Mean ± SD	1190 ± 114	1129 ± 124		56 ± 4	55 ± 3	
Test (AFB_1-fed) mice						
1	914	324	−64.6†	55	46	−16.4
2	1318	773	−41.4‡	59	47	−20.3
3	1508	335	−77.8†	57	54	−5.3
4	1390	289	−79.2‡	60	54	−10.0
5	1081	955	−11.7	54	51	−5.6
Mean ± SD	1242 ± 241	535 ± 307		57 ± 3	50 ± 3	

* Six-month-old male mice were treated with MG: 150 mg/kg body wt once daily for 3 days, i.p. Blood samples were drawn from the tails of the mice before and 4 hr after the last injection of the MG. The blood was clotted, serum separated and analysed for glucose by the O-toluidine method [13] and protein by a modification of the Lowry procedure [10].

† Died within 24 hr after third (last) dose of the MG.

‡ Died at day 7 after the third dose of the MG.

QNS, Quantity not sufficient.

shown). All the animals showed normal appearance. Serum glucose and protein levels [10, 13] were also similar in both groups of animals (Table 1). A single dose of the methylglyoxal was without apparent toxic effects but resulted in higher levels of 2,4-dinitrophenyl hydrazine reacting material (presumably methylglyoxal [14, 15] in the serum (4 hr post i.p.) of the AFB_1-fed mice relative to the controls (data not shown). However, after receiving three doses of the methylglyoxal, serum glucose and protein levels fell in the AFB_1-fed mice whereas those of the controls were not significantly affected (Table 1). The amount of daily food intake also fell to 0.9–1.0 g/day in both test and control mice. The test mice became lethargic and four out of five died (see legend of Table 1). Post-mortem examination showed extensive cutaneous congestion, cirrhotic liver with haemorrhagic points, atrophy of the spleen and whitish substance in the abdomen. These observations are consistent with a decreased capacity to dispose of methylglyoxal in the test mice. The severe hypoglycemia shown in the test mice could, probably, be due to the hyperinsulinemic action of an excess glyoxal [2] and not to the loss of appetite.

Complete loss of glyoxylase activity results in the accumulation of cytotoxic amounts of methylglyoxal [1, 2] in spite of the suggested compensation by α-ketoaldehyde dehydrogenase [2, 11]. The rate limiting role of GLO-I in the disposal of methylglyoxal synthesized *de novo* in *Escherichia coli* by methylglyoxal synthase have been confirmed [14, 15]. This is further stressed by the observation that strains of *E. coli* with low GLO-I activity accumulated cytotoxic amounts of methylglyoxal and subsequently led to their self destruction [15]. Thus the AFB_1 mediated fall in GLO-I activity could lead to decreased disposal of endogenous methylglyoxal against susceptible micro-organisms and alter the established balance of the microflora. This suggest is under investigation. It is worth noting that the amount of AFB_1 ingested 0.498–0.796 ng/kg body wt/day is much lower than the 50% lethal dose level (LD_{50}: 60 mg/kg body wt) for adult mouse [16].

Summary: colon glyoxylase-I (GLO-I) activity was lower (39–50%) in mice fed very low levels of AFB_1 (0.0166 ng/10 g feed/day) when compared to values obtained for unexposed (controls) mice. The activity of liver GLO-I was also lower. The amount of AFB_1 used did not produce overt adverse effects. When the AFB_1-fed and control mice (6-month old) were treated with methylglyoxal (150 mg/kg body wt, i.p. once daily for 3 days), this caused toxic effects (hypoglycemia and hypoproteinemia, lethargy and death) only in the AFB_1-fed mice. This observation indicates that the effect of the injected methyglyoxal is related to decreased capacity to dispose of the glyoxal and is consistent with the fall in GLO-I activity in the AFB_1-fed mice.

Acknowledgements—I am grateful to Mr B. R. Anku for technical assistance and to Dr P. G. Addo for her interest and support. I am also thankful to Prof. J. A. A. Mingle for his helpful suggestions.

