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Heme Oxygenase-1 Protects Interstitial Cells of Cajal from Oxidative Stress and Reverses Diabetic Gastroparesis

Kyoung Moo Choi^{1,3}, Simon J. Gibbons^{1,4}, Tien V. Nguyen^{1,3}, Gary J. Stoltz^{1,4}, Matthew S. Lurken^{1,3}, Tamas Ordog^{1,4}, Joseph H. Szurszewski^{1,4}, and Gianrico Farrugia^{1,2,3,4}

¹ Enteric NeuroScience Program, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA

² Miles and Shirley Fiterman Center for Digestive Diseases, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA

³ Division of Gastroenterology and Hepatology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA

⁴ Department of Physiology and Biomedical Engineering, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA

Abstract

Background and Aims—Diabetic gastroparesis (delayed gastric emptying) is a well recognized complication of diabetes. Diabetic gastroparesis causes considerable morbidity and makes glucose control difficult. Kit-positive interstitial cells of Cajal (ICC) are required for normal gastric emptying. We hypothesized that there is a loss of Kit in diabetic gastroparesis due to elevated oxidative stress and that the elevated oxidative stress is due to low levels of heme oxygenase-1 (HO1), an important cytoprotective molecule against oxidative injury.

Methods—Gastric emptying was measured in non-obese diabetic (NOD) mice and correlated with levels of HO1 expression and activity. Endogenous HO1 activity was increased by administration of hemin and inhibited by chromium mesoporphyrin.

Results—Early in diabetes, HO1 was upregulated in gastric macrophages and remained upregulated in all mice resistant to development of delayed gastric emptying. In contrast, HO1 upregulation was lost in all mice that developed delayed gastric emptying and Kit and neuronal nitric oxide synthase (nNOS) expression was markedly decreased in these mice. Loss of HO1 upregulation resulted in increased reactive oxygen species. Induction of HO1 by hemin decreased reactive oxygen species, rapidly restored Kit and nNOS expression and completely normalized gastric emptying in all mice. Inhibition of HO1 activity in mice with normal gastric emptying resulted in loss of Kit expression and development of diabetic gastroparesis.

Conclusion—Induction of the HO1 pathway appears to be critical to prevent and reverse cellular changes that lead to development of gastrointestinal complications of diabetes and offers a novel therapeutic option.

Keywords

Heme oxygenase-1; diabetic gastroparesis; oxidative stress; hemin; macrophages; interstitial cells of Cajal

Corresponding Author: Gianrico Farrugia, MD, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, Farrugia.gianrico@mayo.edu, Phone: 507-284-4695, Fax: 507-284-0266.

Conflict of Interest: None

Complications of diabetes mellitus^{1, 2} include changes in the function of the gastrointestinal tract including diabetic gastroparesis.³ Diabetic gastroparesis is characterized by a delay in emptying of stomach content and carries significant risk of morbidity and even mortality to the patient.⁴ Glycemic control is significantly more difficult in patients with gastroparesis because of irregular delivery of content to the small bowel and mismatched hormonal responses to food, further predisposing patients to complications. The etiology of diabetic gastroparesis is multifactorial and may involve extrinsic neuropathy and increased muscle fibrosis.^{5, 6} The preponderance of data from recent animal and human studies suggest that two consistent histological findings in diabetic gastroparesis are loss of interstitial cells of Cajal (ICC)⁷ and loss of expression of neuronal nitric oxide synthase (nNOS).^{6, 8} ICC are required to generate and propagate the electrical signal that regulates smooth muscle contraction.^{9, 10} The receptor tyrosine kinase Kit is expressed on ICC and often used to identify these cells. Kit, together with its ligand, stem cell factor¹¹ is required for ICC survival. nNOS generates neuronally derived NO, the major inhibitory neurotransmitter in the gastrointestinal tract. NO is also a survival factor for ICC.¹²

