Differences between the Promoting Activities of the Peroxisome Proliferator WY-14,643 and Phenobarbital in Rat Liver

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ABSTRACT

In order to characterize the promoting activity of the peroxisome proliferator [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid (WY-14,463), male F344 rats which received a single 150-mg/kg dose of diethylnitrosamine (DEN) were fed 0.1% WY-14,643 or 0.05% phenobarbital in the diet for 11, 22, or 54 wk. WY-14,643 promoted the development of ATPase-deficient foci but not GGTase-positive or G6Pase-deficient foci, in contrast to phenobarbital which promoted development of foci detected by all three markers. The mode of promotion of ATPase-deficient foci by WY-14,643 was distinctly different from that of phenobarbital. WY-14,643 primarily increased mean volume of foci at 11 and 22 wk, while phenobarbital primarily increased the numerical density of foci at these time points. At 54 wk, the yield of hepatic neoplasms per liver was higher in rats fed WY-14,643 than in rats fed phenobarbital. To evaluate the possibility that the promotional activity of WY-14,643 was more effective at a later stage in hepatocarcinogenesis, rats receiving a dose of DEN and then phenobarbital in the diet for 11 wk were changed to a diet containing WY-14,643 for an additional 11 or 43 wk. However, WY-14,643 feeding from wk 11 to 22 caused a reduction in volume density of ATPase-deficient foci relative to the volume density of foci at 11 wk. In addition, feeding WY-14,643 from wk 11 to 54 caused similar yields of hepatic neoplasms whether or not phenobarbital was fed for the initial 11 wk. WY-14,643 induced hepatic peroxisome proliferators as indicated by palmitoyl CoA oxidase activity regardless of prior treatment with DEN and/or phenobarbital. The yield of neoplasms in rats not receiving DEN was greater in rats fed WY-14,643 for wk 11 to 54 than in rats fed WY-14,643 for wk 1 to 54. In summary, the peroxisome proliferator WY-14,643 was a more efficient promoter of hepatocarcinogenesis in DEN-initiated rats than phenobarbital. The promotional activity of WY-14,643, when evaluated by stereological analysis and by changing promoters, is distinct from that of phenobarbital, perhaps suggesting different cellular and/or molecular mechanisms of promotion. Understanding the role of promotion by WY-14,643 and other peroxisome proliferators may be important in understanding the mechanism of their hepatocarcinogenicity.

INTRODUCTION

Several different chemicals cause rapid induction of peroxisome proliferation and eventual development of hepatic neoplasia in rats (1). These PP include hypolipidemic drugs such as clofibrate (2) and the plasticizer di(2-ethylhexyl)phthalate (3). The mechanism of the carcinogenicity of PP is not understood. Considerable evidence suggests that, unlike many carcinogens (4, 5), PP (a) are not DNA reactive (6–9), and (b) are nonmutagenic (10, 11). Furthermore, PP did not induce reparative DNA synthesis in rat hepatocytes following treatment in vivo (12, 13). Limited evidence also suggests that PP fail to initiate hepatocarcinogenesis in rats following in vivo treatment (14–16).

Schulte-Herman et al. (17) have suggested that promotion of spontaneously initiated cells may explain the production of tumors following long-term treatment of rats with nonmutagenic compounds. This raises the question of whether the hepatocarcinogenicity of PP could result from promotional activity. Two observations may indirectly support this possibility. (a) PP can induce replicative DNA synthesis and mitosis (18) in hepatocytes, a feature common to other promoting agents such as phenobarbital (19) and partial hepatectomy (20). (b) the role of PP in increasing oxidative injury (21) may also be associated with promotion (22).

