INTRODUCTION

Severe acute gastric inflammation or ulcer disease can occur with high prevalence and with complications such as bleeding in patients with diabetes mellitus with little or no dyspeptic symptoms (1, 2). Experimental diabetes dramatically impairs gastric ulcer healing (3, 4). It has been reported that disturbance of the healing process of gastric ulcer in diabetes is associated with the increases in inflammatory cytokines (interleukin 1β and tumor necrosis factor), the down-regulation of growth factors (vascular endothelial growth factor and insulin-like growth factor-1) and heat shock proteins, and the depletion of insulin (4, 5). Recent studies indicated that hyperglycemia and a number of hyperglycemia-related factors have been linked to impaired diabetic skin wound healing, including advanced glycation end products (AGEs) (6). Methylglyoxal is a reactive dicarbonyl compound produced from cellular glycolytic intermediates that reacts non-enzymatically with proteins to form products such as argpyrimidine at arginine residue. The aim of the present study was to investigate the role of methylglyoxal in the delayed healing of gastric ulcer in diabetes, and to identify the methylglyoxal-modified proteins as a target molecule of this modification. Using male C57BL/6 mice, diabetes was induced by a single i.p. injection of streptozotocin and gastric ulcers were produced by the focal application of 40% of acetic acid to the serosal surface of the stomach. In order to evaluate the effect of OPB-9195, an inhibitor of methylglyoxal modification, on gastric ulcer healing, mice were given orally OPB-9195 (30 mg/kg) twice daily for 14 days, one week before and after the injection of streptozotocin. The area of gastric ulcer on day 7 was significantly increased in diabetic mice compared to non-diabetic mice, indicating delayed ulcer healing. This increase in ulcer area in diabetic mice was significantly reversed by the treatment with OPB-9195 without affecting blood glucose levels. Proteomics analysis showed the methylglyoxal-modification of peroxiredoxin 6 proteins in the diabetic gastric mucosa around gastric ulcer, and this modification was markedly inhibited by the treatment with OPB-9195. In conclusion, the present study suggests a link of increased methylglyoxal modification of proteins including peroxiredoxin 6 to the delayed gastric ulcer healing in diabetes, and also shows the therapeutic potential of the inhibitor of methylglyoxal modification for the treatment of diabetic gastric ulcers.

Key words: diabetes mellitus, gastric ulcer healing, methylglyoxal, peroxiredoxin 6, protein modification
Induction of gastric ulcers

Male C57BL/6 mice weighing 20-30 g were used. Regular mouse chow and tap water were allowed ad libitum. Mice were housed in a cage at room temperature (25±1°C) with a 12:12 h light/dark cycle and humidity of 65-70%. All experimental procedures described below were approved by the Animal Care Committee of the Kyoto Prefectural University of Medicine (Kyoto, Japan). Diabetes was induced by a single i.p. injection of STZ (180 mg/kg). Control animals received an equal volume of saline. Normal or diabetic mice were placed in each cage with raised mesh bottoms to prevent coprophagy and deprived of food for 18 h before experiments. Four weeks after the injection of STZ, the gastric ulcers were produced using the modified acetic acid method originally proposed by Okabe et al. (11, 12). Briefly, under light ether anesthesia, the abdomen was opened, the stomach exposed and gastric ulcers were produced by the focal application of 40% of acetic acid to the serosal surface of the mouse stomach for 40 s. After the application of acetic acid, the animals were allowed to recover from anesthesia and received only water at the day of operation. In order to evaluate the effect of OPB-9195 on gastric ulcer healing, mice were given orally OPB-9195 suspended in 0.5% carboxymethyl cellulose sodium salt solution, twice daily (30 mg/kg of mice weight) for 14 days, one week before and after the injection of STZ. Control mice were given orally the same amount of 0.5% carboxymethyl cellulose sodium salt solution without OPB-9195. Following treatment groups were used: 1) non-diabetic mice with gastric ulcer treated without or with OPB-9195 and 2) diabetic mice with gastric ulcer treated without or with OPB-9195.

