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CHLOROFORM–INDUCED MULTIPLE FORMS OF ORNITHINE DECARBOXYLASE: DIFFERENTIAL SENSITIVITY OF FORMS TO ENHANCEMENT BY DIETHYL MALEATE AND INHIBITION BY ODC–ANTIZYME

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The role of glutathione (GSH) and ornithine decarboxylase-antizyme (ODC-AZ) in the regulation of the chloroform-mediated stimulation of rat hepatic ornithine decarboxylase (ODC) was investigated. We have previously implicated roles for each while examining the chloroform effect on crude cytosolic enzyme preparations. In this study we examined the effect of pretreatment with diethyl maleate (DEM), a GSH-depleting agent, on the chloroform stimulation of the two forms of the rat hepatic ODC enzyme and the sensitivity of these two forms to inhibition by the ODC-AZ. While the pretreatment with DEM provided a greater amount of the two forms of the ODC enzyme, it also resulted in a differential stimulation of each form when compared to chloroform alone. Additionally, Peak II was 20–25% more sensitive to the same amount of ODC-AZ then Peak I ODC activity.

INTRODUCTION

We have previously reported that chloroform induces rat liver ornithine decarboxylase (ODC). In both male and female rat liver this induction is dose-dependent and maximal 18 h after a single intraperitoneal dose (Savage et al., 1982). Two distinct forms of the enzyme are induced in rat liver (Pereira et al., 1983). Pretreatment of animals with diethyl maleate enhances the effect of chloroform (Savage et al., 1987). In addition, chloroform induces hepatic ornithine decarboxylase-antizyme (ODC-AZ) in rat liver (Savage et al., 1988). In this study, we report the effect of diethyl maleate pretreatment on the chloroform

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induction of two different forms of male rat liver ODC and the sensitivity of the two forms of the enzyme to inhibition by ODC-AZ.

MATERIALS AND METHODS

Materials

Glass-distilled chloroform without preservative was purchased from Burdick & Jackson Laboratories (Muskegon, Mich.), DEAE-Sepharose CL-6B from Pharmacia (Uppsala, Sweden), DL-[1-^{14}C]ornithine hydrochloride from New England Nuclear Corp. (Boston, Mass.), and diethyl maleate, dithiothreitol, and pyridoxal 5'-phosphate from Sigma Chemical Co. (St. Louis, Mo.).

Animals

Male Fisher 344 albino rats from the Charles River Co. (Portage, Mich.) weighing 200–250 g were used. They were fed Purina Laboratory Chow (Ralston Purina Co., St. Louis, Mo.) and given distilled water ad libitum. All rats were housed in a certified animal facility featuring constant temperature (22 ± 20°C), constant humidity (40–60%), and 12-h light-dark cycles (6:00 a.m. to 6:00 p.m.). Three animals were used for the preparation of the two forms of liver ODC as described previously (Pereira et al., 1983). Six rats were used for the induction and preparation of hepatic ODC-AZ (Hayashi and Fujita, 1983).

Injection Schedules

We have previously reported that a 3-h pretreatment of animals with diethyl maleate (325 mg/kg, ip) prior to chloroform administration (6.0 mmol/kg, ip for 18 h) enhanced the stimulation by chloroform of ODC (Savage et al., 1987). In this study we utilized this dosing regimen to maximize the amount of resolvable Peaks I and II of ODC enzyme activity eluted from DEAE-Sepharose CL-6B column chromatography. Chloroform and diethyl maleate were administered undiluted.

Separation of Two Species of ODC Activity

Multiple species of ODC were separated by a modification of the procedure of Richards et al. (1981). Briefly, with all subsequent procedures performed at 0–4°C, the livers were homogenized at a concentration of 1 g/ml in a buffer containing 50 m*M* Tris, 5.0 m*M* dithiothreitol, and 0.1 m*M* EDTA (pH = 7.3). The homogenate was sequentially centrifuged at 10,000 × g for 10 min and at 144,000 × g for 60 min. A 10-ml aliquot of the supernatant fraction was applied to a DEAE-Sepharose CL-6B column (1.6 × 40 cm) previously equilibrated with Buffer A containing 50 m*M* Tris, 5.0 m*M* dithiothreitol, 0.1 m*M* EDTA, 0.125 *M* NaCl, and 50μ*M* pyridoxal phosphate (pH = 8.0). The column was eluted with

150 ml of Buffer A followed by a 600-ml gradient of 0.125 *M* to 0.3 *M* NaCl in Buffer A at a flow rate of 0.5 ml/min. Unfractionated cytoplasm was also assayed for ODC activity. Prior to assay, the buffer of the cytoplasm was changed to Buffer A by chromatography on Pharmacia PD-2 disposable columns preequilibrated with Buffer A.

