Carbon monoxide reverses diabetic gastroparesis in NOD mice

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Kashyap PC, Choi KM, Dutta N, Linden DR, Szurszewski JH, Gibbons SJ, Farrugia G. Carbon monoxide reverses diabetic gastroparesis in NOD mice. Am J Physiol Gastrointest Liver Physiol 298: G1013–G1019, 2010. First published April 8, 2010; doi:10.1152/ajpgi.00069.2010.—Diabetic gastroparesis is associated with increased oxidative stress attributable to loss of upregulation of heme oxygenase-1 (HO1), with resultant damage to interstitial cells of Cajal and delayed gastric emptying. These changes can be reversed by induction of HO1. HO1 catalyzes the breakdown of heme into iron, biliverdin and, carbon monoxide (CO). The aim of this study was to determine whether inhalation of CO can mimic the protective effects of HO1. Nonobese diabetic (NOD) mice with delayed gastric emptying were treated with CO inhalation. Serum malondialdehyde was measured as a marker of oxidative stress. Gastric emptying of solids was measured using a 1[14C]octanoic acid breath test. Kit expression levels were determined in immunoblots of protein extracted from the external muscle layers of the gastric body and antrum. The effect of CO on oxidative stress and gastric emptying was also determined in the presence of HO activity inhibitor chromium mesoporphyrin. CO inhalation reduced oxidative stress, restored Kit expression and reversed delayed gastric emptying in diabetic NOD mice with delayed gastric emptying. CO inhalation maintained this effect in the presence of the HO activity inhibitor, chromium mesoporphyrin, also resulting in restoration of the delay in gastric emptying. CO inhalation mimics the protective effect of upregulation of HO1 and decreased oxidative stress, increased Kit expression, and restored delay in gastric emptying. This effect of CO was independent of HO activity, suggesting that its effects were downstream of HO1. CO represents a potential therapeutic option for treatment of diabetic gastroparesis.

Macrophages; interstitial cells of Cajal; stomach; heme oxygenase; nonobese diabetic

GASTROPARESIS IS A COMPLICATION OF DIABETES, characterized by delayed emptying of the stomach in the absence of obstruction. Diabetic gastroparesis is associated with histological changes that include loss of neuronal nitric oxide synthase and interstitial cells of Cajal (ICC) in both humans and in animal models (17, 19, 29). ICC generate electrical signals that regulate smooth muscle contraction in the stomach and help maintain normal gastric function (7). ICC can be identified in tissue by labeling with antisera to receptor tyrosine kinase Kit, a protein expressed on ICC. Kit and its ligand, stem cell factor, are also important survival factors for ICC (25).

The number of ICC in healthy tissue is not static; rather, normal ICC networks are maintained by a balance between cell death, maintenance, and ICC proliferation (14). In disease states such as diabetic gastroparesis, this balance is disturbed with eventual loss of ICC. We have previously shown that increased oxidative stress associated with diabetes can lead to loss or damage to ICC in mice (9). Upregulation of the enzyme heme oxygenase-1 (HO1) is an important cellular defense mechanism against oxidative stress (1). Upon onset of diabetes in the nonobese diabetic (NOD) mouse model of diabetes, there is upregulation of HO1, which protects ICC by lowering oxidative stress. Loss of upregulation of HO1 in these mice leads to increased oxidative stress, which in turn leads to damage and loss of ICC networks and the development of delayed gastric emptying. Induction of HO1 by hemin reverses these effects, decreases oxidative stress, increases Kit expression levels, and normalizes gastric emptying (9).

The underlying mechanism of the cytoprotective effect of HO1 is unknown, but the preponderance of data in the literature suggests that the protective effect of HO1 is mediated by carbon monoxide (CO), a product of heme metabolism by HO1 (33, 35). In the gastrointestinal tract, low-dose CO inhalation has been shown to protect rat intestinal grafts from ischemia/reperfusion injury (27), improve levels of colitis in IL10−/− mice (18), and protect against the development of postoperative ileus and necrotizing enterocolitis (26, 40). CO released from CO-releasing molecules also has similar effects in animal models and has been shown to reduce oxidative stress and reduce postoperative ileus (11).

