**Gamma-Aminobutyric Acid (GABA) Attenuates Ischemia Reperfusion-Induced Alterations in Intestinal Immunity**

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The aims of this study were to determine the effects of gamma-aminobutyric acid (GABA) on immunoglobulin A (IgA) secretion from Peyer’s patch (PP) cells; to assess rat alpha-defensin-5 (RD-5) expression in the rat small intestine; and to determine the effect of GABA on intestinal ischemia reperfusion (I/R) injury-induced intestinal innate immunity. We found that GABA caused an increase in IgA secretion in the presence and absence of lipopolysaccharide (LPS). Moreover, GABA also significantly increased the mRNA levels of RD-5 and superoxide dismutase (Sod) 1, 3. Intestinal I/R was induced by a 30-min occlusion of the superior mesenteric artery followed by a reperfusion for 60-min. This led to a significant decrease in IgA secretion, and mRNA levels of RD-5 and Sod 1-3 in the ileum. On the other hand, administration of GABA before I/R induction had a significant protective effect against oxidative injury and attenuated the effects on intestinal immunity.

**Key words** gamma-aminobutyric acid; defensin; immunoglobulin A; intestinal ischemia–reperfusion; superoxide dismutase

**MATERIALS AND METHODS**

**Chemicals** GABA was purchased from Tokyo Chemical Industry (Tokyo, Japan). Lipopolysaccharide (LPS) from *Escherichia coli* O111 (produced by phenol extraction) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents were of the highest grade available to us, and used without further purification.

**Animals** Male Wistar rats were obtained from Hokudo (Sapporo, Japan). Rats (weighing 190–220 g and aged 7 weeks) were housed in plastic cages under standard laboratory conditions of constant temperature (23±2°C) with a 12:12-h light–dark cycle, with *ad libitum* access to water and food (standard rodent chow diet). All animal experiments were conducted in accordance with the guidelines of the Care and Use of Laboratory Animals of Hokkaido University. The dosage of GABA was 1–30 mg/kg body weight. GABA was dissolved in water and 1 mL/kg was injected orally by sonde. All animals were euthanized 24 h after GABA administration.

**Peyer’s Patch (PP) Cell Preparation and Determination of IgA Levels** PP cell preparation and determination of IgA levels were performed as previously described.

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Intestinal Ischemia–Reperfusion The rats were divided into 3 groups (sham-operated group, I/R group and I/R+GABA group). Intestinal ischemia–reperfusion were performed as previously described.\textsuperscript{18) }

Quantitative Real-Time PCR Quantitative real-time PCR was performed using a LightCycler 480 II System (Roche Diagnostics GmbH, Mannheim, Germany) with a KAPA SYBR Green Fast qPCR kit (KAPA Biosystems, Boston, MA, U.S.A.) as previously described.\textsuperscript{17) } PCR was performed using primers for rat RD-5 and Sods with the following conditions: 40 cycles at 95°C for 3 s, 95°C for 10 s, 58°C for 20 s, and 72°C for 1 s. Primer sequences are shown in Supplementary Table 1. Levels of PCR products were normalized to the internal reference gene, beta-actin.

Histological Examination Samples of the ileum were taken after reperfusion, and the internal portion was washed with phosphate buffered saline ( PBS ) \textsuperscript{ (−) . The tissue was immediately fixed in 10% buffered formalin. Fixed tissue was embedded in paraffin and sectioned. Slides were stained with hematoxylin and eosin to evaluate intestinal morphology and observed under a light microscope for classification.

Statistical Analysis Statistical significance was performed using Student’s \textit{t}-test and Tukey’s test or Dunnett’s test for \textit{post hoc} analysis. Differences were considered statistically significant at \( p < 0.05 \).

