Troglitazone, a Ligand for Peroxisome Proliferator-activated Receptor γ, Inhibits Chemically-induced Aberrant Crypt Foci in Rats

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The biological roles of peroxisome proliferator-activated receptors (PPARs) in various diseases, including inflammation and cancer, have been highlighted recently. Although PPARγ ligand is suspected to play an important role in carcinogenesis, its effects on colon tumorigenesis remain undetermined. The present time-course study was conducted to investigate possible modifying effects of a PPARγ ligand, troglitazone, on the development and growth of aberrant crypt foci (ACF), putative precursor lesions for colon carcinoma, induced by azoxymethane (AOM) or dextran sodium sulfate (DSS) in male F344 rats. Oral troglitazone (10 or 30 mg/kg body weight (b.w.)) significantly reduced AOM (two weekly subcutaneous injections, 20 mg/kg b.w.)-induced ACF. Treatment with troglitazone increased apoptosis and decreased polyamine content and ornithine decarboxylase (ODC) activity in the colonic mucosa of rats treated with AOM. Gastric gavage of troglitazone also inhibited colitis and ACF induced by DSS (1% in drinking water), in conjunction with increased apoptosis and reduced colonic mucosal polyamine level and ODC activity. Our results suggest that troglitazone, a synthetic PPARγ ligand, can inhibit the early stage of colon tumorigenesis with or without colitis.

Key words: Inhibition — Colitis — Aberrant crypt foci — PPARγ ligand — Rat

The physiological and metabolic roles of peroxisome proliferator-activated receptors (PPARs) in various nutritional states and diseases including inflammation and cancer have been highlighted in recent years [for reviews see Refs. 1–4]. In particular, interest in PPARs markedly increased when these receptors were found to be directly activated by a number of medically relevant compounds.41

PPARs are members of the nuclear hormone receptor superfamily and are thought to be involved in the development of colorectal cancer.31 PPARγ is expressed predominantly in adipose tissue and large intestine,60 and its agonists play significant roles in diabetes and obesity. In combination with retinoid X receptor (RXR) α, a 9-cis-retinoic acid receptor, PPARγ forms a heterodimer, which activates gene transcription and mediates terminal differentiation of adipocytes.71 Although there are three possible splice variants (PPARγ1, γ2 and γ3) in PPARγ, no physiological differences have been reported among the isoforms.25 Besides the synthetic ligands, PPARγ can be activated by endogenous ligands, including fatty acids and eicosanoids, such as 15-deoxy-Δ12,14-prostaglandin J2 (15-deoxy-Δ12,14-PGJ2),8 which exhibits antitumor activity.9 Activation of PPARγ leads to cell differentiation and apoptosis.23 PPARγ also induces growth arrest by regulating cell-cycle withdrawal.10 These findings suggest that PPARγ may play a role in cancer development.79 In fact, PPARγ ligands induce growth arrest and differentiation of human colon cancer cells,11, 12 which express PPARγ12, 13. In addition, PPARs, including PPARγ, play certain roles in inflammation.19 PPARγ ligands have been reported to significantly inhibit chemically induced colitis in mice.24 However, oral administration of troglitazone or rosiglitazone increased the frequency of large, but not small, bowel polyps in APCmin/+ mice.15, 16

Oxidative stress may be involved in either the initiation or promotion stage of colon carcinogenesis.27 Chronic colitis including ulcerative colitis (UC) under oxidative stress is reported to increase the frequency of colon cancer in rodents and humans.18, 19 In addition, acute inflammation increases mutagenic oxidative DNA damage.28 Thus, inflammation may be important among the factors involved in oxidative events leading to DNA damage in colorectal epithelium.20 Dextran sodium sulfate (DSS) has been extensively studied as an agent that induces both acute and chronic colonic inflammations, depending on the dose and duration of treatment.23 Long-term administration of DSS is known to induce colorectal cancer while short-term exposure in rats results in the appearance of aberrant crypt foci (ACF), which are putative precursor lesions for colon adenocarcinoma.25 Thus, the DSS-
induced colitis model is useful for studying colitis-associated colorectal neoplasia.\(^\text{19, 26)}\)