The goal of this study was to determine whether, in the NOD mouse model of type 1 diabetes, low-dose CO inhalation mimics the protective effects of HO1 in diabetic mice with delayed gastric emptying by reducing oxidative stress, increasing Kit expression, and normalizing delay in gastric emptying.

MATERIALS AND METHODS

Animals and experimental design. Diabetic female NOD/ShiLtJ (Jackson laboratory, Bar Harbor, ME) mice were studied as previously described (9, 10). All procedures were approved by the Mayo Clinic IACUC. The experimental designs for the two experiments in this study are outlined in Figs. 1 and 2. In the first experiment (Fig. 1), diabetic mice that developed delayed gastric emptying were assigned to two groups. One group (n = 5) was killed, and serum and gastric tissue was collected to obtain tissue as a pretreatment control. The other group (n = 5) was assigned to receive CO inhalation 100 ppm for 6 h/day for a maximum period of 8 wk or less if the mouse had two consecutive normal rates of gastric emptying. At the end of this period, mice were killed, and gastric tissue and serum were collected. In the second set of experiments (Fig. 2), diabetic mice, after 2 wk of diabetes, either received daily intraperitoneal injections of chromium mesoporphyrin (CrMP, 3 μmol/kg per day dissolved in 0.25% ammonium hydroxide, n = 10; Frontier Scientific, Logan, UT), to inhibit HO or daily intraperitoneal injections of 0.25% ammonium hydroxide (vehicle; n = 5). All mice injected with CrMP developed delayed gastric emptying as confirmed by two consecutive gastric emptying readings, and all mice injected with ammonium hydroxide alone had normal gastric emptying. Mice with delayed gastric emptying were assigned to two groups. One group (n = 5) was killed after develop-

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Mice with delayed gastric emptying were either killed soon after development of delayed gastric emptying (CrMP developed delayed gastric emptying as confirmed by 2 consecutive gastric emptying readings; all mice injected with ammonium hydroxide alone had this period, mice were killed and gastric tissue and serum was collected. After a baseline reading, mice were fed 200 mg scrambled cooked egg yolk containing 2.5 mg/kg of [13C]octanoic acid. Air containing the exhaled breath was collected and analyzed to determine the [13C]/[12C] ratio using an Infra Red Isotope Analyzer (IRIS; Wagner Analyisen Technik, Bremen, Germany).

**CO inhalation.** Mice were housed in specialized chambers for the treatment. CO was mixed with compressed air using an air gas mixer (Omega systems) to deliver 100 ppm. Random samples were obtained from the chamber and analyzed using gas chromatography to confirm the CO concentration in the chamber.

**Western blotting.** Western blot analysis was used to detect and quantify Kit protein and GAPDH from protein extracts of the muscle layers of the gastric body as described previously (9). Homogenates of gastric body were lysed in a solution consisting of 50 mM Tris-HCl, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM activated Na3VO4, 1 mM NaF, and the Complete Protease Inhibitors cocktail tablet (Roche, Indianapolis, IN). The total protein content 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. From the tissue lysates, 50 µg of protein were resolved by SDS-PAGE on a 12% gel. Proteins were transferred to Immunoblot PVDF membranes (Bio-Rad Laboratories) for immunolabeling. Rabbit polyclonal anti-Kit (Cell Signaling Technology, Danvers, MA) was used as the primary antibody for Kit detection. The intensity of enhanced chemiluminescence of immunoreactive bands was quantified using the Bio-Rad Gel Doc system (Bio-Rad Laboratories). The intensities of all bands were in the linear range of detection. Kit protein expression levels are expressed as the ratio of relative OD units (rODU) for Kit-immunoreactive bands over rODU for GAPDH-immunoreactive bands.

