Journal of Pharmacological Sciences 143 (2020) 30–38

Contents lists available at ScienceDirect

Journal of Pharmacological Sciences

journal homepage: www.elsevier.com/locate/jphs

Full Paper

The role of nitric oxide in small intestine differs between a single and a consecutive administration of methotrexate to rats

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ARTICLE INFO

Article history:
Received 5 November 2019
Received in revised form
20 January 2020
Accepted 10 February 2020
Available online 20 February 2020

Keywords:
Methotrexate
Chemotherapy-induced mucositis
Nitric oxide
Inflammation

ABSTRACT

The role of nitric oxide (NO) on intestinal mucosal injury induced by single or consecutive administration of methotrexate was investigated in a rodent model. Rats received methotrexate intraperitoneally either as a single administration (50 mg/kg) or as a consecutive administration (12.5 mg/kg/day) for 4 days. N^G-nitro-L-arginine methyl ester (L-NAME) was given subcutaneously to inhibit NO synthase (NOS). Ninety-six hours after the first administration of methotrexate, ileal tissues were collected for analysis. Consecutive administration of methotrexate led to decreased body weight and reduced intake of food and water, which were further worsened by L-NAME. Although a slight mucosal injury resulted from single administration of methotrexate, L-NAME had almost no effect. Consecutive administration of methotrexate caused a significant mucosal injury, which was further worsened by L-NAME. Consecutive, but not single, administration of methotrexate induced mRNA expression of inflammatory cytokines in ileal tissue. Consecutive administration of methotrexate significantly induced constitutive NOS expression in ileal tissue. These results suggest that consecutive administration, rather than single administration, of methotrexate aggravates mucosal injury. Potentiation of constitutive NOS expression by consecutive administration might be one of the main reason to antagonize the intestinal mucosal injury as well as lead to a reduction in rat quality of life.

Introduction

Methotrexate is widely used as a chemotherapeutic agent for leukemia and other malignancies such as malignant lymphoma and sarcoma. Methotrexate affects not only cancer cells but also rapidly proliferating cells such as those found in the gastrointestinal system. Gastrointestinal mucosal damage caused by methotrexate is a major side effect in clinical settings. Many studies using experimental rodent models revealed that the induction of inflammatory responses, that is, the release of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), and the production of nitric oxide (NO) and prostaglandins has become apparent as the mechanism of methotrexate-induced mucosal damage.[1–3]

NO plays a variety of important roles in many physiological and pathophysiological processes. NO is produced from L-arginine by three distinct isoforms of NO synthase (NOS): endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). Beacuse eNOS and nNOS are constitutively expressed, NO derived from constitutive NOS (cNOS) is thought to be involved in physiological roles. However, overproduction of NO derived from iNOS, which is induced by inflammatory cytokines and bacterial endotoxin in the gastrointestinal tract, triggers tissue injury and is therefore thought to have a pathophysiological role.

Our previous study showed that single administration of methotrexate at a dose of 50 mg/kg altered the gastrointestinal 5-hydroxytryptamine (5-HT) metabolism associated with hyperplasia of the mucosal enterochromaffin cells in the intestinal tracts of

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https://doi.org/10.1016/j.jphs.2020.02.006

rats.[4] This single administration of methotrexate significantly induced iNOS expression, but not cNOS expression, in the ileal tissue.[5] We found that NO derived from the induced iNOS is essential for methotrexate-induced 5-HT metabolism, because N^G-nitro-L-arginine methyl ester (L-NAME), a non-specific NOS inhibitor, significantly inhibited 5-HT metabolism.[5] Contrary to many reports showing that consecutive administration of methotrexate, even at lower doses, causes severe gastrointestinal mucosal damage in rats,[1–3,6] in our study no severe histologic injury due to the methotrexate was observed in the ileal tissue at 96 h after administration.[4,5]

We also recently reported that single administration of methotrexate 50 mg/kg to rats potentiates endogenous glucagon-like peptide-2 (GLP-2) dynamics via hyperplasia of L-cells, which contain GLP-2, in rat ileal tissue at 24 h after drug administration.[7] Because intestinal GLP-2 maintains homeostasis, including proliferation of crypt cells, barrier function, nutrient absorption, reduction of epithelial apoptosis, motility, anti-inflammation, and blood flow,[8] the potentiation of GLP-2 dynamics by methotrexate may have a role in tolerance to gastrointestinal injury by counteracting the mucosal injury induced by methotrexate itself.[7]

From these observations, we hypothesized that consecutive administration, rather than dosage, is one of the main reasons that methotrexate causes gastrointestinal mucosal injury. To elucidate the above assumption, we compared the gastrointestinal mucosal injury in rats triggered by a single administration of methotrexate at a dose of 50 mg/kg and by administration of methotrexate 12.5 mg/kg for 4 consecutive days (with an equivalent final total dose of 50 mg/kg). Furthermore, we examined the role of NO and GLP-2 in gastrointestinal mucosal injury with single and consecutive administration of methotrexate.

Materials and methods

Drugs and reagents

Methotrexate was obtained from Pfizer Inc. (New York, NY, USA). L-NAME was purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). The other reagents used in this study were of special grade and purchased from local suppliers unless otherwise noted.

Animals

Male Wistar rats weighing 180–200 g were purchased from Japan SLC, Inc. (Shizuoka, Japan). All animals were housed under constant conditions at a room temperature of 22 ± 2 °C and humidity of 50 ± 10% with a regular 12-h light (8:00–20:00)-dark (20:00–8:00) cycle and free access to water and food. All animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals by the Animal Research Committee of Health Sciences University of Hokkaido.