In the present study, we evaluated the modifying effects of PPAR\(\gamma\) ligand troglitazone on azoxymethane (AOM) or DSS-induced ACF in rats. Furthermore, we measured polyamine contents and ornithine decarboxylase (ODC) activity in colonic mucosa, since certain chemopreventive agents exert their inhibitory action by controlling cell proliferation in target tissues\(^\text{27)}\) and these biomarkers are known to be modified in colitis.\(^\text{28)}\)

**MATERIALS AND METHODS**

**Animals, chemicals, and diets** Male F344 rats (Shizuoka Laboratory Animal Center, Shizuoka) aged 4 weeks were used. The animals were maintained in the Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. All animals were housed in plastic cages (three or four rats/cage) with free access to drinking water and a basal diet, CE-2 (CLEA Japan Inc., Tokyo), under controlled conditions of humidity (50±10%), light (12-h light/dark cycle) and temperature (23±2°C). They were quarantined for 7 days then randomized according to body weight into experimental and control groups. DSS, with a molecular weight of 40 000, was purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). For induction of colitis, DSS was dissolved in water at a concentration of 1% (w/v). AOM for ACF induction was purchased from Sigma Chemical Co. (St. Louis, MO). Powdered CE-2 diet was used as basal diet throughout the study. Troglitazone, a PPAR\(\gamma\) ligand, was kindly supplied by Sankyo Co. (Tokyo).

**Experimental procedure** A total of 78 male F344 rats were divided into eight experimental and control groups (Fig. 1). Groups 1 through 3 were treated with AOM by two weekly subcutaneous injections (20 mg/kg body weight (b.w.)), one week after the commencement of the experiment. Groups 4 through 6 were given drinking water containing 1% DSS \(\text{ad libitum}\) for 7 days, one week after the commencement of the experiment. Groups 4 through 6 also received troglitazone (10 mg/kg b.w. for groups 2 and 5 or 30 mg/kg b.w. for groups 3 and 6) suspended in 0.75% methyl cellulose containing normal physiological solution every other day, 3 times/week for 3 weeks, starting 24 h after the commencement of the study. Group 7 was given troglitazone (30 mg/kg b.w.) alone. Group 8 consisted of untreated rats. Three or four rats of each group were sacrificed on weeks 4, 8, and 12 to count ACF. The duration of the experiment protocol was 12 weeks. At sacrifice, the colon was flushed with saline, dissected out, cut open longitudinally along the main axis, and washed with saline.

**ACF count** The lower two-thirds of the large intestine was fixed in 10% buffered formalin for at least 24 h, followed by ACF counting. Fixed colons were dipped in a 0.5% solution of methylene blue in distilled water for 30 s, briefly washed with distilled water, and placed on a microscope slide with the mucosal surface facing upward. Using a light microscope at a magnification of \(\times40\), ACF were distinguished from the surrounding “normal-appearing” crypts by their large size.\(^\text{25)}\) Histological examination was also performed using paraffin-embedded sections after hematoxylin and eosin staining.

**Polyamine content and ODC assay** The remaining upper one-third of the large intestine was used for polyamine and ODC assay after scraping the mucosa using a stainless steel disposable microtome blade (S35; Feather Safety Razor Co., Osaka).\(^\text{29, 30)}\) Total protein concentration was determined using the Bradford protocol.\(^\text{31)}\)

**Measurement of apoptotic cryptal cells** At week 12, the apoptotic index was estimated by counting the number of nuclei with apoptotic bodies in non-lesional crypts. Apoptosis is characterized by DNA fragmentation and cleavage into 180–200-bp internucleosomal-sized fragments. In general, the appearance of a “ladder” of nucleosomal-sized fragments on agarose gel electrophoresis is used as a hallmark of apoptosis. However, a ladder of DNA fragments is also associated with necrosis in some types of cells. The most commonly encountered histological manifestation of apoptosis is the presence of apoptotic bodies. Therefore, in the present study, we counted the number of apoptotic nuclei with apoptotic bodies in non-lesional colonic crypts. To identify apoptotic cryptal cells, sections were stained with Feulgen/fast green and the number of apoptotic cells was quantified using a light microscope (\(\times40\)). Apoptotic cells containing apoptotic bodies in their nuclei also showed cell shrinkage and nuclear condensation on
hematoxylin and eosin-stained histological sections. The apoptotic index, which represents the percentage of cells exhibiting apoptosis, was determined for each sample, by counting at least 20 crypts. Samples were scored by a single investigator (T.T.) who was blinded to the treatment protocol.