**Oxidative stress.** Oxidative stress was determined as previously reported (9) from the concentration of thiobarbituric acid-reactive substances, calculated as malondialdehyde equivalents using a commercial kit (Oxi-Tek; Zeptometrix, Buffalo, NY). Five microliters of serum sample were mixed with an equal volume of sodium dodecyl sulfate solution and 125 µl of 5% thiobarbituric acid/acetic acid reagent. Samples were incubated for 60 min at 95°C. After centrifugation at 1,600 g, supernatants from samples were read at 532 nm using a spectrophotometer (NanoDrop Technology, Wilmington, DE). A serum sample was not available for one mouse, and therefore measurement could not be performed on this mouse.
**RESULTS**

Mice with blood glucose levels consistently higher than 250 mg/dl were considered to be diabetic. Twelve percent of NOD mice developed delayed gastric emptying (at a mean time of 4.7 wk after development of diabetes; range 3–7 wk). Of the 10 mice with delayed gastric emptying included in the study, five mice were assigned to receive low-dose CO inhalation for 6 h daily (100 ppm; mean period of 16 days; range 14–19 days) after development of delayed gastric emptying. The remaining five untreated mice were killed after development of delayed gastric emptying confirmed on two consecutive gastric emptying readings (Fig. 1).

Serum malondialdehyde levels were significantly lower in untreated mice that were killed soon after development of delayed gastric emptying compared with serum malondialdehyde levels in mice after treatment with CO inhalation (273.4 ± 48.8 vs. 41.1 ± 13.9 nmol/ml; n = 4; \( P < 0.05 \), one-way ANOVA with Tukey’s posttest; Fig. 3).

Western blot analysis of protein extracts from the gastric body of mice with delayed gastric emptying detected a protein that was the molecular weight of Kit (145 kDa). This was similar to our previously described finding using a different rabbit polyclonal antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) (9). In the gastric body, Kit expression was significantly decreased in all untreated mice with delayed gastric emptying that were killed after development of delayed gastric emptying, whereas CO inhalation increased the expression of Kit protein in mice with delayed gastric emptying compared with untreated mice (0.23±0.20–0.29 vs. 0.57±0.54–0.69 nmol; \( n = 5 \); \( P < 0.05 \), Mann-Whitney; Fig. 4).

As evident by the lower time to half emptied (1/2) values, the rate of gastric emptying was increased in mice following CO inhalation compared with both untreated mice with delayed gastric emptying and the 1/2 values recorded for the same mice before initiation of CO treatment (Fig. 5; \( n = 5 \); \( P < 0.05 \), one-way ANOVA with Tukey’s posttest). The 1/2 values following CO treatment were similar to values obtained before the onset of diabetes as well as before the development of delayed gastric emptying. Thus CO treatment normalized the rate of gastric emptying.

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**Fig. 3.** Reduced oxidative stress in mice treated with CO inhalation. Levels of oxidative stress as measured by serum malondialdehyde levels were measured from diabetic mice with delayed gastric emptying killed after development of delayed gastric emptying (untreated) and diabetic mice with delayed gastric emptying before CO inhalation and after CO inhalation. Open symbols represent individual values, whereas bars represent means ± SE, \( *P < 0.05 \), one-way ANOVA with Tukey’s posttest.