ODC Assay

The cytoplasmic sample or fractions collected from the eluate of the DEAE-Sepharose CL-6B column (500 μl) were added to Kontes incubation flasks containing 50 μl of the incubation medium at a final concentration of 50 m*M* sodium phosphate (pH = 7.5), 3.5 m*M* dithiothreitol, 2.0 m*M* EDTA, 8.0 m*M* DL-ornithine monohydrochloride, and 1.0 μCi of DL-[1-^{14}C]ornithine (51.3 mCi/mmol). After incubation for 30 min at 37°C, the reaction was terminated by adding 0.3 ml of 5.0 *M* H_2SO_4 and by allowing it to stand for an additional 45 min. The liberated $^{14}CO_2$ was absorbed by filter paper saturated with hyamine hydroxide. The filter paper with the $^{14}CO_2$ trapped onto it was transferred to a liquid scintillation vial containing 15.0 ml ACS and 0.41 ml of 0.5 *M* HCl. The radioactivity in the samples was measured in a Beckman model 8000 liquid scintillation counter (Beckman Instruments, Fullerton, Calif.). ODC activity was expressed as dpm $^{14}CO_2$ liberated per 30-min incubation at 37°C.

Preparation of Peaks I and II for ODC-AZ Assay

Two peaks of ODC activity were eluted from the DEAE Sepharose CL-6B column as previously reported (Pereira et al., 1983). The first 20 fractions containing Peak I activity were pooled, while the last 20 fractions containing Peak II activity were similarly pooled. This left an intermediate set of 10 fractions that were excluded from the study, since they constituted a mixture of the two peaks. The two sets of pooled fractions were dialyzed overnight against Buffer A (500 volumes, 3 buffer changes). Following dialysis, aliquots of the isolated peaks were assayed for ODC activity.

Preparation of ODC-AZ Assay

Antizyme was partially purified by DEAE-cellulose chromatography and Sephadex G-75 gel filtration from livers of rats injected intraperitoneally (ip) with putrescine and sacrificed 4 h later as described by Hayashi and Fujita (1983). ODC-AZ activity was determined by the inhibition of renal ODC activity (Savage et al., 1988).

Assay for Peak Sensitivities to ODC-AZ

Following the establishment of a known amount of ODC activity associated with each of the two sets of pooled peaks from the DEAE-Sepharose column, the sensitivity of each of the sets of pooled peaks of

ODC activity to inhibition by ODC-AZ was determined. This was accomplished by conducting the ODC assay in a Kontes reaction flask containing (1) varying amounts of ODC-AZ active protein as described by Bradford (1976), (2) the incubation media, and (3) a fixed amount of Peak I or Peak II ODC activity.

RESULTS

Separation of Two Species of ODC Activity

The elution profiles from DEAE-Sepharose CL-6B column chromatography of a cytoplasmic fraction from the livers of control, chloroform-treated, and diethyl maleate chloroform-treated male rats are shown in Fig. 1. Treatment of animals with chloroform alone resulted in stimulations of both Peak I and Peak II activities (5.5- and 12.0-fold increases over controls, respectively, when comparing maximum activities observed with each peak), as we previously reported (Pereira et al., 1983). However, pretreatment with diethyl maleate followed by the same dose of chloroform resulted in a minor increase in Peak I activity (25% increase when compared to chloroform alone) but a continued increase in the ODC activity associated with Peak II (3.0-fold over chloroform alone and 36.0-fold over controls). This latter finding is further evidenced by the fact that with chloroform alone, Peak II activity constituted 35% of the total ODC activity recoverable from the cytoplasm, while pretreatment of animals with diethyl maleate followed by chloroform resulted in Peak II activities that accounted for 70% of the total ODC recoverable activity. When diethyl maleate was administered without chloroform there was no change in the ODC activity or ODC activity profiles when compared to control animals.

Measurement of Form Sensitivity to ODC-AZ

Isolated established amounts of ODC activity derived from either Peak I or Peak II were incubated with varying protein concentrations of an ODC-AZ active preparation. The results of this study are depicted in Fig. 2. From this particular study it appears that the Peak II form of ODC isolated from male rat liver is slightly more sensitive to inhibition by ODC-AZ than the Peak I form of the enzyme. This is evident at all three different concentrations of the ODC-AZ examined in this study.