Sample preparation and proteomic assays

The frozen tissue samples (200 mg) were homogenized in 2 mL homogenization buffer (8 M Urea, 4% CHAPS, 40 mM Tris) containing nuclease and protein inhibitors (GE Healthcare UK Ltd., Buckinghamshire, England) using a homogenizer at 25000 r/min. Homogenized samples were transferred to a ultracentrifuge tube, and the nucleic acids were removed by centrifugation (20 min at 20000 × g, 25°C). Samples were precipitated using the Plus One 2D Clean-up kit as recommended by the manufacturer (GE Healthcare UK Ltd., Buckinghamshire, England). The protein concentration in the supernatant fraction was determined by a Bradford assay, using bovine serum albumin as a standard. Samples were solubilized in 6 M urea, 20 mM dithiothreitol, 30% glycerol, 45 mM Tris base, 1.6% LDS (Invitrogen Japan K.K., Tokyo, Japan), and 0.002% bromophenol blue and then were heated at 70°C for 10 min. Protein lysates (50 µg) were separated by two-dimensional PAGE. IPG strips, pH 4-7 (Invitrogen Japan K.K., Tokyo, Japan), were rehydrated overnight with protein samples. Proteins were separated on the basis of their isoelectric point by IEF using the ZOOM IPG Runner (Invitrogen Japan K.K., Tokyo, Japan) with a maximal voltage of 2000 V and 50 µA per gel. Following IEF, IPG strips were incubated in equilibration buffer I (6 M urea, 130 mM dithiothreitol, 30% glycerol, 45 mM Tris base, 1.6% LDS, 0.002% bromophenol blue; Genomic Solutions) and once in equilibration buffer II (6 M urea, 135 mM iodoacetamide, 30% glycerol, 45 mM Tris base, 1.6% LDS, 0.002% bromophenol blue; Genomic Solutions) for 15 min.

Equilibrated IPG strips were applied to 4-12% Bis-Tris gradient gels (Invitrogen Japan K.K., Tokyo, Japan), and proteins were separated in the second dimension based on their molecular size using NuPAGE MOPS buffer (Invitrogen Japan K.K., Tokyo, Japan) at 200 V for 55 min. Following electrophoresis, gels were transferred onto nitrocellulose and immunoblotted with antibody. Ten µg samples were applied to 12% Bis-Tris gels using NuPAGE MOPS buffer (Invitrogen Japan K.K., Tokyo, Japan) at 200 V for 55 min. Following electrophoresis, gels were transferred onto nitrocellulose and immunoblotted with antibody.

Immunoprecipitation

Gastric mucosa proteins (50 µg) were incubated for 16 h at 4°C with 10 µg of antibody specific to peroxiredoxin-6 (Prx6), and were captured by 50 µl gel suspension of protein G-sepharose (GE Healthcare UK Ltd., Buckinghamshire, England) at 4°C for 4 h. After centrifugation the immunoprecipitates were subsequently washed three times with PBS, boiled with the SDS–PAGE sample buffer for 5 min and subjected to Western blot analysis as described above.

Immunohistochemical staining

After 24-h of fixation in formalin, the samples were embedded in paraffin, and sections were cut at 5 mm thickness using a microtome cryostat, and mounted on MAS-coated slides. We performed antigen retrieval using Proteinase K solution, and the sections were rinsed with distilled water for 5 min, and then incubated with 3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. After incubation, the sections were washed in PBS-Tween for 5 min each. Non-specific binding was blocked by incubating the slides with Dako cytometry protein block (Dako, Tokyo, Japan) for 30 min at room temperature. The sections were then incubated with primary antibody (anti-Prx6 monoclonal antibody) diluted 1:1000 in antibody dilution (Dako) for one night at 4°C. The sections are then washed three times in PBS-Tween for 5 min each, and incubated with secondary antibody (Histfine Simple Stain mouse MAX PO (Rabbit), Nichirei Biosciences Inc., Tokyo, Japan) for 30 min at room temperature. Unbound antibodies were washed away by three washes in PBS for 5 min and the bound antibodies were visualized using DAB as chromogen substrate reagent. Negative controls for non-specific binding incubated with secondary antibodies were also processed and revealed no signal. All sections are counterstained with hematoxylin. The sections were finally dehydrated, cleared, and coverslipped.

