Dietary Induction of Some Enzymes of Carbohydrate Metabolism during 2-Acetylaminofluorene Feeding

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SUMMARY

The dietary induction of the rat liver enzymes glucokinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, malic enzyme, and citric cleavage enzyme in 3-day-fasted rats before and after a 27-hour refeeding period with 30% protein diet was investigated in rats fed a grain diet or the same diet containing 0.03% 2-acetylaminofluorene. The metabolic responses of all the enzymes studied was found to be absent in the animals fed the carcinogenic diet for 2–4 weeks; the responses of each of these enzymes in the control animals remained normal. The chronic administration of 2-acetylaminofluorene appeared to increase the base level of glucose-6-phosphate dehydrogenase; the base level of no other enzyme investigated appeared to be affected by either the control or the carcinogen-containing diet. The data indicate that the normal metabolic responses of these enzymes of carbohydrate and lipid metabolism were lost in the livers of rats fed 2-acetylaminofluorene. This loss of response is markedly similar to the absence of the control mechanisms of the same enzymes in certain hepatomas and of the amino acid-metabolizing enzymes in the livers of carcinogen-fed rats.

INTRODUCTION

Altered control mechanisms of several amino acid-metabolizing enzymes have been reported in the livers of rats fed various hepatocarcinogens for several weeks (2, 4, 6, 9). In general, the altered enzymatic control mechanisms bore a resemblance to similar alterations observed in hepatomas (9). To date, however, very few studies have been reported purporting to demonstrate abnormal controls for enzymes metabolizing substrates other than amino acids in the livers of carcinogen-fed rats. That such alterations may be expected is seen from the fact that several adaptive carbohydrate-metabolizing enzymes show abnormal responses to altered environmental conditions in a variety of hepatomas. Thus, the hormonal induction of glucose-6-phosphatase was found to be absent in both hepatomas (15) and in the livers of rats fed 3'-methyl-DAB\textsuperscript{4} (5). Although the levels of GPD were found to be elevated in most hepatomas investigated (15), no dietary induction of this enzyme by the Tepperman fasting-refeeding regime could be detected in any hepatomas investigated (10, 15). In normal liver the Tepperman regime produces marked increases in the hepatic levels of glucokinase (ATP: D-glucose-6-phosphotransferase, EC.2.7.1.2), PGD, malic enzyme (L-malate: NADP oxidoreductase (decarboxylating), EC.1.1.1.40) and CCE; no such responses can be elicited in the Morris 7800 and 5123 or in the Reuber H-35 hepatomas (unpublished observations). Recently Sabine et al. (11) have reported that, in four highly differentiated hepatocellular carcinomas, fatty acid metabolism was essentially unaffected by the fat composition of the diet. Siperstein et al. (13) have shown that dietary cholesterol does not inhibit cholesterol synthesis in vivo in hepatomas as it does in normal or host liver. The present investigations were undertaken to determine whether the loss of metabolic increases observed with amino acid-metabolizing enzymes in both neoplastic and preneoplastic liver and with carbohydrate-metabolizing enzymes in hepatomas could be extended to carbohydrate-metabolizing enzymes in preneoplastic livers.

MATERIALS AND METHODS

Animals and Diets

Young adult male rats (170–190 gm) (Holtzman Rat Co., Madison, Wisconsin) were housed in galvanized wire-mesh cages (5 rats/cage) and were fed food and water ad libitum. In order to minimize possible enzymatic changes due to dietary alterations after shipment, for 12 days prior to the start of the