Diabetes is associated with increased oxidative stress.¹³ Oxidative stress has been implicated in the development of end organ damage from diabetes such as cardiomyopathy¹⁴ and nephropathy.¹⁵ Induction of heme oxygenase 1 (HO1), the inducible isoform of HO, has been recently identified as an important cellular defense mechanism against oxidative stress.¹⁶ HO1 also regulates cell survival and proliferation.¹⁷ HO1 is induced by many mechanisms including exposure to heavy metals¹⁸ and inflammatory cytokines¹⁹ and by oxidative stress.²⁰ The goals of this study were to determine, in non-obese diabetic (NOD) mice, an animal model of type 1 diabetes,²¹ if activity and expression of HO1 was linked to nNOS and Kit expression and markers of oxidative stress and to determine if manipulation of HO activity changes the course of delayed gastric emptying.

Materials and Methods

Animals and experimental design

Eight-week-old female NOD/ShiLtJ mice were received from Jackson laboratory (Bar Harbor, ME). Glucose levels were measured in whole blood from the tail vein or from the vascular bundle located at the rear of the jaw bone using an ACCU-CHEK device (Roche, Indianapolis, IN). Blood glucose was measured on a weekly basis and mice were considered diabetic when the glucose levels were over 250 mg/dl. Once mice became diabetic, their glucose levels were measured daily. The incidence of diabetes in NOD/ShiLtJ mice younger than 26 weeks was approximately 60% (supplementary Figure 1). In initial experiments only 4% of diabetic mice survived for longer than 4 weeks after onset of diabetes and none had developed delayed gastric emptying at this point. Therefore sub-therapeutic insulin (humulin 50/50, Eli Lilly and company, Indianapolis, IN) was injected twice daily i.p. after 4 weeks of diabetes to keep the diabetic mice alive yet also keep blood glucose between 400–600 mg/dl to allow complications of diabetes to develop. After onset of diabetes gastric emptying was determined weekly. When the mice developed diabetes they were assigned to 4 groups. Mice that did not develop delayed gastric emptying were studied at 3 time points: 2 weeks after development of diabetes (a time-point when all diabetic mice developed accelerated gastric emptying as previously shown,²² at 4–5 weeks and at 10 weeks of diabetes. Twenty percent of diabetic mice developed delayed gastric emptying before reaching the 10 week time point (mean 5.5 weeks after development of diabetes) and these mice were placed in a separate group. No mice developed delayed gastric emptying after 8 weeks of diabetes and therefore the mice with normal gastric emptying in the 10 week group was considered resistant to the development of gastroparesis. Six mice were assigned to each group. Two additional mice were assigned to the 10 week time point for

immunohistochemistry. Eighteen non-diabetic NOD mice were selected as age-matched controls for the diabetic groups.

HO1 expression was induced in vivo by i.p. injection of hemin (40 $\mu\text{mol/kg}$; Sigma-Aldrich, St Louis, MO). HO activity was inhibited by injection with chromium mesoporphyrin (CrMP, 3 $\mu\text{mol/kg}$; Frontier Scientific, Inc., Logan, Utah). All animal protocols were approved by the Mayo Foundation Institutional Animal Care and Use Committee. Additional methods are presented in supplementary materials and methods.

Gastric Emptying

Gastric emptying of solids (baked egg yolk) was measured after an overnight fast using a [^{13}C]-octanoic acid breath test as described previously.²² Three baseline gastric emptying tests were obtained after habituation of the mice to the chamber. After onset of diabetes, gastric emptying was measured once a week. Values for normal, delayed and accelerated gastric emptying were determined previously.²²

Western blotting

Lysates extracted separately from the muscle layers of the gastric body and antrum from each of the experimental groups were used to detect proteins by Western blot analysis as described previously¹² and in the supplemental material. Rabbit polyclonal anti-Kit (Santa Cruz Biotech. Inc., Santa Cruz, CA), anti nNOS (NOS1) antibodies (Abcam Inc., Cambridge, MA) or anti-HO1 (Stressgen Biotechnologies Corporation, Victoria BC Canada) were used as primary antibodies. Changes in expression levels of HO1, Kit and nNOS protein normalized by GAPDH were determined from optical densitometry of immunoblots and shown as relative OD units (rODU) using the Bio-Rad Gel Doc system (Bio-Rad Laboratories, Hercules, CA).