DISCUSSION

In our previous studies examining the two major forms of ODC induced by chloroform, we were unable to demonstrate any form specific inhibition by alpha-difluoromethylornithine (DFMO) (Pereira et al.,

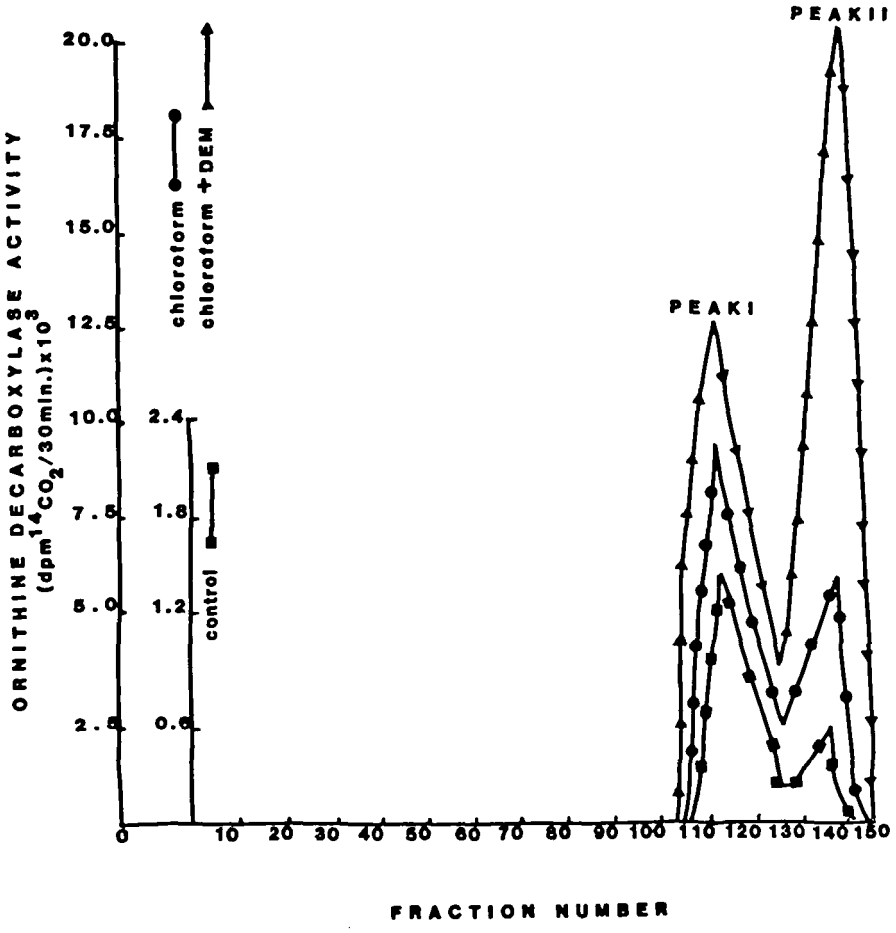

FIGURE 1. DEAE-Sepharose (CL-6B column chromatography of the ODC activity in a liver cytoplasmic fraction. The livers of three rats were pooled and homogenized. A 10-ml aliquot of the cytoplasmic fraction was applied to a DEAE-Sepharose CL-6B column (1.6 × 40 cm) and chromatographed as described in Materials and Methods. Key: (■) control rats (illustrated on inner scale), (●) rats treated with 6.0 mmol/kg chloroform and killed 18 h later, and (>) rats treated with 325 mg/kg diethyl maleate (3 h prior to chloroform), treated with 6.0 mmol/kg chloroform, and killed 18 h later (chloroform and chloroform plus DEM rats chromatography profiles are depicted using outer scale).

1983), This observation was substantiated by Mitchell et al., (1985) using rat hepatoma tissue culture (HTC) cells. Furthermore, we reported that the two isolatable forms of ODC exhibited different half lives. Our findings that Peak I was the more labile ($t_{1/2}$ = 10 min) and Peak II the more stable ($t_{1/2}$ = 50 min) were also supported by Mitchell et al. (1985). In this current report, however, the data suggest that although Peak I has a shorter half life, Peak II is the more sensitive (by 20–25%) to inhibition

