Protective role of adiponectin against ethanol-induced gastric injury in mice

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1Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine; 2Department of Gastroenterology and Hepatology, Itami City Hospital; 3Department of Biomedical Informatics, Departments of 4Metabolism and Atherosclerosis and 5Metabolic Medicine, Osaka University Graduate School of Medicine, Osaka; and 6Kansai Rosai Hospital, Hyogo, Japan

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Yamamoto S, Watabe K, Araki H, Kamada Y, Kato M, Kizu T, Kiso S, Tsutsui S, Tsujii M, Kihara S, Funahashi T, Shimomura I, Hayashi N, Takehara T. Protective role of adiponectin against ethanol-induced gastric injury in mice. Am J Physiol Gastrointest Liver Physiol 302: G773–G780, 2012. First published February 9, 2012; doi:10.1152/ajpgi.00324.2011.—Adiponectin is an anti-inflammatory molecule released from adipocytes, and serum adiponectin concentrations are reduced in obesity. We previously reported that gastric erosion occurs in association with obesity and low serum adiponectin levels. In the present study, we examined adiponectin-knockout (APN-KO) mice to elucidate the role of adiponectin in gastric mucosal injury. Gastric injury was induced by oral administration of ethanol in wild-type (WT) and APN-KO mice. Ethanol treatment induced severe gastric injury in APN-KO mice compared with WT mice. In APN-KO mice, increased apoptotic cells and decreased expression of prostaglandin E2 (PGE2) were detected in the injured stomach. We next assessed the effect of adiponectin on the cellular response to ethanol treatment and wound repair in rat gastric mucosal cells (RGM1). Adiponectin induced the expression of PGE2 and cyclooxygenase 2 (COX-2) in ethanol-treated RGM1 cells. RGM1 cells exhibited efficient wound repair accompanied by increased PGE2 expression in the presence of adiponectin. Coadministration of adiponectin with celecoxib, a COX-2 inhibitor, inhibited efficient wound repair. These findings indicate that adiponectin has a protective role against ethanol-induced gastric mucosal injury in mice. This effect may be partially mediated by the efficient wound repair of epithelial cells via increased PGE2 expression.

prostaglandin; cyclooxygenase

THE BALANCE BETWEEN AGGRESSIVE and defensive factors determines the development of gastric mucosal injury. Gastric mucosa is continuously exposed to noxious factors: ethanol, gastric acid, pepsin, reactive oxygen species, nonsteroidal anti-inflammatory drugs, and Helicobacter pylori. If the balance of these factors is disturbed, gastric erosion occurs (12). Various endogenous defense systems, such as (PGs), nitric oxide, and antiferent neurons protect the gastric mucosa from these factors (21, 26).

Obesity is currently a major health concern worldwide, because it leads to various diseases such as diabetes mellitus, dyslipidemia, and cardiovascular disease. Obesity has a documented association with gastrointestinal diseases such as reflux esophagitis, gallstone disease, and tumors of the pancreas, esophagus, and colon. Morbidly obese patients have a higher prevalence of gastritis than nonobese subjects (3, 39). In the general population, an increase in the body mass index is significantly associated with erosive gastritis and gastric ulcers (7, 16). We (50) previously reported that the incidence of endoscopically observed gastric erosion is increased in obese subjects.

Adiponectin, an adipose tissue-derived secretory factor, circulates abundantly throughout the body, and its plasma concentrations are lower in obese individuals (23, 27). Adiponectin has anti-inflammatory, antiatherosclerotic, and anti-inflammatory properties in various organs (37, 47). In addition, adiponectin has anti-inflammatory and antiobiotic properties in liver fibrosis, colitis, pancreatitis, and gallstone disease (1, 14, 32, 34, 49). We analyzed the relation between adiponectin and gastric erosion and revealed that low serum adiponectin is an independent risk factor for gastric erosion (50). Based on clinical findings, we hypothesized that adiponectin has a protective role against gastric mucosal injury. We considered that gastric erosion is a certain type of gastric mucosal injury caused by several irritants such as nonsteroidal anti-inflammatory drugs, alcohol, and steroids, as well as gastric acid (43). In the present study, we aimed to clarify the role of adiponectin in gastric injury induced by oral administration of ethanol using adiponectin-knockout (APN-KO) mice.