**Statistical analysis** All data are expressed as mean±SD. Differences between groups were examined for statistical significance using Student’s t test or Welch’s t test. A P value less than 0.05 denoted the presence of a statistically significant difference.

**RESULTS**

**General observations** As illustrated in Fig. 2, gains in b.w. during the study were comparable in all groups, although the mean b.w. of group 4 (DSS) at week 4 was significantly lower (P<0.01) than that of group 8 (untreated). Thus, all animals well tolerated the allocated treatments. At week 4, DSS treatment (group 4) caused severe colitis with ulceration affecting about 50% of the distal large intestine. However, rats in other groups, including groups 5 (DSS+10 mg troglitazone) and 6 (DSS+30 mg troglitazone) did not develop such colitis. Histological examination revealed no significant pathological findings indicative of troglitazone toxicity in other organs (liver and kidney).

**ACF count** With regard to ACF formation, AOM exposure resulted in the appearance of 83±6 ACF/colon at week 4, 127±13 ACF/colon at week 8, 108±21 ACF/colon at week 12. As indicated in Fig. 3, treatment with troglitazone significantly reduced the frequency of ACF at all sacrifice time points: 54±7 ACF/colon at week 4 (P<0.05), 62±13 ACF/colon at week 8 (P<0.005), and 49±12 ACF/colon at week 12 (P<0.005) by 10 mg/kg b.w. troglitazone; and 51±7 ACF/colon at week 4 (P<0.005), 39±5 ACF/colon at week 8 (P<0.001), and 26±6 ACF/colon at week 12 (P<0.005) by 30 mg/kg b.w. troglitazone. Similarly, administration of troglitazone reduced the number of aberrant crypts (ACs) per focus, with a significant difference between groups 1 (AOM) and 3 (AOM+30 mg/kg b.w. troglitazone) was noted at week
8: 2.82±0.16 for group 1 vs. 2.05±0.16 for group 3 (P<0.005). Furthermore, the number of ACF with more than 4 ACs was decreased by administration of troglitazone by gastric gavage, and significant differences were observed at week 12: 27±11 in group 1 vs. 10±8 in group 2 (P<0.05) or 5±3 group 3 (P<0.05). The frequency of ACF/colon in rats treated with DSS (group 4: 31±10 at week 4; 35±13 at week 8; 51±11 at week 12) was less than that of rats treated with AOM (group 1: 83±6 at week 4; 127±13 at week 8; 108±21 at week 12) at each sacrifice time point (Fig. 4). The mean numbers of ACF/colon in groups 5 (DSS+10 mg/kg b.w. troglitazone) and 6 (DSS+30 mg/kg b.w. troglitazone) were less than that of group 4. A significant reduction was noted at week 12: 51±11 in group 1 vs. 24±13 in group 5 (P<0.001) or 10±3 in group 6 (P<0.05). In addition, the number of ACs per focus in rats treated with DSS and troglitazone (groups 5 and 6) was lower than in those treated with DSS alone (group 1) at weeks 4, 8, and 12. These values were significantly lower in groups 2 (1.97±0.09, P<0.001) and 3 (1.56±0.37, P<0.05) at week 12 than in group 1 (2.58±0.08). In rats given DSS (groups 4–6), a few ACF with more than 4 ACs were found.

