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Heme deficiency of soluble guanylate cyclase induces gastroparesis

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Abstract

Background—Soluble guanylate cyclase (sGC) is the principal target of nitric oxide (NO) to control gastrointestinal motility. The consequence on nitrergic signaling and gut motility of inducing a heme-free status of sGC, as induced by oxidative stress, was investigated.

Methods—sGC β_1^{H105F} knock-in (apo-sGC) mice, which express heme-free sGC that has basal activity, but cannot be stimulated by NO, were generated.

Key Results—Diethylenetriamine NONOate did not increase sGC activity in gastrointestinal tissue of apo-sGC mice. Exogenous NO did not induce relaxation in fundic, jejunal and colonic strips, and pyloric rings of apo-sGC mice. The stomach was enlarged in apo-sGC mice with hypertrophy of the muscularis externa of the fundus and pylorus. In addition, gastric emptying and intestinal transit were delayed and whole-gut transit time was increased in the apo-sGC mice, while distal colonic transit time was maintained. The nitrergic relaxant responses to electrical field

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CONFLICT OF INTEREST

The authors have no disclosures relevant to this subject or any conflicts of interest.

AUTHOR CONTRIBUTION

S. Cosyns and I. Dhaese performed the experiments and analyzed the data. R. Lefebvre designed the study, and wrote the manuscript with S. Cosyns and I. Dhaese. P. Brouckaert, E.S. Buys and R. Thoonen generated and provided the sGC β_1^{H105F} knock-in mice; E. Buys measured sGC activity; A. Vral did the histologic analysis; all checked the final version of the manuscript.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Drug application protocol for fundus, jejunum and colon strips.

Figure S2. Drug application protocol for pyloric rings.

Figure S3. Influence of ODQ and L-NAME on response to EFS in fundic strips from apo-sGC mice. Representative traces showing the inhibitory response to EFS (40 V; 0.1 ms; 1–8 Hz) occasionally occurring in PGF $_{2\alpha}$ -pre-contracted circular muscle strips of the gastric fundus of apo-sGC mice before and after incubation with 10 $\mu\text{mol L}^{-1}$ ODQ (A, B) or 300 $\mu\text{mol L}^{-1}$ L-NAME (C). The initial part of the contractile response to PGF $_{2\alpha}$ after L-NAME was out of range for 10 s, therefore the trace is not visible during this time.

Table S1. EC $_{50}$ and E_{max} of the contractions to carbachol and PGF $_{2\alpha}$

Data S1. Methods.

stimulation at 1–4 Hz were abolished in fundic and jejunal strips from apo-sGC mice, but in pyloric rings and colonic strips, only the response at 1 Hz was abolished, indicating the contribution of other transmitters than NO.

Conclusions & Inferences—The results indicate that the gastrointestinal consequences of switching from a native sGC to a heme-free sGC, which cannot be stimulated by NO, are most pronounced at the level of the stomach establishing a pivotal role of the activation of sGC by NO in normal gastric functioning. In addition, delayed intestinal transit was observed, indicating that nitrergic activation of sGC also plays a role in the lower gastrointestinal tract.

Keywords

gastrointestinal motility; nitric oxide; soluble guanylate cyclase

INTRODUCTION

Nitric oxide (NO), synthesized by neuronal NO synthase (nNOS, NOS-1) and released from non-adrenergic non-cholinergic (NANC) neurons, induces smooth muscle relaxation and contributes to the control of gastrointestinal motility, as evident from the delay in gastric emptying and intestinal transit upon NOS inhibition or in nNOS knockout mice.^{1–6} The principal intracellular target of NO is the heme-protein soluble guanylate cyclase (sGC), generating the second messenger cyclic guanosine 3′–5′-monophosphate (cGMP) to induce smooth muscle relaxation.⁷ However, sGC-independent relaxant effects of NO involving activation of small conductance Ca^{2+} -dependent K^{+} channels have been described in duodenum^{8,9} and colon.¹⁰ In addition, sGC can be activated by other stimuli than NO such as carbon monoxide (CO). Even though CO is a very weak activator of purified sGC (three-fold),¹¹ CO-induced relaxation in gastrointestinal smooth muscle is inhibited by the sGC inhibitor ODQ.¹² CO has been proposed as a gastrointestinal inhibitory neurotransmitter¹³ or as an endogenous hyperpolarizing factor.¹⁴

The physiologically active isoforms of sGC are $\text{sGC}\alpha_1\beta_1$, the predominant isoform in the gastrointestinal tract, and $\text{sGC}\alpha_2\beta_1$.¹⁵ We previously reported that in the gastrointestinal tract, $\text{sGC}\alpha_2\beta_1$ can compensate at least partially for the absence of $\text{sGC}\alpha_1\beta_1$ as gastric emptying was only mildly impaired and small intestinal transit was not influenced in $\text{sGC}\alpha_1$ knockout mice.^{16,17} Full knockout of sGC, eliminating activation of both sGC isoforms by NO but also basal sGC activity, is associated with systemic hypertension, severely delayed gut transit, and premature death.¹⁸ Smooth muscle-specific deletion of sGC was associated with hypertension and loss of vascular muscle responsiveness to NO,¹⁹ but the responsiveness to NO of gastrointestinal muscle was only mildly reduced, suggesting that sGC in gastrointestinal muscle is dispensable for nitrergic relaxation.²⁰ Selective deletion of sGC in the interstitial cells of Cajal (ICC) did not induce a decrease in NO responsiveness of gastrointestinal muscle. Deletion of sGC in both smooth muscle and ICC resulted in an impairment of nitrergic relaxation and an increase in gut transit time that was similar to that in mice lacking sGC ubiquitously.²¹ Together, these results suggest a redundant action of sGC in ICC and smooth muscle cells to induce gastrointestinal nitrergic relaxation.