**Fig. 4.** Increased Kit expression in the stomach of mice treated with CO inhalation. Western blot analysis of Kit protein expression in the gastric body. A: representative images from Kit and GAPDH blots. B: relative protein expression (medians with interquartile ranges) obtained by densitometric analysis normalized to GAPDH. \( *P < 0.05 \), Mann-Whitney test. X-axis legends apply to both panels. Mr, relative molecular weight.
The decreases in serum malondialdehyde and improvement of Kit levels and gastric emptying were not due to lowering of blood glucose levels in mice treated with CO. There was no difference in the blood glucose levels measured for three days before starting CO treatment in mice with delayed gastric emptying and blood glucose levels measured for three days after normalization of gastric emptying with CO treatment (474 ± 91 mg/dl vs. 549 ± 100 mg/dl; n = 5; P > 0.05, paired t-test). Also, there was no difference in insulin amount administered during this period (4.4 ± 2.4 U vs. 3.3 ± 1.8 U; n = 5; P > 0.05, paired t-test).

Given our previous observation that HO1 upregulation reverses delayed gastric emptying (9), we tested our hypothesis that normalized gastric emptying following CO inhalation was a direct result of CO effects, rather than a result of a potential feedback upregulation of HO1 by CO, using CrMP to inhibit any HO that might have become active following CO treatment. Diabetic mice treated daily with an intraperitoneal injection of 3 μmol/kg CrMP developed delayed gastric emptying compared with vehicle-treated mice (t1/2 values 167.5 ± 10.1 min, n = 10, vs. 95 ± 4.9, n = 5; P < 0.05, unpaired t-test) after mean period of 1.9 wk (range 1–3 wk) similar to that previously described (9). Of the 10 mice that developed delayed gastric emptying following CrMP treatment, five mice were assigned to receive low-dose CO inhalation for 6 h daily (100 ppm; mean period of 16 days; range 14–19 days). The mice continued to receive daily intraperitoneal injections of CrMP during treatment with CO to continually inhibit HO activity. The remaining five untreated mice were killed after development of delayed gastric emptying on two consecutive gastric emptying readings (Fig. 2). Mice with delayed gastric emptying had significantly lower serum malondialdehyde levels following CO inhalation (169.1 ± 38.1 to 45.8 ± 7.8 nmol/ml; n = 5; P < 0.05, one-way ANOVA with Tukey’s posttest; Fig. 6) in the presence of maintained HO inhibition. Serum malondialdehyde levels were significantly higher in untreated mice that were killed after development of delayed gastric emptying with CrMP treatment compared with serum malondialdehyde levels in CrMP-treated mice after CO inhalation (382.6 ± 46.1 vs. 45.8 ± 7.8 nmol/ml; n = 5; P < 0.05, one-way ANOVA with Tukey’s posttest; Fig. 6). CO inhalation significantly shortened t1/2 of gastric emptying and normalized gastric emptying in mice with delayed gastric emptying (t1/2 values 156.7 ± 6.1 to 83.2 ± 5.7 min; n = 5; P < 0.05, one-way ANOVA with Tukey’s posttest; Fig. 7) in the presence of HO inhibition. The t1/2 of gastric emptying in untreated mice killed after development of delayed gastric emptying with CrMP treatment was significantly higher than mice treated with CO inhalation (t1/2 values 178.6 ± 21.6 vs. 83.2 ± 5.7 min; n = 5; P < 0.05, one-way ANOVA with Tukey’s posttest; Fig. 7).

HO activity measured in the spleen was significantly inhibited in CrMP-treated mice killed soon after development of delayed gastric emptying compared with vehicle-treated mice (3,450 ± 1,494 vs. 9,250 ± 1,212 pmol bilirubin/mg per h; n = 5; P < 0.05, one-way ANOVA with Tukey’s posttest; Fig. 8). HO activity was also significantly inhibited in CrMP-treated mice that developed delayed gastric emptying and were treated with CO inhalation compared with vehicle-treated mice (3,000 ± 1,287 vs. 9,250 ± 1,212 pmol bilirubin/mg per h; n = 5; P < 0.05, one-way ANOVA with Tukey’s posttest; Fig. 8). There was no statistical difference in the HO activity among the CrMP-treated mice killed soon after development of delayed gastric emptying and the CrMP-treated mice with delayed gastric emptying, which received CO inhalation (Fig. 8). There was no difference in blood glucose levels measured for three days after starting CO treatment in mice with delayed gastric emptying and blood glucose levels measured for three days after normalization of gastric emptying with CO treatment.