\textsuperscript{4}The following abbreviations are used: 3'-methyl-DAB, 3'-methyl-4-dimethylaminoazobenzene (Chem. Abstracts nomenclature: \(N,N\)-diiethyl-p-(m-tolylazo)-aniline); 2-methyl-DAB, 2-methyl-4-diso-(phenylazo)-m-toluidine; AAF, 2-acetylaminofluorene (Chem. Abstracts: N-fluore-2-ylacetamide); GPD, glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP oxidoreductase, EC.1.1.1.49); PGD, 6-phosphogluconate dehydrogenase (6-phospho-D-glucuronate: NADP oxidoreductase (decarboxylating), EC.1.1.1.44); CCE, citrate cleavage enzyme (ATP: citrate oxaloacetate-lyase (CoA-acetylating and ATP-dephosphorylating), EC.4.1.3.8); EDTA, ethylenediaminetetraacetate.
experiment, the rats were placed on the modified grain diet (1) that was subsequently used during the experiment. At the start of the experiment, the carcinogen-treated rats were placed on the grain diet containing 0.03% AAF (Mann Research Laboratories, N. Y.) (m.p. = 192—194°C). The control rats were maintained on the grain diet. Control rats were sacrificed by decapitation 0, 2, 4, and 5 weeks after the start of the experiment; AAF-fed rats were killed at 2, 4, and 5 weeks. The Tepperman regime (14) was used to manipulate the hepatic enzyme levels in vivo. The rats to be killed were fasted in individual cages for 3 days prior to sacrifice. Water was provided ad libitum throughout the fast. At zero-time (6:00 A.M.) groups of 5—6 rats were sacrificed. Similar groups were fed a 30% protein—60% carbohydrate (sucrose) diet (General Biochemicals, Chagrin Falls, Ohio) for 27 hours, at which time the latter rats were also sacrificed. Following sacrifice, the livers were quickly excised and homogenized in 4 volumes of 0.2 M KC1 containing 0.1 M Tris buffer (pH 8.2), 10−3 M Tris buffer (pH 8.2), 10−3 M EDTA, and 10−4 M dithiothreitol by means of an Ultraturrax homogenizer (Janke and Kunkel, Stauffen, Germany). Ten-ml portions of each homogenate were centrifuged at 100,000 X g for one hour, and the individual clear supernatants were collected and stored at —70°C for the later assays of hexokinase, glucokinase, PGD, GPD, CCE, and malic enzyme. All assays were performed within 2 weeks, during which time there was negligible loss in the activity of these enzymes as long as the liver extracts were not thawed and refrozen. The body and liver weights of the rats used in these experiments were measured at the time of sacrifice; the results are illustrated in Charts 1 and 2 respectively. Chart 1 demonstrates the body weights of the rats fed the grain diet with and without the AAF. Food intake was not determined, and it is probable that some of the weight change is related to differences in food intake. Since the body weights after the 27-hour refeeding period were very similar to those prior to the refeeding, only the latter weights are listed. Each point represents the average of 5—6 rats; the vertical lines represent the standard error of the mean of the weight at each of the points. The asterisks indicate a statistically significant difference (P < 0.01) in average weight between the control and the AAF-fed rats at any given week. Since the fasting-refeeding regimen did produce striking increases in the liver weights of the control animals at least, the average liver weights both before and after the 27-hour period of refeeding are recorded in Chart 2. The solid line indicates the average liver weights of the control or of the AAF-fed rats following a 3-day fast which was initiated at the time shown on the abscissa. The dotted lines represent the liver weights at the end of the 27-hour refeeding period following the 3-day fast. The vertical lines again denote the standard errors of the mean; the asterisks indicate that there is a significant statistical difference (P < 0.01) between the base (zero-time or solid line) liver weights and the induced (27-hour, dotted line) liver weight averages. The enzyme levels before and after the refeeding of a 30% protein diet are represented exactly as has been done with the liver weights.

Enzyme Assays

The double enzyme assays of hexokinase and glucokinase and of PGD and GPD were performed using an automated combination unit as previously described (3, 8). All points on the graphs reported here represent the average values obtained from 5 or 6 rats. The standard deviation and the standard error of the mean were calculated for each point. All comparisons between groups and all conclusions drawn were based on the significance of the statistical analysis by the t test with P values of less than 0.01. All statistical calculations were carried out through the use of a Control Data Corporation 1604 computer of the University of Wisconsin Computing Center.

Malic enzyme and CCE were assayed automatically with the combination unit (8). The components of the systems were as follows:

**Malic Enzyme.** Reservoir: 15 mg L-malic acid/100 ml in 0.05 M Tris, pH 7.4. Sampler Tubes (for 100 ml of 0.05 M Tris, pH 7.4): 185 mg MgCl2, 80 mg triphosphopyridine nucleotide (General Biochemicals). The sampler tubes containing the liver extract (0.1 ml in 3 ml of the above solution) were preincubated 5 minutes at 37°C before assay. Total cycle time was 8 minutes (8).

**Citrate Cleavage Enzyme.** Reservoir: 100 ml of 0.08 M Tris, pH 7.3, containing 406 mg MgCl2 and 400 mg ATP; immedi-
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Sampler Tubes: 1.29 gm potassium citrate and 15 mg dithiothreitol (Calbiochem.), 80 mg reduced diphosphopyridine nucleotide (Sigma), 150 µg malate dehydrogenase (Calbiochem.), 48 mg Li CoA (Pabst), 150 µg malate dehydrogenase (Calbiochem.), 80 mg reduced diphosphopyridine nucleotide (Sigma), 150 µg malate dehydrogenase (Calbiochem.), 48 mg Li CoA (Pabst), 150 µg malate dehydrogenase (Calbiochem.), 80 mg reduced diphosphopyridine nucleotide (Sigma). All enzyme assays were performed under conditions of substrate saturation or zero order kinetics. All enzyme units are expressed as µmoles of pyridine nucleotide reduced/hour.

