Effect of selective and non-selective muscarinic blockade on baclofen inhibition of gastric carcinogenesis induced by N'-methyl-N'-nitro-N-nitrosoguanidine in Wistar rats

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The effects of baclofen, a γ-amino-n-butyric acid receptor B agonist, on gastric carcinogenesis induced by N'-methyl-N'-nitro-N-nitrosoguanidine and how its effects are influenced by selective (M₁) and non-selective (M₁ and M₂) pharmacological blockade of muscarinic receptors were investigated in inbred Wistar rats. Rats were given s.c. injections of 8 mg/kg body wt baclofen with and without 0.5 mg/kg body wt atropine (non-selective M₁ and M₂ muscarinic receptor antagonist) or 1.0 mg/kg body wt pirenzepine (selective M₁ muscarinic receptor antagonist) every other day after a 25 week carcinogen treatment. At week 52 baclofen significantly decreased the incidence of gastric cancers. Concomitant treatment with atropine significantly attenuated the inhibition by baclofen of gastric carcinogenesis, but combined use with pirenzepine had no significant effect on the inhibition by baclofen of gastric carcinogenesis. Baclofen also significantly decreased the labeling index of the antral mucosa. Baclofen plus atropine attenuated the decrease in the labeling index of the antral mucosa due to baclofen, but baclofen plus pirenzepine had no significant effect on the labeling index. These results suggest that the inhibition of gastric carcinogenesis by baclofen is mediated through muscarinic receptors and M₂ receptors, but not M₁ receptors, are involved in this response.

Introduction

Previously we found that baclofen, a γ-amino-n-butyric acid receptor B agonist, significantly decreased the incidence and number of gastric cancers of the glandular stomach induced by N'-methyl-N'-nitro-N-nitrosoguanidine (MNNG*) in Wistar rats (1). However, the exact mechanism of this effect remains unknown.

Baclofen has a marked stimulatory effect on gastric motility and acid secretion in several species, including rats (2), dogs (3) and man (4). Its stimulatory effects were completely abolished by surgical and chemical vagotomy (5–8). These findings suggest that a muscarinic receptor mechanism participates in these actions of baclofen.

The existence of both M₁ and M₂ muscarinic receptor subtypes is now well established (9). However, it is not known which muscarinic receptor is involved in the inhibition of gastric carcinogenesis by baclofen. Therefore, in the present work we examined the effects of pirenzepine, a selective muscarinic receptor (M₁) antagonist, and atropine, a non-selective muscarinic receptor (M₁ and M₂) antagonist, on baclofen inhibition of gastric carcinogenesis induced by MNNG in Wistar rats.

Materials and methods

Animals

One hundred and fifty inbred young (6-week-old) male Wistar rats were purchased from Japan SLC (Shizuoka, Japan). The animals were housed in suspended cages with a wire bottom in a room maintained at 21 ± 1°C and 40 ± 10% humidity with a 12 h light/12 h dark cycle. Regular chow pellets (Nihon-Nosan, Yokohama, Japan) were available ad libitum.

Experimental design

Animals were given drinking water containing 50 µg/ml MNNG (Aldrich Chemical Co., Milwaukee, WI) for 25 weeks. From week 26 rats were given normal tap water ad libitum and were randomly divided into six groups of 25 rats each. Each group received s.c. injections of 8 mg/kg body wt baclofen (Sigma, St Louis, MO), 0.5 mg/kg body wt atropine (Sigma) and 1.0 mg/kg body wt pirenzepine (Sigma), alone or in combination, every other day until the end of the experiment at week 52. Dosages were based on results of studies by Garrigues et al. (10) and Hashimoto et al. (8). Agents were administered in a 0.9% NaCl solution between 2 and 3 p.m.. Group 1, the control group, was given only 0.9% NaCl. Group 2 was given baclofen alone. Group 3 was given both baclofen and atropine. Group 4 was given both baclofen and pirenzepine. Group 5 was given atropine alone. Group 6 was given pirenzepine alone.