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Biochemical Pharmacology, Vol. 39, No. 7, pp. 1263–1266, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

Antiproliferative effects of glutathione *S*-transferase inhibitors on the K562 cell line

(*Received* 11 *April* 1989; *accepted* 28 *September* 1989)

Glutathione *S*-transferases (GSTs* EC 2.5.1.18) are enzymes which catalyze the reaction of reduced glutathione (GSH) with a variety of electrophilic compounds [1, 2]. They are distributed not only in mammalian tissues but also in microorganisms and even in plants [3]. In spite of its wide distribution, little is known about its physiologic function (e.g. detoxification of xenobiotics [4], leukotriene C_4 synthesis [5] and transport of heme [6] and bilirubin [7]). Recently, there have been a few reports suggesting the possible involvement of GST in cell proliferation. Senjo and Ishibashi [8] reported that GST activity in C6 astroglioma cells changes significantly during the cell cycle and that the cell growth is inhibited in a dose-dependent manner by the GST inhibitors ethacrynic acid (EA) and caffeic acid. Tew *et al.* [9] reported that in rat breast carcinoma cell lines and human colon carcinoma cell lines EA and piriprost inhibit their colony forming ability and cytosolic GST activity.

The K562 cell is from a human leukemia cell line which has been established from the blast cells in pleural effusion of a patient with chronic myelogenous leukemia in blast crisis [10]. To investigate whether GST may play a role in the proliferation of K562 cells, we examined the effects of GST inhibitors, EA, bromosulfophthalein (BSP) and ferulic acid (FA) [11, 12], on cell proliferation.

Materials and methods

Materials. EA, BSP, FA, DL-buthionine[*S*,*R*]-sulfoximine (BSO) and GSH were purchased from the Sigma Chemical Co. (St Louis, MO). RPMI 1640 medium, fetal calf serum and penicillin–streptomycin were obtained from Flow Laboratories (U.S.A.), and [^{3}H]thymidine (50 Ci/mmol) was obtained from New England Nuclear (Boston, MA). 1-Chloro-2,4-dinitrobenzene (CDNB) was purchased from Nakarai Chemicals, Ltd (Kyoto, Japan). All other chemicals were of analytical grade.

Cell culture. The K562 cell line was supplied by the Japanese Cancer Research Resources Bank. K562 cells were routinely grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, penicillin (50 units/mL) and streptomycin (50 μg/mL) at 37° in a humidified atmosphere with 5% CO_2.

Effects of GST inhibitors on cell proliferation. EA, BSP and FA were dissolved in RPMI 1640 medium by vigorous shaking. K562 cells were cultured in the medium containing various concentrations of a GST inhibitor. Cells were counted in a hemocytometer, and viability was estimated by the trypan blue exclusion assay.

To examine the effects of GST inhibitors on DNA synthesis, after cells were incubated for 24 hr with various concentrations of a GST inhibitor on 96-well microtiter plates, [^{3}H]thymidine was added at a final concentration of 5 μCi/mL. After a 2-hr incubation, the trichloroacetic acid insoluble counts were counted on filter paper (Labo Science Corp., Japan) in toluene-based scintillant.

GST assay. K562 cells were washed twice with ice-cold phosphate-buffered saline and sonicated in 10 mM potassium phosphate buffer (pH 7.0). After centrifugation by an Eppendorf centrifuge at 4000 *g* for 10 min, GST activity in the supernatant fraction was assayed by the method of Habig *et al.* [1] using CDNB as a substrate at 37°. The inhibitory effects of EA, BSP and FA were examined in the presence of 1 mM GSH and 1 mM CDNB.

Measurement of intracellular glutathione. Intracellular glutathione was measured by the method of Griffith [13], with concurrent standards being monitored.

* Abbreviations: GST, glutathione *S*-transferase; EA, ethacrynic acid; BSP, bromosulfophthalein; FA, ferulic acid; BSO, DL-buthionine[*S*,*R*]-sulfoximine; GSH, reduced glutathione; and CDNB, 1-chloro-2,4-dinitrobenzene.