Results

Biologic

The chronic administration of AAF to rats led to marked symptoms of toxicity. The AAF-fed rats gained only 49 gm during the 5-week experimental period while the control rats gained 136 gm (Chart 1). In addition, although the control rats consistently showed a 70–90% increase in liver weights after a 27-hour period on 30% protein diet, this response was significantly diminished (P < 0.01) in the rats fed AAF for 2 weeks and was lost altogether in the rats treated with AAF for 4 or 5 weeks (Chart 2). In contrast to the situation previously reported for the liver weights of AAF-treated rats placed on a 0% protein diet for 5 days (9), the liver weights of rats fed AAF for 5 weeks and fasted for 3 days were not significantly different from the corresponding controls. However, significant differences were found between the zero-time liver weights of rats fed AAF for 2 and 4 weeks and those of rats fed the control diet for similar periods (Chart 2).

Enzymatic

AAF produced losses in the adaptive responses of all the inducible carbohydrate metabolizing enzymes investigated: glucokinase, PGD, GPD, CCE, and malic enzyme. A marked decrease in the induced level of glucokinase could be observed in the livers of rats fed AAF for 2 weeks when compared to those of the corresponding levels in the control rats (Chart 3). After 4 and 5 weeks of AAF administration, no response to refeeding could be observed; in contrast, the fasting-refeeding regimen always elicited a 300–500% increase in the control rats. A significant decrease (P < 0.01) was observed in the base levels of glucokinase in the rats fed either the AAF or the control diet for 2 and 4 weeks when compared to the base level at zero weeks of feeding. The physiologic significance of this observation remains obscure. Sharma et al. (12) have demonstrated a lowering of glucokinase and an increase in hexokinase during azo-dye feeding, but no specific attempts were made to investigate the metabolic responses of these enzymes. These authors correlated the changes in enzyme levels with changes in cell population. Similarly, the base and induced glucokinase levels in the rats fed the control diet for 5 weeks were significantly higher than the same levels in rats fed the control diet for 4 weeks. Hexokinase (ATP:D-hexose-6-phosphotransferase, EC.2.7.1.1), which is assayed along with glucokinase, appeared to be unaffected by either the control or the AAF-containing diet. This finding was somewhat surprising in view of the observation by Sharma et al. (12) as well as by ourselves that the chronic administration of 3'-methyl-DAB produces marked increases in the hepatic level of hexokinase.

The administration of AAF in the diets of rats for 2 weeks led to the complete loss of response of hepatic PGD and GPD to dietary alteration (Charts 4 and 5 respectively). The control
The increase in the levels of hepatic malic enzyme normally observed following the fasting-refeeding regimen employed in these experiments produced a normal response in the control rats; in the rats fed 0.03% AAF in the diet for 2, 4 or 5 weeks, no such increase could be observed (Chart 6). The 5-week administration of either the control or the AAF diet did not, however, affect the base levels of malic enzyme at any of the weeks investigated. The levels of CCE were affected in a similar manner. Throughout the 5-week feeding of the control diet, the 27-hour feeding following a 3-day fast led to approximately a 500% increase in the levels of CCE in rat liver. This increase, however, was lost in the rats fed AAF for 2 to 5 weeks (P < 0.01), but the physiologic significance of this phenomenon is unknown.

**DISCUSSION**

These investigations have uncovered a remarkable similarity between the altered control mechanisms noted in certain hepatocellular carcinomas and those of livers of AAF-treated rats. Each of the 5 adaptive enzymes investigated, all of which were inducible in liver by the fasting-refeeding regimen described above, completely or partially lost their adaptive responses during the chronic administration of AAF; a similar loss or decrease of the adaptive responses of each of these enzymes to dietary alteration was noted in certain hepatomas investigated. Thus, glucokinase, PGD, GPD, malic enzyme, and CCE do not respond to acute dietary alteration in the 5123, 7800, and H-35 hepatomas (unpublished observations). The response of GPD to dietary alteration is also lost in the 7794A hepatoma (15). The loss of enzyme response to dietary alteration during hepatocarcinogenesis extends previous observations made with amino acid-metabolizing enzymes (6, 8). The inducibility of ornithine-transaminase and of histidase was shown to be lost, and that of serine dehydratase and tryptophan pyrrolase was
greatly diminished during the chronic administration of AAF (9). The same amino acid-metabolizing enzymes had altered control mechanisms in a majority of the hepatomas investigated (7). Unpublished results from this laboratory have shown that, where rats were fed an AAF diet for 5 weeks then placed on a 30% protein diet for 4 weeks, after which they were fasted for 3 days and refed the 30% protein diet for 27 hours, the metabolic responses of glucokinase, PGD, malic enzyme, and CCE returned to normal. This finding plus the similarity observed between the altered enzyme responses noted in the preneoplastic liver and those found in its ultimate product, the hepatocellular carcinoma, are consistent with the view that the essential neoplastic transformation occurs in a population of cells with altered enzymatic control mechanisms and that the neoplastic transformation renders these alterations permanent.

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