Fig. 5. Gastric emptying normalizes following CO inhalation. Individual mean ± SE t1/2 values of gastric emptying for each mouse (open symbols) and the grouped data (bars) are shown. *P < 0.05, one-way ANOVA with Tukey’s posttest. The 2 horizontal dashed lines indicate the normal range of gastric emptying.

Fig. 6. Reduced oxidative stress in mice treated with CO inhalation in the presence of HO inhibition by CrMP. Serum levels of malondialdehyde were measured from CrMP-treated diabetic mice with delayed gastric emptying killed soon after development of delayed gastric emptying (untreated), with delayed gastric emptying before CO inhalation and after CO inhalation. Open symbols represent individual values, whereas bars represent means ± SE. *P < 0.05, one-way ANOVA with Tukey’s posttest.
DISCUSSION

In this study we show that administration of low-dose CO via inhalation to diabetic mice with delayed gastric emptying reduces oxidative stress, increases gastric Kit expression, and restores gastric emptying to normal rates. Therefore, CO inhalation has the same effect as upregulation of HO1 by hemin as previously described (9). Because the effects of CO inhalation persist in animals that are treated with CrMP to inhibit HO activity, the effects of CO are downstream of HO. Thus our data show that it is not necessary to have functional HO1 expressed in the muscularis propria to ameliorate the effects of diabetes on gastric function; the presence of one of the products of its activity (CO) is sufficient.

The therapeutic effect of CO is consistent with previous studies demonstrating the anti-inflammatory and antiapoptotic roles of this product of heme metabolism (23, 28, 30, 34). There are studies indicating that the expression of both HO1 (33) and HO2 (36) proteins protects cells from injury. Although bilirubin, one of the other products of heme metabolism, is an antioxidant with cytoprotective properties (13, 33), our study shows that CO alone was sufficient to restore gastric emptying to normal in diabetic mice. Normalization of gastric emptying by CO inhalation was observed even in the presence of an inhibitor of HO activity. By demonstrating that HO activity was significantly inhibited in the spleen by CrMP, regardless of CO treatment status, we established that the ability of CO to induce HO1 expression by itself (22) is not necessary for the positive response to CO administration. Therefore, the beneficial effects of CO are independent of HO1 activity, and CO is likely the product of HO1 activity responsible for HO1-mediated restoration of normal gastric function in diabetes. These results of the independent action of CO are similar to the action of CO in suppression of rejection in mouse to rat cardiac transplant (35) and protection from the effects of hyperoxic lung injury (31).

CO is a well-known environmental toxin. However, CO was used in this study at doses of 100 ppm for 6 h per day, and this dose is known to result in low levels (3%) of carboxyhemoglobin in humans (32). By comparison, smokers commonly have carboxyhemoglobin levels of 6% (39), and the toxic effects attributable to hypoxia are observed in humans when levels exceed 20% although some ill effects can be seen at 10–20% (8). Other studies have administered CO at doses around 1,000 ppm and have reported no significant adverse effects (39). Thus there seems to be a reasonable window of safety between doses of CO that are potentially therapeutic and the doses that are toxic, and the dose of CO used in this study can be considered a low dose.