**Polyamine content** Table I shows the polyamine contents of colonic mucosa of each treatment group at each sacrifice point. AOM or DSS treatment significantly increased

### Table I. Polyamine Content of the Colonic Mucosa

| Group | Treatment | Wk 4 | Wk 8 | Wk 12 |
|-------|-----------|-----|-----|------|
| 1     | AOM       | 58±8⁺⁻ (3) | 38±3⁻⁻ (3) | 27±5⁻⁻ (4) |
| 2     | AOM+troglitazone (10 mg/kg b.w.) | 27±3⁻⁻ (3) | 21±8⁻⁻ (3) | 21±7 (4) |
| 3     | AOM+troglitazone (30 mg/kg b.w.) | 28±2⁻⁻ (3) | 27±5⁻⁻ (3) | 21±8 (4) |
| 4     | DSS       | 217±17⁻⁻ (3) | 118±12⁻⁻ (3) | 36±3⁻⁻ (4) |
| 5     | DSS+troglitazone (10 mg/kg b.w.) | 168±33 (3) | 124±15 (3) | 38±8 (4) |
| 6     | DSS+troglitazone (30 mg/kg b.w.) | 142±8⁻⁻ (3) | 120±16 (3) | 28±6 (4) |
| 7     | Troglitazone (30 mg/kg b.w.) | 14±3 (3) | 17±2 (3) | 13±1 (3) |
| 8     | None      | 11±2 (3) | 14±2 (3) | 13±3 (3) |

All values are mean±SD.
Numbers in parentheses represent numbers of rats examined.

*a, b, g* Significantly different from group 8 by Student’s *t* test or Welch’s *t* test (*a* P<0.001, *b* P<0.01, and *g* P<0.005).

*c, d* Significantly different from group 4 by Student’s *t* test (*c* P<0.001 and *d* P<0.05).

*e, f* Significantly different from group 1 by Student’s *t* test (*e* P<0.005 and *f* P<0.05).

*h* Significantly different from group 4 by Student’s *t* test (P<0.005).

### Table II. ODC Activity of the Colonic Mucosa

| Group | Treatment | ODC activity (pmol/14 CO₂/h/mg protein) |
|-------|-----------|-----------------------------------------|
|       |           | Wk 4 | Wk 8 | Wk 12 |
| 1     | AOM       | 92±10⁻⁻ (3) | 63±10⁻⁻ (3) | 58±8⁻⁻ (4) |
| 2     | AOM+troglitazone (10 mg/kg b.w.) | 82±4 (3) | 38±2 (3) | 24±3⁻⁻ (4) |
| 3     | AOM+troglitazone (30 mg/kg b.w.) | 63±9⁻⁻ (3) | 32±4⁻⁻ (3) | 21±7⁻⁻ (4) |
| 4     | DSS       | 408±23⁻⁻ (3) | 91±5⁻⁻ (3) | 68±6⁻⁻ (4) |
| 5     | DSS+troglitazone (10 mg/kg b.w.) | 300±37⁻⁻ (3) | 84±4 (3) | 66±4 (4) |
| 6     | DSS+troglitazone (30 mg/kg b.w.) | 165±41⁻⁻ (3) | 57±4⁻⁻ (3) | 47±5⁻⁻ (4) |
| 7     | Troglitazone (30 mg/kg b.w.) | 23±2 (3) | 8±3 (3) | 9±1 (3) |
| 8     | None      | 20±1 (3) | 5±2 (3) | 8±1 (3) |

All values are mean±SD.
Numbers in parentheses represent numbers of rats examined.

*a, c, e, i* Significantly different from group 8 by Student’s *t* test or Welch’s *t* test (*a* P<0.01, *c* P<0.02, *e* P<0.002, and *i* P<0.001).

*b, d* Significantly different from group 4 by Student’s *t* test (*b* P<0.001 and *d* P<0.005).

*f, g, h* Significantly different from group 1 by Student’s *t* test (*f* P<0.001, *g* P<0.05, and *h* P<0.01).

*j, k, l* Significantly different from group 4 by Student’s *t* test (*j* P<0.02, *k* P<0.001, and *l* P<0.002).
polyamine content; the increase by DSS administration was much greater than that by AOM at all sacrifice time points. Administration of troglitazone (groups 2 and 3) significantly decreased polyamine levels compared with AOM alone (group 1) at weeks 4 and 8. Troglitazone (30 mg/kg b.w., 142±8) caused a significant reduction in colonic polyamine level compared with that of rats treated with DSS (217±17) only at week 4.