Drug treatment and measurement of food/water intake

Rats were randomly assigned to two groups: single administration of methotrexate group and consecutive administration of methotrexate group (Fig. 1A and B). Each group was further divided into four sub-groups: basal, L-NAME alone, methotrexate alone, and methotrexate + L-NAME. The drug schedule for the single administration group is shown in Fig. 1A. Rats were intraperitoneally injected with a single administration of methotrexate (50 mg/kg), or physiological saline. In experiments using L-NAME, rats were subcutaneously injected with 20 mg/kg L-NAME, 10 min before methotrexate or physiological saline administration, and then again every 24 h for three consecutive days (Fig. 1A). The drug schedule for the consecutive administration group is shown in Fig. 1B. Rats were intraperitoneally injected with methotrexate (12.5 mg/kg), or physiological saline for four consecutive days. Thus, the total dose of methotrexate finally administered to each rat was 50 mg/kg. In experiments using L-NAME, rats were subcutaneously injected with 20 mg/kg L-NAME, 10 min before first administration of methotrexate or physiological saline, and then again every 24 h for three consecutive days (Fig. 1B).

To measure food and water intake, the rats were placed in individual cages and allowed access to food during the 6-day adaptation period before drug administration. Food and water intake

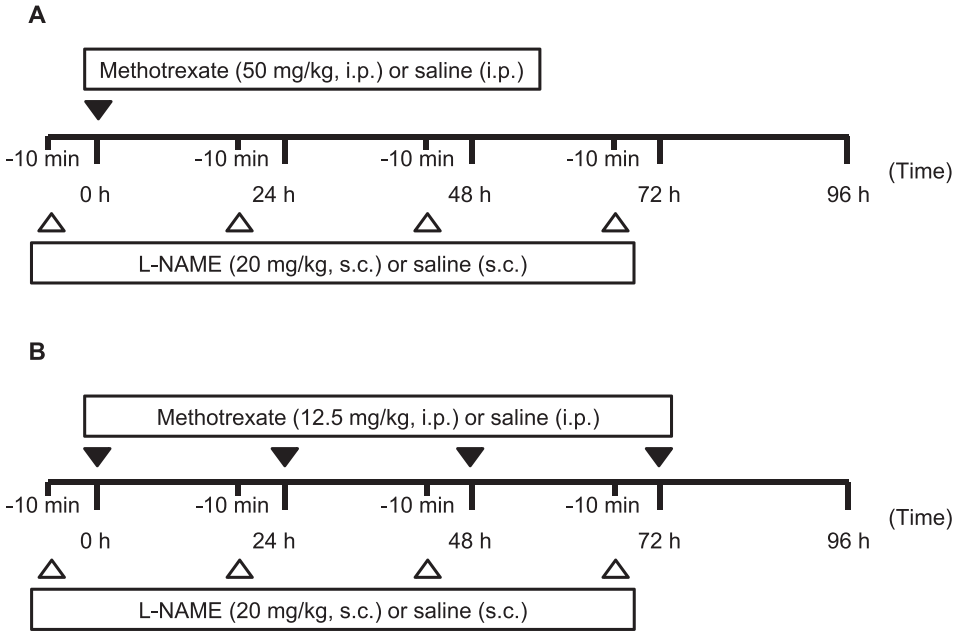

Fig. 1. Schedule for the single (A) and consecutive (B) administration of methotrexate to rats.

and body weight were measured every 24 h until the end of the experiment (96 h after first injection of methotrexate).

At 96 h after first injection of methotrexate, the rats were euthanized by exsanguination under light anesthesia using isoflurane. Ileal tissues were dissected in approximately 3 cm long segments and frozen rapidly in liquid nitrogen and stored until further analysis. Fresh ileal tissues were also fixed with 4% paraformaldehyde in 0.1 M phosphate buffer and embedded in paraffin for immunohistochemical analysis.

Immunohistochemical analysis

For histopathological analysis, specimens were stained with hematoxylin and eosin and blindly examined under light microscopy. Assessment of villus and crypt integrity, inflammatory cell influx, vacuolization, and edema were made according to the following scale: normal = 0; mild damage = 1; moderate damage = 2, and severe damage = 3.[1]

The expression and localization of myeloperoxidase and GLP-2 in rat ileum were analyzed by immunohistochemistry, as previously described.[7,9] The myeloperoxidase-immunopositive area stained in brown within 1920 x 1080 pixels were calculated in three different fields of view by using ImageJ (ver. 2.0.0) and a mean area was determined for each specimen. The number of anti-GLP-2 antibody-positive cells was counted under a light microscope, as previously described.[7]

RNA extraction and reverse transcription-polymerase chain reaction

Messenger RNA expression was quantified by real-time RT-PCR using a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) and PrimeScript™ One Step RT-PCR kit (Takara Bio, Shiga, Japan), as previously described.[7] The primers for proglucagon and glyceraldehyde 3-phosphate dehydrogenase were described previously.[7] The sense primer for eNOS was 5′ CATCACCTACGATACCCTCAG 3′ and the antisense primer was 5′ CGGCTCTGTAACTTCCTTG 3′. The sense primer for nNOS was 5′ TTTCTGTCCGTCTCTTCAAACGCAAAGTGG 3′ and the antisense primer was 5′ GACATCATTCTCGCAGTCAA 3′. The sense primer for iNOS was 5′ TCGAGCCCTGGAAGACCCACATCTG 3′ and the antisense primer was 5′ GTTGTTCCTCTTCCAAGGTGTTTGCCTTAT 3′. The sense primer for TNF-α was 5′ GTGATCGGTCCCAACAAGGA 3′ and the antisense primer was 5′ AGGGTCTGGGCCATGGAA 3′. The sense primer for IL-1β was 5′ CACCTCTCAAGCAGAGCACAGA 3′ and the antisense primer was 5′ ACGGGTTCCATGGTGAAGTC 3′. The sense primer for IL-6 was 5′ TCCTACCCCAACTTCCAATGCTC 3′ and the antisense primer was 5′ TTGGATGGTCTTGGTCCTTAGCC 3′. The PCR products were calculated relative to glyceraldehyde 3-phosphate dehydrogenase.