Determination of oxidative stress

The concentration of thiobarbituric acid reactive substances was calculated as malondialdehyde(MDA) equivalents using a commercial kit (Oxi-Tek®, Zeptometrix Corporation, Buffalo, NY). One hundred μl of serum sample were mixed with an equal volume of sodiumdodecyl sulfate solution and 2.5 ml of 5% thiobarbituric acid/acetic acid reagent. Samples were incubated for 60 min at 95°C. After centrifugation at 1600g, supernatants from samples were read at 532 nm.

HO activity assay

Tissue was washed twice with phosphate-buffered saline (pH 7.4) and homogenized in 100 mM potassium phosphate buffer (pH 7.4). After sonification, the homogenate was centrifuged at 3000 g for 15 min. The supernatant solution was centrifuged at 12,000 g for 20 min followed by ultra-centrifugation at 105,000 g for 1h. Microsomal fractions were resuspended in 100 mM potassium phosphate buffer (pH 7.4) containing 2 mM MgCl_2 . In 500 μl reaction mixture, 500 μg microsome proteins were incubated with 20 μM hemin, 3 mg of liver cytosol, 0.2 U glucose-6-phosphate dehydrogenase, 2 mM glucose-6-phosphate, and 0.8 mM NADPH for 1h at 37°C in the dark. The bilirubin produced was extracted with chloroform, and the absorbance of bilirubin at 464 nm was measured against a baseline absorbance at 530 nm (extinction coefficient, 40 $\text{mM}^{-1} \text{cm}^{-1}$ for bilirubin). Heme oxygenase activity was expressed as pmol of bilirubin produced/mg protein/h. The total protein content of microsomes was determined using a Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA) by comparison with a standard curve obtained with bovine serum albumin.

Immunohistochemistry

Whole mounts were obtained from the greater curve of the gastric body¹² from 2 mice each in the non-diabetic and 10-week diabetes groups. To identify the HO1 positive cells in the muscle layers, we double labeled the tissue for the expression of the macrophage marker F4/80 and for HO1 as described in the supplementary materials and methods.

Statistical analysis

Data are presented either as the medians with interquartile ranges (IQR) for the box graphs and means $\pm$ SEM for the bar graphs. The statistical methods used in this study were paired t-test and one-way ANOVA with Tukey's post-test. A probability of less than 0.05 was considered significant. Nonparametric alternatives (Wilcoxon signed rank test and Kruskal-Wallis with Dunn's post-test) were used when the datasets did not meet criteria for parametric analyses.

Results

Blood glucose levels were higher in all diabetic mice compared to non-diabetic controls (102;93–115 mg/dl, $n = 14$, $p < 0.001$, Kruskal-Wallis test with Dunn's post-test) (Figure 1). The blood glucose level was 381;324–548 mg/dl ($n = 6$) at 2 weeks and 600;569–600 mg/dl ($n = 6$) at 4–5 weeks ($P < 0.001$ Kruskal-Wallis test with Dunn's post-test). 20% of diabetic mice developed delayed gastric emptying (at a mean time of 5.5 weeks after development of diabetes, range 4.5–7.5 weeks). The $T_{1/2}$ for gastric emptying was accelerated (56 ± 3.4 min, $n=6$) at 2 weeks of diabetes as previously reported²² and was normal at 4–5 weeks of diabetes (94 ± 3.7 min, $n=6$). The $T_{1/2}$ of gastric emptying for the mice with delayed gastric emptying was 152 ± 7.5 min ($n = 6$) as compared to 94 ± 1.2 min ($n=6$) in age matched controls ($P < 0.0001$, paired t-test, Figure 1). The mean gastric emptying curves are also shown in Figure 1. No mice developed delayed gastric emptying after 8 weeks, therefore the mice with 10 weeks of diabetes were considered resistant to the development of diabetic gastroparesis. The $T_{1/2}$ of gastric emptying was 110 ± 2.0 min ($n = 6$) in these mice as compared to 93 ± 1.3 min ($n = 6$, $P > 0.05$, paired t-test) in age matched controls (Figure 1). There was no difference in glucose levels or ketone levels (β -hydroxybutyrate levels) between diabetic mice resistant to development of delayed gastric emptying and those that developed delayed gastric emptying (Figure 1).