MATERIALS AND METHODS

Animals. APN-KO mice were generated as described previously and backcrossed to the C57BL/6J strain for five generations (24). C57BL/6J mice were purchased as wild-type (WT) controls from Clea Japan (Tokyo, Japan). The Ethics Committee for Animal Experimentation of Osaka University Graduate School of Medicine approved all of the animal experiments reported in this study.

Ethanol-induced gastric mucosal injury in mice. The gastric ulcerogenic response to ethanol was examined using conventional methods with some modifications (33, 45). Concentrated ethanol at the indicated dose was administered to male mice (8–10 wk old, weighing 20–25 g) that had been fasted in wire-bottom cages for 6 h. At the indicated time after administration (15 min and 1 and 4 h), the animals were killed while they were under deep anesthesia and subjected to analysis. For macroscopic analysis of the gastric lesions, the organs were perfused transcardially with PBS (→) to flush out the blood cells and then with 4% paraformaldehyde. The stomachs were opened along the greater curvature and photographed using a digital camera (Powershot A720IS; Canon, Tokyo, Japan). The area of visible erosive lesions was measured using ImageJ 1.40f (National Institutes of Health, Bethesda, MD). The gastric lesion index was expressed as the percent damage of the total glandular mucosa (33). For the other analyses, different mouse groups were prepared. The stomachs were removed without using the perfusion process, sectioned horizontally, and either fixed in 10% buffered formalin for histochrome or immediately frozen in liquid nitrogen for the anal-
ysis of RNA, protein, and PGE2. The histologic extent of the gastric mucosal injury was evaluated by a pathologist as the percentage of the length of microscopically identified mucosal injury, which comprised hemorrhage and disruption of gastric epithelial cells to the length of the circumference of the gastric corpus, according to the method described by Miyake et al. (28).

To determine the optimum dose of ethanol, we performed several preliminary experiments. Treatment with 25 and 50% ethanol induced more severe gastric injury in APN-KO mice compared with WT mice. Because the difference in the gastric injury between WT and APN-KO mice was significantly greater following treatment with 50% ethanol compared with 25% (data not shown), the experiments in this study were conducted with 50% ethanol.

Cell culture. Nontransformed rat gastric mucosal cells (RGM1) derived from rat gastric mucosa were maintained in DMEM/F-12 medium (Invitrogen, Carlsbad, CA) containing 10% FBS (Sigma Chemical, St. Louis, MO) and 1% antibiotic-antimycotic (Invitrogen) in a humidified atmosphere of 95% air with 5% CO2 at 37°C (5, 17).

Ethanol treatment of gastric mucosal cells. RGM1 cells were inoculated in 24-well plates, cultured until subconfluent, starved for 24 h in a medium containing 0.5% FBS, and exposed to ethanol at the indicated dose for 4 h in the presence or absence of recombinant human adiponectin (R&D Systems, Minneapolis, MN). Cell viability was indicated dose for4hi nt h epresence or absence of recombinant human adiponectin (R&D Systems, Minneapolis, MN). Cell viability was determined by WST assay using Cell Count Reagent SF (Nacalai Tesque, Kyoto, Japan). Caspase activity was measured in the supernatant of cultured RGM1 cells with a luminescent substrate assay for caspase-3 and -7 (Caspase-Glo assay; Promega, Madison, WI) according to the manufacturer’s protocol.

Wound repair assay using gastric mucosal cells. Confluent monolayers of RGM1 cells grown in 24-well plates were starved overnight in medium containing 0.5% FBS, wounded linearly with a 1,000-μl pipette tip in the center of each well, and further cultured for 8 h. Photographs of the wound were taken at 0, 4, and 8 h after wound formation. The cells were cultured under the presence or absence of either adiponectin (10 μg/ml) or celecoxib (5 μM; Sigma Chemical) throughout the experiment. The repaired area was defined as the area covered by migrated cells based on a comparison of the photographs using ImageJ 1.40f software.