Western blot analysis

NOS protein expression was analyzed via Western blot as described by Takano et al.[5] iNOS (130 kDa), nNOS (155 kDa), and eNOS (140 kDa) bands corresponding to the appropriate positive controls were analyzed by densitometry and calculated relative to β-actin. Protein content was determined using a BCA protein assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Statistical analysis

Statistical analysis of the results was performed using either the *F*-test followed by Student's *t*-test or Welch's *t*-test, or one-way or two-way analysis of variance (ANOVA) with a post-hoc Tukey's test for multiple comparison, or Kruskal–Wallis and Dunn's test for comparison for histopathological scores. P values < 0.05 were considered significant.

Results

Changes in body weight and food/water intake

We first examined the effect of consecutive 12.5-mg/kg administration of methotrexate for four days on body weight and food/water intake. As shown in Fig. 2, consecutive administration of methotrexate caused significant body weight loss and suppression of food/water intake in a time-dependent manner. When L-NAME was co-treated with methotrexate, methotrexate-induced body weight loss and anorexia was further significantly potentiated. L-NAME alone had no significant effect on body weight and food/water intake.

Morphological observation

Consistent with our previous results,[4,5] morphologic observation of the ileal tissue at 96 h after a single 50-mg/kg administration of methotrexate revealed mild, but not severe, mucositis (Fig. 3A, Table 1). L-NAME co-treated with methotrexate had no significant effect on histological changes compared with methotrexate alone

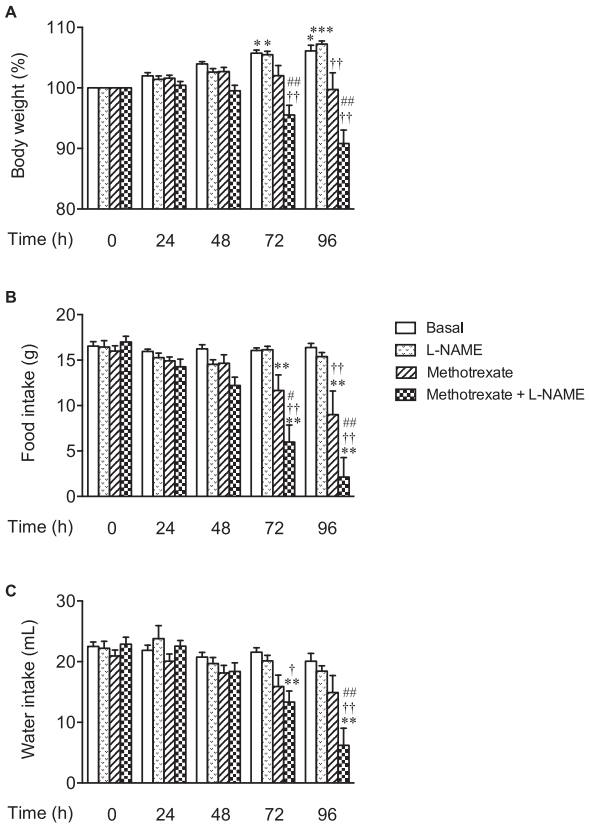

Fig. 2. Effects of L-NAME on consecutive administration of methotrexate-induced change in body weight and food/water intake in rats. After the first administration of physiological saline (basal) or methotrexate, body weight (A) was measured every 24 h up to 96 h, and expressed as a percentage with 100% representing Time 0 h. Food (B) and water (C) intake were also measured every 24 h up to 96 h, and are shown as cumulative daily amounts (g or mL) during the 24-h periods up to 96 h. Each column represents the mean ± S.E. (n = 6). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus Time 0 h; †$p < 0.05$ and ††$p < 0.01$ versus basal; #$p < 0.05$ and ##$p < 0.01$ versus methotrexate.