RESULTS AND DISCUSSION To investigate the innate intestinal immuno-stimulatory activity of GABA, we first evaluated the levels of IgA secretion from rat PP cells. We found that GABA increased IgA secretion with and without LPS, in a concentration-dependent manner (Fig. 1A), and had no effect on cell viability (Supplementary Fig. 1A). This shows that GABA activates natural immunity in the small intestine. Next, we measured mRNA levels of RD-5 in the rat ileum in response to GABA (30 mg/kg) and found that RD-5 expression was significantly increased (Fig. 1B) compared to that in the jejunum (Supplementary Fig. 1B). It has been reported that Paneth cells, which are the primary producers of RD-5, are present in higher concentrations in the ileum than in the jejunum.
numbers in the ileum than in the jejunum. Thus, our results correlate with the occurrence of a higher number of Paneth cells in the ileum.

It has been reported that GABA prevents oxidative stress-induced damage. We previously reported that oxidative stress causes a significant decrease in the mRNA levels of human α-defensin 5 (HD-5) in Caco-2 cells, which serve as a model of human intestinal epithelial cells. We therefore hypothesize that the mechanism by which GABA induces these changes is related to the activity of antioxidant enzymes, such as SOD. To test this, we assessed the expression levels of $Sods$ in the rat intestine. We found that the mRNA levels of $Sods\,1$ and $3$ were significantly increased in response to treatment with 30 mg/kg GABA (Fig. 1C). These results correlate with IgA production and RD-5 mRNA levels. On the other hand, we examined the effect of another factor such as Toll-like receptor (TLR) 2, 4, tumor necrosis factor-alpha (TNF-$\alpha$) and transforming growth factor-beta (TGF-$\beta$). GABA had no effect on TLR2, 4, TNF-$\alpha$ and TGF-$\beta$ mRNA level (Supplementary Table 2). Since GABA induced the secretion of IgA as well as changes in the expression levels of mRNA in RD-5 and Sods, we speculate that GABA attenuates oxidative stress-induced alterations in innate immunity.

Intestinal I/R occurs in a number of clinical settings, including small intestine transplant, intestinal surgery, circulatory shock, and strangulation ileus. It has been shown that the small intestine is highly sensitive to I/R injury. Reactive oxygen species (ROS) play an important role in acute intestinal I/R injury. ROS induce membrane lipid peroxidation, which is accompanied by a loss of intestinal barrier function. We previously reported that intestinal I/R injury causes an increase in malondialdehyde levels. Therefore, in the present study, we used a rat model of I/R. IgA secretion from rat PP cells and RD-5 mRNA levels in the rat ileum were significantly decreased compared with the sham-operated group (Figs. 2A, B). Moreover, $Sods$ mRNA levels were also significantly decreased in the rat ileum (Fig. 2C). These results suggest that the ROS-induced reduction in innate immunity is associated with mRNA levels of $Sods$ in the rat’s small intestine.

Finally, we investigated the effect of GABA on I/R-induced injury and innate immunity. In addition to our findings that GABA suppresses I/R, induces IgA secretion, and affects $RD-5$ mRNA levels (Figs. 2A, B), we also found that pretreatment with GABA prevents damage to the crypt (Fig. 3). There were no significant differences in the mRNA levels of $Sods$ between the I/R plus GABA treatment group and the sham-operated group (Fig. 2C). These results are consistent with a previous report showing that GABA reduces hepatic I/R injury-mediated oxidative stress.
epithelium. GABA content has an antioxidant capacity.

Next, we examined direct effect of GABA on I/R-induced IgA secretion in cultured rat PP cells. Intestinal concentration of orally administered GABA was predicted according to the previous report. Based on these results, we selected 5 mM GABA concentration in vitro study. A direct treatment of 5 mM GABA in PP cells significantly increased IgA secretion. Although I/R significantly reduced IgA secretion, there were no significant differences in IgA secretion between I/R plus 5 mM GABA treatment group and the sham-operated group (data not shown). We therefore suggest that the protective effect of GABA on I/R-induced injury can be caused by inducing an antioxidant enzyme such as SODs and a direct immuno-stimulatory activity. The results of this study provide further scientific evidence of the value of GABA in treating oxidative injury and attenuating alterations in intestinal immunity.

In conclusion, we focused on the protective effect of GABA on intestinal I/R-induced alterations in innate immunity. We showed that GABA, an antioxidant, significantly reduced oxidative injury and relieved the effects of I/R on innate immunity.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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