Western blot analysis using an antibody to nNOS on tissue from the gastric body and antrum (Figures 2 and 3) detected a protein band at approximately 160 kDa that corresponded to the molecular weight of nNOS protein. In the gastric body (Figure 2), nNOS expression was not different ($P > 0.05$, Wilcoxon signed rank test) in 2-week diabetic mice (0.72;0.60–0.84 rODU) compared to controls (0.91;0.81–1.07 rODU). nNOS expression was lower in 4–5 week and 10 week diabetic mice with normal gastric emptying (0.47;0.35–0.54 and 0.40;0.28–0.43 rODU) compared with 0.95;0.83–1.07 and 0.90;0.81–1.04, respectively, in their controls ($P < 0.05$). nNOS expression was also decreased in the mice with delayed gastric emptying (0.26;0.22–0.31 vs 0.88;0.81–1.09 rODU in controls $P < 0.05$, Wilcoxon signed rank test). Similar data were obtained for the antrum (Figure 3 and Supplementary Table 1).

In contrast, Kit expression in both body and antrum was maintained in all mice that did not develop delayed gastric emptying but was markedly decreased in every mouse that developed delayed gastric emptying (Figures 2 and 3). In the gastric body (Figure 2) Kit expression was not different ($P > 0.05$, Wilcoxon signed rank test) in diabetic mice with normal gastric emptying (0.99; 0.97–1.28, 0.99;0.88–1.20, 0.99;0.92–1.00 rODU at 2, 4–5 and 10 weeks respectively) compared to age-matched non-diabetic controls (0.97;0.81–1.23, 1.02;0.84–1.13, 0.93;0.84–1.09 rODU at 2, 4–5 and 10 weeks respectively). However, Kit expression was markedly decreased in diabetic mice with delayed gastric emptying (0.24;0.18–0.27 vs

0.91;0.84–1.12 rODU in age matched non-diabetic controls, $P < 0.05$, Wilcoxon signed rank test). Similar results were obtained for the antrum (Figure 3 and supplementary table 1).

Serum malondialdehyde levels were higher in diabetic mice at 10 weeks of diabetes (51 ± 3.3 nmol/ml, $n = 6$) compared to controls (18 ± 0.83 nmol/ml, $n = 9$, $P < 0.05$, one-way ANOVA with Tukey's post-test, Figure 4). Serum malondialdehyde levels were substantially higher in the mice that developed delayed gastric emptying (290 ± 39 nmol/ml, $n = 6$, $P < 0.001$, one-way ANOVA with Tukey's post-test) compared to the mice with 10 weeks of diabetes with normal gastric emptying (Figure 4).

We next studied HO1 expression in the different mouse tissues. Western blots showed a protein band at 32 kDa that corresponds to the molecular weight of HO1 protein. In both the gastric body and antrum expression of HO1 was low in non-diabetic mice (Figure 5) suggesting low baseline expression of HO1. In the gastric body (Figure 5A), HO1 expression did not change at 2 weeks of diabetes (0.43;0.42–0.74 vs 0.60;0.49–0.63 rODU in controls, $p > 0.05$, Wilcoxon signed rank test). HO1 expression was up-regulated in all mice with normal gastric emptying at the 4–5 week and 10 week time points (2.26;1.87–2.66 and 2.05;1.98–2.18 rODU respectively vs 0.61;0.49–0.73 and 0.56;0.48–0.67 rODU respectively in controls. In contrast, HO1 up-regulation was lost in all mice that developed delayed gastric emptying (0.27;0.22–0.36 vs 0.54;0.48–0.78 rODU in controls, $P < 0.05$, Wilcoxon signed rank test). Similar results were seen in the antrum (Figure 5B and Supplementary Table 1) except that HO1 was already up-regulated at the 2 week time point.

