Modulations of Hepatic γ-Glutamyltranspeptidase and N-Hydroxy-N-2-fluorenylacacetamide Sulfotransferase Activities following Treatment of Rats with a Hepatocarcinogenic Regimen: Effect of Partial Hepatectomy

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The feasibility of using biochemical assays of γ-glutamyltranspeptidase (γ-GTP) and N-hydroxy-N-2-fluorenylacacetamide sulfotransferase (N-OH-2-FAA ST) activities to monitor the effects of treatment of male Sprague-Dawley rats with a two-stage hepatocarcinogenic regimen was investigated. One week after initiation with diethylnitrosamine (200 mg/kg of bw), the rats were treated with 10 oral doses within 2 weeks of N-2-fluorenylacacetamide (2-FAA) at 0.05 mmole/kg or vehicle (corn oil) at 5 ml/kg of body weight. After five doses of 2-FAA or corn oil, half of the rats in each group underwent partial (70%) hepatectomy (PH). Three days after completion of 2-FAA treatment, γ-GTP activity increased approximately 8-fold in the livers of both the nonhepatectomized (−PH) and hepatectomized (+PH) groups. After 17 days, the enzyme activity decreased to the control level in the −PH group but increased 3.1-fold above the control level in the +PH group. After 31, 66, and 87 days, γ-GTP activity increased only 1.4- to 2.6-fold in the +PH group, whereas that of +PH group increased 15- to 32-fold. N-OH-2-FAA ST activity, determined 3 days after completion of 2-FAA treatment, decreased by approximately 60% in the −PH and +PH groups. After 17 days, the effect of PH became evident in that the losses of N-OH-2-FAA ST activity were smaller (20%) in the −PH than in the +PH group (45.5%). After 31, 66, and 87 days, the respective decreases of 27, 29, and 41% in the +PH group were significant. Nodules excised from the livers of the +PH group 87 days after treatment with 2-FAA showed 25% of N-OH-2-FAA ST activity of the control livers. The data suggested that early increases in γ-GTP and decreases in N-OH-2-FAA ST activities, which were independent of PH, were affected by hepatic metabolism of 2-FAA. After 17 days, the presumed clearance of 2-FAA, the effect of PH was manifested in that it prevented return to control levels of both enzyme activities in 2-FAA-treated rats. The time and PH dependency of the extent of modulations of γ-GTP and N-OH-2-FAA ST activities support their assays as markers of the promotion/progression of hepatocarcinogenesis. —Environ Health Perspect 102(Suppl 6):105-108 (1994)

Key words: hepatocarcinogenesis, initiation/promotion, hepatocytoc, 2-FAA, γ-glutamyltranspeptidase, N-hydroxy-N-2-fluorenylacacetamide sulfotransferase

Introduction

Two-stage models for studies of initiation and promotion of hepatocarcinogenesis (HC) in the rat include the Solt-Farber protocol employing diethylnitrosamine (DEN) as a single dose initiator and N-2-fluorenylacacetamide (2-FAA) at 0.02% in diet as promoter (I). Although the mechanism of tumor promotion by 2-FAA is not as yet clearly understood, the available evidence strongly suggests that normal hepatocytes are more susceptible to cytotoxic effects of 2-FAA-derived metabolite(s) than DEN-initiated preneoplastic cells, which results in preferential growth of the latter (2). Partial hepatectomy (PH), usually performed during the course of treatment with the promoter, provides a stimulus to cell proliferation. Promotion usually has been monitored by histochemical and/or biochemical determinations of the increased expression of γ-glutamyltranspeptidase (γ-GTP) since γ-GTP-positive foci of the initiated cells increase in size (volume), but not number, in response to a promoter (3). In the liver of the susceptible male rat, the suspected cytotoxic metabolite of 2-FAA is suggested via N-hydroxylation to N-hydroxy-N-2-fluorenylacacetamide (N-OH-2-FAA) and sulfation of the latter to N-O-sulfate. The extremely reactive N-O-sulfate of N-OH-2-FAA binds to DNA and causes liver necrosis (4). N-O-Sulfation is catalyzed by N-OH-2-FAA sulfotransferase (ST) (5), an isozyme of arylsulfotransferase IV (6). Inhibition of ST by pentachlorophenol in vivo resulted in decreased volume of hepatic γ-GTP-positive foci in DEN-initiated rats treated with N-OH-2-FAA, suggesting that N-O-sulfate has a role in promotion (7). Decreases in N-OH-2-FAA ST activity following administration of 2-FAA have been reported by several investigators (5,6,8–11). The enzyme assays were usually carried out during or within 1 week after completion of treatment with 2-FAA. Ringer et al. (6) found that N-OH-2-FAA ST activity returned to the level of intact liver within 1 week following feeding of 2-FAA at subcarcinogenic levels. However, carcinogenic regimens of 2-FAA led to a permanent decrease in N-OH-2-FAA ST activity, and the resulting nodules and tumors were devoid of the enzyme. This suggested that a decrease in N-OH-2-FAA ST activity might be progressive and serve as a marker of promotion/progression of HC. Hence, in this study, we examined N-OH-2-FAA ST activity in the livers of rats.
at timed intervals within a 12-week period following treatment with a modified Solt-Farber regimen of two-stage HC. The modification, based on the protocol employed by Möller et al. (12), concerned replacement of 2-FAA-containing diet with intragastric intubations of 2-FAA in corn oil. In parallel assays, we determined γ-GTP activity, an established marker of promotion of HC (3). We also determined the effect of PH, performed in the middle of a 2-week treatment with 2-FAA, on both enzyme activities.

