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DRUG-INDUCED ALTERATIONS IN HEPATIC FATTY ACID DESATURASE ACTIVITY*

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Abstract—A significant inhibition *in vitro* of the hepatic fatty acid desaturase system was observed after pretreatment of animals with 3-methylcholanthrene or fenfluramine. In contrast to previous reports, no effect on the desaturase system was observed after pretreatment of rats with phenobarbital. The drug-treated animals which demonstrated decreased desaturase activities also ate less food, drank less water, and had lower body weight gains than did control or phenobarbital-treated animals. Similar inhibition of fatty acid desaturase activity was achieved by restricting food intake of non-drug-treated animals. Decreased desaturase activity occurred concomitant with elevated serum free fatty acid levels, regardless of the mechanism of fatty acid elevation. These results suggest that the hepatic fatty acid desaturase system is very sensitive to drug-induced alterations in the nutritional status of animals and that this nutritional factor must be closely evaluated in studies examining the interaction *in vivo* of drugs with this enzyme system. These results also suggest that the mechanism of this nutritional alteration may be related to the serum concentration of free fatty acids.

The hepatic microsomal oxidative Δ^9 desaturation of fatty acids is a mixed function oxidase which catalyzes the conversion of saturated fatty acyl CoA's to the corresponding monoenoic acid ester; the conversion most often studied is the metabolism of stearyl-CoA to oleoyl-CoA. The electrons required for this desaturase are transferred from NADH or NADPH through cytochrome b_5 to a cyanide-sensitive factor [1]. This factor is thought to be the rate-limiting component for desaturation.

The activity of the fatty acid desaturase is very sensitive to dietary manipulation [1–6]. Microsomal preparations from animals fed a fat-free diet exhibit activities of 4–8 nmoles oleic formed/min/mg of microsomal protein [1–4], while those from starved animals have activities of only 0·06 to 0·30 nmole oleic formed/min/mg of protein [1, 4]. Preparations from animals fed normal rat chow exhibit intermediate activities [1, 2, 4].

Similarly, the activity of the desaturase system has also been reported to decrease after pretreatment of animals with phenobarbital [7, 8]. This was most intriguing in light of our previous observation that phenobarbital pretreatment of rats resulted in the disappearance of a particular nonheme iron species from the hepatic microsomes [9]. Since the cyanide-sensitive factor has been postulated to be an iron-containing compound [10], and may also be the rate-limiting step in the desaturation process [8], a possible relationship between the cyanide-sensitive factor and this iron species [9] was envisioned. In this study, we have found that the activity of the desaturase is not related to the concentration of this iron species but rather to the serum concentration of free fatty acids.

MATERIALS AND METHODS

Male, Charles River CD rats (180–220 g) were given *ad lib.* access to a fat-free diet ("Fat Free" Test Diet, Nutritional Biochemicals, Cleveland, Ohio) and tap water for 4 days prior to sacrifice, unless otherwise stated. Phenobarbital sodium (1 mg/ml, Merck Chemical Division, Rahway, N.J.) *d*-amphetamine sulfate (0·05 or 0·20 mg/ml, gift of Sigma Chemical Co., St. Louis, Mo.) or fenfluramine hydrochloride (0·05 or 0·20 mg/ml, gift of Robbins Research Laboratory, Richmond, Va.) was administered in the drinking water for 4 days. 3-Methylcholanthrene (40 mg/kg in corn oil, i.p., Eastman Kodak Co., Rochester, N.Y.) was administered on days 3 and 4. 'Starved' and 'limited' animals received the fat-free diet *ad lib.* for days 1 and 2 and then either no food or graded aliquots, respectively, of fat-free diet for days 3 and 4. All animals were sacrificed by decapitation between 9:00 and 10:00 a.m., the livers removed [9] and homogenized in a motor-driven Teflon-glass homogenizer with 0·10–0·15 mm clearance in 3 vol. of 0·02 M Tris–0·15 M KCl buffer, pH 7·4. The homogenate was centrifuged at 9000 *g* for 15 min. The supernatant was centrifuged at 165,000 *g* for 38 min, the resultant pellet was resuspended in the Tris–KCl buffer and resedimented at 165,000 *g* for 38 min. The microsomal pellet was then resuspended in Tris–KCl at a final concentration of 10–15 mg microsomal protein/ml. Microsomal protein was determined by the method of Sutherland *et al.* [11].