Up-regulation of HO1 was maintained in all mice that were resistant to development of delayed gastric emptying and reduced to control levels in all mice that developed delayed gastric emptying. To determine if restoration of elevated HO1 levels would reverse delayed gastric emptying, a separate set of mice were followed until delayed gastric emptying developed and then assigned to receive hemin, an established inducer of HO1 expression,²³ or vehicle ($n = 5$ in each group). The mean duration of diabetes before development of delayed gastric emptying in the mice was 5.6 weeks (Supplementary Figure 2) and the mean duration of hemin treatment was 4.4 weeks. Median (IQR) blood glucose levels were 489 (441–529) mg/dl in hemin-treated mice and 476 (437–513) mg/dl in vehicle treated mice. The average $T_{1/2}$ s of the mice with delayed gastric emptying were 155 ± 13 min and 162 ± 9 min before treatment with hemin or vehicle respectively. HO1 expression and activity increased in mice treated with hemin. In hemin-treated mice, HO activity (Figure 6) was 103.7 ± 8.8 pmol bilirubin/mg/h compared to 7.5 ± 1.2 pmol bilirubin/mg/h in vehicle treated mice ($P < 0.0005$, Paired t-Test). HO1 protein levels (supplementary figure 3) were higher in hemin treated mice compared to vehicle treated mice (5.24;3.86–5.84 rODU vs. 0.25;0.24–0.31 rODU, $P < 0.05$, Wilcoxon signed rank test). Oxidative stress levels (serum malondialdehyde levels, Figure 6) were lower in hemin treated mice compared to vehicle treated mice (19 ± 8.3 nmol/ml vs. 686 ± 46.9 nmol/ml, ($P < 0.0005$, paired t-test). Hemin treatment did not change glucose levels nor β -hydroxybutyrate levels (Supplementary Figure 4). Gastric emptying reversed back to normal values in all mice treated with hemin ($T_{1/2} = 95 \pm 10$) but remained delayed (166 ± 12 min) in all mice treated with vehicle (Figure 6). Kit expression (Figure 6) increased in hemin-treated mice compared to vehicle-treated mice (1.00;0.94–1.14 vs. 0.18;0.13–0.20 rODU, $P < 0.05$, Wilcoxon signed rank test). nNOS expression (Figure 6) also increased in hemin-treated mice compared to vehicle-treated mice (0.44;0.38–0.51 vs. 0.28;0.24–0.31 rODU, $P < 0.05$, Wilcoxon signed rank test).

Given the significant effects of HO1 upregulation on gastric emptying we confirmed the finding by treating mice with 2 week of diabetes with chromium mesoporphyrin, an inhibitor of HO activity (CrMP, 3 μ mol/kg) ip (treatment regime in supplementary Figure 5) for 3 weeks. CrMP inhibits activity but not expression of HO.²⁴ Median (IQR) blood glucose levels were 435 (403–476) mg/dl in CrMP treated mice and 451 (410–482) mg/dl in vehicle treated mice ($n = 5$,

$P > 0.05$, Wilcoxon signed rank test). Treatment with CrMP did not change glucose or β -hydroxybutyrate levels (Supplementary Figure 4). HO activity was inhibited in CrMP treated mice as compared to vehicle treated mice (13.7 ± 2.5 vs. 64.6 ± 2.5 pmol bilirubin/mg/h, $P < 0.005$, paired t-test, Figure 7). Oxidative stress levels (serum malondialdehyde levels, Figure 7) were higher in CrMP treated mice compared to vehicle treated mice (686 ± 46.9 vs. 37 ± 4.9 nmol/ml, $p < 0.0005$, paired t-test). Mice treated with CrMP all developed delayed gastric emptying after 3 weeks of treatment ($T_{1/2}$ 195 ± 36 min), whereas none of the mice treated with vehicle developed delayed gastric emptying ($T_{1/2}$ 95 ± 5 min) (Figure 7). Expression of Kit (Figure 7) was reduced ($P < 0.05$, Wilcoxon signed rank test) in CrMP treated mice compared to vehicle treated mice ($0.26; 0.20-0.37$ rODU vs. $1.19; 1.09-1.23$ rODU) but no change in nNOS expression (Figure 7) was found ($0.32; 0.23-0.34$ rODU vs. $0.39; 0.31-0.50$, $P > 0.05$, Wilcoxon signed rank test). Neither hemin or CrMP changed gastric emptying in non-diabetic mice (Supplementary Tables 2 and 3 and Supplementary figure 6).