The promotional activity of PP in rats has been studied using a variety of chemicals and protocols. In those studies which demonstrated promoting activity of PP, initiation was achieved by continuous administration of DEN or 2-acetylaminofluorene for 2 or more wk. Using these protocols, feeding clofibrate (23, 24) or WY-14,643 (24) after initiation increased the yield of tumors. However, feeding nafenopin (25) and BR-931 (26) after initiation did not increase numbers of foci of altered hepatocytes, and clofibrate (25) was only marginally active. In those studies where initiation consisted of a single administration of DEN, feeding di(2-ethylhexyl)phthalate (27–29), nafenopin (30), or BR-931 (31) resulted in no promotional activity. When the variability in promotional activity of PP is considered with regard to the initiation protocol (single dose versus continuous administration) and end point (foci versus tumors), two possible characteristics of PP promotion are suggested. (a) Prolonged administration of the initiating carcinogen may be necessary for PP promotion, because PP might only act at a later phase in hepatocarcinogenesis. (b) Promotional activity may involve generation of tumors but not foci.

To evaluate these possibilities, the promotional activity of the highly carcinogenic PP, WY-14,643 (32), was measured in rats following a single administration of DEN. In addition, the possibility that WY-14,643 has promotional activity only at a later phase of promotion was suggested by studies in which the carcinogenic process was advanced by prolonged administration of initiating carcinogen. For this reason, the “late” promoting activity of WY-14,643 was evaluated in rats administered a single dose of DEN followed by a brief period of phenobarbital. In order to evaluate the role of hepatic foci in development of hepatic neoplasia in PP promotion, foci as well as neoplasms were quantitated. Furthermore, these results were compared to the promotional activity of phenobarbital, which is known to increase foci and neoplasms when fed after initiation by a single administration of DEN (33).

MATERIALS AND METHODS

Animal Treatments. Male F344 rats (Charles River Breeding Laboratories, Kingston, NY), approximately 180 to 200 g of body weight, were housed 3 to 4 per filter top polycarbonate cage. Distilled and filtered water and diet were available ad libitum. The cages were maintained in a mass air displacement room (Hazelton Systems, Inc., Vienna, VA) with automatically controlled temperature (22°C), relative humidity (50%), and lighting (12-hr light/dark cycle).

Rats were randomly assigned to the treatment groups indicated in
The fraction of liver volume consisting of foci (i.e., volume density) was calculated as the total focal cross-sectional area divided by the total section area (36). The number of foci per unit volume of liver and the mean focal volume were determined by a stereological procedure (37).

For the final 54-wk sacrifice, hepatic neoplasms were enumerated and measured in formalin-fixed livers sliced at 4-mm intervals. Representative hematoxylin- and eosin-stained sections of formalin-fixed, paraffin-embedded tissue were examined by light microscopy to confirm the gross identification of neoplasms. Treatment effects were analyzed by comparing selected groups using the t test, and differences were considered statistically significant using P = 0.05.

Enzyme Assays. A 20% liver homogenate was prepared from a piece of grossly normal left lobe in 50 mM Tris-HCl containing 154 mM KCl (pH 7.2) and frozen at −20°C. The activity of palmitoyl CoA oxidation (38) was determined in supernatant (2500 x g, 5 min) and normalized to protein content determined by the biuret method with a commercially available kit (Abbott Laboratories, North Chicago, IL).

RESULTS

The effects of feeding WY-14,643 or phenobarbital for 11 wk on volume densities of GGTase-positive, ATPase-deficient, and G6Pase-deficient foci are depicted in Fig. 2. Compared to control diet, phenobarbital increased the volume densities of foci identified by each enzyme alteration, while WY-14,643 only increased the volume density of ATPase-deficient foci in DEN-initiated rats (P < 0.05). The numerical density and mean volume of ATPase-deficient foci from the livers of rats fed WY-14,643, phenobarbital, and control diet for 11 wk are depicted in Fig. 3. WY-14,643 increased the mean focal volume but not the numerical density of foci relative to control diet (P < 0.05). Phenobarbital increased the numerical density but not the mean focal volume of ATPase-deficient foci relative to control diet (P < 0.05). Irrespective of the stain used, induction of foci in noninitiated rats was negligible (Fig. 2).