Materials and Methods

Chemicals

DEN, p-nitrophenylsulfate, $3', 5$'-adenosine diphosphate, γ-L-glutamyl-p-nitroanilide, glycylglycine, sodium nitrite, N-1-naphthylethenediamine, and corn oil were from Sigma Chemical Co. (St. Louis, MO). Metofane (methoxyflurane) was from Pittman-Moore (Mundelein, IL). 2-FAA, melting point 192 to 196°C, was from Aldrich (Milwaukee, WI). N-OH-2-FAA, melting point 150 to 151°C, was prepared by the published method (13). 2-FAA and N-OH-2-FAA were recrystallized until pure by HPLC.

Treatment of Rats and Preparation of Liver

Male Sprague-Dawley rats, 6 weeks old, weighing 150 to 174 g (Harlan Sprague-Dawley, Inc., Indianapolis, IN), were maintained on a pelleted semisynthetic 30% casein diet (Teklad Premier Diets, Madison, WI) and water ad libitum. After 1 week, the rats were given a single ip injection of DEN, 200 mg/kg bw, in saline. One week later, the rats started a 2-week treatment of 10 intragastric intubations of 2-FAA in corn oil at 0.05 mmole/kg or corn oil at 5 ml/kg of body weight (Figure 1). Three days after completion of five doses of 2-FAA or corn oil, half of the rats in each group underwent PH (70%) under Metofane anesthesia. On day 3 after PH, the treatment resumed. On the days shown in Figure 1, rats from each group were killed by decapitation under CO$_2$, and livers were removed. After washing with icold saline, livers were homogenized in 4 volumes of 50 mM Tris-HCl 154 mM KCl buffer, pH 7.4, as described previously (14). Whole homogonates and the cytosolic fractions (105,000 g supernatants) were used for enzyme assays. The protein content was determined by the method of Lowry et al. (15) with a bovine serum albumin standard.

Enzyme Assays

γ-GTP activity was determined according to the method of Igarashi et al. (16). The final volume consisted of 0.5 ml 100 mM Tris-HCl buffer, pH 7.4, containing γ-L-glutamyl-p-nitroanilide (0.051 mM) as the substrate, and glycylglycine (1.1 mM) as the acceptor for the glutamyl group released by γ-GTP of liver homogenate (0.6 mg protein). After 20 min at 37°C the liberated p-nitroaniline was diazotized with 0.1% sodium nitrite and 0.05% N-1-naphthylethenediamine to a pink azo dye measured spectrally at 540 nm. The activity of γ-GTP was calculated from a standard curve obtained with p-nitroaniline and expressed as nmole of p-nitroaniline per minute per milligram protein.

N-OH-2-FAA ST activity was determined in cytosolic fractions by the procedure of Ringer and Norton (17). Briefly, in a final volume of 0.5 ml, p-nitrophenylsulfate (10 mM), $3', 5$'-adenosine diphosphate (0.02 mM) in 100 mM Tris-HCl buffer, pH 8.0, 0.3 mg cytosolic protein, and the acceptor substrate, N-OH-2-FAA (0.55 mM) in ethanol (5%), were mixed in a cuvette, and the increase in absorbance of p-nitrophenol at 405 nm (ε, 18,380 M$^{-1}$ cm$^{-1}$) was recorded every 30 sec at 31°C for 10 min, during which time the rates were linear. Control incubations contained 5% ethanol without N-OH-2-FAA. The rate of p-nitrophenol formed in the control was subtracted from the rate measured with N-OH-2-FAA as the acceptor. The activity of N-OH-2-FAA ST was expressed as nmole of p-nitrophenol per minute per milligram protein. Duplicate assays were carried out with liver preparations from two to four rats per each time point. The data were analyzed using Student’s two sample t-test. The level of significance was set either at $p<0.005$ or $p<0.05$.

Results

Following treatment of rats with DEN (one dose) and 2-FAA (10 doses), according to the regimen in Figure 1, the average cumulative dose of 2-FAA was 0.125 mmole per rat. This regimen had no significant effect on the weight gain of rats except for temporary (~2-day) decreases of ≤10% body weight following ip injection of DEN and PH. On day 66 after completion of treatment with 2-FAA, the livers had many tiny nodules (<1 mm diameter), which increased to 2 to 3 mm diameter, some to 3 to 5 mm, by day 87. The largest nodules were excised for determination of enzyme activities. The levels of hepatic γ-GTP and N-OH-2-FAA ST activities assayed on days 3, 17, 31, 66, and 87 (including nodules) after completion of 2-FAA treatment are shown in Figures 2 and 3, respectively.