Blood was collected at the time of decapitation, the serum separated and immediately frozen for subsequent analysis of serum free fatty acids by the colorimetric assay of Novak [12].

Desaturation of [1-^{14}C]stearyl-CoA was determined as described by Oshino *et al.* [7]. One-tenth ml of the microsomal suspension (containing 1·0–1·5 mg microsomal protein) was added to the incubation mixture containing 1 mM NADH and 70 μM [1-^{14}C]stearyl-CoA in a final volume of 0·50 ml of 0·1 M Tris–HCl buffer, pH 7·25. [1-^{14}C]stearyl-CoA was obtained from New England Nuclear, Boston, Mass., with a sp. act. of 51·8 mCi/m-mole (Lot No. 678–279). Two-tenth mg of this (0·010 mCi) was added to

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10 mg cold stearyl-CoA (Sigma Co.) in Tris–HCl, pH 7·25, for use in the incubation mixtures. All incubations were performed in duplicate for 4 min at 37° in a shaking water bath. The reaction was stopped by the addition of 1·0 ml of 10% KOH in methanol, followed by saponification at 80° for 30 min. The mixture was then acidified with 2·0 ml of 4 N HCl, the fatty acids were extracted with 30 ml petroleum ether, and converted to methyl esters with 3 ml of 14% boron trifluoride in methanol [13]. The methyl esters were extracted into petroleum ether and separated by thin-layer chromatography on Silica gel GF plates (Analtech Inc., Newark, Del.) containing 10% $AgNO_3$ by developing in ether–hexane (1:9, v/v) [7]. It should be noted that separation of mono-unsaturated from the saturated esters was greatly enhanced by prewashing the plates with ethyl acetate. The spots were identified under ultraviolet light after spraying of 0·05% Rhodamine B (in methanol) and compared to authentic standards. The spots were scraped and counted in a toluene scintillator (4 g PPO and 0·25 g POPOP/liter of toluene)* in a Beckman LC-100 scintillation counter. Desaturation activity was determined by dividing the radioactivity found in the mono-unsaturated ester by the sum of the radioactivities in both the saturated and unsaturated esters; this ratio was then converted to nmoles oleic formed/min/mg of microsomal protein. Background activity for the assay was determined by addition of the microsomes after addition of the KOH.

Weight gain of animals was determined as the percentage weight increase after the 4 days of treatment as compared to the initial weight. Statitistical significance was taken as $P < 0·05$ by the Student *t*-test or by linear regression.

RESULTS

In order to establish the effect of phenobarbital on the fatty acid desaturase activity, a group of rats was treated with phenobarbital for 4 days while on the fat-free diet (Fig. 1). In contrast with previous studies which have reported complete [7] or partial [8] inhibition of fatty acid desaturase by phenobarbital, it can be seen that no significant difference was observed between the drug-treated and control rats in the activity of the microsomes to desaturate stearyl-CoA to oleate.

Furthermore, both the control and drug-treated preparations exhibited identical sensitivity to inhibition by 0·10 mM cyanide. Control incubations (N = 5) were inhibited 31·3 ± 1·5 per cent (S.E.M.), while preparations from phenobarbital-treated rats (N = 5) were inhibited 27·8 ± 1·6 per cent ($P > 0·1$). Thus, it appears that the cyanide-sensitive factor of fatty acid desaturase remains fully intact after phenobarbital pretreatment of rats. This would suggest that the iron species which disappears from the microsomes during phenobarbital treatment [9] is unrelated to the cyanide-sensitive factor, since neither the control rate of desaturase activity nor the cyanide sensitivity was affected by phenobarbital treatment. Presumably, if the concentration of the rate-limiting component (the cyanide-sensitive factor) had been decreased, phenobarbital treatment would have resulted in a decreased over-all desaturation activity and a significant or complete disappearance of the cyanide sensitivity. Neither was observed under these experimental conditions.