The relative safety of the effective dose of CO, together with the independence of CO effects from activation of HO, makes CO a potential therapy for restoring normal gastric emptying in diabetic gastroparesis. An alternative approach would be to use compounds that induce HO1. These compounds include hemin, aspirin, and statins (2), with only hemin currently being shown to be effective in humans at standard doses (6). Hemin is FDA approved for treatment of intermittent porphyria (4), also making it attractive as a potential drug for the treatment of gastroparesis. Potential but not absolute limitations for use of hemin include the need for slow venous infusion and its side-effect profile (12, 16, 21). CO can be administered by inhalation safely in humans as shown in previous clinical trials, making it an attractive therapeutic option (5). In addition, an alternative is also available in the form of CO-releasing mol-

![Fig. 7. Gastric emptying normalizes following CO inhalation in the presence of HO inhibition by CrMP. Individual means ± SE $t_1/2$ values of gastric emptying for each mouse (open symbols) and the grouped data (bars) are shown. *$P < 0.05$, one-way ANOVA with Tukey's posttest. The 2 horizontal dashed lines indicate the normal range of gastric emptying.](http://ajpgi.physiology.org/)

Fig. 7. Gastric emptying normalizes following CO inhalation in the presence of HO inhibition by CrMP. Individual means ± SE $t_1/2$ values of gastric emptying for each mouse (open symbols) and the grouped data (bars) are shown. *$P < 0.05$, one-way ANOVA with Tukey’s posttest. The 2 horizontal dashed lines indicate the normal range of gastric emptying.

($447 ± 35$ mg/dl vs. $414 ± 125$ mg/dl; $n = 5$; $P > 0.05$, paired t-test). Also, there was no difference in insulin administered during this period ($1.2 ± 0.9$ U vs. $1.2 ± 1.0$ U; $n = 5$; $P > 0.05$, paired t-test).

![Fig. 8. CrMP treatment inhibits HO activity. Individual values (open symbols) and means ± SE (bars) are shown. HO activity as measured by picomoles bilirubin per milligram of protein per hour. *$P < 0.05$, one-way ANOVA with Tukey’s posttest.](http://ajpgi.physiology.org/)

Fig. 8. CrMP treatment inhibits HO activity. Individual values (open symbols) and means ± SE (bars) are shown. HO activity as measured by picomoles bilirubin per milligram of protein per hour. *$P < 0.05$, one-way ANOVA with Tukey’s posttest.
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ecleres (CORMs), which slowly release the CO in aqueous solution or in response to changes in pH (3). CO released from CORMs has been shown to reduce oxidative stress and reduce the development of postoperative ileus (11), but CORMs are not available for clinical use yet.

The cellular mechanism of action for CO was not established in this study. We focused on the changes in Kit protein in response to CO, as we and others have previously reported that, although changes in other markers, particularly nNOS expression, are observed in most models of diabetic gastroparesis (9, 17, 19, 38), it is the expression of Kit, the marker for ICC, that tracks most closely with development of delayed gastric emptying (9). Normalization of gastric emptying and Kit expression was clearly associated with a reduction in overall oxidative stress as indicated by malondialdehyde levels, a reporter molecule for lipid peroxidation (20). Thus, although blood glucose levels were not different between the groups and insulin was only administered to maintain the glycemia levels between 400 and 600 mg/dl, the lowering of oxidative stress was sufficient to restore Kit levels and gastric emptying to normal. The amount of insulin required to maintain the level of blood glucose between 400 and 600 mg/dl before and after administration of CO inhalation was not different, making it unlikely that CO altered insulin sensitivity.

Because Kit expression is the marker that best correlates with the changes in gastric emptying during diabetes in NOD mice (9), we propose that CO may be acting on ICC or ICC progenitors to restore gastric emptying to normal. The mechanism for this effect might be via modulation of p38 MAPK (30). A recent study demonstrated that oxidative stress and the development of postoperative ileus are reduced primarily because of activation of p38 MAPK following treatment of the animals with CORMs (11). In this respect, CO inhalation may act to correct an imbalance between the processes that injure and those that protect gastric emptying (135: 2055–2064, 2008).

In summary, we show that low-dose CO inhalation reversed damage to ICC in diabetic mice by countering the effects of increased oxidative stress associated with diabetes. These data, if confirmed in humans, hold promise as a new treatment in disorders associated with loss of ICC, such as diabetic gastroparesis, by preventing or reversing loss of ICC.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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