Altered Heme Pathway Regulation and Drug Metabolizing Enzyme System in a Mouse Model of Hepatocarcinogenesis: Effect of Veronal

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ABSTRACT. 1. Male CF 1 mice were fed p-dimethylaminoazobenzene (DAB) for 35 days and received 5,5-diethylbarbituric acid, before or after DAB treatment, with the purpose of investigating whether the onset of the preinitiation stage of carcinogenesis alters the natural regulatory mechanism of the heme pathway.

2. Changes detected in drug metabolizing enzymes are likely to be the consequence of a primary deregulation mechanism of heme metabolism, shown by an increase in δ-aminolevulinic acid synthetase activity and a decrease in microsomal heme oxygenase, which would finally lead to a great enhancement of cytochrome P450 levels.

3. The alterations found here would give rise to a pattern distinctive to that usually observed in the so-called resistant hepatocyte.

KEY WORDS. Hepatocarcinogenesis, Azo-dye, porphyrinogenic drug, heme metabolism, drug metabolizing enzyme system

INTRODUCTION

One of the characteristics of the rat preneoplastic liver focus is its relative resistance to hepatotoxins. This feature would enable these cells to proliferate rapidly in response to chemically induced hepatocellular injury and ultimately give rise to malignancy (Farber, 1984; Gindi et al., 1994). This resistance arises partly from metabolic alterations that diminish the cells’ capacity to activate xenobiotics to reactive species (Stout and Becker, 1978), and it is largely attributable to reduced availability of cytochrome P450 (cyt P450), the terminal electron acceptor of the monoxygenase system (Cameron et al., 1976).

Carcinogenesis is a multistep, multistage process that begins with irreversible but heritable damage to a single cell. It is now clear that many forms of irradiation and chemical carcinogens produce alterations of all the major macromolecules of the target cells, including proteins, RNA and DNA (Miller and Miller, 1976). Although some chemicals are active per se, the majority require prior metabolic activation to highly reactive derivatives (Kanduc et al., 1994; Tredaniel et al., 1995).

Many hepatic tumors show changes in the drug metabolizing system due to a large decrease in the activities of phase I enzymes, in which the main components are the microsomal mixed-function oxidase enzymes. The levels of various cyt P450 proteins have been reported to be decreased to varying degrees in chemically induced hepatocyte nodules (Cameron et al., 1976; Habib et al., 1974). Furthermore, the biochemical pattern observed in hepatocarcinogenesis induced by chemicals consisted not only of low levels of phase I components, but also increased activities of phase II enzymes (Farber, 1984; Harrison, 1995; Tredaniel et al., 1995).

Stout and Becker (1987) suggested that the decline in heme protein levels during hepatocarcinogenesis may result from a diminution of the intracellular heme pool, due either to reduced heme synthesis or to increased heme catabolism or to both.

In a previous report (Polo et al., 1992), we proposed a mechanism for the onset of hepatocarcinogenesis that postulates a primary activating liver status involving the whole organ, the so-called preinitiation stage, resulting in a biochemical aberration in heme metabolism, which then leads to the actual initiation stage of hepatocarcinogenesis.

The present study was undertaken to determine whether any alteration in heme regulation occurs before the appearance of hyperplastic nodules. For this, δ-aminolevulinate synthetase (ALA-S) and microsomal heme oxygenase (MHO) activities were examined in animals exposed to dietary carcinogens. In the same group of animals, activities of both oxidative (phase I) and conjugative (phase II) enzymes were measured. The same enzymes were studied in two groups of animals treated with veronal (VER), a known porphyrinogenic drug, with the purpose of investigating whether the onset of the preinitiation stage alters the natural regulatory mechanism of the heme pathway.

MATERIALS AND METHODS

Chemicals

Chemicals were reagent grade and were purchased from Sigma (St. Louis, MO).