To determine the cell type or types that up-regulate HO1 in diabetes, whole mount tissues were obtained from the gastric body of diabetic mice resistant to development of delayed gastric emptying and immunolabeled with an antibody to HO1. HO1 appeared to be expressed in a distinct cell type with morphological features suggestive of macrophages. We therefore double labeled whole mounts from non-diabetic mice, and diabetic mice resistant to development of delayed gastric emptying (Figure 8) with antibodies to HO1 and to F4/80 (a marker for macrophages). There were very few HO1 positive cells in non-diabetic mice (Figure 8). In the mice resistant to development of delayed gastric emptying nearly all HO1 positive cells were F4/80 positive suggesting that most of the up-regulation of HO1 occurred in macrophages. The majority, but not all, F4/80 positive cells were HO1 positive (Figure 8).

Discussion

Data presented in this study show that HO1 expression was up-regulated after development of diabetes and the concurrent increased oxidative stress. Up-regulation of HO1 was lost in all mice that went on to develop delayed gastric emptying and maintained in all mice that did not develop delayed gastric emptying. Failure to maintain up-regulation of HO1 and development of delayed gastric emptying was associated in all mice with high levels of markers of oxidative stress and loss of expression of Kit, a marker for ICC. There appears to be a loss of up-regulation of HO1 rather than a failure to up-regulate HO1 in the mice that go on to develop delayed gastric emptying as we have always seen an up-regulation of HO1 in all mice tested at the 4–5 week time point. Furthermore, the development of delayed gastric emptying was reversed by induction of HO1 in all diabetic mice receiving hemin, which is a remarkable finding given the intractability of the disease. In the hemin-treated mice, decreased nNOS and Kit expression was restored to normal levels. Conversely, inhibition of HO1 activity caused development of delayed gastric emptying and loss of Kit in all CrMP treated mice, suggesting that maintaining normal gastric function requires ICC and HO1 is essential to protect ICC against oxidative injury.

nNOS generated nitric oxide is an important inhibitory neurotransmitter in the gastrointestinal tract.²⁵ Together with the role of NO as an inhibitory neurotransmitter, NO is also a survival factor for ICC.¹² Several studies^{8, 26, 27} have shown that diabetic gastroparesis is associated with loss of expression of nNOS, a finding that was confirmed in our study. However, these studies did not compare nNOS expression in diabetic animals with and without delayed gastric emptying. The current study suggests that nNOS expression is lost early in the course of diabetes in NOD mice, before the onset of delayed gastric emptying and there was no direct correlation between loss of nNOS and development of delayed gastric emptying although the lowest levels of nNOS expression were seen in the mice that did develop delayed gastric emptying. Loss of nNOS was also not seen in the mice that developed delayed gastric emptying

induced by inhibition of HO1. These data suggest that nNOS loss is associated with delayed gastric emptying but on its own does not cause delayed gastric emptying. Partial loss of nNOS expression may have no overt effect on gastric emptying but may make mice more susceptible to injury from other factors such as loss of HO1 expression.

