Dietary glycine prevents the development of liver tumors caused by the peroxisome proliferator WY-14,643

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Introduction

WY-14,643 is a member of the class of compounds known as peroxisome proliferators, which include a wide variety of structurally unrelated chemicals that increase both the number and size of peroxisomes in rodent liver (1). Chronic exposure to peroxisome proliferators leads to hepatocellular carcinomas in rats and mice (2). The mechanism by which WY-14,643 and other peroxisome proliferators cause cancer remains unknown; however, several studies suggest that they act via non-genotoxic mechanisms involving increased cell replication (3–5).

Stimulated rates of hepatocyte proliferation most likely play a key role in the development of liver cancer caused by these chemicals. For example, their potency as carcinogens has been associated with their ability to sustain cell proliferation. WY-14,643, one of the most potent carcinogens in this class, increased cell proliferation for as long as the compound was administered while the much less potent carcinogen diethylnyl phthalate did not, even at doses 12-fold higher (3). Elevated rates of hepatocyte replication may be important in the promotion of previously initiated cells, which are more numerous in livers of older rats (4,5). Thus, it has been proposed that peroxisome proliferators act as tumor promoters by stimulating proliferation of previously initiated cells (3).

Since Kupffer cells, the resident hepatic macrophages, are a rich source of mitogenic stimuli in the liver (6), it was hypothesized that peroxisome proliferators act as tumor promoters to release cytokines that stimulate cell replication in the liver. In support of this hypothesis, WY-14,643 was shown to activate the production of the hepatocyte mitogen tumor necrosis factor (TNFα) which is produced predominantly by Kupffer cells in liver (7). Moreover, antibodies to TNFα prevented WY-14,643-induced cell proliferation (7). Furthermore, inactivation of Kupffer cells with methyl palmitate prevented TNFα production and cell proliferation in response to WY-14,643 (8). Since inactivation of Kupffer cells with methyl palmitate requires daily i.v. injections, long-term studies are not practical. Therefore, a dietary method of Kupffer cell inactivation was employed. A glycine-enriched diet prevented both the initial increase in cell proliferation 24 h after a single dose and the sustained elevation caused by WY-14,643-induced TNFα production after 3 weeks of dietary treatment (9). Therefore, these studies were designed to test the hypothesis that dietary glycine would prevent the development of preneoplastic foci and tumors caused by long-term dietary exposure to WY-14,643. Preliminary accounts of this work have been presented elsewhere (10).

Materials and methods

Animal treatment

Male F344 rats (Charles River Breeding Laboratories, Raleigh, NC) were housed two per cage in biologically clean rooms with filtered air and a 12 h day/night cycle. Animals were quarantined for 2 weeks upon arrival and maintained on NIH-07 chow and purified water ad libitum. Temperature and relative humidity were held at 22 ± 2°C and 50 ± 5%, respectively. Treated animals were given the same rodent chow blended with WY-14,643 (ChemSyn Science Laboratories, Lenexa, KS) at a target concentration of 0.1%. The concentration of WY-14,643 in the feed was assayed after each blending and
the measured values were within 20% of the target concentration. Glycine or
valine (as a nitrogen balance) were added to the same rodent chow at a target
concentration of 5% (Table I). Seven days prior to the 22 week killing, Alzet
osmotic pumps (Palo Alto, CA; flow rate 10 µl/h) containing 16 mg/ml 5-
bromo-2'-deoxyuridine (BrdU; Sigma) were implanted s.c. in six rats per
group. Food consumption and body weights were monitored weekly throughout
the study.

**Histopathology and immunohistochemistry**

After 22 weeks (six animals per group) or 51 weeks (10–14 animals per
group) on diets, rats were killed by exsanguination under pentobarbital
anesthesia. At the time of necropsy, terminal body weights and liver weights
were recorded. Liver sections were made for hematoxylin and eosin, and
immunohistochemical staining for BrdU and proliferating cell nuclear
antigen (PCNA). Incorporation of BrdU was determined as described elsewhere
(11). A section of ileum was included from each rat as a positive control for
BrdU incorporation. Labeled and unlabeled hepatocyte nuclei were counted
in randomly generated, high-power (400×) fields of tissue sections from
preneoplastic foci or tissue sections from ‘normal’ surrounding parenchyma.
At least 1000 hepatocyte nuclei were counted per rat to calculate the percentage
of labeled nuclei (labeling index). In animals not given osmotic pumps
containing BrdU (51 week animals), labeling index was determined using
antibodies to PCNA. The percentage of labeled nuclei was determined in
tumors and in normal parenchyma as described for BrdU incorporation above.