**ODC activity** The results of ODC assay of colonic mucosa at each sacrifice point are summarized in Table II. AOM or DSS treatment significantly increased colonic mucosal ODC activity; the increase induced by DSS administration was much greater than that by AOM at weeks 4 and 8. High-dose troglitazone (group 3) significantly decreased ODC activity compared with AOM alone (group 1) at weeks 4 and 8. At week 12, administration of troglitazone at both doses (groups 2 and 3) significantly lowered ODC activity compared with AOM alone. Troglitazone treatment at both doses significantly reduced colonic ODC activity compared with that of rats treated with DSS at week 4. At weeks 8 and 12, the higher dose of troglitazone significantly lowered ODC activity compared with that of group 4.

**Apoptotic index** Data on apoptotic index in non-lesional crypts are illustrated in Fig. 5. AOM (group 1: 1.58±0.80, P<0.05) or DSS treatment (group 4: 2.22±0.64, P<0.005) significantly increased the apoptotic index compared with that of group 8 (untreated controls, 0.22±0.19). Treatment with troglitazone (10 or 30 mg/kg b.w.) significantly increased the apoptotic index; groups 2 (3.31±0.67, P<0.02) and 3 (4.62±2.19, P<0.05) vs. group 1. The apoptotic indices of groups 5 and 6 were also higher compared with that of group 4, albeit without statistical significance. Troglitazone alone (group 7, 0.20±0.18) did not affect the apoptotic index.

**DISCUSSION**

The main findings of the present study indicate that troglitazone, a PPARγ ligand, inhibited the development and growth of ACF induced by either AOM or DSS. The anti-proliferative action of troglitazone may explain its inhibitory action on AOM-induced development and growth of ACF. As reported by Su et al., troglitazone also inhibited DSS-induced colitis. This anti-inflammatory and/or anti-ulcer activity of troglitazone in large intestines through the modification of trefoil factors in colonic mucosa may contribute to the inhibition of development and growth of colitis-related ACF. Our results support the findings with human colon cancer cell lines and differ somewhat from the effect of PPARγ on the spontaneous development of intestinal tumors, whereas the xenograft model is better suited to the investigation of the antiproliferative action of PPARγ activation in carcinoma cells. However, the data reported, including ours, suggest that the effects of PPARγ on cell cycle, apoptosis, differentiation, and carcinogenesis might depend on the cell type and/or the contingency of mutational events that predispose tissues to cancer development.

Our time-course studies of ACF development and growth indicate that troglitazone has inhibitory and regressive effects. Several investigators have reported that some chemopreventive agents possess such regressive effects on ACF. They postulated induction of apoptosis as well as inhibition of cell proliferation as possible mechanisms of the regressive effects of chemopreventive agents, although no evidence was presented. In the present study, we determined the apoptotic index in non-lesional crypts, since colonic mucosa exposed to a genotoxic (AOM) or non-genotoxic (DSS) colon carcinogen exhibit certain genetic and/or phenotypic changes. Oral administration of troglitazone induced apoptosis in AOM- or DSS-treated colonic mucosa. The induction was statistically significant in rats treated with AOM and troglitazone, but not in those treated with DSS and troglitazone. This may be due to the difference in the type of colon carcinogen or method of administration. It has been reported that troglitazone can induce apoptosis. With respect to cell proliferation, troglitazone treatment in the present study decreased colonic ODC activity at all sacrifice points and polyamine content at weeks 4 and 8. Intestinal mucosa is one of the most rapidly proliferating tissues. Cell division and growth are known to depend on polyamine. Therefore, troglitazone may exert a regressive effect on ACF growth by lowering polyamine content in the colonic mucosa.