Histological examination

Rats that survived for more than 50 weeks were included in the effective numbers, because the first tumor of the glandular stomach was found in a rat from group 1 that died at week 50. All rats were killed at the end of the experiment at week 52. All rats were carefully examined, especially the stomach and other organs. The stomach was opened along the greater curvature, pinned flat on a cork mat and fixed with a buffered picric acid/formaldehyde solution for histological examination. The stomach was cut into longitudinal strips of 3 mm width. Specimens were embedded in paraffin and serial sections of 5 µm thickness were stained with hematoxylin and eosin. Sections were examined without knowledge of which group they belonged to.

Definition and classification of gastric cancers

Histologically adenocarcinomas were defined as lesions in which neoplastic glandular tissue had penetrated the muscularis mucosae to involve the submucosa or deeper layers. As previously reported (11), adenocarcinomas were classified as very well-differentiated, well-differentiated or poorly differentiated.

Measurement of labeling index of gastric mucosa

The labeling index of the gastric mucosa was measured at weeks 30 and 52 in five rats of each group with an immunohistochemical analysis kit for assaying bromodeoxyuridine (BrdU) incorporated (Becton-Dickinson, Mount View, CA) (12, 13). For this purpose rats were fasted for 12 h and then received their scheduled injections. One hour later the animals were given an i.p. injection of 20 mg/kg BrdU and after another hour were killed with ether. The stomach was removed and fixed in 10% ethanol for 4 h. Thin sections of 3 µm thickness were immersed in 2 N HCl solution for 30 min and then in 0.1 M Na₂B₄O₇. Sections were placed on slides and immersed in 0.3% H₂O₂ in methanol for 30 min to block endogenous peroxidase activity and then treated with 10% porcine serum. The specimens were incubated with anti-BrdU monoclonal antibody (diluted 1:20) for 2 h, washed, stained with biotin-conjugated horse anti-mouse antibody (diluted 1:200; Vector Laboratories, Burlingame, CA) for 30 min and then stained by the avidin–biotin–peroxidase complex method (Vector Laboratories) for 30 min. The reaction product was visualized with 3,3'-diaminobenzidine tetrahydrochloride. Cells that contained BrdU were identified by the presence of a dark pigment over the nucleus.

To determine the BrdU labeling index of the gastric mucosa the number of

*Abbreviations: MNNG, N'-methyl-N'-nitro-N-nitrosoguanidine; BrdU, bromodeoxyuridine.
baclofen and atropine (group 3) had a significantly increased

Statistical analysis

Measurement of antral pH

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Rats that received both but not their number per tumor-bearing rat, was significantly

Week 52 the weights of animals that had received baclofen

BrdU-labeled and unlabeled cells (14) were counted without knowledge of

**Table I. Incidence and number of gastric cancers in MNNG-treated rats**

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Treatment</th>
<th>Body wt (g)</th>
<th>Effective no. of rats</th>
<th>No. of rats with gastric cancer (%)</th>
<th>No. of gastric cancers per tumor-bearing rat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Week 26</td>
<td>Week 52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>371 ± 4</td>
<td>401 ± 7</td>
<td>20</td>
<td>19 (95)</td>
</tr>
<tr>
<td>2</td>
<td>Baclofen</td>
<td>373 ± 4</td>
<td>353 ± 7</td>
<td>20</td>
<td>10 (50)</td>
</tr>
<tr>
<td>3</td>
<td>Baclofen + atropine</td>
<td>368 ± 4</td>
<td>341 ± 6</td>
<td>20</td>
<td>17 (85)</td>
</tr>
<tr>
<td>4</td>
<td>Baclofen + pirenzepine</td>
<td>369 ± 6</td>
<td>361 ± 8</td>
<td>19</td>
<td>9 (47)</td>
</tr>
<tr>
<td>5</td>
<td>Atropine</td>
<td>375 ± 5</td>
<td>406 ± 12</td>
<td>19</td>
<td>17 (89)</td>
</tr>
<tr>
<td>6</td>
<td>Pirenzepine</td>
<td>363 ± 6</td>
<td>396 ± 12</td>
<td>18</td>
<td>16 (89)</td>
</tr>
</tbody>
</table>

*After 25 weeks of MNNG treatment each groups of rats was given s.c. injections in 0.9% NaCl of 8 mg/kg body wt baclofen, 0.5 mg/kg body wt atropine and 1.0 mg/kg body wt pirenzepine, alone or in combination, every other day. Control rats received only 0.9% NaCl.