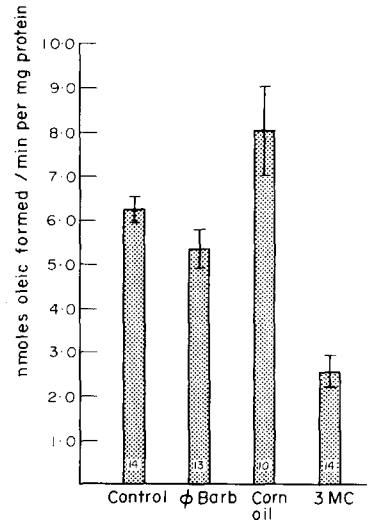

Fig. 1. Effect of phenobarbital and 3-methylcholanthrene on hepatic fatty acid desaturase activity. All animals received a fat-free diet *ad lib.* as described in Methods. ϕ Barb = phenobarbital in drinking water (1 mg/ml) for 4 days prior to sacrifice; 3-MC = 3-methylcholanthrene (40 mg/kg in corn oil) given in 1 ml volume, i.p., on days 3 and 4 of dietary treatment; and corn oil = 1 ml of vehicle only on days 3 and 4. Values are mean ± 1 S. E.; number within bars = N.

However, as can also be seen in Fig. 1, treatment of rats with 3-methylcholanthrene resulted in a highly significant inhibition of the desaturase ($P < 0·01$). The corn oil vehicle alone was not responsible for this inhibition (Fig. 1). It was, therefore, apparent that the two compounds, phenobarbital and 3-methylcholanthrene, both of which are inducers of the hepatic, microsomal, mixed function oxidase [14, 15], may have totally different effects on the activity of the cytochrome b_5-dependent microsomal desaturase system.

While examining the variously treated rats prior to sacrifice, it was noted that the 3-methylcholanthrene-treated animals tended to eat and drink less than the control, phenobarbital or corn oil-treated animals. The over-all body weight changes of the variously treated animals exhibited a pattern very similar to the desaturase activity (Fig. 2). When all the animals were grouped together and the fatty acid desaturase activity was plotted solely as a function of body weight change for each individual animal, a highly significant correlation was found ($P < 0·01$). Thus, it appeared that the alterations in desaturase activity may be unrelated to the drugs themselves, but rather were the result of an alteration in the nutritional status of the animals which was expressed grossly by impaired growth.

If this were the case, then it was reasoned that similar alterations in desaturase activity should result from a restrictive feeding where a group of rats is given various aliquots of the fat-free diet. The well known depressive effect of starvation has been pre-

* PPO = 2,5-diphenyloxazole; and POPOP = 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)] benzene.

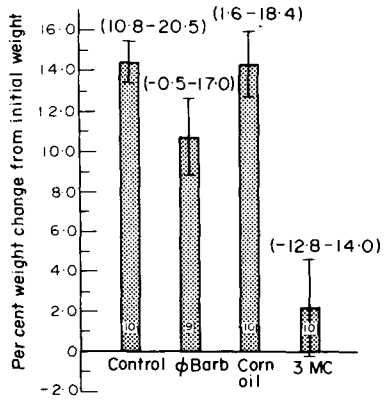

Fig. 2. Effect of phenobarbital and 3-methylcholanthrene on body weight change in rats. Treatments are identical to Fig. 1. Data (mean ± 1 S. E.) represent the percentage of the initial body weight either gained or lost during the 4 days of treatment prior to sacrifice. Numbers in parentheses represent the range of values for each group; number within bars = N.

viously documented; this experiment was designed to investigate intermediate nutritional status between that of fully fed and totally starved, similar to those nutritional states resulting from treatment of animals with 3-methylcholanthrene. The results indicated that with graded feedings there was a significant correlation ($r = 0{\cdot}94$, N = 20) between a wide range of body weight changes and fatty acid desaturase activity. The similarity between the results was highly suggestive that the 3-methylcholanthrene effect might be related to a nutritional factor. This possibility became even more apparent when the results of drug-treated animals from Fig. 2 were combined with the data from the graded feeding experiment. The results of these (Fig. 3) demonstrated a high degree of correlation between the ability of microsomal preparations to desaturate stearic acid to oleic acid and the nutritional status of the animal, regardless of how alterations in that nutritional status were achieved.

