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CD206-positive M2 macrophages that express heme oxygenase-1 protect against diabetic gastroparesis in mice

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Abstract

Background & Aims—Gastroparesis is a well-recognized complication of diabetes. In diabetics, up-regulation of heme oxygenase-1 (HO1) in gastric macrophages protects against oxidative stress-induced damage. Loss of up-regulation of HO1, the subsequent increase in oxidative stress, and loss of Kit delays gastric emptying; this effect is reversed by induction of HO1. Macrophages have pro- and anti-inflammatory activities, depending on their phenotype. We investigated the number and phenotype of gastric macrophages in *NOD/ShiLtJ* (NOD) mice after onset of diabetes, when delayed gastric emptying develops, and after induction of HO1 to reverse delay.

Methods—Four groups of NOD and *db/db* mice were studied: non-diabetic, diabetic with normal emptying, diabetic with delayed gastric emptying, and diabetic with delayed gastric emptying reversed by the HO1 inducer hemin. Whole-mount samples from stomach were labeled in triplicate with antisera against F4/80, HO1, and CD206 and macrophages were quantified in stacked confocal images. Markers for macrophage subtypes were measured by quantitative PCR.

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Results—Development of diabetes was associated with an increased number of macrophages and up-regulation of HO1 in CD206+ M2 macrophages. Onset of delayed gastric emptying did not alter the total number of macrophages, but there was a selective loss of CD206+/HO1+ M2 macrophages. Normalization of gastric emptying was associated with re-population of CD206+/HO1+ M2 macrophages.

Conclusion—CD206+ M2-macrophages that express HO1 appear to be required for prevention of diabetes-induced delayed gastric emptying. Induction of HO1 in macrophages might be a therapeutic option for patients with diabetic gastroparesis.

Keywords

Interstitial cells of Cajal; immune cells; gastrointestinal tract

Introduction

Gastroparesis is defined as a delay of emptying of gastric content in the absence of obstruction. Diabetes is one of the more common causes of gastroparesis. Diabetic gastroparesis is associated with increased morbidity and mortality.¹ In a mouse model of diabetic gastroparesis, ² development of diabetes is associated with up-regulation of heme oxygenase-1 (HO1) that protects against oxidative stress and loss of Kit-positive interstitial cells of Cajal (ICC), required for normal gastric emptying. Loss of up-regulation of HO1 results in increased oxidative stress, loss of Kit expression and neuronal nitric oxide synthase, and development of delayed gastric emptying. Re-induction of HO1 expression results in complete reversal of the delay in gastric emptying and the cellular defects.² HO1 catalyzes the degradation of heme to biliverdin, iron and carbon monoxide. Under normal conditions the gastric muscularis propria has very low levels of expression of HO1. Once diabetes develops in non-obese diabetic mice, HO1 is markedly up-regulated in muscularis propria macrophages.²

Macrophages are scavenger cells that play a role in homeostasis and defense. Resident macrophages are present in the muscle layers of the gastrointestinal tract.³ They can be polarized in response to the microenvironment. Polarized macrophages are classified in two main groups: classically activated macrophages (M1) and alternatively activated macrophages (M2). Polarization results in a change in phenotype and their physiology, including alterations in the expression of surface proteins and the production of cytokines.⁴ Phenotypic switch in macrophages is mediated by both innate and adaptive immune responses. The combination of two innate signals, interferon- γ (INF γ) and tumor necrosis factor α from immune cells such as natural killer cells and T helper 1 (T_H1) cells convert resident macrophages into a population of cells that secrete high levels of pro-inflammatory cytokines and mediators (M1 phenotype). The pro-inflammatory cytokines that are produced by M1 macrophages are important for host defense, but can cause considerable damage to the host.

Other innate signals such as interleukin 4 (IL-4) released from immune cells including basophils, mast cells, and T_H2 cells as a result of tissue injury convert resident macrophages into a population of cells that are programmed to promote wound healing (M2 phenotype) with up-regulation of the mannose receptor (CD206). CD206 is not expressed in M1 macrophages and therefore serves as a useful marker for M2 macrophages.⁵ M2 macrophages can be further subdivided into 3 sub-populations - M2a, M2b and M2c.⁶ M2a macrophages are induced by IL-4 or IL-13. M2b macrophages are induced by immune complexes or toll-like receptors. M2c macrophages are induced by IL-10. These sub-phenotypes can be differentially activated in diseases such as muscular dystrophy.⁷

The phenotype of a macrophage is not fixed and macrophages are functionally plastic in response to their microenvironment and can reversibly switch between M1 and M2 phenotypes.