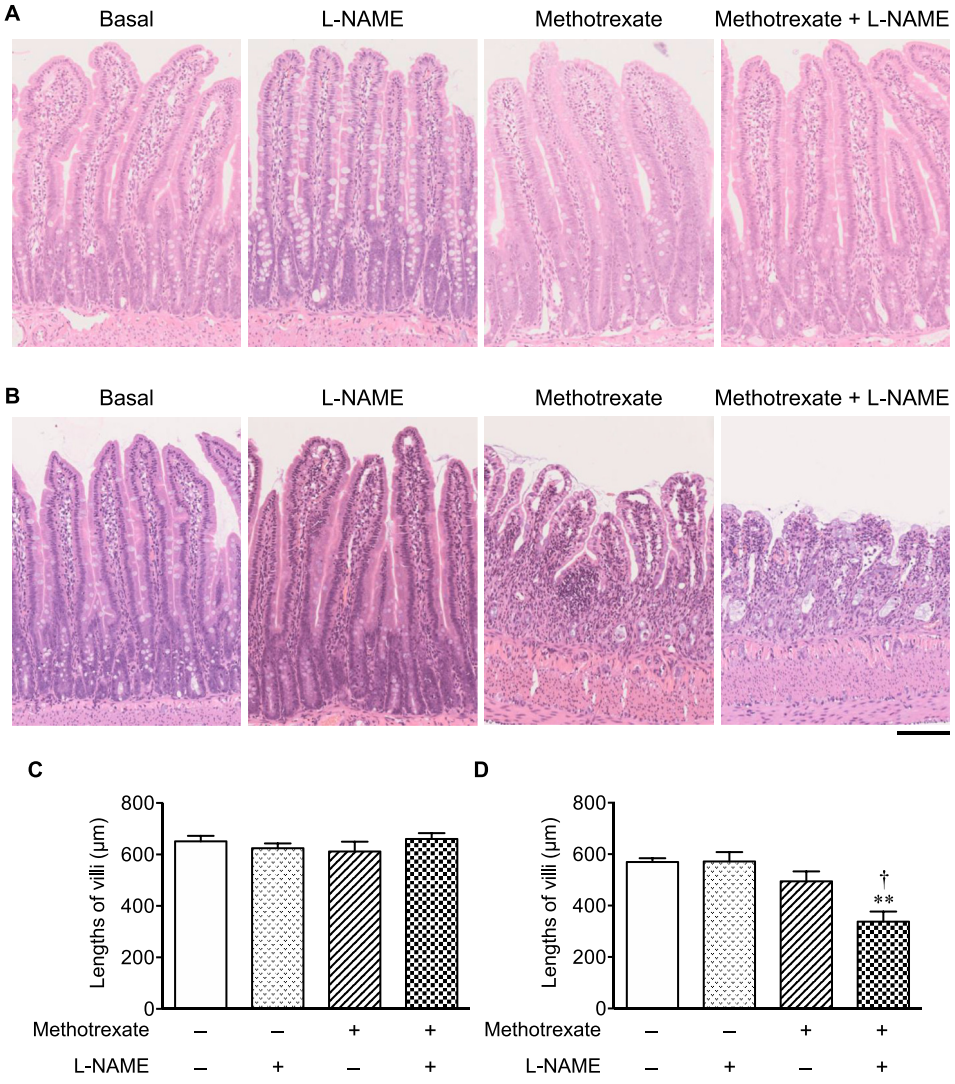

Fig. 3. Hematoxylin-eosin stained sections from rat ileal mucosa. A and C: The single administration group. B and D: The consecutive administration group. A and B: Representative images of Hematoxylin-eosin stained sections. Scale bar = 100 μm. C and D: Lengths of villi. Each column represents the mean ± S.E. (n = 5 each for C and 6 each for D). $^{**}p < 0.01$ versus basal; $\dagger p < 0.05$ versus methotrexate.

Table 1
Effect of methotrexate and L-NAME on histopathological scores of damage to rat intestinal tissue induced by single or consecutive administration of methotrexate.

Group	Median	Range
Single administration		
Basal	0	0–0
Methotrexate	1	1–2*
+ L-NAME	1	1–1
Consecutive administration		
Basal	0	0–1
L-NAME	0	0–1
Methotrexate	2	0–2*
+ L-NAME	2	1–2***

Each value represents results of 4 animals in the single administration group and 8 animals consecutive administration group. *p < 0.05, ***p < 0.001 versus basal level.

(Fig. 3A, Table 1). Also, no villus atrophy was found by single administration of methotrexate with or without L-NAME (Fig. 3C). In contrast, consecutive 12.5-mg/kg administration of methotrexate caused moderate mucositis (Fig. 3B, Table 1). L-NAME further worsened methotrexate-induced mucositis (Fig. 3B, Table 1). In fact, villus atrophy was significantly present when L-NAME was co-treated with consecutive administration of methotrexate (Fig. 3D). None of the tissue samples were scored as grade 3 because neutrophils did not infiltrate the muscle layer.[1]

Inflammatory response

As shown in Fig. 4A and C, a single 50-mg/kg administration of methotrexate had no effect on myeloperoxidase expression in the ileal tissue; L-NAME also had no effect. In contrast, numerous myeloperoxidase-immunopositive area was isolated in the lamina propria of the ileum after consecutive administration of methotrexate, although it was not significant (Fig. 4B and D). When L-NAME was co-treated with methotrexate, myeloperoxidase expression was significantly increased (Fig. 4B and D).

Next, we examined the mRNA expression of representative inflammatory cytokines in the ileal tissue isolated from both single and consecutive administration of methotrexate. Single administration of methotrexate had no effect on the expression of TNF-α, IL-1β, and IL-6 mRNA (Fig. 5A, C, and E). When L-NAME was co-treated with methotrexate, IL-1β mRNA expression was rather