At the 22 week time point, grossly visible lesions on the liver surface were
sized and quantitated. At the 51 week time point, livers were sectioned at
1–2 mm intervals for quantification of grossly observable lesions. Frozen and
formalin-fixed sections of lesions were collected for histological evaluation.

**Enzyme assays**

Liver from the left lobe was used to prepare 20% homogenates in 50 mM
Tris–HCl, 154 mM KCl, pH 7.2. Samples were kept on ice until freezing at
~80°C. The post-nuclear supernatant of the thawed liver homogenate was
prepared by the day of enzyme assays by centrifugation at 2500 g for 10 min.
Acyl CoA oxidase activity was assayed by measurement of hydrogen
peroxide production in the presence of 25 µM palmitoyl-CoA (12). Enzyme activity
was normalized per g of protein (13).

**Measurement of serum glycine levels**

Blood was harvested by cardiac puncture for glycine determination in serum
as described previously (14). Briefly, glycine was extracted, benzoylelated and
the resulting hippuric acid was extracted and dried. Subsequently, the
concentration of hippuric acid was determined spectrophotometrically at 458
nm (15).

**Results**

**Effects of WY-14,643 and dietary glycine on body and liver weights, serum glycine concentration, and induction of peroxisomes**

Food consumption (w/w) did not differ among the four dietary
groups studied (data not shown). However, body weights of
both WY-14,643 and WY-14,643 + glycine-fed rats were
significantly lower than control and glycine-fed rats between 4 and 51 weeks, as expected (Figure 1). Rats on WY-14,643
or WY-14,643 + glycine diets weighed from 10 to 25% less
than those on control or glycine diets alone. This effect of
WY-14,643 on body weight confirms results obtained in other
long-term feeding studies with WY-14,643 (3,9). Reasons for
this phenomenon remain unknown. The glycine-containing
diets increased serum glycine levels 4–5-fold after 22 weeks
of feeding (Table II) consistent with results from other studies
(16). Interestingly, WY-14,643 alone caused an almost 2-fold
increase in serum glycine levels for reasons that also remain
unknown (Table II).

*Fig. 1. Effect of glycine and WY-14,643 on body weight. Body weights were measured weekly for the first 10 weeks and every 2 weeks thereafter.

*Statistical difference from both the control and glycine groups (repeated measures ANOVA with Student–Newman–Keuls post-hoc tests; P < 0.05, n = 14–20 in each group).
Glycine prevents tumors

A defining characteristic of all peroxisome proliferators is an induction of peroxisome-specific enzymes such as acyl CoA oxidase (1). Following 22 weeks of dietary treatment, WY-14,643 increased peroxisomal acyl CoA oxidase activity ~10-fold over control values, an increase which was unaffected by dietary glycine (Figure 3). The increases in peroxisomal enzymes measured in these studies are similar to those reported in previous chronic feeding studies with WY-14,643 (3).

Effects of WY-14,643 and dietary glycine on preneoplastic foci and cell proliferation following 22 weeks of treatment

At time of death, visible lesions (preneoplastic foci) on the liver surface were sized and counted. As expected, no foci were detected in livers from rats fed control and glycine diet alone (data not shown). However, WY-14,643 treatment led to formation of preneoplastic foci that were separated into three categories based on size: ≤1 mm diameter, 1–2 mm diameter or >2 mm diameter (Table III). WY-14,643 lesions of 1–2 mm diameter per liver (Table III). The addition of 5% glycine to the WY-14,643 diet did not affect the development of preneoplastic foci in either of these groups. Furthermore, lesions >4 mm in diameter were rare (Table III).