^{8, 9} This functional plasticity of macrophages may play a critical role in response to tissue damage and infection, and in adaptation to an immune response and to resolution of wound healing.

We have previously shown that macrophages are the dominant source of HO1 in diabetic mouse stomach. HO1 induction is required to protect against the development of delayed gastric emptying in diabetic mice.² The aim of this study was to determine if the number and type of gastric macrophages changes in NOD mice and db/db mice after onset of diabetes, when delay in gastric emptying develops, and after delay in gastric emptying is reversed by induction of HO1. Our data suggest a new role for macrophages in the gastrointestinal tract, as CD206-positive, HO1 positive macrophages appear to be critical to the prevention of development of delayed gastric emptying in diabetic mice.

Materials and Methods

Animals and experimental design

Eight-week-old female NOD mice (a model of type 1 diabetes) and 6-week-old female db/db mice (a model of type 2 diabetes),¹⁰ were received from Jackson laboratory (Bar Harbor, ME). Glucose levels were measured using an ACCU-CHEK device (Roche, Indianapolis, IN) in whole blood from the vascular bundle located at the rear of the jaw bone. 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 mice younger than 26 weeks was 68%² while the incidence of diabetes in the db/db studied was 100%. Sub-therapeutic insulin (Lantus insulin glargine, Sanofi-Aventis U.S. LLC, Bridgewater, NJ) was injected once daily i.p. when the glucose levels were over 500 mg/dl to keep the diabetic NOD mice alive yet also keep blood glucose levels higher than 400 mg/dl to allow complications of diabetes to develop.

Gastric emptying of solids (baked egg yolk) was measured after an overnight fast using a [¹³C]-octanoic acid breath test as described previously.¹¹ Three baseline gastric emptying tests were obtained after habituation of the mice to the chamber. After onset of diabetes, gastric emptying was measured weekly. Four groups of mice were studied and 4 mice assigned to each group for NOD mice. Of the 20 db/db mice, two mice were euthanized before onset of diabetes and served as non-diabetic controls. Of the 18 mice that developed diabetes, 4 developed delayed gastric emptying of which two were assigned to group 3 and 2 to group 4. Two mice that developed diabetes but not delayed gastric emptying at 9 weeks after onset of diabetes were assigned to group 2 (see below). The groups were as follows - Group 1: non-diabetic control mice. Group 2: diabetic mice that did not develop delayed gastric emptying (after 10 weeks of diabetes for NOD mice and 9 weeks of diabetes for db/db mice) and therefore were resistant to the development of delayed gastric emptying.² Group 3: diabetic mice that developed delayed gastric emptying and were then treated with vehicle. Group 4: mice that developed delayed gastric emptying and were then treated with hemin. After two consecutive measurements of gastric emptying showing delay ($T_{1/2} > 129$ min), HO1 expression was induced by i.p. injection of hemin (40 μ mol/kg; Sigma-Aldrich, St Louis, MO) daily for 5 weeks as previously described.² For NOD mice, 16% of 61 total mice developed delayed gastric emptying with a mean of 5.3 weeks after development of diabetes. After injection of hemin to the mice with delayed gastric emptying, the first normal gastric emptying occurred at the 4 week time point of treatment with hemin. Mice in group 3, after measurement of 2 consecutive delayed gastric emptying times received daily i.p. injections of the vehicle used to formulate the hemin given to group 4 (0.25% ammonium hydroxide in 0.9% NaCl). For NOD mice, 4 additional diabetic mice received chromium mesoporphyrin to inhibit heme oxygenase activity and 4 received vehicle as previously published.²

All animal protocols were approved by the Mayo Foundation Institutional Animal Care and Use Committee.

Immunohistochemistry

Whole mounts were obtained from the greater curve of the anterior distal gastric body for NOD mice. Antibodies used were a rat monoclonal raised against mouse F4/80 conjugated to ALEXA FLUOR® 488 (AbD serotec, Raleigh, NC), a rabbit polyclonal anti-HO1 (Stressgen Biotech. Corp., Victoria BC Canada) and a goat polyclonal anti-CD206 (Santa Cruz Biotech Inc., Santa Cruz, CA). The F4/80 monoclonal antibody, which recognizes a 160-kDa glycoprotein on the surface of most mouse macrophage populations is widely used as a marker for mouse macrophages.¹² CD206-immunoreactivity is a marker for M2 macrophages.¹³ CD206-positive M2 macrophages were assessed in db/db mice on 12µm sections obtained from the distal gastric body.