Statistics

All values are expressed as means±SEM. The data were compared by one-way analysis of variance (ANOVA) followed by Bonferroni’s Multiple Comparison Test. A probability value less than 5% was considered statistically significant.

RESULTS

Delayed ulcer healing and methylglyoxal-modified proteins of gastric mucosa in diabetic mice

Blood glucose levels increased in non-fasting mice after STZ injections, reaching at 3-fold higher levels (320±38 mg/ml) than those in sham-treated mice on day 7 after the injection of STZ, and maintaining these levels during experiments. Four
weeks after the injection of STZ, the gastric ulcers were produced by the application of acetic acid. The area of gastric ulcer on day 3 after inducing ulcers did not significantly differ between control mice and diabetic mice, the ulcer areas being 43.0±1.7 mm² and 41.2±1.9 mm², respectively. Fig. 1 shows the effects of the STZ-induced diabetes with respect of gastric ulcer area and gastric mucosal proteomics at ulcer margin on day 7 after inducing ulcers in these mice. In the control mice, the gastric ulcer rapidly tended to heal, and the ulcer area on day 7 was reduced to approximately 30% of the initial value observed on day 3, namely 12.0±0.7 mm² (Fig. 1a, b). In contrast, the ulcer area of diabetic mice on day 7 was significantly higher (35.5±3.3 mm²) than control mice.

In a search for protein modification by methylglyoxal, we used a proteomic approach on gastric mucosal tissue obtained from the marginal mucosa around gastric ulcers in control mice and diabetic mice. Whole protein extracts from the tissues were separated by two-dimensional electrophoresis, followed by immunoblot analysis with anti-methylglyoxal-modified protein monoclonal antibody, which specifically recognized argpyrimidine (7). There were no significant differences in total protein profiles in gastric mucosa between two groups (Fig. 1c). The immunoreactivity of proteins in gastric mucosa around ulcer was increased in diabetic mice compared to control mice (Fig. 1d).

Effects of OPB-9195 on blood glucose levels and gastric ulcer area in non-diabetic mice and diabetic mice

Fig. 2a showed the non-fasting blood glucose levels on day 7 after the production of acetic acid ulcer with/without OPB-
9195 treatment. Blood glucose levels increased in the diabetic mice, reaching 5-fold higher levels than those in non-diabetic mice. OPB-9195 treatment did not affect blood glucose levels in non-diabetic mice or diabetic mice. Fig 2b showed the area of gastric ulcer on day 7 after the production of acetic acid ulcer with/without OPB9195 treatment. The area of gastric ulcer on day 7 was significantly increased in diabetic mice compared to non-diabetic mice, indicating delayed ulcer healing. This increase in ulcer area in diabetic mice was significantly reversed by the treatment with OPB-9195.

**Detections of methylglyoxal-modification and Prx-6 in the gastric mucosa of mice**

To identify the methylglyoxal-modified proteins in gastric mucosa of diabetic mice with gastric ulcer, two-dimensional gel electrophoresis and Western blot analysis were used. As shown in Fig 3a-d, two-dimensional electrophoresis on a strip that focuses proteins between pI4.0 and 7.0 followed by immunoblot analysis revealed methylglyoxal-modified proteins with molecular weights between 20 and 30 kDa (indicated by arrow). As shown in Fig. 3e and 3f, protein that reacted with antibody to Prx6 showed immunoreactivity to argpyrimidine antibody.