Since the inhibitory effect of various fatty acids on the desaturase system has been demonstrated *in vitro* [4, 7, 16], the concentration of free fatty acids in the serum of variously treated animals was examined next. There was a significant correlation ($P < 0{\cdot}01$) between the serum free fatty acid concentration and the desaturase activity for control, drug-treated, and the limited fed animals. As can be seen in Fig. 4, a serum free fatty acid concentration below 0·70 mM (700 μ-equiv/liter) is associated with desaturase activities between 4·0 and 11·0 nmoles/min/mg of protein, while fatty acid concentrations in excess of 0·70 mM result in progressive inhibition of the enzymatic activity.

In order to further examine the relationship between desaturase activity, nutritional status, and serum free fatty acid concentrations, several groups of animals were treated with two different doses (0·05 mg/ml and 0·20 mg/ml in the drinking water) of either *d*-amphetamine sulfate or fenfluramine hydrochloride. The drugs were administered for 4 days prior to sacrifice. Both of these compounds are clinically used anorexic drugs which are postulated to have different mechanisms for their inhibitory effects upon the central nervous system for eating and drinking behavior [17]. The treatment of rats with these drugs resulted in decreased rates of weight gain, consistent with the known anorexic effect. However, for both doses of *d*-amphetamine sulfate (0·05 mg/ml and 0·20 mg/ml) and for the lower dose of fenfluramine hydrochloride (0·05 mg/ml), the desaturase activity was in the normal range of 8·0–12·0 nmoles oleic formed/mg of protein/min and the serum free fatty acid concentrations were within the normal range of 0·40–0·65 mM. Only with the higher dose of fenfluramine (0·20 mg/ml) was a depression of desaturase activity observed. Two animals in this group demonstrated markedly depressed activities of 0·78 and 0·31 nmole oleic/mg/min and these lower activities were correlated with elevated serum free fatty acid levels of 0·76 mM and 0·92 mM respectively. Thus, in this experiment it appears that the correlation of body weight gain with desaturase activity is not substantiated. This may be attributed to the observation that, while the animals recorded an over-all decreased weight gain for the total 4-day treatment period, during the last day they had apparently developed a tolerance for the drug effect and, therefore, were eating and drinking almost the same amount as the control rats. Thus, in this case the over-all weight gain did not reflect the nutritional state of the animals

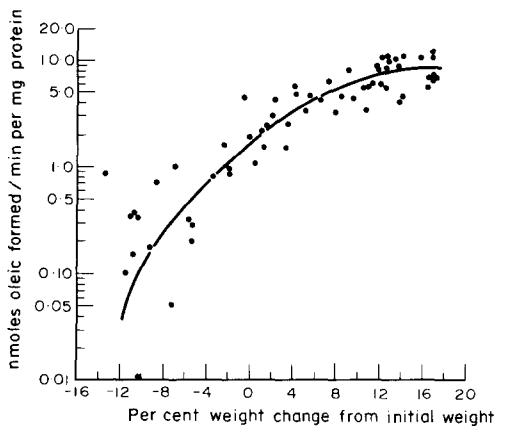

Fig. 3. Correlation between hepatic desaturase activity and body weight changes for drug-treated and limited fed animals. Data from Fig. 2 are combined with data from rats which were given graded aliquots (between total starvation and *ad lib.* access) of fat-free diet on days 3 and 4 of dietary treatment. Total N = 62.

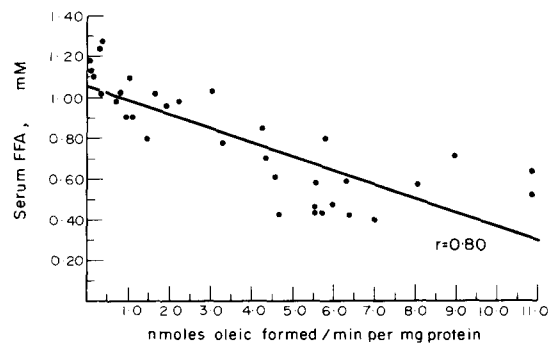

Fig. 4. Correlation between serum free fatty acid concentration and hepatic desaturase activity. Sera obtained at sacrifice from limited fed animals and animals treated with drugs as explained in Fig. 1. Total N = 34.