Macrophages were identified using a modified triple fluorescent immunolabeling technique.¹⁴ Tissues were incubated in F4/80 antibody diluted to 0.4 µg/ml with Ca²⁺-containing, HEPES-buffered physiological salt solution (CaPSS) containing 135 mM NaCl, 5mM KCl, 2 mM CaCl₂, 1.2 mM MgCl₂, 10 mM glucose, and 10 mM HEPES, adjusted to pH 7.4 with Tris and applied at 37°C for 1 h in dark. The tissue was then fixed in 4% paraformaldehyde. After blocking the tissue in 10% normal donkey serum (NDS)/1× phosphate buffered saline (PBS)/0.3% Triton overnight at 4°C, HO1 (0.125 µg/ml) and CD206 (0.5 µg/ml) antibodies were applied by incubation in 5% NDS/1× PBS/0.3% Triton overnight at 4°C. After rinsing in PBS, donkey anti-rabbit cy3 (3 µg/ml, Chemicon, Temecula, CA) and donkey anti-goat cy5 (3 µg/ml, Chemicon, Temecula, CA) were added to incubate in 2.5% NDS/1× PBS/0.3% Triton at room temperature for 2 h in dark. After rinsing in PBS, nuclei in the tissue were stained by incubating the tissue for 30 min at 4°C with 0.3 µM 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI, Molecular Probes, Inc., Eugene, OR) in 1× PBS.

For NOD mice, labeled tissues were examined using a laser scanning confocal microscope using a 25× (NA 0.81) Zeiss Plan Neofluar objective in Zeiss LSM510 (Carl Zeiss, Inc., Oberkochen, Germany) with a field size of 368.5 µm × 368.5 µm. Stacks of confocal images across the full thickness of the muscularis propria were collected from 9 regions of each whole mount tissue. The total numbers of immunolabeled cells were counted in stacked confocal images of the 9 defined regions using Analyze™ (Mayo Foundation, Rochester, MN) and expressed as cells/mm³. For db/db mice, examination of labeled tissues was carried out using a laser scanning confocal microscope using a 40× (NA1.00 Oil) Olympus UPlanApo objective on an Olympus FLUOVIEW 1000 (Olympus America Inc., Center Valley, PA) microscope with a field size of 401 µm × 200µm. Stacks of confocal images across the full 12 µm thickness of muscularis propria were collected from 12 randomly generated regions from each sectioned tissue and examined by 2 investigators blind to the tissue source.

Real-time quantitative PCR

NOD mice were used for this study. Six mice from each of the 4 groups were studied. RNA was extracted from full-thickness gastric body tissues using RecoverAll™ (Ambion, Austin, TX). SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) was used to generate cDNA from extracted RNA and real-time PCR was carried out on cDNA using commercial primer sets and RT²SYBR®Green/ROX™ qPCR master mix according to the manufacturer's instructions (SABiosciences, Frederick, MD). Data were normalized to the expression of GAPDH, a housekeeping gene, by transforming the difference in threshold cycle (ΔC_T) to the ratio of the gene of interest to the housekeeping gene (2^{-ΔC_T}), and expressed as the mean ± SEM for six animals.

Statistics

Data are presented either as medians with interquartile ranges (IQRs) for nonparametric data and means $\pm$ SEM for parametric data. The statistical methods used for parametric data were unpaired t-tests or one-way analysis of variance with Tukey's post-test for multiple comparisons and for non-parametric data, Mann-Whitney tests when comparing two groups and Kruskal-Wallis when comparing more than two groups. A probability of less than 0.05 was considered significant.