The tight correlation of high levels of serum malondialdehyde with the presence of delayed gastric emptying, both in the mice that spontaneously developed delay as well as the mice that received CrMP, corresponds with an increasing appreciation that oxidative stress mediates many of the deleterious effects of diabetes on organ function.²⁸ Serum malondialdehyde levels reflect increased lipid peroxidation due to increased oxidative stress. Increased oxidative stress in diabetes is associated with an increased risk of developing complications.²⁹ HO1 induction is now recognized as an important generalized response to oxidative stress and failure to induce HO1 is associated with several disease states.³⁰ HO1 is highly expressed in tissues directly involved in erythrocyte or hemoglobin metabolism but otherwise is usually expressed at low to undetectable levels under basal conditions. However diverse chemical and physical stimuli including oxidative stress induce rapid transcriptional up-regulation of expression of HO1.³¹ The pathways and mechanisms by which HO1 is induced and remains up-regulated and the pathways that lead to loss of up-regulation of HO1 were not determined in the present study. Multiple stress response elements are located in promoter region of the *hmx1* gene³² and a number of transcription factors mediate the induction of HO1 in response to external and internal stimuli.³³ For example, the transcription factor Nrf2 protects against oxidative stress through antioxidant response element (ARE) -directed induction of antioxidant enzymes, including HO1.³⁴ Another important regulator of HO1 is Bach 1. Bach1 is a heme binding protein and forms heterodimers with small proteins of the Maf family. These heterodimers repress transcription of the HO1 gene by binding to the ARE in the HO1 promoter.³⁴ Future studies will need to be directed towards understanding which transcription factors are operative in the induction of HO1 seen in diabetes and why some mice do not maintain HO1 up-regulation.

Up-regulation of HO1 did not occur in every cell type in the muscle wall of the stomach. Rather, HO1 up-regulation was noted primarily in macrophages. A dense network of resident macrophages are distributed in the intestinal muscularis³⁵ and resident macrophages have been shown to come in very close proximity to enteric nerves and ICC.³⁶ While macrophages play a pro-inflammatory role³⁷ they may also play an anti-inflammatory role in the gut.^{38, 39} There are two main activation pathways for macrophages. The first is through the 'classical' immune pathway associated with their microbiocidal function, their role in tissue damage and cellular immunity and in delayed-type hypersensitivity.^{40, 41} The second activation pathway is the alternative pathway that is not only associated with humoral immunity but also results in tissue protection and repair.^{40, 41} Our present data suggest a potential role for alternatively activated macrophages in protecting ICC through up-regulation of HO1.

In summary, the data presented in this report suggest that induction of HO1 protects ICC from damage by countering the effects of increased oxidative stress associated with diabetes. Loss of Kit expression is the result of an alteration in the balance between factors deleterious to ICC and factors that promote their survival. Loss of up-regulation of HO1 and subsequent increased oxidative stress may tip the balance toward ICC loss and delayed gastric emptying. An increase in pro-survival factors such as induction of HO1 restores the balance resulting in increased expression of Kit and normalization of delayed gastric emptying. These data, if confirmed in humans, suggest the possibility that strategies that target induction of the HO1 pathway may be of benefit to patients by preventing or reversing loss of ICC and normalizing gastric emptying with subsequent improved glycemic control and quality of life.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Glucose and β -hydroxybutyrate levels and gastric emptying. Panels A and B shows the glucose and β -hydroxybutyrate levels in the different groups of mice. Boxes are medians with IQRs. Bars represent means $\pm$ SEMs while the symbols represent individual mice. * $P < 0.01$, ** $P < 0.001$, Kruskal-Wallis test with Dunn's post-test for glucose levels, * $P < 0.001$, one-way ANOVA with Tukey's post-test for β -hydroxybutyrate levels ($n = 9$ for non-diabetic controls and $n = 6$ for others). Panel C shows average gastric emptying curves for each group (solid lines with circles are controls, dashed lines with squares are diabetic animals). Panel D shows individual mean $\pm$ SEM $T_{1/2}$ values for each mouse and the grouped data. * $P < 0.0001$, Paired t-Test. Gastric emptying was accelerated after 2 weeks of diabetes as previously shown²² and delayed in the mice assigned to the delayed gastric emptying group. The two horizontal dashed lines indicate normal range of gastric emptying.

Figure 2.

Western blot analysis of nNOS and Kit protein expression in the gastric body. Panel A shows representative images from nNOS, Kit and GAPDH blots. Predicted molecular weights are shown by arrows. Panel B shows relative protein expression (medians with IQRs) obtained by densitometric analysis normalized to GAPDH. nNOS protein expression was significantly decreased at 4–5 weeks and 10 weeks as well as in the mice with delayed gastric emptying. Kit expression was decreased only in the mice that developed delayed gastric emptying. Wilcoxon matched pairs test, * $P < 0.05$ ($n=6$). X axis legends apply to both panels.