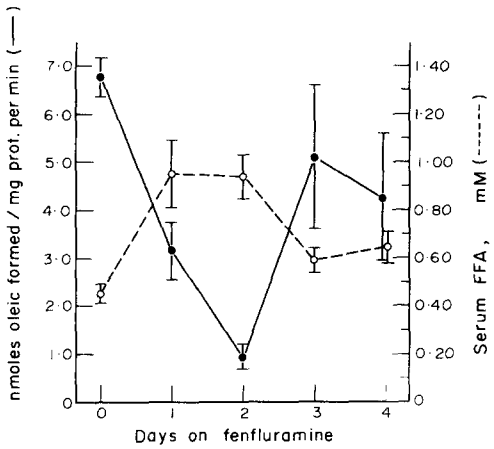

Fig. 5. Time course of fenfluramine effect on hepatic fatty acid desaturase and serum free fatty acids. Separate groups (N = 6) of rats were given fenfluramine hydrochloride, 0·20 mg/ml in drinking water, for indicated periods. Desaturase activity (——), serum free fatty acids (- - - -). Data are mean ± S. E. M.

at the time of sacrifice. This actual nutritional state was reflected by normal levels of serum free fatty acids which again correlated with control desaturase activities. Only those two animals with elevated free fatty acid levels exhibited microsomal desaturase activities which were depressed.

To further elucidate this point, the time course for the fenfluramine effect was examined for the entire 4-day treatment period. Individual groups of six rats each were given either no drug (controls) or fenfluramine (0·20 mg/ml in drinking water) for 1, 2, 3 or all 4 days prior to sacrifice. All animals had *ad lib.* access to fat-free diet for all 4 days. The results (Fig. 5) show that those animals receiving fenfluramine for 1 or 2 days had markedly depressed desaturase activities and significantly elevated serum free fatty acid levels. However, by days 3 and 4 of drug treatment, some animals began to demonstrate tolerance to the drug as indicated by a return of the desaturase activity toward control levels. This occurred concomitantly with a decrease in serum free fatty acid levels. There was also a significant correlation between the individual free fatty acid levels and the corresponding desaturase activity (N = 30, $r = 0{\cdot}63$, $P < 0{\cdot}01$). The regression line for this correlation (slope ± S. D. = $-0{\cdot}059 \pm 0{\cdot}014$, y intercept = 0·954) was not different than that determined for the data presented in Fig. 4 (slope ± S. D. = $-0{\cdot}067 \pm 0{\cdot}009$, y intercept = 1·050). Thus it appears that a significant and similar correlation exists between hepatic fatty acid desaturase activity and the concomitantly occurring level of serum free fatty acids under a variety of chemical treatments (3-methylcholanthrene, phenobarbital, fenfluramine) or during artificially generated alterations in feeding behavior (restricted feeding experiment).

DISCUSSION

These studies clearly suggest that a number of drugs and nutritional manipulations may affect the hepatic, microsomal Δ^9 fatty acid desaturase activity and that these effects may be correlated to the serum free fatty acid concentrations. The effects of these drugs and nutritional treatments on body weight seem to be of secondary importance. Hence, it may be postulated that a primary control mechanism for the desaturase activity may involve inhibition by certain free fatty acids which are present in serum. This mechanism has been suggested by other investigators on the basis of inhibition of desaturase activity by certain unsaturated fatty acids [4, 18]. Physiological alterations, such as starvation or stress, and certain metabolic diseases, such as diabetes mellitus, are known to result in depression of activity of this enzyme system [19 20]. It is well known that during these conditions fatty acids are readily mobilized from the adipose tissue and may, therefore, serve as a ready source for this enzymatic inhibition.

These results could also easily explain the discrepancy between our observation that phenobarbital has no effect on the desaturase and the results of Oshino *et al.* [7, 8] who found significant inhibition. In our study, the animals received the phenobarbital in their drinking water and thus never received a single bolus of drug, while in the latter studies the animals were given this agent by i.p. injection. It has been our experience that the latter mode of administration has a much more marked effect on the animals' feeding habits than does administration in the drinking water.

Our studies also eliminate the possibility that the cyanide-sensitive factor is the nonheme iron which we previously observed in microsomes from control animals.

While these studies do not absolutely preclude the possibility of a direct drug effect on the desaturase system, they do reaffirm the need to closely scrutinize the physiological and nutritional results of pretreatment of animals with various agents before suggesting that these agents have a direct effect on an enzymatic system. The organism is a complex system which maintains the animal in a delicate homostatic balance. Often, as in this study, the agents administered upset this balance and indirectly alter the enzyme system which is being investigated.

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