Results

The $T_{1/2}$ of gastric emptying for non-diabetic control NOD mice (group 1) was 96 ± 4 minutes ($n=4$), 100 ± 3 minutes ($n=4$) for group 2 (10 weeks of diabetes with normal gastric emptying), 165 ± 14 minutes before treatment with vehicle and 158 ± 9 minutes after treatment with vehicle ($P>0.05$, $n=4$) for group 3 (delayed gastric emptying $P<0.001$, one-way ANOVA with Tukey's post-test compared with groups 1 and 2). The $T_{1/2}$ of the NOD mice in group 4 was 173 ± 25 minutes before treatment with hemin and reversed to normal (99 ± 11 minutes) levels after treatment (Figure 1A). The gastric emptying results observed in NOD mice and with treatment of hemin are similar to previously published.² To extend these observations beyond the NOD mouse we also investigated gastric emptying in db/db mice, a type 2 model of diabetes.¹⁰ Gastric emptying in db/db mice was similar to NOD mice. The $T_{1/2}$ values pre-diabetes for all db/db mice ($N=20$) was between 64 and 131 minutes, similar to non-diabetic NOD mice.¹¹ The $T_{1/2}$ values for the two db/db mice euthanized prior to the development of diabetes were 84 and 109 minutes. Four diabetic db/db mice developed delayed gastric emptying (22%). Two of these mice, with $T_{1/2}$ values of 183 and 178 minutes (above the normal gastric emptying range) were assigned to receive vehicle treatment (group 3) and the rates of gastric emptying remained elevated (202 and 145 minutes) after vehicle treatment. The other two mice with $T_{1/2}$ values of 165 and 139 minutes were assigned to receive hemin treatment (group 4) and the rates of gastric emptying were reduced (116 and 91 minutes) after hemin treatment. Of the 14 db/db mice that did not develop delayed gastric emptying after 10 weeks of diabetes, two were randomly chosen to serve as controls (group 2). The $T_{1/2}$ values for these mice were 98 and 93 minutes.

Blood glucose levels did not differ between the three diabetic NOD groups but were significantly elevated compared to the non-diabetic controls ($P<0.05$). Blood glucose was (median with IQR) 110;99-120 mg/dl in the non-diabetic controls (group 1), 528;373-813 mg/dl in diabetic mice with normal gastric emptying (group 2). Blood glucose for the NOD mice that developed delayed gastric emptying (group 3) was 613;473-959 mg/dl before treatment with vehicle and 611;480-952 mg/dl after treatment with vehicle. Blood glucose of the mice in group 4 was 598;485-668 mg/dl before the mice received hemin and 601;489-665 mg/dl after the mice received hemin ($P>0.05$, Kruskal-Wallis with Dunn's post-test for the 3 diabetic groups, Figure 1B). Like in the NOD mice, blood glucose levels in three groups of diabetic db/db mice were higher than non-diabetics (481;406-532 mg/dl) but not different from each other.

Resident macrophages were present in all gastric tissues and distributed through circular (CM) to longitudinal muscle layers (LM) (Figure 2A). The thickness of the muscle layers were similar in all NOD groups (52 ± 8 μ m in non-diabetic controls, 56 ± 7 μ m in diabetic mice with normal gastric emptying, 60 ± 7 μ m in diabetic mice with delayed gastric emptying and 61 ± 8 μ m in the mice that received hemin, $P>0.05$, one-way ANOVA with Tukey's post-test).

The number of macrophages in NOD diabetic mice with normal gastric emptying, as determined by F4/80 immunoreactivity, was higher than in non diabetic controls (5154 ± 262 cells/mm³ vs 3806 ± 133 cells/mm³, $P<0.001$, one-way ANOVA with Tukey's post-test, Figure

2B and 2C). Development of delayed gastric emptying did not further change the number of macrophages (5577 ± 234 cells/mm³). Hemin treatment also had no effect (5261 ± 231 cells/mm³, both $P > 0.05$ compared with diabetic mice with normal gastric emptying, Figure 2B and 2C, $P < 0.05$ compared to non diabetic controls). Most macrophages in the non diabetic animals were F4/80-positive, CD206-negative with only 248 ± 27 cells/mm³ CD206-positive, that is, approximately 7% (Figure 3A, 3B and 3C). In contrast, CD206-positive macrophages (4107 ± 250 cells/mm³) accounted for approximately 80% of all macrophages present (Figure 3A, 3B and 3C) once diabetes developed ($P < 0.05$, Figure 3B and 3C).

Triple staining showed that the HO1-positive macrophages were CD206-positive, M2 macrophages (Supplementary figure 1A). In non-diabetic controls, approximately 7% (248 ± 28 cells/mm³) of all F4/80-positive macrophages were HO1-positive (Supplementary figure 1A, 1B and 1C). In diabetic mice with normal gastric emptying, approximately 82% of all F4/80-positive macrophages were positive for HO1 (Supplementary figure 1A and 1B). The number of HO1-positive macrophages was markedly higher in diabetic mice with normal gastric emptying (4241 ± 267 cells/mm³) compared to the non-diabetic controls (248 ± 28 cells/mm³, t test, $P < 0.05$, Supplementary figure 1A and 1C). In non-diabetic controls, all of the few HO1-positive macrophages present were CD206-positive and all of CD206-positive macrophages were HO1-positive. In diabetic mice with normal gastric emptying, approximately 97% of the HO1-positive macrophages were CD206-positive M2 macrophages and 3% were CD206-negative, whereas all of CD206 positive macrophages were HO1-positive.