γ-GTP activity determined 3 days after completion of 2-FAA treatment showed significant increases, 9.5- and 7.4-fold, respectively, in the livers of both -PH and +PH rats. On day 17 after the treatment, the enzyme activity in the -PH rats had returned to the control level but was 3.1-fold above the control level in the livers of +PH rats. On days 31, 66, and 87 after treatment with 2-FAA, γ-GTP activity had increased only 1.4- to 2.6-fold in the livers of -PH rats; whereas, it had increased 15- to 32-fold in the livers of +PH rats. The level of γ-GTP on day 87 was similar in the nodules and the nodular liver.

N-OH-2-FAA ST activity was inversely affected compared to γ-GTP within the same time frame. N-OH-2-FAA ST activity also significantly decreased (by 48%) 3 days after five doses of 2-FAA; whereas that of γ-

![Figure 1. Experimental protocol. Initiation consisted of a single ip injection of DEN, 200 mg/kg of body weight, in saline, to 50-day-old male rats. Promotion consisted of ten intragastric doses of 2-FAA in corn oil at a dose level of 0.05 mmole/kg of body weight. Control rats received corn oil at a dose level of 5 ml/kg of body weight. Three days after five doses of 2-FAA or corn oil, half of the rats in each group underwent partial (70%) hepatectomy (PH). The enzyme activity was determined in individual livers of two to four rats on the days shown. * denotes enzyme assay in the livers removed during PH.](image-url)
GTP was as yet unaffected. Three days after completion of 2-FAA treatment (10 doses), N-OH-2-FAA ST activity significantly decreased by 55 and 62% in the −PH and +PH rats, respectively. On day 17 after the completion of 2-FAA treatment, the effect of PH started to become evident in that the decreases of N-OH-2-FAA ST activity, although still significant, were smaller (20%) in the −PH than +PH rats (45.5%). On days 31 and 66 only the decreases in the +PH rats of 27 and 29%, respectively, were significant. By day 87 N-OH-2-FAA ST activity in the livers of +PH and −PH groups had decreased by 41 and 16%, respectively. The nodules found only in 2-FAA-treated +PH group had 25% of N-OH-2-FAA ST activity of the control liver.

**Discussion**

Following treatment of male rats with DEN (1 dose) and 2-FAA (10 doses), the effect of PH on the increase in hepatic γ-GTP and decrease in N-OH-2-FAA ST activities (Figures 2, 3) was time-dependent. At 3 days after the completion of treatment, the changes in these activities were independent of PH. Between 3 and 17 days or 3 and 31 days after the treatment, γ-GTP or N-OH-2-FAA ST activities, respectively, approached the levels of control livers, especially in −PH rats. Later, between 31 and 87 days after the treatment, greater differences in activities between DEN + 2-FAA- and DEN + corn oil-treated rats were determined in the +PH groups. The data suggested that the early and late responses to 2-FAA treatment, differing in dependency on PH, are mediated by different mechanisms.

The early increases in γ-GTP activity in the livers of DEN + 2-FAA-treated rats of both −PH and +PH groups suggested an increased rate of detoxication involving the γ-glutamyl cycle (18). The progressively larger increases of γ-GTP activity determined at later stages after treatment with 2-FAA of +PH rats reflected an increase in volume of γ-GTP-positive foci (nodules) and confirmed the enzyme as a marker of promotion/progression of HC (3).

The early decreases in N-OH-2-FAA ST activity in the livers of DEN + 2-FAA-treated rats of both −PH and +PH groups may result from inactivation of the enzyme by the reaction product, N-O-sulfate of N-OH-2-FAA (19). The decreases in N-OH-2-FAA ST activity determined at later stages may be due to the progressive development of nodules observed between 66 and 87 days after completion of 2-FAA treatment of +PH rats. Although the nodules contained 25% of N-OH-2-FAA ST activity of the control liver, this might result from contamination by extra-nodular liver tissue, since Ringer et al. (6) showed that 2-FAA-derived hepatic nodules and tumors were devoid of N-OH-2-FAA ST activity and the corresponding protein. The mechanism of irreversible loss of this enzyme activity in hepatocellular carcinoma is not yet understood. However, it has been postulated, especially for +PH rats, that the decrease in sulfation activity towards N-OH-2-FAA in (pre)neoplastic cells is due to their high rate of proliferation rather than genetic changes (20).

The inverse modulation of γ-GTP and N-OH-2-FAA ST activities determined between 4 and 12 weeks (31 and 87 days) in the livers of rats exposed to PH and the modified Solt-Farber regimen of HC suggested that these enzymes are useful markers of promotion/progression of HC and the above time period is suitable for mechanistic studies of HC at the molecular level.

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