Whole protein extracts from the tissue were separated by SDS-PAGE, followed by immunoblot analysis with anti-methylglyoxal modified protein monoclonal antibody. Prx6 in protein extracts obtained from each gastric mucosa was confirmed by immunoblotting using anti-Prx6 antibody, as shown in Fig. 4. The immunoreactivity of a ~25 kDa protein in diabetic mice and diabetic mice with gastric ulcer was identified by an antibody to argpyrimidine (Fig. 4a). The expression level of Prx6 was similar...
among control, diabetic, and diabetic with gastric ulcer groups (Fig. 4b). Forced expression of Prx6 was observed in gastric mucosa proteins from control mice with gastric ulcer. Moreover, the extra band of Prx6 around 40 kDa was appeared in mucosa proteins from control mice with gastric ulcer, and this band was detected by using anti-argpyrimidine antibody (Fig. 4a and 4b).

To establish the extent of argpyrimidine modification of Prx6 in gastric mucosa, proteins from control mice with gastric ulcer and diabetic mice with gastric ulcer were immunoprecipitated with Prx6 antibody. We tested for argpyrimidine in immunoprecipitated pellets with Western blotting. As expected, the pellets from diabetic mice with gastric ulcer displayed stronger immunoreactivity for argpyrimidine compared to control mice with gastric ulcer (Fig. 5a). Furthermore, two extra bands were detected in the precipitated pellet from diabetic mice with gastric ulcer. These results indicate that methylglyoxal-modified Prx6 abounds in diabetic mice with gastric ulcer. Oral administration of OPB-9195 markedly reduced methylglyoxal-modification of Prx6. The expression level of Prx6 in gastric ulcer was similar between control and diabetic mice (Fig. 5b). The migrated band due to post-translational modification in diabetic mice with gastric ulcer was observed by using anti-Prx6

![Fig. 4. Detections of argpyrimidine and peroxiredoxin-6 (Prx6) in the gastric mucosa of mice. Methylglyoxal-modified proteins were identified by immunoblot analysis using anti-argpyrimidine monoclonal antibody (a). Prx6 was identified by immunoblot analysis using anti-Prx6 polyclonal antibody (b).](image)

![Fig. 5. Argpyrimidine immunoprecipitated with peroxiredoxin-6 (Prx6) antibody. Gastric mucosa protein was immunoprecipitated by anti-Prx6 polyclonal antibody. After centrifugation, the immunoprecipitated pellet were washed three times with PBS and then analyzed by SDS-PAGE followed by Western blotting with anti-argpyrimidine monoclonal antibody (a) and anti-Prx6 monoclonal antibody (b).](image)
antibody. Oral administration of OPB-9195 to diabetic mice caused this migrated band to disappear.

**Localization of Prx6 in gastric tissue**

To investigate the expression of Prx6 in gastric mucosa, we performed Prx6 immunohistochemical staining of the gastric mucosa. Prx6-immunoreactivity was observed in epithelial cells in the gastric mucosal layer in normal mucosa. After the induction of gastric ulcer, Prx6 immunopositivity are stronger in epithelial cells, especially in regenerated mucosal cells around ulcer margin than background mucosa.

**DISCUSSION**

In the present study, we confirmed the delayed ulcer healing in the model of diabetes induced by STZ, and detected methylglyoxal-modified proteins in the gastric mucosa around ulcer in diabetic mice. Secondary, oral administration of OPB-9195, an inhibitor of both glycoxidation and lipoxidation reactions, reversed the delayed ulcer healing without affecting blood glucose levels. Finally, we demonstrated the methylglyoxal-modification of Prx6 protein by proteomic analysis, and this modification was markedly inhibited by the treatment with OPB-9195.