Development of delay in gastric emptying in NOD mice is preceded by loss of HO1 expression.² Once delayed gastric emptying occurred, development of delayed gastric emptying was accompanied by a marked reduction in the number of CD206-positive macrophages (470 ± 99 cells/mm³, $P < 0.001$, one-way ANOVA with Tukey's post-test compared to diabetic mice with normal gastric emptying Figure 4A and 4B) even though the total number of macrophages did not change (see above, Figure 2). Treatment with hemin to re-induce HO1 and restore normal gastric emptying also did not increase the total number of macrophages but markedly increased the number of CD206-positive macrophages (2598 ± 99 cells/mm³, Figure 4A and 4B, $P < 0.001$, one-way ANOVA with Tukey's post-test compared to diabetic mice with delayed gastric emptying). This number of CD206-positive M2 macrophages in mice treated with hemin was approximately 63% of the number of CD206-positive macrophages present in diabetic NOD mice with normal gastric emptying and 553% of the number of CD206-positive macrophages present in mice with delayed gastric emptying. The stomachs of the diabetic mice that developed delayed gastric emptying were dilated compared to non-diabetic control mice. Treatment with hemin did not restore the size of the dilated stomachs suggesting that the changes seen in the number of M2 macrophages and total number of macrophages (Figure 2 and 4B) was not due to a change in stomach size.

Expression of HO1 in macrophages showed a similar pattern to the number of CD206 positive macrophages (Figure 4A). The number of HO1-positive macrophages markedly decreased in mice that developed delayed gastric emptying (79 ± 18 cells/mm³) compared to the diabetic mice with normal gastric emptying (4241 ± 267 cells/mm³, oneway ANOVA with Tukey's post-test, $P < 0.001$, Figure 4C). However, this number was lower than the number of CD206-positive macrophages suggesting that there was a loss of HO1 expression even in this small subpopulation of macrophages. There was an approximately 89% reduction in the number of CD206-positive macrophages with onset of delayed gastric emptying with an approximately 98% reduction in the number of HO1-positive macrophages with onset of delayed gastric emptying. Only 16% of CD206-positive macrophages were HO1-positive in diabetic mice with delayed gastric emptying as compared with 100% in diabetic mice with normal gastric emptying ($P < 0.05$, Mann-Whitney test, Figure 5A and 5B). Hemin treatment increased the number of CD206-positive macrophages to 3126 ± 96 cells/mm³ (Figure 4A and 4B), 73% of

the number found in diabetic mice with normal gastric emptying. Inhibition of heme oxygenase activity, which induced delayed gastric emptying in diabetic mice,² decreased the number of CD206 positive macrophages (Supplementary figure 2) suggesting that HO1 activity contributes to macrophage phenotype switching. In db/db mice, the CD206 positive macrophage numbers changed in the same way as for NOD mice, more CD206 positive macrophages were present in diabetic mice with normal gastric emptying compared to non-diabetic controls, but numbers were markedly decreased in the diabetic mice that developed delayed gastric emptying. The decrease of CD206 positive macrophages was reversed after treatment of hemin (Figure 6).

Expression of transcripts from genes associated with HO1 expression and the different M2 subtypes was determined using real-time quantitative PCR. Arginase-1 mRNA levels increased early in diabetes (2 wks) compared to control mice (Figure 7A, $258 \times 10^{-4} \pm 40 \times 10^{-4}$ vs. $8 \times 10^{-4} \pm 1 \times 10^{-4}$, fold difference, mean $\pm$ SEM, one-way ANOVA with Tukey's post-test, $P < 0.001$, $n = 6$). Arginase-1 mRNA was no longer increased in the mice resistant to development of delayed gastric emptying and in the mice with delayed gastric emptying (Figure 7A, $46 \times 10^{-4} \pm 4 \times 10^{-4}$ and $9 \times 10^{-4} \pm 1 \times 10^{-4}$, fold difference, mean $\pm$ SEM, one-way ANOVA with Tukey's post-test, $P < 0.001$, $n = 6$). The high expression of arginase-1 at 2 weeks suggests that M2a macrophages were the dominant M2 macrophage subtype in early diabetes but not later in the duration of the disease.