The effects of dietary promotion for 22 wk on volume densities of ATPase-deficient foci in DEN-initiated and noninitiated rats are depicted in Fig. 4, A and B, respectively. Continued feeding of WY-14,643, phenobarbital, or control diet for the additional 11 wk increased the volume densities of ATPase-deficient foci ~ 9x, 3x, and 3x, respectively (P < 0.05). The mean focal volume and numerical density of ATPase-deficient foci of these groups are presented in Fig. 5. As at 11 wk, WY-14,643 continued to increase the mean focal volume of ATPase-
The mean volumes of ATPase-deficient foci were increased in rats which continued to be fed phenobarbital or control diet for an additional 11 wk (P < 0.05), but no difference was observed between these groups. However, the numerical density was not further increased at 22 wk (versus 11 wk) of promotion in either the WY-14,643, phenobarbital, or control groups. Thus, the differing effects of WY-14,643 and phenobarbital promotion on ATPase-deficient foci at 22 wk reflected those effects observed at 11 wk. Foci were infrequently observed at 22 wk in all groups not receiving DEN initiation (Fig. 4B). However, in rats fed WY-14,643 for 22 wk, 3 of 12 rats each had a single large focus.

Changing rats fed phenobarbital for the first 11 wk to WY-14,643 diet for an additional 11 wk resulted in an ~80% reduction in volume density of ATPase-deficient foci when compared to the effect of phenobarbital for the initial 11 wk (P < 0.05) (Figs. 2 and 4A). Also at 22 wk, macroscopic hepatic neoplasms were observed in 12 of 12 WY-14,643-, 3 of 8 phenobarbital-, 4 of 10 phenobarbital → WY-14,643-, 2 of 10 phenobarbital → control-, and 5 of 10 control → WY-14,643-fed rats previously given DEN.

The incidences of rats with (a) hepatocellular carcinoma or (b) hepatocellular carcinoma or neoplastic nodule after 54 wk of promotion are given in Table 1. The effects of dietary promotion for 54 wk on multiplicity of hepatic neoplasms in DEN-initiated and noninitiated rats are depicted in Fig. 6, A and B, respectively. In DEN-initiated rats fed WY-14,643 for 54 wk, livers contained more large (greater than 8 mm in diameter) neoplasms (~8 per liver), in contrast to rats receiving control diet (<1 per liver) or phenobarbital diet (~2 per liver) after initiation (P < 0.05) (Fig. 6A). The yield of neoplasms (greater than 4 mm in diameter) in rats fed WY-14,643 for 43 wk after feeding phenobarbital for the initial 11 wk was the same as in rats fed WY-14,643 for 43 wk after feeding control diet for the initial 11 wk (~11 neoplasms per liver) after initiation (Fig. 6A). In rats not given DEN initiation, only WY-14,643 feeding resulted in hepatic neoplasms (Fig. 6B). The yield of neoplasms (greater than 4 mm in diameter) in rats fed WY-14,643 for 54 wk (5 per liver) was less than in rats fed WY-14,643 for 43 wk after initially being fed control diet for 11 wk (9 per liver), although this difference was not statistically significant (P = 0.06).

The histological characteristics of phenobarbital- and WY-14,643-promoted foci and neoplasms differed in paraffin-embedded, hematoxylin/eosin-stained sections. Phenobarbital-promoted foci tended to be composed of eosinophilic or clear cells. WY-14,643-promoted foci were basophilic. In contrast to phenobarbital, WY-14,643-promoted foci caused modest compression of adjacent hepatic parenchyma even at the 11-wk time point. Staining of neoplasms promoted by these chemicals was similar to that of foci. In addition, spongiosis hepatitis was observed in several of the neoplasms from phenobarbital-treated rats, but not in neoplasms from WY-14,643-treated rats.