Animals and treatments

The schematic representation of the protocol used is shown in Figure 1. Male CF 1 mice weighing 25 g were employed. Four groups of animals were placed on dietary p-dimethylaminoazo-benzene (DAB, 0.5%, w/w) for 35 days. Two groups of animals received VER (5, 5-diethylbarbituric acid, sodium salt) in saline (167 mg/kg body
liver was homogenized (1:10, w/v) in 0.05 M sodium phosphate buffer, pH 7.4, and was centrifuged for 10 min at 9,500 g; the resulting supernatant was used for measuring catalase activity. β-Glucuronidase, sulfatase and glutathione-S-transferase (GST) activities were measured in a homogenate (5%, w/v) prepared from perfused liver in 2 mM TrisHCl buffer, pH 8.1, containing 230 mM mannitol and 70 mM sucrose.

**Assays**

ALA-S activity was measured as described by Marver et al. (1966) and MHO activity according to Yoshida and Kikuchi (1978). Cyt P450 content was determined in the microsomal fraction according to Omura and Sato (1964). Tryptophan pyrrolase (holoenzyme) was measured as described by Knox (1970). Catalase was measured as described by Chance and Maehly (1955). β-Glucuronidase and sulfatase were determined as described by El-Mouelhi et al. (1987), and enzymatic activities were estimated fluorometrically and expressed as fluorescence relative units (FRU). GST was determined by the method of Habig et al. (1974). Protein concentration was determined by the method of Lowry et al. (1951).

Enzyme units (U) were defined as the amount of enzyme producing 1 nmol of product under standard incubation conditions. Specific activity (Sp. Act.) was expressed as units per milligram of protein.

**Statistical analysis**

Newman-Keuls test was used to assess the degree of significance. A probability level of 0.01 was used in testing for significant differences between controls and treated animals.

**RESULTS**

As expected, ALA-S activity in animals treated with DAB (groups S+DAB and DAB+S, respectively) was increased about 100% in accordance with previously reported data (Polo et al., 1992). VER treatment of animals fed with SLD provoked the known induction of ALA-S activity (36%, group VER+SLD, and 63%, group SLD+VER); however, it did not further increase the effect due to DAB (groups VER+DAB and DAB+VER; Fig. 2A).

MHO activity was diminished about 40–60% in all groups. VER injection produced no changes in animals receiving DAB, but a differential diminution in this enzyme activity was observed in animals receiving SLD (64%, group VER+SLD, and 35%, group SLD+VER; Fig. 2B).

Cyt P450 levels were dramatically increased in all groups. In groups VER+SLD and VER+DAB, the enhancement was about 400%, whereas, in groups S+DAB and DAB+S, it was about 200% and 100%, respectively (Fig. 2C).

The levels of GST activity were increased 100% in all groups of DAB-fed animals. The response was different for animals fed the SLD; no variation was observed when VER was injected at the end of the assay period, but an enhancement of 45% was found when VER was injected before it (Fig. 2D).

Tryptophan pyrrolase (holoenzyme) activity was between 35% and 90% augmented in all groups studied (Fig. 3A).

No alteration was detected in catalase activity when SLD-fed animals were treated with VER, but the activity of this enzyme was 60% diminished in all groups receiving DAB (Fig. 3B). β-Glucuronidase and sulfatase activities were diminished in