IL-10 is both a potent inducer of HO1¹⁵ and an inducer of M2c macrophages.⁶ IL-10 levels were not changed in early diabetic mice ($9 \times 10^{-4} \pm 1 \times 10^{-4}$ compared with $5 \times 10^{-4} \pm 1 \times 10^{-4}$, fold difference, mean $\pm$ SEM), but were increased in the mice resistant to development of delayed gastric emptying compared to non-diabetic controls (Figure 7B, $368 \times 10^{-4} \pm 189 \times 10^{-4}$ vs. $9 \times 10^{-4} \pm 1 \times 10^{-4}$, fold difference, mean $\pm$ SEM, one-way ANOVA with Tukey's post-test, $P < 0.05$, $n = 6$). This increase in IL-10 was not present when the diabetic mice developed delayed gastric emptying (Figure 7B, $15 \times 10^{-4} \pm 12 \times 10^{-4}$, fold difference, mean $\pm$ SEM). These high levels of IL-10 will favor a phenotypic shift from M2a to M2c macrophages as diabetes progressed. This observation was strengthened by high expression level of CD163, a hemoglobin scavenger receptor¹⁶ and a surface marker up-regulated during differentiation into M2c macrophages¹⁷ in the mice resistant to development of delayed gastric emptying (10 weeks of diabetes with normal gastric emptying). CD163 mRNA levels were not increased at the 2 week time point ($7 \times 10^{-4} \pm 1 \times 10^{-4}$, fold difference, mean $\pm$ SEM), but higher at the 10 week time point compared to non-diabetic controls (Figure 7C, $63 \times 10^{-4} \pm 16 \times 10^{-4}$ vs. $9 \times 10^{-4} \pm 1 \times 10^{-4}$, fold difference, mean $\pm$ SEM, one-way ANOVA with Tukey's post-test, $P < 0.001$, $n = 6$). The increase in CD163 mRNA was not present in the mice that developed delayed gastric emptying ($8 \times 10^{-4} \pm 1 \times 10^{-4}$, fold difference, mean $\pm$ SEM). Arginase-1 competes for L-arginine with inducible nitric oxide synthase (iNOS), a marker of M1 macrophages⁷ with the balance between iNOS and arginase-1 corresponding to the balance between M1 and M2 macrophages.¹⁸ Levels of iNOS mRNA (Figure 7D) were not changed with onset of diabetes ($439 \times 10^{-4} \pm 116 \times 10^{-4}$, fold difference, mean $\pm$ SEM at 2 weeks) nor at 10 weeks of diabetes with normal gastric emptying ($10 \times 10^{-4} \pm 5 \times 10^{-4}$ fold difference, mean $\pm$ SEM, in 10 weeks diabetic). However, iNOS mRNA levels were increased in the mice with delayed gastric emptying ($965 \times 10^{-4} \pm 237 \times 10^{-4}$, fold difference, mean $\pm$ SEM) compared to non-diabetic controls ($15 \times 10^{-4} \pm 3 \times 10^{-4}$, fold difference, mean $\pm$ SEM, one-way ANOVA with Tukey's post-test, $P < 0.001$, $n = 6$). The high expression level of iNOS in mice with delayed gastric emptying is consistent with the decrease in the number of CD206 positive/HO1 positive M2 macrophages and the consequential increase in the number of M1 macrophages.

Discussion

We previously demonstrated in a NOD mouse model of type 1 diabetes that induction of HO1 prevents and also reverses the cellular changes that lead to development of delayed gastric emptying.² We now show that expression of HO1 occurs in a specific subset of macrophages, M2 macrophages, suggesting a novel role for these muscularis propria macrophages. Furthermore, we demonstrate that similar events contribute to delayed gastric emptying in a db/db mouse model of type 2 diabetes. The overall pattern of expression of HO1 in macrophages is consistent with previous immunoblots showing up-regulation of HO1 expression with onset of diabetes, loss of HO1 expression in mice with delayed gastric emptying and re-expression of HO1 with hemin treatment.²