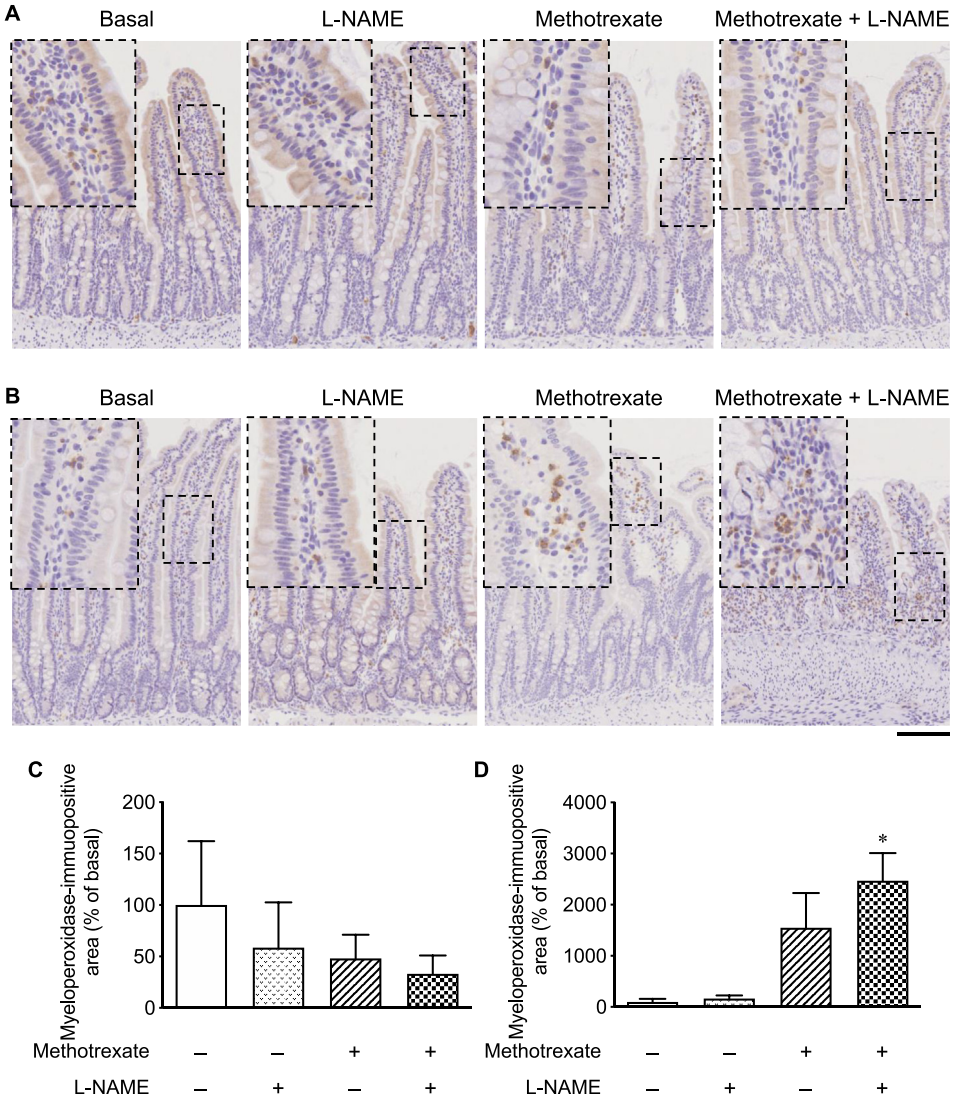

Fig. 4. Effect of methotrexate and L-NAME on myeloperoxidase expression in rat ileal tissue. Ileal tissues were dissected and fixed with 4% paraformaldehyde for immunohistochemical examination with an anti-myeloperoxidase antibody. A: The single administration group. B: The consecutive administration group. Scale bar = 100 μm. The inset in each photo is a higher magnification view of the dotted square. C and D: The myeloperoxidase-immunopositive area. Each column represents the mean ± S.E. (n = 4). *$p < 0.05$ versus basal.

decreased (Fig. 5C). Consecutive administration of methotrexate significantly induced mRNA expression of TNF-α and IL-1β, but not of IL-6 (Fig. 5B, D, and F); L-NAME tended to increase mRNA expression of TNF-α and IL-6, but to decrease IL-1β.

NOS expression

We examined the effect of consecutive 12.5-mg/kg administration of methotrexate for four days on NOS expression in the ileal tissue. As shown in Fig. 6, consecutive administration of methotrexate slightly induced iNOS mRNA expression, although not to a statistically significant level (Fig. 6A). Consecutive administration of methotrexate significantly induced expression of nNOS and eNOS mRNAs (Fig. 6C and E); L-NAME tended to decrease mRNA expressions of nNOS and eNOS. Consecutive administration of methotrexate also significantly increased nNOS and eNOS protein expression (Fig. 6D and F). In addition, iNOS protein expression tended to be increased by consecutive administration of methotrexate ($p = 0.058$ by Welch's *t*-test, Fig. 6B).

GLP-2 expression

The effect of methotrexate on the number of anti-GLP-2 antibody positive cells (i.e., L-cells) was investigated. Both single and consecutive administration of methotrexate significantly increased the number of L-cells (Fig. 7A and B), whereas L-NAME had no significant effect. Single administration of methotrexate in the presence or absence of L-NAME had no effect on the mRNA expression of proglucagon, a precursor of GLP-2 (Fig. 7C). Consecutive administration slightly, but not significantly, increased proglucagon mRNA expression (Fig. 7D). When L-NAME was co-treated with methotrexate, proglucagon mRNA expression significantly increased compared with the basal level (Fig. 7D).

Discussion

In this study, we demonstrated that the mechanisms of anorexia and mucosal injury induced by methotrexate are clearly different between single and consecutive administration, even when the