In the present study, DSS-induced colitis was inhibited by oral administration of troglitazone. These results con-
fermed the findings of Su et al.\textsuperscript{14} We recently demonstrated that troglitazone decreased the amount of trefoil factors (TFF) 2 in the colonic mucosa.\textsuperscript{32} It is well known that TFF2 and TFF3 protect against gastrointestinal injury.\textsuperscript{30} Although TFF3 but not TFF2 is expressed in normal and pathologically abnormal large intestines,\textsuperscript{31} our results showed that TFF2 was decreased during the healing stage in DSS-induced colitis. Similar results were reported using a different colitis model.\textsuperscript{10} Polyamine contents in colonic mucosa increased soon after ischemic damage and gradually decreased with recovery of intestinal morphology, indicating that polyamine is involved in mucosal repair.\textsuperscript{29} Our results are consistent with those of Kummerlen et al.\textsuperscript{20} Thus, alteration in polyamine level as well as TFF2 content by troglitazone may contribute to its inhibitory action in DSS-induced colitis. Recent studies showed that troglitazone treatment increased the expression of two cyclin-dependent kinase inhibitors, p21\textsuperscript{WAF1/CIP1} and p18\textsuperscript{INK4a}, and reduced cyclin D1 expression, consistent with G1 arrest,\textsuperscript{39} suggesting the involvement of PPAR\textgamma in the cell cycle.

A second form of the cyclooxygenase enzyme, COX-2, is inducible by cytokines and growth factors.\textsuperscript{39} This inducible COX-2 is linked to inflammatory cell types and tissues and is believed to be the target enzyme for the anti-inflammatory activity of nonsteroidal anti-inflammatory drugs (NSAIDs). NSAIDs currently available for clinical use inhibit both COX-1 and COX-2.\textsuperscript{38} Given the correlation between increased COX-2 expression and colonic carcinoma and/or inflammation\textsuperscript{39, 40}, the chemopreventive effects of NSAIDs appears to be mediated, at least in part, by COX inhibition.\textsuperscript{39} In this regard, we have previously demonstrated that indomethacin, a NSAID, inhibits colon tumorigenesis as well as colitis induced by 1-hydroxyanthraquinone.\textsuperscript{41} It is known that some NSAIDs act as peroxisome proliferators,\textsuperscript{42} suggesting that they might also regulate gene expression as a part of their chemopreventive mechanism. Inhibition of colonic inflammation and reduced cell proliferation by PPAR\textgamma ligands might be responsible for their chemopreventive effects on colitis-associated colon carcinogenesis. Damage to DNA by reactive oxygen and/or nitrogen species contributes to inflammatory diseases, including colitis-related colon tumorgenesis.\textsuperscript{43} PPAR\textgamma \textsuperscript{44} involves inflammation control and inhibits inducible nitric oxide synthase.\textsuperscript{45, 46} Several NSAIDs bind to PPAR\textgamma.\textsuperscript{42} Their anti-inflammatory activities might be mediated through inhibition of COX-1 and/or COX-2. PPAR\textgamma is aberrantly expressed in chemically induced rodent colon cancer and in several human colon cancer cell lines.\textsuperscript{13} PPAR\textgamma can be upregulated by treatment with butyrate, which induces differentiation of Caco-2 cells.\textsuperscript{47} Development of colon cancer in humans seems to be commonly associated with mutations in PPAR\textgamma gene.\textsuperscript{48} Lehmann et al.\textsuperscript{42} reported that NSAIDs could bind and activate PPAR\textgamma, providing a molecular basis for the preventive effect of this drug in colon carcinogenesis. Recently, ligands (GW7845 and troglitazone) for PPAR\textgamma were reported to inhibit rat mammary carcinogenesis.\textsuperscript{49, 50} Thus, activation of PPAR\textgamma may play an important role in tumorigenesis. It will be interesting to investigate whether PPAR\textgamma influences COX-2 expression through NF-kB and/or prostaglandins receptors.\textsuperscript{51} Recently, it was shown that PPAR\textgamma\textsuperscript{52} is involved in carcinogenesis in vivo. Also, PPAR\delta was found to be a target of adenomatous polyposis coli (APC) in human colorectal cancer cells.\textsuperscript{53} Thus, additional work is needed to clarify the role of PPARs, including PPAR\gamma, in cancer development, the role played by cofactors, and whether mutations or modulation of expression of coactivators or corepressors could be responsible for PPAR\gamma-dependent tumor formation.

In conclusion, we have demonstrated in the present study that ACF, putative precursor lesions of colonic carcinoma, induced by AOM or DSS, were inhibited by treatment with troglitazone, possibly through modulation of cell proliferation, apoptosis, and/or inflammation in colonic mucosa.

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