To investigate the contribution of methylglyoxal to delayed gastric ulcer healing in diabetes, we firstly compared the methylglyoxal-modified proteins in the gastric mucosa around ulcer between control mice and diabetic mice using a monoclonal antibody, which specifically recognized argpyrimidine (7). The present study clearly demonstrated that argpyrimidine-immunoreactivity of proteins increased in diabetic gastric mucosa around ulcer compared to control mice, without differences in whole protein profiles between these two groups. This is the first report that detected methylglyoxal modification proteins in the gastric mucosa as far as we know it.

OPB-9195 belongs to a group of thiazolidine derivatives and inhibits the formation of AGEs (13). OPB-9195 inhibits most, if not all, carbonyl reactions with proteins including not only glycoxidation but also lipoxidation reactions (14, 15). A plausible explanation for the inhibitory mechanism of OPB-9195 on the carbonyl reactions might be the trapping of reactive carbonyl compounds, via the free base upon hydrolysis, by the hydrzone formation. We therefore speculate that OPB-9195 could accelerate ulcer healing by entrapping reactive carbonyl compounds and quenching carbonyl reactions in the gastric mucosa. Because OPB-9195 may interact carbonyl compounds derived from physiological oxidative reactions, whether it also applies to humans remains to be investigated. Another important observation of this study is to confirm argpyrimidine modification of Prx6 in gastric mucosa from diabetic mice with gastric ulcer. It has been reported that methylglyoxal reacts with arginine residues of proteins to form argpyrimidine (17), and the presence of this adduct in the human serum and cornea has also been demonstrated (18). Methylglyoxal can bind and modifies a number of proteins, including ribonuclease A, lysozome, collagen, and heat shock protein 27, however, this is a first observation that confirmed the modification of Prx6 by methylglyoxal. Prx6 has a single redox-active cysteine and use glutathione to catalyze the reduction of hydrogen peroxides and various organic peroxides (19, 20). Previous studies have shown that overexpression of Prx6 in different cell types protects from oxidative-stress-induced cytotoxicity (21-23), whereas knockdown of this enzyme resulted in enhanced sensitivity to oxidative injury (24, 25). Interestingly, Prx6 levels are rate limiting in the wound epidermis of aged animal because keratinocyte-specific overexpression of Prx6 enhanced wound closure in old mice (26). However, the role of Prx6 in the healing process of gastric ulcer is as yet known. The present study have demonstrated a strong up-regulation of Prx6 after gastric ulcer induction, in particular in regenerated gastric epithelial cells around ulcers, and the correlation between the inhibition of the methylglyoxal modification of Prx6 and the reverse of delayed ulcer healing in diabetic mice by the treatment with OPB-9195. These data suggest that methylglyoxal-modified Prx6 in the epithelial cells may play a crucial role in the delayed ulcer healing in a diabetes condition. Of course, Prx6 may be one of candidates involved in delayed ulcer healing among many methylglyoxal-modified proteins in diabetic gastric mucosa. It will be extremely necessary to investigate the changes in the function of Prx6 protein after this modification.

In conclusion, the present study suggests a link of increased methylglyoxal modification of proteins including Prx6 to the delayed gastric ulcer healing in diabetes. Further studies will be undoubtedly required to evaluate the usefulness of the inhibitors of this modification for the treatment of diabetic gastric ulcer.

**Acknowledgements:** This research was partially supported by FY 2009 Grant-in-Aid for Scientific Research for young scientists (B) to T. O.–I. from Japan Society for the Promotion of Science, by FY 2010 Research for Promoting Technological Seeds A (discovery type) to T. O.–I. from Independent Administrative Corporation Japan Science and Technology Agency, and by the 170th Redox Life Sciences Committee to T. Y. from Japan Society for the Promotion of Science.

Conflicts of interests: None declared.
REFERENCES


Received: October 15, 2009
Accepted: December 11, 2009

Author’s address: Prof. Yuji Naito; E-mail: ynaito@koto.kpu-m.ac.jp