Cell proliferation was assessed using incorporation of BrdU in both foci and the surrounding parenchyma at 22 weeks. In the surrounding liver tissue, rates of cell proliferation in control and glycine-fed rats were generally <1%, rates that were increased ~4-fold by WY-14,643 (Table IV). The increase in

Fig. 2. Effect of glycine and WY-14,643 on liver weights. Livers were excised from animals and weighed at time of killing after 22 weeks (A) (n = 6) or 51 weeks (B) (n = 14) on diets. Data are reported as means ± SEM for control (CON), glycine (GLY), WY-14,643 (WY) and WY-14,643 + glycine (WY + GLY) groups. *, statistical differences from control and glycine groups (P < 0.05).

Fig. 3. Acyl CoA oxidase activity after treatment with WY-14,643. Acyl CoA oxidase activity was determined after treatment with diets as described in Materials and methods. Data are reported as means ± SEM for groups described in Figure 2. *, statistical differences from control and glycine groups (P < 0.05, ANOVA with Tukey’s post-hoc tests, n = 6 per group).
cell proliferation caused by WY-14,643 at 22 weeks was unaffected by the addition of 5% glycine to the diet (Table IV). Similarly, proliferation rates in preneoplastic foci from rats fed WY-14,643 did not differ from those in the WY-14,643 + glycine group at this time point (Table IV). Rates of proliferation in foci were much higher than in the surrounding parenchyma as demonstrated in previous studies (17).

Effect of dietary glycine on WY-14,643-induced tumor formation

After 51 weeks on diets, animals were killed and tumors identified. Rats on control and glycine diets did not have liver tumors as expected (data not shown). However, WY-14,643 caused tumors of varying sizes in all rats (Figure 4). Tumors were sized and placed into the following groups: 0–5 mm diameter, 5–10 mm diameter, >10 mm diameter. WY-14,643 caused an average of nearly 32 tumors per liver of 0–5 mm diameter which was reduced significantly by the addition of 5% glycine to the diet (Figure 4A). Only about four tumors per liver of 5–10 mm were found in WY-14,643-treated rats and glycine reduced this value ~3-fold (Figure 4B). The addition of 5% glycine to the diet also caused a significant reduction in the number of tumors >10 mm in diameter (Figure 4C). Interestingly, the inhibitory effect of dietary glycine was greater as the tumor size increased (Figure 5). It inhibited the development of the largest tumors (>10 mm diameter) by nearly 80%, was ~65% effective on tumors from 5–10 mm in size, and inhibited formation of the smallest tumors (0–5 mm) by only ~23%. While dietary glycine inhibited the development of the large tumors, cell proliferation in the tumors did not differ between the WY-14,643 and WY-14,643 + glycine groups (Table IV).

Discussion

Previous studies demonstrated that dietary glycine blocked WY-14,643-induced increases in hepatocyte proliferation (9). The initial 8-fold burst in cell replication and the sustained 5-fold increase following 3 weeks of dietary treatment were both prevented by the addition of 5% glycine to the diet (9). Since hepatocyte replication has been hypothesized to play a key role in peroxisome proliferator-induced liver cancer (3), these studies were designed to determine if dietary glycine would prevent WY-14,643-induced hepatic cancer in rodents.

The addition of 0.1% WY-14,643 to the diet suppressed weight gain compared with rats fed control diet (Figure 1). While this phenomenon has been observed in several long-term feeding studies with peroxisome proliferators, the cause remains unknown (3,9). One possibility is that TNFα, which is also known as cachectic factor and is responsible for the wasting phenomenon in cancer patients, plays a role since WY-14,643 causes a 2–3-fold increase in TNFα in liver (7). Importantly, glycine had no effect on body weight gain in these studies (Figure 1).

Feeding glycine at 5% in the diet resulted in a 4–5-fold increase in blood glycine levels (Table II). While glycine-supplemented diet increased blood glycine levels, the absolute concentration was not as high as the nearly 1.5 mM levels obtained in previous studies (16). Although the exact reason is unknown, several possibilities exist. First, other studies with dietary glycine were for shorter periods of time (e.g. 3 days to 4 weeks) than the 22 and 51 week feeding studies reported here, and there may be mechanisms to regulate blood glycine levels after prolonged periods of time (16,18). Second, the base diet used in this study (NIH-07) is different from the semi-synthetic AIN 76-based diets used previously (16). However, more interesting is the 2–3-fold increase in serum glycine levels caused by WY-14,643 (Table II). While reasons for this finding remain unknown, one possibility is that the increase in serum glycine is caused by TNFα. WY-14,643 has been shown to increase TNFα following both a single i.g. dose and 3 weeks of feeding WY-14,643 at 0.1% in the diet (7,9). In other studies, TNFα given as a single dose to rats caused an increase in circulating amino acids, including glycine (19). Therefore, it is possible that the increase in serum glycine levels in rats fed WY-14,643 alone is mediated by TNFα.