Figure 3.

Western blot analysis of nNOS, Kit protein from the gastric antrum. Panel A shows representative images and panel B the relative protein expression. Similar results were obtained as in the body with decreased nNOS protein expression after 2 weeks of diabetes and Kit expression was decreased only in the mice that developed delayed gastric emptying. Wilcoxon matched pairs test, * $P < 0.05$ ($n=5$). X axis legends apply to both panels.

Figure 4.

Levels of oxidative stress as measured by serum malondialdehyde levels. Serum levels of malondialdehyde were measured from non-diabetic controls, diabetic NOD mice after 10 weeks of diabetes (mice resistant to development of delayed gastric emptying) and diabetic NOD mice with delayed gastric emptying. Bars are means $\pm$ SEM, one-way ANOVA with Tukey's post-test, * $P < 0.05$, ** $P < 0.001$ ($n = 9$ for non-diabetic controls and $n = 6$ for the others).

Figure 5.

Western blot analysis of HO1 protein from mouse gastric body and antrum using the same tissues as in Figures 2 and 3. Panel A shows representative HO1 immunoblots from gastric body and relative protein expression normalized to GAPDH from same blots as in Fig 2 (medians with IQRs). Panel B shows the corresponding blots and graphs from the antrum. HO1 protein expression was significantly increased in all mice at 4–5 weeks (and 2 weeks in the antrum) and in mice resistant to the development of diabetic gastroparesis (10 weeks of diabetes). However HO1 expression dropped back to baseline in all mice with delayed gastric emptying. Wilcoxon matched pairs test, * $P < 0.05$ ($n=6$).

Figure 6.

Hemin treatment reversed delayed gastric emptying. Panel A shows time course of mean $T_{1/2}$ values before and after development of delayed gastric emptying (week 0) and after treatment with hemin ($\square$ -started 2 weeks after development of delayed gastric emptying) or vehicle ($\circ$). Panel A also shows the mean kinetics of gastric emptying taken from the last gastric emptying for each mouse in each group (dotted line: vehicle; solid line: hemin). Panel B shows $T_{1/2}$ values (means $\pm$ SEM) for the last gastric emptying for each mouse in each group ($\circ$ vehicle, $\square$ hemin). Paired t-test, * $P < 0.05$ ($n=5$). Panel C shows HO activity (individual values and mean $\pm$ SEM, Paired t-test, * $P < 0.005$, $n=5$). Panels D and E show representative blots and relative protein expression (median with IQRs) for Kit and nNOS (Wilcoxon matched pairs test, * $P < 0.05$, $n=5$). Panel F shows malondialdehyde levels (mean $\pm$ SEM, Paired t-test, * $P < 0.0005$, ($n=5$). Hemin treatment returned Kit expression and nNOS expression to normal values, reduced oxidative stress and normalized gastric emptying in all mice treated. (DGE = delayed gastric emptying).

Figure 7.

CrMP treatment resulted in delayed gastric emptying. CrMP treatment was started 2 weeks after development of diabetes. The layout of this figure is similar to figure 5. CrMP treatment decreased Kit expression, increased oxidative stress and delayed gastric emptying in all mice treated. nNOS expression was not changed by CrMP.

Figure 8.

HO1 staining from whole mount tissues obtained from the gastric body. Panel A shows double-labeled whole mount tissues with antibodies to HO1 (red) and to F4/80 (green) from non-diabetic mice and panel B from diabetic mice resistant to development of delayed gastric emptying. The bottom images are the merged images. There was little HO1 expression in the non-diabetic mice. HO1 was up-regulated in the diabetic mice resistant to delayed gastric emptying and most of the HO1 co-localized in macrophages (F4/80 positive cells). Most but not all macrophages expressed HO1. Scale bar 100 μ m for all images.