Immunohistochemistry. For evaluation of the gastric mucosa, the tissues were stained with periodic acid-Schiff reaction and then counterstained with hematoxylin. Apoptotic cells were identified by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (ApopTag; Chemicon International, Temecula, CA) following the manufacturer’s protocol.

For cyclooxygenase 2 (COX-2) immunocytochemistry, RGM1 cells were cultured on culture slides coated with collagen I (Becton-Dickinson, Franklin Lakes, NJ). After overnight culture in medium containing 0.5% FBS in the presence or absence of adiponectin (10 μg/ml), a linear wound was made with a 1,000-μl pipette tip. Four hours later, the cells were fixed with acetone/methanol, incubated with anti-rat COX-2 antibody (Cayman Chemical, Ann Arbor, MI), stained with fluorescein isothiocyanate-labeled secondary antibody (Invitrogen), and counterstained with 4',6-diamidino-2-phenylindole (Southern-Biotech, Birmingham, AL). Cells were visualized using a fluorescent microscope (PROVIS AX80; Olympus, Tokyo, Japan) with a constant exposure time so that signal intensity could be compared among groups.

Real-time RT-PCR analysis. Total RNA was extracted using QIAshredder and RNeasy Mini kits (Qiagen, Hilden, Germany). Samples (210 μg of RNA) were reverse-transcribed using a QuantiTect reverse transcription kit (Qiagen). Quantitative real-time PCR was performed using a QuantFast SYBR Green PCR kit with specific primers (Qiagen) on a LightCycler (Roche Diagnostics, Indianapolis, IN). Primers used were Cox2-2 antibody (Cayman Chemical, Ann Arbor, MI), stained with fluorescein isothiocyanate-labeled secondary antibody (Invitrogen), and counterstained with 4’,6-diamidino-2-phenylindole (Southern-Biotech, Birmingham, AL). Cells were visualized using a fluorescent microscope (PROVIS AX80; Olympus, Tokyo, Japan) with a constant exposure time so that signal intensity could be compared among groups.

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PGE2 measurement. Whole stomach and cultured cells were subjected to PGE2 measurement. For analysis of the whole stomach, frozen tissue was placed in 0.1 M phosphate buffer containing 1 mM EDTA and 10 μM indomethacin, pH 7.4. The tissues were then homogenized with a TissueRuptor (Qiagen) and centrifuged at 8,000 g for 10 min at 4°C. PGE2 expression of was measured using a PGE2 enzyme immunoassay kit (Cayman Chemical). To analyze the cultured cells, cultured medium was subjected to the PGE2 enzyme immunoassay.

Statistical analysis. Results are expressed as means ± SE. Comparisons between groups were performed using the Wilcoxon rank test for nonparametric data, and more than three groups were compared by Tukey’s honestly significant difference test. A P value <0.05 was considered statistically significant. All data analysis was performed with the JMP 8 statistical package (Statistical Analysis Systems, Cary, NC).