Twenty-two week study

Surprisingly, the elevated blood glycine levels achieved with dietary glycine did not block hepatomegaly caused by dietary exposure to WY-14,643 in these studies (Figure 2). Hepatomegaly caused by peroxisome proliferators is due to both hepatocyte hypertrophy and hyperplasia (20). A major contributor to hypertrophy is peroxisome proliferation which was unaffected by dietary glycine in these (Figure 3) and other studies (9). While previous studies demonstrated that glycine prevented sustained hepatocyte proliferation caused by 3 weeks of feeding

Table III. Visible lesions on the liver surface after 22 weeks of dietary treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of livers without lesions</th>
<th>Foci visible at 22 weeks (total in six livers)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 mm diameter</td>
</tr>
<tr>
<td>WY-14,643</td>
<td>0/6</td>
<td>3</td>
</tr>
<tr>
<td>WY-14,643 + glycine</td>
<td>1/6</td>
<td>3</td>
</tr>
</tbody>
</table>

Lesions visible on the surface of the livers were sized and counted. Data are reported as the total number of lesions of each size (i.e. 1 mm, 1–2 mm, 4 mm) found in all six livers in each group.

Table IV. Hepatocyte proliferation after treatment with WY-14,643 and glycine in the diet

<table>
<thead>
<tr>
<th>Hepatocyte proliferation</th>
<th>Control</th>
<th>Glycine</th>
<th>WY-14,643</th>
<th>WY-14,643 + glycine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal liver (22 weeks)</td>
<td>1.0 ± 0.0</td>
<td>0.6 ± 0.1</td>
<td>3.8 ± 0.6a</td>
<td>4.8 ± 0.5a</td>
</tr>
<tr>
<td>Prenecoplastic foci (22 weeks)</td>
<td>ND</td>
<td>ND</td>
<td>52.0 ± 4.0</td>
<td>45.2 ± 3.6</td>
</tr>
<tr>
<td>Tumors (51 weeks)</td>
<td>ND</td>
<td>ND</td>
<td>40.6 ± 9.8</td>
<td>48.2 ± 6.5</td>
</tr>
</tbody>
</table>

Cell proliferation was assessed in the normal parenchyma and in preneoplastic foci after 22 weeks of dietary treatment and in tumors after 51 weeks of treatment as described in Materials and methods. Foci and tumors were not detected in livers from control and glycine-treated animals (ND = not detected). Data are reported as means ± SEM for groups described in Table 2.

aStatistical differences from control and glycine groups (ANOVA with Student–Newman–Keuls post-hoc tests; P < 0.05, n = 6 in each group).
WY-14,643 in the diet (9), hyperplasia was unaffected by glycine in studies presented here (Table IV).
Based on the results of previous studies, which demonstrated that glycine prevented sustained hepatocyte proliferation and blunted hepatomegaly caused by WY-14,643 after 3 weeks (9), these findings were surprising. The mechanism by which glycine prevented the increase in cell proliferation caused by WY-14,643 in previous studies most likely involved its actions on Kupffer cells. In support of this hypothesis, inactivating Kupffer cells with methyl palmitate prior to treatment with WY-14,643 completely prevented the initial burst in hepatocyte proliferation caused by WY-14,643 (8) demonstrating that Kupffer cells are causally responsible for the stimulation in cell replication. Since antibodies to TNFα also prevented WY-14,643-stimulated cell proliferation, and inactivating Kupffer cells with methyl palmitate blocked the increase in TNFα caused by WY-14,643, Kupffer cell-derived TNFα is most likely responsible for WY-14,643-induced cell proliferation. In addition, dietary glycine also prevented WY-14,643-stimulated TNFα production and cell proliferation (7).
The mechanism by which glycine prevented Kupffer cell TNFα production is likely due to its actions on a glycine-
gated chloride channel (9). Glycine binds to its receptor allowing extracellular chloride to flow into the cell causing hyperpolarization of the membrane (21). This prevents increases in intracellular calcium that are required for TNFα production (21). However, Kupffer cells isolated from rats treated with dietary glycine (5%) for 4 weeks were not inhibited by glycine treatment in vitro and increases in intracellular calcium were identical to controls (data not shown). This suggests that long-term dietary glycine causes down-regulation of the glycine-gated chloride channel on Kupffer cells which may explain the lack of effect of glycine on WY-14,643-induced cell proliferation and hepatomegaly (Figure 2; Table IV).