The induction of peroxisomal palmitoyl CoA oxidase activity...
initiation. While this finding is consistent with recent reports of PP promotion (39, 40), the present study also demonstrates that the effect of WY-14,643 on developing foci of altered hepatocytes during promotion is different than that of phenobarbital. (a) Phenobarbital promoted foci that could be identified by all three stains used, while WY-14,643 promoted only ATPase-deficient foci. The phenobarbital promotion of multiple-staining foci using different markers was previously described (41). (b) Even more interesting was the dissimilarity of WY-14,643 and phenobarbital promotion with respect to size and numbers of foci. WY-14,643 increased the size but not number of ATP-deficient foci, while phenobarbital increased the number but not the size of these foci, after 11 and 22 wk of promotion. The dietary level of phenobarbital (0.05%) used in the present study was previously reported to be the optimal dietary concentration (42). In another report, phenobarbital did increase the mean volume of hepatic foci after 6 to 8 mo of feeding (43). This apparent discrepancy regarding the effect of phenobarbital on size of hepatic foci may be explained by the duration of dietary promotion. The relatively early time points evaluated in the present study are consistent with a previous report that phenobarbital did not increase the mean volume of hepatic foci after 3 mo of dietary administration (27). Furthermore, the development of foci and neoplasms in rats changed from phenobarbital to control diet after 11 wk (Figs. 2, 4, and 6) is consistent with reported increases in the percentage of liver volume occupied by foci following phenobarbital withdrawal (41).

The dissimilar effects of WY-14,643 and phenobarbital on the size versus number of hepatic foci as described in the present study have not been described in any other studies of PP promotion. The results suggest that PP promotion may involve a different mechanism than phenobarbital. The possibility that WY-14,643 promotion may involve to a greater degree some later phase in carcinogenesis was not confirmed by changing dietary promotional agents in rats previously given DEN initiation. Changing from phenobarbital to WY-14,643 at 11 wk did not increase or even maintain the volume density of ATPase-deficient foci. Furthermore, feeding either control or phenobarbital diet for the initial 11 wk resulted in similar yields of neoplasms when WY-14,643 was subsequently fed for 43 wk. WY-14,643 failed to further promote carcinogenesis following DEN initiation and phenobarbital promotion even though peroxisome proliferation (as indicated by enzyme activity) was induced. It therefore appears that foci induced by DEN and phenobarbital are insensitive to WY-14,643 promotion and undergo regression. Other PP, BR-931 and di(2-ethylhexyl)phthalate, caused a reduction in GGT-positive foci when fed after DEN initiation and choline-devoid dietary promotion (26). Taken together, these observations suggest a distinct mechanism of promotion by PP and not merely a phase-related effect.

While feed consumption was not markedly affected, rats in groups fed WY-14,643 had reduced body weight gain when compared to other groups (data not shown). It has generally been shown that reduction in weight gains (by dietary restriction) diminishes the carcinogenic activity of chemicals in rodents (44). However, in the present study WY-14,643 feeding was clearly associated with an augmented carcinogenic response in rats receiving DEN initiation, despite diminished body weight gain.

PP induce a variety of biological responses, making it difficult to determine which responses may be responsible for promotional activity. Considerable attention has focused on genera-
tion of reactive oxygen intermediates in PP-treated rats (21), as these intermediates may play a role in promotion (22). Induction of cell replication by PP (18), a response common to several promoters (45), has also received attention. In regards to the latter, we have recently observed that replicative DNA synthesis (and presumably cell turnover) is elevated for up to 1 yr of continuous WY-14,643 feeding (46). Consequently, an interesting comparison of the promoting activity of WY-14,643 (present study) and 4-DAB (47) can be made. Both WY-14,643 and 4-DAB were very effective at increasing the size of foci of these intermediates may play a role in promotion (22). (present study) and 4-DAB (47) can be made. Both WY-14,643

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