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**Homogenate preparation**

A fraction of the liver was scissored and immediately homogenized (1:3, w/v) in a solution containing 0.9% NaCl, 0.1 mM TrisHCl (pH 7.4) and 0.5 mM EDTA, for ALA-S determination. The rest of the organ, previously perfused with sterile ice-cold saline, was removed. A fraction was homogenized (1:3, w/v) in ice-cold 0.25 M sucrose. After differential centrifugation of the homogenate the 18,000 g supernatant was used for measuring MHO activity. An aliquot of the 18,000 g supernatant was centrifuged at 105,000 g and this pellet was used for measuring cyt P450. Another fraction of the perfused liver was homogenized (1:1, w/v) in ice-cold 0.14 M KCl, pH 7.5, containing 2.5 mM NaOH and was centrifuged for 10 min at 9,500 g; the resulting supernatant was used for measuring tryptophan pyrrolase (holoenzyme). Yet another fraction of the perfused liver was homogenized (1:10, w/v) in 0.05 M sodium phosphate buffer, pH 7.4, and was centrifuged for 10 min at 9,500 g; the resulting supernatant was used for measuring catalase activity.
FIGURE 2. Effect of DAB and VER on hepatic (A) ALA-S, and (B) MHO activities, (C) cyt P450 levels and (D) GST activity. Animals were treated with DAB (0.5%, w/w, for 35 days). Control animals received a standard laboratory diet (SLD). Nontreated animals and DAB-treated animals were injected (SC) every 24 h for 3 days with VER (167 mg/kg body weight) or saline (S), before (black) or after (hatched) the assayed period, as described in Figure 1. The data represent mean values ±SD of at least six animals and are expressed as percentage of mean control values of SLD-fed animals without any other treatment. Mean control value: ALA-S = 1.4 × 10^−3 ± 0.3 × 10^−4 U/mg protein; MHO = 2.25 ± 0.11 U/mg protein; cyt P450 = 0.34 ± 0.03 U/mg protein; GST = 1.45 ± 0.25 U/mg protein. Other experimental conditions are as indicated in the section on materials and methods. *P < 0.01.

In view of the ability of PB to affect liver ultra-structure and function without producing hepatoma, Peraino et al. (1971) undertook experiments to determine whether PB treatment could alter the incidence of neoplasia induced by the hepatocarcinogen 2-acetylamino-fluorene (AAF). They found that, when PB was fed simultaneously with AAF, the appearance of tumors was delayed. However, when PB was fed after the rats had been exposed to AAF, tumor production was enhanced. Kitagawa et al. (1979) investigated the promoting effect of PB after the short-term administration of the weak carcinogen 2-methyl-N,N-dimethyl-4-aminoazobenzene and suggested that the promoting agent administration appeared to cause an acceleration of cancer formation rather than an increase in the number of enzyme-altered foci, which are the putative precursors of hepatocellular carcinomas.

In this study, the porphyrinogenic effect of VER, a PB analog, was observed only when this agent was administered immediately before sacrifice. However, cyt P450 levels were significantly increased when VER was given at the beginning of the assay period. Considering that induction capability of barbiturates was directly related to their long half-life in plasma (Waxman and Azaroff, 1992), the high levels of cyt P450 found at the end of the period could be ascribed to a sustained induction produced by the drug. Furthermore, it is interesting to point out that the activities of other enzymes related to heme-pathway regulation (MHO, tryptophan pyrrolase), except ALA-S, were altered; this effect could be likely due to a modification in heme regulatory pool content as a consequence of altered levels of cyt P450.
posed as a possible marker for hepatocarcinogenesis (Tatematsu et al., 1986). It is of great interest that treatment with the carcinogen that increases ALA-S and decreases MHO activity produces a paradoxical reduction in catalase, which is a heme-requiring enzyme. However, the rise in cyt P450 levels under these regimens indicates that there is no general deficiency of heme. Perhaps, the diminution of catalase activity may reflect the formation of reactive oxygen intermediates, which contributes to the pathogenesis of liver tumors.

Toyokuni et al. (1995) suggested that the concept of "persistent oxidative stress in cancer" may open up a new research area, explaining part of the characteristic tumor biology of cancer. Further study is necessary to establish the role of oxidative stress and natural antioxidant enzymes in this model of hepatocarcinogenesis induced by DAB.

These findings further support our hypothesis put forward for a probable mechanism for the onset of hepatocarcinogenesis, in which we postulated that the inactive carcinogen gets its reactive form in a hypothetical activating liver status; here a novel but harmful biochemical change is produced, which then triggers the initiation stage. Only at this stage of development is the liver able to complete the transforming sequence, leading to the putative resistant hepatocyte (Polo et al., 1992).

References


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Veronal Effect in Hepatocarcinogenesis