Significantly different from the value for group 1 at *P* < 0.001.

Significantly different from the value for group 2 at *P* < 0.001.

**Table II. Histologic type and depth of involvement of gastric cancers in MNNG-treated rats**

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Treatment</th>
<th>No. of gastric cancers</th>
<th>Histology (%)</th>
<th>Depth of involvement (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Very well-differentiated</td>
<td>Well-differentiated</td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>31</td>
<td>25 (81)</td>
<td>6 (19)</td>
</tr>
<tr>
<td>2</td>
<td>Baclofen</td>
<td>17</td>
<td>10 (59)</td>
<td>7 (41)</td>
</tr>
<tr>
<td>3</td>
<td>Baclofen + atropine</td>
<td>20</td>
<td>18 (90)</td>
<td>2 (10)</td>
</tr>
<tr>
<td>4</td>
<td>Baclofen + pirenzepine</td>
<td>15</td>
<td>13 (87)</td>
<td>2 (13)</td>
</tr>
<tr>
<td>5</td>
<td>Atropine</td>
<td>29</td>
<td>20 (69)</td>
<td>9 (31)</td>
</tr>
<tr>
<td>6</td>
<td>Pirenzepine</td>
<td>19</td>
<td>19 (100)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*For an explanation of the treatments see Table I.

BrdU-labeled and unlabeled cells (14) were counted without knowledge of which treatment group the sample was from. The zone of proliferating cells in the fundic mucosa was defined as a rectangle of 250 μm width between the highest and lowest cells in a well-oriented section. Ten such areas were selected in each rat. In the antral mucosa all cells below the highest labeled cell in each gland were regarded as being within the zone of proliferating cells. We selected 100 well-oriented glands in each rat. From these measurements we derived the BrdU labeling index as the number of BrdU-labeled cells per total number of cells within the zone of proliferation.

**Measurement of antral pH**

The antral pH was examined at week 52 in 10 rats of each group. To measure antral pH rats were starved for 12 h and then received their usual treatment. One hour later the rats were killed and the stomach was opened, pinned flat on a cork mat and antral pH was measured with a fine needle electrode.

**Statistical analysis**

Data were analyzed by the χ² test, Fisher's exact probability test or one-way analysis of variance with Dunn's multiple comparison (15,16). Data are given as means ± SE. Differences were considered as significant at *P* < 0.05.

**Results**

**Incidence, number, histological type and depth of involvement of gastric cancers**

One rat in each of groups 4 and 5 and two in group 6 died before experimental week 42. Tumors were not found in these rats and they were excluded from the effective numbers. In week 52 the weights of animals that had received baclofen with or without atropine or pirenzepine (groups 2–4) were significantly lower than those of the control group (Table I).

The incidences of gastric cancers and their number per tumor-bearing rat in each group are summarized in Table I. In group 1 (control) gastric cancers were found in 19 (95%) of 20 rats and the mean number per tumor-bearing rat was 1.6 ± 0.2. In group 2 (baclofen) the incidence of gastric cancers, but not their number per tumor-bearing rat, was significantly lower than in the control group. Rats that received both baclofen and atropine (group 3) also had a significantly increased incidence of gastric cancers compared with those in group 2. However, administration of both baclofen and pirenzepine (group 4) had no significant influence on the incidence of gastric cancer compared with that in group 2. The incidence and mean number of gastric cancers in rats treated with atropine alone (group 5) or pirenzepine alone (group 6) was not significantly different from those of the control group.

The histological type and depth of involvement of gastric cancers are summarized in Table II. All tumors induced in the glandular stomach were histologically identified as adenocarcinomas. There were no significant differences in the distribution of different histological types and depth of involvement of gastric cancers. All cancers were found in the antral mucosa and no metastases were detected in any rats.

**Labeling index of gastric mucosa and antral pH**

Table III summarizes data on the labeling index of the gastric mucosa and antral pH at weeks 30 and 52. At both weeks 30 and 52 administration of baclofen (group 2) caused a significant decrease in the labeling index of the antral mucosa, but not of the fundic mucosa, as compared with the control group. The labeling index of the antral mucosa, but not of the fundic mucosa, was significantly higher after administration of both baclofen and atropine (group 3) than after administration of baclofen alone (group 2). However, in rats that received both baclofen and pirenzepine (group 4) there was no significant difference in labeling index of the antral mucosa compared with rats in group 2. Administration of atropine alone (group 5) or pirenzepine alone (group 6) had no significant influence on the labeling index of either fundic or antral mucosa compared with those of the control group. Table III also shows that there were no significant differences in proliferative zone thickness in the fundic mucosa among these six groups.