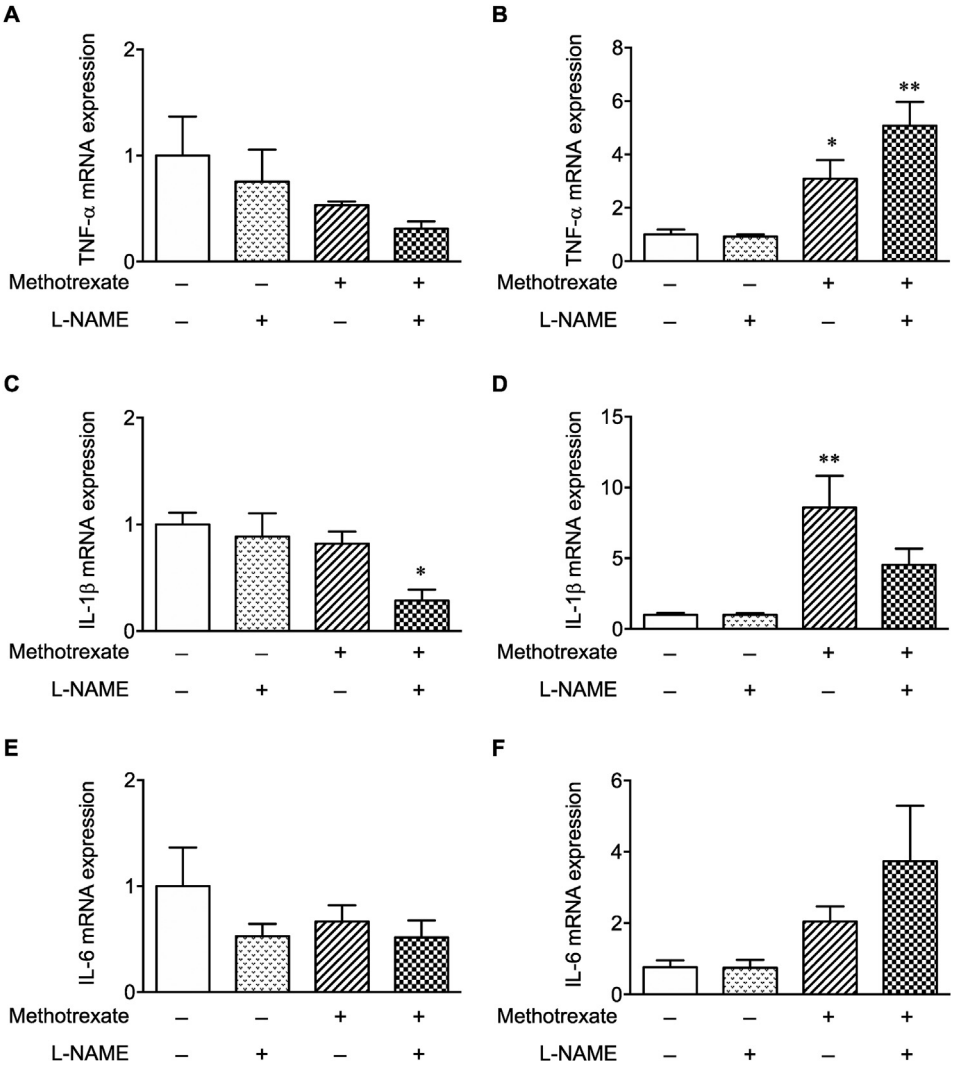

Fig. 5. Effects of methotrexate and L-NAME on the inflammatory cytokine mRNA expression in rat ileal tissue. A, C, and E: The single administration group. B, D, and F: The consecutive administration group. Each column represents the mean ± S.E. (n = 4–5). *$p < 0.05$, **$p < 0.01$ versus basal.

total dose is the same. We previously reported that transient anorexia within 96 h was observed with single administration of 50 mg/kg methotrexate.[5] In the present study, significant anorexia was observed at 72 and 96 h with consecutive administration of methotrexate. Anorexia and body weight loss began gradually from the first day of administration, suggesting that anorexia occurred in a time-dependent manner due to the accumulation of the pharmacological effect of methotrexate. Although L-NAME had no effect on anorexia and body weight loss induced by single administration of methotrexate,[5] the same dose of L-NAME markedly exacerbated anorexia and body weight loss induced by consecutive methotrexate administration. These results suggest that a consecutive administration of methotrexate may change endogenous NO dynamics, and that NO protects against reduced quality of life induced by consecutive administration of methotrexate. We also found that consecutive administration of methotrexate exacerbates gastrointestinal injury compared with single administration. L-NAME worsened methotrexate-induced mucositis, including the villus atrophy, after consecutive, but not single, administration of methotrexate. These observations may be due to gastrointestinal inflammation induced by consecutive administration of methotrexate, because the morphological changes induced by methotrexate correlate with the results of myeloperoxidase staining. In fact, consecutive administration of methotrexate clearly induced the mRNA expression of pro-inflammatory cytokines (i.e., TNF-α and IL-1β), although a single administration of methotrexate had no obvious effect, again suggesting that consecutive administration, rather than the dose, is a more important factor in gastrointestinal injury. It is worth noting that there was no significant effect on the mRNA expression of pro-inflammatory cytokines at 24 h after single administration of 50-mg/kg methotrexate (data not shown). L-NAME tended to change mRNA expressions of these pro-inflammatory cytokines induced by consecutive administration of methotrexate. NO plays an important role in the regulation of IL-10 production, and a major role for IL-10 is to inhibit expression of proinflammatory cytokines.[10,11] Clarifying the intestinal IL-10 alteration by the treatment of methotrexate and L-NAME might be help to identify the mechanisms of the change of the mRNAs in future study. Recent studies have shown that, in rats, anorexia resulting from anti-cancer chemotherapy is caused by the central regulation of food intake, which involves appetite-regulating neuropeptidergic agents and inflammatory mediators in the hypothalamus.[12,13] Therefore, it is also possible that anorexia and body weight loss induced by consecutive administration of