Fifty-one week study
Although dietary glycine did not block WY-14,643-stimulated hepatocyte proliferation (Table IV) or prevent the formation of preneoplastic foci (Table III), it inhibited the development of tumors by 25–80% depending on tumor size (Figures 4 and 5). In previous tumor studies with peroxisome proliferators (22), the development of hepatocellular neoplasia was more sensitive than foci for detecting promoting activity. This effect was explained by the observation that peroxisome proliferator-induced promotion resulted in few foci that rapidly increased in size. Because they are present in low numbers, foci are more likely to be missed because of necessarily blind and limited sampling of the entire liver. Therefore, inhibition of tumor development is a more sensitive measure of the effect of dietary glycine on WY-14,643-induced liver cancer than morphometry of foci.

The multi-stage model of cancer growth is generally divided into three processes: initiation, promotion and progression. Peroxisome proliferators have been classified as tumor promoters because they have been negative in most widely used assays for determining genotoxic agents (23–25), and they lack initiating activity in classic initiation–promotion protocols (26,27). They have been hypothesized to act on spontaneously initiated cells since older rats with more spontaneously initiated cells develop more tumors than younger ones (4,5). Since glycine had no effect on the development of preneoplastic foci <2 mm diameter (Table III), it is concluded that glycine does not prevent either the initiation or promotion phases of tumor development. While it is possible that glycine simply delays the onset of tumor development, this seems unlikely since it did not prevent WY-14,643-induced preneoplastic foci formation. Therefore, it is likely that dietary glycine inhibits the growth of these rapidly growing lesions present after 22 weeks of dietary treatment to larger tumors.

Working hypothesis: glycine inhibits tumor growth by preventing angiogenesis
Tumor progression is dependent on angiogenesis for the supply of oxygen and nutrients to the tumor (28). The development of tumors greater than ~2 mm in diameter requires neovascularization (29). For example, tumors grown in systems where blood vessels do not proliferate, such as isolated perfused organs, reach a size of only ~2 mm but expand quickly to nearly 2 cm following transplantation in vivo after vascularization occurs (30). In these studies, glycine inhibited the growth of larger tumors to a greater extent than smaller tumors (Figure 5). These data are consistent with the hypothesis that glycine inhibits tumor growth by preventing neovascularization.

In support of this hypothesis, dietary glycine inhibited the growth of mouse tumors that are dependent on vascularization for development in vivo (31). Specifically, tumor growth resulting from s.c. implantation of B16 melanoma cells in vivo has been shown to be dependent on vascularization of the implanted tumor (32). Treatments that inhibit tumor vascularization also prevent tumor growth (33). In previous studies, dietary glycine blocked the formation of arteries by 70% in B16 tumors and tumor growth was inhibited by 65% in vivo (31). However, the technique used to identify arteries in experimental mouse tumors (i.e. van Gieson’s elastic stain) is not applicable in liver because only thin strands of elastin fibers are present in portal triad areas instead of the thick ring present in artery walls in experimental tumors (31,34).

The mechanism by which glycine inhibited angiogenesis in experimental mouse tumors is unclear (31); however, several possibilities exist. Proliferation of endothelial cells is a key step in the process by which new blood vessels grow from established ones (28), and glycine inhibited endothelial cell proliferation in vitro in a dose-dependent manner in a previous study (31). Taken together, these data support the hypothesis that dietary glycine inhibits tumor growth by preventing neovascularization by inhibiting endothelial cell proliferation. Therefore, it is likely that dietary glycine inhibits WY-14,643-induced hepatic cancer by preventing neovascularization and suggests that glycine may be an effective anti-cancer agent.

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References
Glycine prevents tumors


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