At week 52 the antral pH was significantly lower after
administration of baclofen (group 2) compared with that of the control group. The antral pH after treatment with both baclofen and atropine (group 3) or pirenzepine alone (group 4) were significantly higher than that after treatment with baclofen alone (group 2).

**Discussion**

In the present work we found that at week 52 baclofen significantly decreased the incidence of gastric cancers and treatment with both baclofen and atropine significantly attenuated the inhibitory effect of baclofen on gastric carcinogenesis. These findings suggest that a vagal-dependent mechanism is involved in the inhibition of gastric carcinogenesis by baclofen.

Previously we found that prolonged administration of atropine resulted in a significant increase in the number of gastric cancers per rat, but not their incidence (20). However, promotion of MNNG carcinogenesis by atropine was not reproduced in the present study. The reason for this discrepancy may be the fact that atropine was used in the depot form as a suspension in olive oil in the previous study, but in the present study we used atropine in 0.9% NaCl.

Baclofen alone caused a significant decrease in the number of tumor-bearing rats, but did not have any effect on the number of tumors per tumor-bearing rat. In the group given baclofen in combination with atropine the incidence of gastric tumors was similar to the control group, but the number of gastric tumors per tumor-bearing rat was lower than that in the other groups. However, the reason was still unclear.

The autonomic nervous system may be intimately involved in the development of cancers in various organs (17). Gurkalo and Volfson (18) suggested that pharmacological compounds that enhance the activity of sympathetic nerves stimulate carcinogenesis, whereas those that enhance cholinergic activity inhibit carcinogenesis. Acetylcholine has been recognized as a neurotransmitter and its capacity to influence the development of gastrointestinal cancers has been demonstrated. Gurkalo and Volfson (18,19) found that nicotine inhibited and atropine stimulated the carcinogenic effect of MNNG. Previously we found that prolonged administration of the acetylcholinesterase inhibitor neostigmine after MNNG treatment resulted in a significantly decreased incidence of MNNG-induced gastric cancers and that prolonged blockade of cholinoreceptor activity by atropine caused a significant increase in the number of gastric cancers (20).

The existence of multiple muscarinic receptor subtypes is now well established. Originally muscarinic receptors exhibiting a high affinity for pirenzepine were referred to as M2 receptors and receptors with a low affinity for pirenzepine were referred to as M3 receptors (21). Biologically the M1 and M2 muscarinic receptors behave differently. Garrigues et al. (10) examined the effect of atropine and pirenzepine on cholecystokinin-induced gallbladder emptying and found that atropine significantly reduced both ejection period and ejection fraction, whereas pirenzepine reduced ejection period, but had no effect on ejection fraction. Therefore, the findings suggest that M2 receptors, but not M1 receptors, are involved in this response. In the present work we found that administration of both baclofen and atropine significantly attenuated inhibition of gastric carcinogenesis by baclofen, but administration of both baclofen and pirenzepine had no significant effect on such inhibition. These findings suggest that M2 receptors, but not M1 receptors, are involved in inhibition of gastric carcinogenesis by baclofen.

The M2 receptors are heterogeneous. Receptors in the atrium which are selectively blocked by AF-DX 116 (22) and methoctramine (23) are referred to as M2alpha, whereas those on glands and smooth muscle which are sensitive to the antagonists 4-DAMP (24) are referred to as M2beta. It is still unknown which subtype of M2 receptor is involved in inhibition of gastric carcinogenesis by baclofen.

In the present work we found that treatment with both baclofen and atropine significantly attenuated the inhibitory effect of baclofen on gastric carcinogenesis, but treatment with both baclofen and pirenzepine had no significant influence on gastric carcinogenesis. These results suggest that the inhibition by baclofen of gastric carcinogenesis is mediated through muscarinic receptors and M2 receptors, but not M1 receptors, are involved in this response.

**References**


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