RESULTS

Severe gastric mucosal injury in APN-KO mice after ethanol treatment. We first examined the stomach of untreated WT and APN-KO mice histologically. Hematoxylin and eosin staining revealed no significant differences in the structure of the epithelium or the distribution of inflammatory cells between WT and APN-KO mice (data not shown). Mucus covering the epithelium was evaluated by periodic acid-Schiff staining, and the distribution patterns were similar between groups (data not shown). We then treated the stomach of WT and APN-KO mice with 50% ethanol and evaluated the resulting gastric injury macroscopically. The lesion index, a well-characterized tool for quantifying the area of erosive lesions (21), was comparable between APN-KO and WT mice at 15 min and 1 h after ethanol administration but 5.3-fold higher in APN-KO mice compared with WT mice at 4 h (Fig. 1, A and B). Visible erosive lesions at 4 h were microscopically identified as epithelial cell death, edema, and hemorrhage in the gastric mucosa, and the histologic extent of the gastric mucosal injury was significantly greater in APN-KO mice compared with WT mice (Fig. 1, C and D). Analysis of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining of the serial sections revealed that the number of apoptotic cells per gastric lesion was significantly greater in APN-KO mice compared with WT mice (Fig. 1, E and F). These findings indicate that APN-KO mice were susceptible to gastric ethanol injury, which was accompanied by an increase in apoptotic cells. We investigated whether ethanol treatment increased the release or production of adiponectin in the stomach. The expression of adiponectin mRNA was not detected in WT mice before or after ethanol treatment (data not shown). Immunohistochemistry for adiponectin protein revealed no signals for adiponectin in the gastric mucosa either before or after ethanol treatment in WT and APN-KO mice. Adiponectin signals were observed in the vascular intima of gastric submucosal vessels only in the stomachs of WT mice, with a signal intensity that did not differ between pretreated and ethanol-treated stomachs (data not shown). To investigate the epithelial cellular proliferation, we performed immunohistochemistry for Ki-67 and found that the number of Ki-67-positive cells was comparable between WT and APN-KO mice after ethanol administration (data not shown).
Expression of molecules related to gastric mucosal defense in the ethanol-treated stomach. To investigate the molecular mechanisms underlying severe gastric mucosal injury in ethanol-treated APN-KO mice, we examined the expression profile of molecules known to affect gastric defense mechanisms. PGE$_2$ expression was equivocal between untreated WT and APN-KO mice. After ethanol treatment, WT mice exhibited significantly higher PGE$_2$ expression compared with untreated controls. In contrast, the increase in PGE$_2$ was blocked in APN-KO mice, suggesting the involvement of PGE$_2$ in severe gastric mucosal injury of APN-KO mice (Fig. 2A). COX-2 mRNA expression was comparable between untreated WT and APN-KO mice and induced similarly after ethanol treatment (Fig. 2B). We further examined the expression of mRNA for heat shock protein 70, endothelial nitric oxide synthase, and inducible nitric oxide synthase after ethanol treatment and found no significant difference between WT and APN-KO mice (Fig. 2B).

Adiponectin induced the expression of PGE$_2$ and COX-2 in ethanol-treated RGM1 cells. The surface epithelial cells of the stomach withstand frequent exposure to damaging factors. We investigated the expression of COX-2 and PGE$_2$ in RGM1 cells after ethanol treatment. We selected RGM1 cells in this study for the following reasons. We considered it important to examine the effect of adiponectin on cells that constitute the first line of gastric mucosal defense under the superficial mucous layer. Second, RGM1 cells are often used to investigate the mechanism of gastric mucosal injury (11, 29, 41).

First, we examined the viability of RGM1 cells after a 4-h treatment with several concentrations of ethanol and detected a cytotoxic effect of ethanol at concentrations $>2\%$ (Fig. 3A). Caspase-3/7 activity in ethanol-treated RGM1 cells peaked at 2\% ethanol (Fig. 3A). These findings suggest that necrosis and apoptosis occur at ethanol concentrations of 2\% or more. To examine the role of adiponectin under the condition that most efficiently induces apoptosis, we conducted the following experiments at an ethanol concentration of 2\%. We confirmed by RT-PCR that RGM1 cells express mRNA for both adiponectin receptor 1 (AdipoR1) and 2 (AdipoR2; Ref. 13; data not shown).

We examined PGE$_2$ expression in RGM1 cells. Ethanol treatment of RGM1 cells increased PGE$_2$ expression 2.5-fold, and supplementation with 10 $\mu$g/ml adiponectin enhanced this increase (Fig. 3B). We then examined COX-2 mRNA expression in the same experimental setting. Ethanol treatment significantly increased the expression of COX-2 mRNA by 2.2-fold, and supplementation with adiponectin enhanced this increase (Fig. 3B). We further examined the effect of adiponectin on the viability of ethanol-treated RGM1 cells. Supplementation with 10 or 20 $\mu$g/ml adiponectin did not lead to improved cell viability after 2\% ethanol treatment (data not shown).