Macrophages in mice have been classified into M1 and M2 phenotypes. M1 phenotype (classically activated) macrophages are characterized by production of nitric oxide, reactive oxygen intermediates and IL-12 and IL-23 at high levels. M2 phenotype (alternatively activated) macrophages are characterized by IL-4 and IL10 production. The F4/80 monoclonal antibody has been used widely as a pan macrophage marker in mice^{7, 19} since it was first developed¹² and CD206 is a widely accepted marker for M2 macrophages.^{5, 13, 20-22} In this study we now have determined that expression of HO1 is not found in all macrophages, rather it is largely limited to a class of macrophage that expresses CD206, the M2 macrophage marker.⁵ We also found that development of diabetes is associated with an increased number of macrophages and up-regulation of HO1 in these macrophages. These macrophages appeared to be M2 macrophages as they were CD206-positive. Given that M2 macrophages are usually considered to have anti-inflammatory function,⁸ these data suggest that the role of the increased number of macrophages with onset of diabetes is protective rather than pro-inflammatory. This protective role is mediated, at least in part, through expression of HO1 in M2 macrophages. HO1 activity results in the production of bilirubin, carbon monoxide and iron.²³ Recent data suggests that carbon monoxide treatment reverses delayed gastric emptying in NOD mice²⁴ suggesting carbon monoxide is the cytoprotective molecule produced by M2, HO1-expressing macrophages. An increase of macrophages in other disorders appears to also be correlated with the up-regulation of HO1 as an anti-oxidant for cytoprotection against environmental stresses.^{25, 26}

Macrophages differentiate from circulating mononuclear cells. Monocytes circulate in peripheral-blood and migrate into tissue to become resident macrophages. Resident macrophages can be activated by various endogenous and exogenous stimuli and change their phenotypes and their physiology, including alterations in the expression of surface proteins and the production of cytokines. While our studies did not directly determine the fate of CD206 positive macrophages in mice that went on to develop delayed gastric emptying, the data suggest that they did not leave the gut wall or die as the total number of macrophages remained the same irrespective of development of delayed gastric emptying, but rather remained in the gastric wall and lost expression of CD206. This is consistent with the known low turnover of macrophages as determined from peritoneal macrophages.²⁷ Furthermore, treatment of the mice with hemin to re-induce HO1 did not result in an overall further increase in macrophages; rather, there was an increase in the percentage of CD206-positive type 2 macrophages again suggesting a phenotypic switch. Interestingly, the trigger for this switch may be HO1 itself.

Up-regulation of HO1 expression occurred in CD206-positive M2 macrophages and up-regulation of HO1 may directly induce a phenotypic change to the alternatively activated M2 phenotype.²⁸ The data presented in this manuscript are consistent with the hypothesis that HO1 is directly involved in the change of the macrophage phenotype to M2. Firstly, the number of CD206-positive M2 macrophages increased in diabetic mice, where HO1 expression was up-regulated. Secondly, CD206-positive macrophages were markedly decreased when the up-

regulation of HO1 expression was lost with onset of delayed gastric emptying. Thirdly, CD206 positive macrophages were reduced when heme oxygenase activity was inhibited. Fourthly, induction of HO1 by hemin in diabetic mice reversed the decrease of CD206-positive macrophages. And lastly, a subset of macrophages were HO1-positive, but not CD206 positive in hemin treated mice, suggesting that HO1 up-regulation occurs before the phenotypic switch from M1 macrophages to M2. Therefore HO1 may play a dual role, acting both as a signal to initiate phenotypic switching and to protect against oxidative stress.

The mRNA data suggest that not only are M2 macrophages critical to the prevention of development of diabetes-induced delayed gastric emptying but that different M2 subtypes are activated along the course of diabetes. The high expression of arginase-1 early in diabetes suggests that the M2 macrophages present at this time point are predominantly M2a macrophages.^{7, 29} The IL-10 and the CD163 data suggest that there is a phenotypic shift from M2a to M2c macrophages as diabetes progresses. Furthermore, the decreased expression of IL-10 and CD163 mRNA coupled with an increase in mRNA levels of iNOS in the stomachs of mice that developed delayed gastric emptying are consistent with a loss of all subtypes of M2 macrophages and a dominance of M1 macrophages seen in these mice.

The data presented in this manuscript suggest a novel role for macrophages in the gastrointestinal tract. CD206 positive M2 macrophages expressing HO1 appear to be critical to the prevention of development of delayed gastric emptying in diabetic mice and induction of HO1 in macrophages results in an increased number of HO1 expressing M2 macrophages. These data were seen in two independent models of diabetes, the NOD mouse as a model of type 1 diabetes and the db/db mouse as a model for type 2 diabetes. It is not known whether a similar increase in M2 macrophages occurs in human diabetes. This information will be important to determine in order to assess whether induction of HO1 in M2 macrophages or delivery of a product of heme metabolism by HO1 may present a therapeutic option in human diabetic gastroparesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations not mentioned in Style Guide