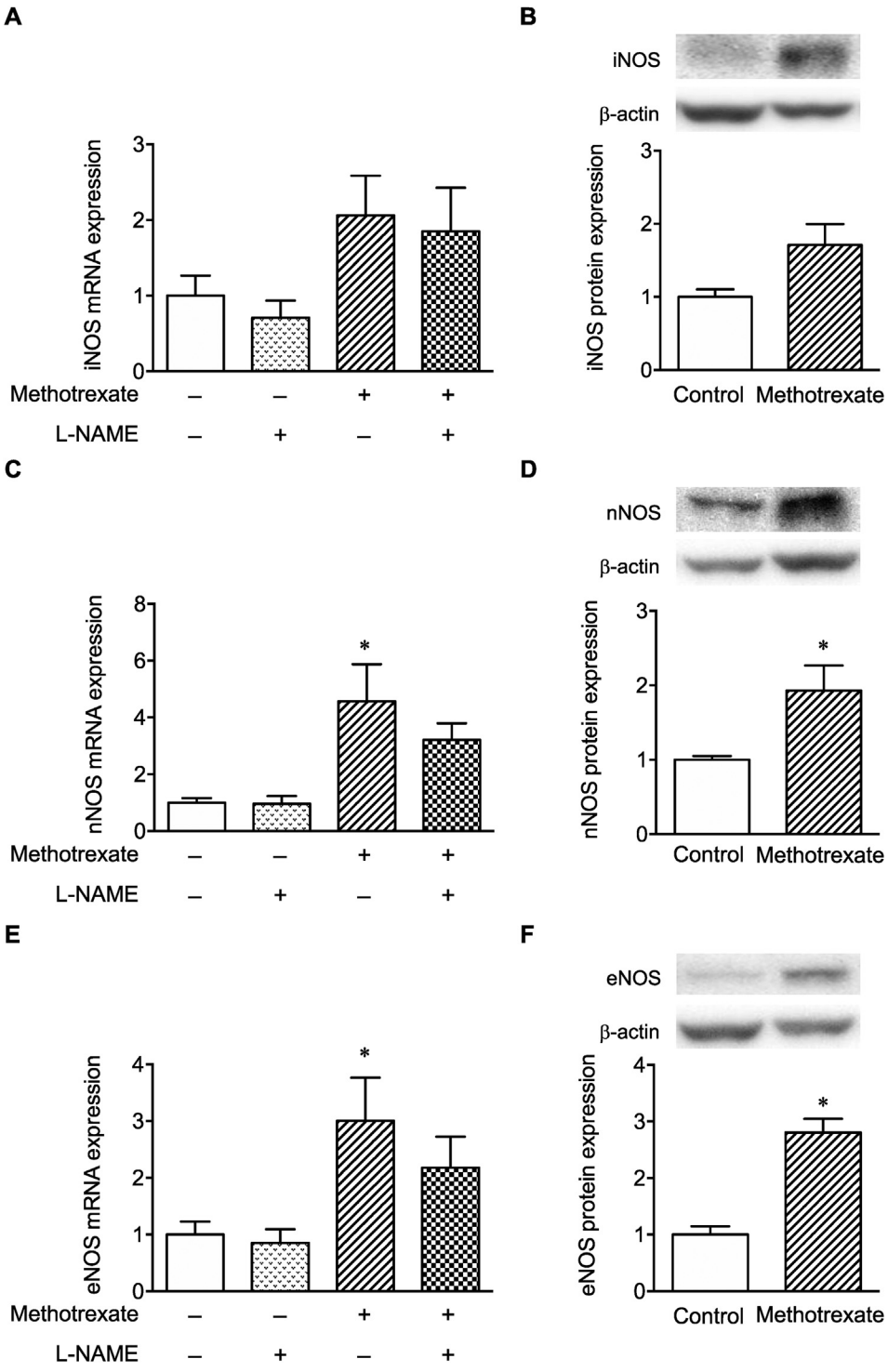

Fig. 6. Effects of consecutive administration of methotrexate on NOS mRNA and protein expression in rat ileal tissue. A, C, and E: NOS mRNA expression. B, D, and F: NOS protein expression. Each column represents the mean ± S.E. (n = 5–6 for A, C, and E; n = 6 for B; n = 4–5 for D; and n = 4 for F). *$p < 0.05$ versus basal.

methotrexate may be due primarily to central regulation and secondarily to mucosal damage and dysfunction.

Prior administration of L-NAME to rats is also reported to exacerbate gastrointestinal injury created by either indomethacin or trinitrobenzene sulphonic acid (TNBS).[14–16] These gastrointestinal injuries are prevented when L-NAME is administered 6 h after administration of indomethacin.[15,16] From these reports, L-NAME has been shown to exhibit a biphasic effect depending on the time of administration; pre-administration of L-NAME inhibits mainly the NO produced by cNOS, while post-administration of L-NAME inhibits mainly the NO produced by iNOS in the intestinal mucosa. In fact, Tanaka et al.[15] reported that prior administration of aminoguanidine, a selective iNOS inhibitor, significantly reduces indomethacin-induced gastrointestinal injury. Therefore, it is speculated that the effect of L-NAME found in this study may be brought about by inhibition of NO production due to cNOS but not