Adiponectin accelerates wound repair of RGM1 cells via the COX-2/PGE$_2$ pathway. Cellular migration is an essential part of early gastric mucosal repair after ethanol injury (19, 46). Therefore, we examined the effect of adiponectin on cellular migration in RGM1 cells using a wound repair assay. Adi-
ponectin accelerated wound repair at 4 and 8 h after wound formation and increased PGE2 production in the medium after 8 h compared with untreated cells (Fig. 4A). Adiponectin-induced wound repair and PGE2 production were inhibited by coadministration of adiponectin with celecoxib, a selective COX-2 inhibitor. To further investigate the role of COX-2 in wound repair, we used immunohistochemistry to examine COX-2 expression in the cells neighboring the wound. Supplementation with 10 µg/ml adiponectin enhanced COX-2 expression, particularly in cells located at the leading edge of the wound (Fig. 4B). The results suggest that adiponectin accelerates wound repair via activation of the COX-2/PGE2 pathway.

**DISCUSSION**

We (50) previously reported that low serum adiponectin was a risk factor for gastric erosion independently of body mass index in subjects undergoing a health check-up program. Adiponectin is a key molecule that attenuates obesity-related diseases in various tissues, including the alimentary system (37, 38, 42). In the present study, we demonstrated that a deficiency in adiponectin exacerbated gastric lesions induced in mice by oral administration of ethanol. The gastric lesion index was comparable between WT and APN-KO mice in the earlier phase (15 min and 1 h) but greater in APN-KO mice compared with WT mice in the later phase (4 h), suggesting that repair after injury was impaired in the absence of adiponectin. Although we did not directly show the protective effect of adiponectin by supplementation of adiponectin, the findings of this study suggest that adiponectin attenuates the severity of gastric injury probably by facilitating mucosal repair.

Adiponectin mRNA was not detected in the stomachs of WT mice before or after ethanol treatment, suggesting that adiponectin is not locally produced in the murine stomach. Because we (30, 35) previously reported that the accumulation of circulating adiponectin in vessels has protective effects on injured organs, we considered that circulating adiponectin might protect the stomach in our gastric injury model. The mechanism of adiponectin release following gastric mucosal injury, however, requires further investigation.

In this study, the induction of PGE2 expression in the stomach was blocked in APN-KO mice after ethanol administration, whereas there was no difference between WT and APN-KO mice before ethanol administration. Prostaglandins are crucial for maintenance of the mucosal integrity and protection against ulcerogenic and necrotizing agents (21, 48). PGE2 stimulates mucus secretion, elevates mucosal blood flow, protects mucosal cells from apoptosis, and accelerates epithelial wound repair and mucosal healing (9, 26, 45). In an acid-induced or ischemia-reperfusion gastric mucosal injury model, inhibition of COX-2, which is a predominant PGE2 enzyme, aggravates the gastric injury (4, 8, 25). Therefore, we considered that impaired PGE2 production in APN-KO mice is involved in severe mucosal injury. To further investigate the role of PGE2 in the pathogenesis of gastric injury in adiponectin-deficient mice, we conducted two in vitro experiments with gastric epithelial cells: 1) cellular response to ethanol treatment and 2) wound repair.

Gastric mucosa is continuously exposed to irritants, and its structural integrity is maintained through several mechanisms, including the generation of PGs. In the present study, ethanol induced the expression of PGE2 in RGM1 cells and adiponectin enhanced the induction. Gastric epithelial cells could contribute to increase PGE2 in ethanol-treated gastric tissue. In the same experimental setting, we found that the expression pattern of COX-2 mRNA resembled that of PGE2 after ethanol and/or adiponectin treatment, suggesting that the expression of PGE2 is regulated by COX-2. This finding is consistent with our previous observation (10, 44) that adiponectin increases the expression of PGE2 together with COX-2 in cardiac myocytes. We (41) previously reported that COX-2 expression increases after HCl administration in RGM1 cells. These results indicate that gastric epithelial cells induce PGE2 production upon stimulation by irritants. Collectively, the present in vitro findings suggest that adiponectin protects the gastric mucosa from ethanol-induced injury, partially through activation of the COX-2/PGE2 pathway in gastric epithelial cells. Kusunoki et al. (18) showed that adiponectin-induced PGE2 production is mediated partially by adiponectin receptors.
AdipoR1 and AdipoR2 in rheumatoid arthritis synovial fibroblasts. Lee et al. (22) showed that adiponectin activates AdipoR1 to increase the expression of COX-2 in mesenchymal progenitor cells. These studies suggest that AdipoR1 and R2 have an important role in RGM1 cells to increase COX-2/PGE2 expression upon stimulation by adiponectin. Further studies are required to elucidate the role of these receptors in the induction of COX-2 and PGE2 by adiponectin in RGM1 cells.