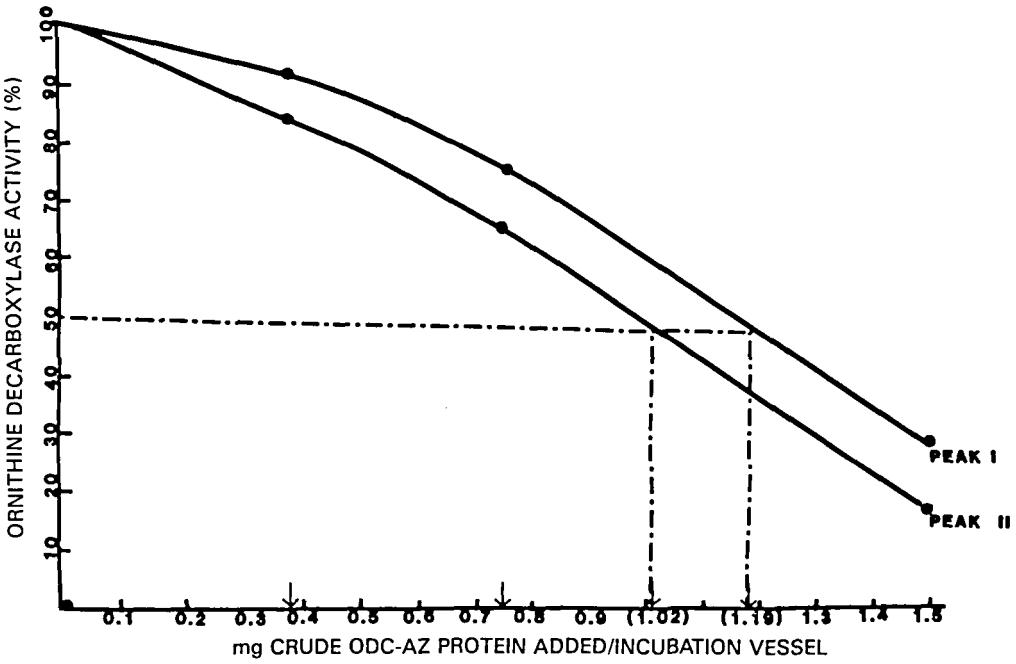

FIGURE 2. Sensitivity of isolated peaks of ODC activity to inhibition by ODC-AZ. Peak I and Peak II ODC activity was isolated and determined as described in Materials and Methods. Established amounts of activity from each peak were incubated with varying amounts of a partially purified ODC-AZ preparation. Each data point thus represents the mean ODC activity for that peak in the presence of a particular ODC-AZ protein concentration when compared to similar peak activity in the absence of ODC-AZ protein (buffer only; 100%). Furthermore, each point represents an average of triplicate assays with an error of less than 10% calculated from three separate experiments. Statistically significant differences ($p > .05$) between Peak I and Peak II inhibition by similar protein concentrations of ODC-AZ were observed at all ODC-AZ protein concentrations evaluated (Student's *t*-test).

by ODC-AZ. To our knowledge this is the first such report of form sensitivities to ODC-AZ.

Pretreatment of male rats with diethyl maleate (a glutathione-depleting agent) led to a further enhancement of the already dramatic chloroform response (Savage et al., 1987). We report here that this enhancement is a preferential stimulation of Peak II activity. During the course of these studies we examined the effect of diethyl maleate administered alone. At the time examined in our studies (21 h) and at the dosage of 325 mg/kg we were not able to observe any detectable change in hepatic ODC activity or the elution pattern of the two forms from DEAE-Sepharose CL-6B column chromatography. Recently, Yoshida et al. (1988) have reported that diethyl maleate is a powerful stimulator of rat hepatic ODC and appears to stimulate both forms equally well. However, the peak of diethyl maleate-mediated ODC stimulation occurs at 6 h following administration and declines rapidly

after 12 h. At 24 h post treatment with 642 mg/kg diethyl maleate (roughly twice the dose employed in our study) Yoshida reported only a 75% elevation over controls. Most of Yoshida's report featured doses of diethyl maleate at fourfold (1284 mg/kg) those used in our study. Although we did not conduct a time course study, it would appear that after 21 h at a diethyl maleate dose of 325 mg/kg, the activity of ODC would have returned to control values, which is indicative of the data generated and reported in our previous study (Savage et al., 1987). The direct contribution of diethyl maleate to the observed changes in hepatic ODC at 21 h is not discernable from controls (or untreated animals). However, an indirect effect is noted when diethyl maleate-pretreated rats are given chloroform. Pretreatment of male rats with diethyl maleate results in a cellular environment that favors the stimulation of Peak II ODC activity when the animals are administered chloroform. This is demonstrated by the relatively small change in Peak I activity (25% increase over chloroform alone) but a significant threefold increase over the chloroform stimulation if pretreated with diethyl maleate.

In summary, we have presented novel information on the sensitivity of the two different forms of ODC to ODC-AZ and on the differential induction of these two forms by the combined treatment of diethyl maleate and chloroform. Although the significance of these results is not obviously clear at this time; these findings could add further information to the data on chloroform effects on liver toxicology, biochemical effects of diethyl maleate on the liver, and various aspects of cellular regulation of hepatic ODC.

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