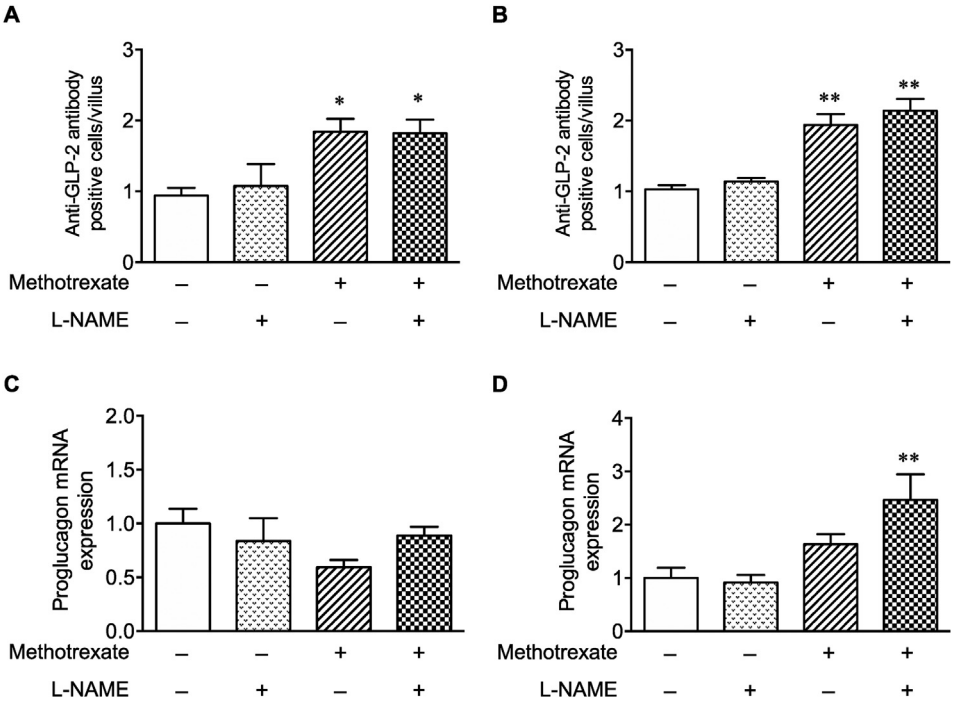

Fig. 7. Effects of methotrexate and L-NAME on the number of anti-GLP-2 antibody positive cells (A and B) and proglucagon mRNA expression (C and D) in rat ileal tissue. A and C: The single administration group. B and D: The consecutive administration group. Each column represents the mean ± S.E. (n = 5–6 for A and C; n = 6 for B; and n = 7 for D). $*p < 0.05$, $**p < 0.01$ versus basal.

iNOS. In contrast to our finding, Leião et al.[1] reported that prior administration of L-NAME prevented villus atrophy and myeloperoxidase activation by administration of methotrexate for 3 consecutive days at a dose of 2.5 mg/kg/day. They also showed that methotrexate caused severe mucosal injury (grade 3). In the present study, none of the tissues were given a grade of 3, despite following the histopathological grading method based on Leião et al.[1] Although at present we have no clear explanation for these discrepancies, it is possible that the types of NOS expression in the rat intestine under the experimental conditions used by Leião et al.[1] may differ from our experimental conditions (e.g., the total administered dose of methotrexate was higher in the present study). In fact, we observed that not only the expression of iNOS but also that of nNOS and eNOS was upregulated by consecutive administration of methotrexate. Since L-NAME is an L-arginine analog that suppress NO from L-arginine by competitive binding to NOS and does not affect NOS expression[17], the tended decrease of cNOS mRNA expression induced by methotrexate might be secondary effects of aggravated mucosal injury by the combination of methotrexate and L-NAME. The mechanisms of cNOS upregulation induced by consecutive administration of methotrexate are still unknown. It was reported that the mRNA expression of not only iNOS but also eNOS in colon, as isolated in both TNBS-treated and dextran sulfate sodium-treated rats, was upregulated compared with control rats.[18] It was also reported that nNOS mRNA expression in guinea pig ileum was increased 60 min after inducing ischemia and in the following 5 min of reperfusion (i.e., ischemia and reperfusion condition).[19] Therefore, upregulating the expression of nNOS and eNOS in intestine by consecutive administration of methotrexate may function primarily as a defensive and compensatory action. In the rat small intestine, most of nNOS and eNOS distribute in the myenteric plexus and the endothelium of submucosa vessels, respectively. Chen et al.[20] showed that there are some cells express nNOS or eNOS in the lamina propria of the villi, and more than 80% of the cells were positive for both nNOS and eNOS. Therefore, it is possible that the consecutive administration of methotrexate promotes the cells co-express nNOS and eNOS in the lamina propria. Further studies are required to determine what types of cells increase cNOS expression and the mechanisms that induce cNOS expression.

Intestinal GLP-2 has multiple physiological roles for maintaining homeostasis.[8] We previously showed that a single administration of 50 mg/kg methotrexate to rats potentiates the number of anti-GLP-2 antibody positive cells (i.e., L-cells) and proglucagon mRNA expression in rat ileal tissue at 24 h after drug administration.[7] In the present study, we found that a single administration of 50 mg/kg methotrexate to rats also potentiated the number of L-cells, but not proglucagon mRNA expression. These results suggest that 50 mg/kg of methotrexate leads to an acute hyperplasia of L-cells within 24 h that persists for at least 96 h after administration. The results also suggest that proglucagon mRNA expression may be transient and have already converged at 96 h after administration. Consecutive administration of methotrexate also potentiated the number of L-cells. Proglucagon mRNA expression was significantly potentiated when L-NAME was co-administered. Although a time-dependent study is required to determine the GLP-2 dynamics, it is speculated that the consecutive administration of methotrexate under our experimental conditions led to delayed hyperplasia of L-cells as a compensatory response to intestinal injury. In fact, Kissow et al.[21] reported that endogenous GLP-2 is upregulated after onset of mucosal injury when 5-fluorouracil, another anti-cancer drug, was administered. Because severe mucosal injury was observed when methotrexate was combined with L-NAME, it is considered that proglucagon mRNA expression was induced most strongly. The relationship between NO and GLP-2 on anti-inflammatory effect is largely unknown. Aykan et al.[22] showed that anti-inflammatory effect of GLP-2 is not directly related to NO based on the formalin-induced paw edema test. Pini et al.[23] reported that

administration of [Gly^2] GLP-2, a GLP-2 analog, increased nNOS expression in cisplatin-treated rats. Further studies are required to determine whether GLP-2 and NO cooperate in the anti-inflammation or independently.

In conclusion, the present study suggests that consecutive administration, rather than single administration, of methotrexate aggravates mucosal injury. Potentiation of cNOS expression by consecutive administration might be one of the main reason to antagonize the intestinal mucosal injury as well as lead to a reduction in rat quality of life.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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