HO1	heme oxygenase-1
NOD	non-obese diabetic
ICC	interstitial cells of Cajal
INF γ	interferon- γ
T _H	T helper
IL	interleukin
PBS	phosphate buffered saline
ANOVA	analysis of variance

Figure 1. Gastric emptying and glucose levels of the mice studied. Panel A shows individual mean $\pm$ SEM $T_{1/2}$ values for each mouse and the grouped data (* $P<0.001$, oneway ANOVA with Tukey's post-test). The two horizontal dashed lines indicate the age-adjusted normal range of gastric emptying. Bars represent means $\pm$ SEM while the symbols represent individual mice. Panel B shows glucose levels in the four groups of mice. Boxes are medians with IQRs. * $P<0.001$, Kruskal-Wallis with Dunn's post test ($n = 4$).

Figure 2.

Number of macrophages is increased in diabetic mice. Panel A shows a representative Z-axis image from whole mount tissue labeled with antibodies to F4/80 in a non-diabetic mouse. Panel B shows representative XY field images from whole mount tissues labeled with antibodies to F4/80. Left to right panels are from non-diabetic mice, diabetic mice with normal gastric emptying, diabetic mice with delayed gastric emptying and diabetic mice treated with hemin after development of delayed gastric emptying. Panel C shows the number of F4/80 positive macrophages counted in stacked confocal images from 9 defined regions from each tissue (expressed as cells/mm³). Bars are means + SEM, one-way ANOVA with Tukey's post-test, * $P < 0.001$, (n = 4). Scale bar 100 μ m for all images.

Figure 3.

CD206 positive macrophages in diabetic mice. Panel A shows the representative images from whole mount tissues labeled with antibodies for F4/80, CD206 and the merged images. Panel B shows the percentage and panel C the number of CD206 positive macrophages counted in stacked confocal images from 9 defined regions from each tissue (expressed as cells/mm³). Bars for panel B are medians with IQRs, (Mann Whitney test, * $P < 0.05$, $n = 4$). Bars for panel C are means + SEM, t test, * $P < 0.05$, ($n = 4$). Scale bar 100 μm for all images

Figure 4.

Expression of CD206 and HO1 after development of delayed gastric emptying and after hemin treatment to reverse the delayed gastric emptying. The representative images from whole mount tissues are labeled with HO1 and CD206 antibodies using tissue from diabetic mice with normal gastric emptying (left), diabetic mice with delayed gastric emptying (middle) and delayed gastric emptying after hemin treatment (right). Panel B shows the number of CD206 positive macrophages and Panel C HO1 positive macrophages counted from stacked confocal images from 9 defined regions of the tissue (expressed as cells/mm³). Development of delayed gastric emptying was associated with loss of HO1, CD206 positive macrophages and this loss was reversed after treatment with HO1. Bars are means + SEM, ANOVA with Tukey's post test * P<0.001, (n = 4). Scale bar 100 μ m for all images.

Figure 5.

HO1 expression in CD206 positive macrophages. Panel A shows representative images from whole mount tissues labeled with antibodies to CD206 (left), HO1 (right) from a diabetic mice with normal gastric emptying (top) and a diabetic mice with delayed gastric emptying (bottom). Panel B shows the percentages of HO1 positive and CD206 positive macrophages in the two groups. Development of delayed gastric emptying was associated with loss of CD206 macrophages and the residual macrophages had decreased HO1 expression. The white ovals show two residual CD206 macrophages that did not express HO1. Bars are medians with IQRs, Mann-Whitney test, * $P < 0.001$, ($n = 4$). Scale bar 100 μm for all images.

Figure 6.

CD206 positive macrophages in db/db mice. Representative images from sections labeled with an antibody to CD206 (top) and DAPI (bottom). Panel A shows a representative image from a non-diabetic control, panel B a representative image from a diabetic mouse with normal gastric emptying, panel C a representative image from a diabetic mouse with delayed gastric emptying and panel D a representative image from a diabetic mouse with that initially had delayed gastric emptying and gastric emptying was normalized with hemin treatment.

Figure 7.

Expression levels of arginase-1, IL-10, CD163 and iNOS mRNA. Panel A shows mRNA expression level of arginase-1, panel B IL-10, panel C CD163 and panel D iNOS in each of the 4 groups of NOD mice. The data are expressed as the fold difference of levels of the target mRNA over the levels of GAPDH mRNA. Bars are means + SEM, one-way ANOVA with Tukey's post-test, *P<0.05 and ** P<0.001, (n = 6).