In the gastric mucosa, apoptosis is induced physiologically and pathologically. In human gastric mucosa, apoptotic cells are physiologically observed as a result of epithelial turnover and pathologically as a result of *Helicobacter pylori* infection and subsequent gastritis (40). In the present study, apoptotic cells were rare in the gastric mucosa of WT mice after ethanol treatment. In contrast, apoptotic cells were abundant in APN-KO mice, indicating that gastric epithelial cells are susceptible to apoptosis after ethanol treatment in the absence of adiponectin. PGE2 inhibits apoptosis in gastric mucosal cells isolated from guinea pig fundic glands after ethanol treatment (9). Adiponectin suppresses stress-induced apoptosis in myocardial cells and salivary gland epithelial cells (15, 44). In the present study, however, adiponectin did not suppress apoptosis of ethanol-treated gastric epithelial cells, suggesting that the susceptibility of gastric epithelial cells to apoptosis in the absence of adiponectin is not mediated by the direct actions of adiponectin in mice. Further studies are required to clarify and confirm this issue.

At the injured site in the gastric mucosa, rapid migration of preserved epithelial cells located in the neck of gastric glands occurs within minutes, thereby promoting rapid repair of the surface epithelium (19, 21, 46). Our wound repair assay suggests that adiponectin enhances epithelial wound repair via activation of the COX-2/PGE2 pathway. Adiponectin promotes migration of endothelial cells and endothelial progenitor cells (31, 36). In endothelial progenitor cells, activation of Cdc42/Rac1 is the molecular basis for adiponectin-promoted migration activity (31). Cdc42/Rac1 plays a central role in cellular polarization and is involved in tissue development, chemotaxis, and wound healing (6). Migrating cells, in general, form a distinct actin organization, called “lamellipodium” at the leading edge of migration (20). Because we demonstrated that adiponectin induced expression of COX-2, especially at the leading edge of the wound, Cdc42/Rac1 might be involved in the migration activated by the COX-2/PGE2 system.

In the present study, RT-PCR assay analysis revealed comparable COX-2 expression in the stomach between WT mice.
and APN-KO mice after ethanol treatment, whereas PGE\textsubscript{2} expression in these mice was different. To investigate this discrepancy between the expression of COX-2 and PGE\textsubscript{2}, we performed the following two experiments. First, we examined the localization of COX-2-expressing cells in the stomach of ethanol-treated mice to determine the cells expressing COX-2 in vivo. We expected to detect COX-2 protein in ethanol-treated gastric mucosa as shown by Miyake et al. (28) and Bhandari et al. (2). We were, however, unable to obtain a clear COX-2 signal in the mucosa of our gastric tissue specimens (data not shown). We then tried to optimize the immunohistochemistry by using another antibody (COX-2, N-20; Santa Cruz Biotechnology, Santa Cruz, CA), preparing frozen tissue of the stomach, and utilizing microwave antigen retrieval. These modifications, however, did not improve the detection of COX-2 expression in the mucosa (data not shown). From these results, we could not explain our in vivo findings regarding PGE\textsubscript{2} and COX-2.

In conclusion, the findings of the present study provide evidence for a protective role of adiponectin against gastric injury in mice. This effect may be partially mediated by the efficient wound repair of epithelial cells via increased PGE\textsubscript{2} expression.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