The prosthetic heme group that interacts with the β_1 subunit of sGC is essential for the activation of both sGC isoforms by NO. Oxidation of sGC by reactive oxygen species results in an NO-insensitive heme-free enzyme²²; the latter status of sGC might contribute to disturbed vasodilatation under oxidative stress.²³ Oxidative stress is also involved in diabetic gastroparesis leading to dysfunction of nitrenergic nerves and ICCs²⁴; however, it remains unclear whether oxidation of sGC contributes to disturbed gastric nitrenergic relaxation in this condition. The histidine 105 residue of the β_1 subunit is a crucial amino acid for the binding of the heme group to sGC.²⁵ Recently, sGC β_1 ^{H105F} knock-in (apo-sGC) mice were developed²⁶; the resulting heme-deficient sGC isoforms retain their basal activity, but can no longer be activated by NO.²⁷ The apo-sGC mice are characterized by a reduced life span, growth retardation, and elevated blood pressure.²⁶ In this study, we investigated the consequence of switching native sGC to heme-free sGC, which cannot be stimulated by NO, in apo-sGC mice on gastrointestinal nitrenergic signaling and motility.

MATERIALS AND METHODS

Ethical approval

All experimental procedures were approved by the Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University.

Animals

sGC β_1 ^{H105F} knock-in mice (apo-sGC) were generated by homologous recombination in which the targeting vector introduces a mutation of the histidine residue at position 105 (exon 5) to phenylalanine, as well as five silent mutations. Correct recombination and germline transmission was confirmed using PCR and Southern Blot.

Homozygous sGC β_1 ^{H105F} knock-in (apo-sGC) mice and wild-type (WT) controls were derived from a heterozygous breeding on a mixed background (129/SvJ-C57Bl/6J). WT and apo-sGC mice of both sexes [male: $n = 79$ (WT) and 84 (apo-sGC), 7–15 weeks; female: $n = 36$ (WT) and 36 (apo-sGC), 7–16 weeks] had free access to regular drinking water and Transbreed Chow (SDS). However, when investigating transit using the phenol red, fluorescein-labeled dextran, or the colonic bead expulsion method (see below), food was withheld for 16 h overnight with free access to water.

Muscle tension experiments

Tissue preparation and isometric tension recording (Data S1)—Animals were sacrificed by cervical dislocation and the gastrointestinal tract was put in aerated (5% CO₂ in O₂) Krebs solution (pH 7.4; composition in mmol L⁻¹: NaCl 118.5, KCl 4.8, KH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 1.9, NaHCO₃ 25.0, and glucose 10.1). Circular muscle strips [2 × 11 mm (fundus) and 4 × 5 mm (jejunum and colon)] were prepared from the gastric fundus, jejunum, and distal colon, for the latter two after removal of the mucosa; one full wall thickness ring (2 mm) was prepared from the pylorus. Fundus, jejunum, and colon strips were mounted under optimal load (L_o as determined from the maximal response to the contractile agent carbachol) and pyloric rings under a load of 0.25 g, for isometric tension recording in classic organ baths, containing aerated (5% CO₂ in O₂) Krebs solution, between

two platinum electrodes, allowing electrical field stimulation (EFS). The medium was then switched to Krebs solution containing $1 \mu\text{mol L}^{-1}$ atropine and $4 \mu\text{mol L}^{-1}$ guanethidine to obtain NANC conditions and tissues were allowed to equilibrate for 60 min with flushing every 15 min in Krebs solution.

Protocol in fundic, jejunal, and colonic strips—All relaxant stimuli were examined after precontraction of the strips with 300 nmol L^{-1} (fundus and jejunum) or $3 \mu\text{mol L}^{-1}$ (colon) prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$); relaxations were induced when the contractile response to $\text{PGF}_{2\alpha}$ was stable for at least 2 min (10–15 min after adding $\text{PGF}_{2\alpha}$). In a first series, relaxations were induced by application of EFS [40 V, 0.1 ms, 1–2–4–8 Hz for 10 s (jejunum), 30 s (colon) or 60 s (fundus) at 5-min interval] via the platinum plate electrodes, then by application of exogenous NO ($1\text{--}10\text{--}100 \mu\text{mol L}^{-1}$ with an interval of at least 5 min during which the effect of a given concentration of NO had disappeared) and finally by vasoactive intestinal polypeptide (VIP; 100 nmol L^{-1} ; 5-min contact time). Strips were washed for 30 min, and were subsequently incubated with the sGC inhibitor 1H[1,2,4,]oxadiazolo [4,3-a]quinoxalin-1-one (ODQ; $10 \mu\text{mol L}^{-1}$) for 30 min. $\text{PGF}_{2\alpha}$ was then applied again and the responses to EFS, NO, and VIP were studied again in the presence of ODQ. In a second series, cumulative contractile responses to carbachol (1 nmol L^{-1} – $30 \mu\text{mol L}^{-1}$) or $\text{PGF}_{2\alpha}$ (1 nmol L^{-1} – $0030 \mu\text{mol L}^{-1}$) were first obtained in the absence of atropine and guanethidine. In jejunal and colonic strips, the contact time for each concentration of carbachol and $\text{PGF}_{2\alpha}$ was fixed at 2 min; in fundic strips, a higher concentration of carbachol and $\text{PGF}_{2\alpha}$ was given when the former concentration reached its maximal contractile effect. After that, the influence of ODQ ($10 \mu\text{mol L}^{-1}$) vs $10 \mu\text{mol L}^{-1}$ (fundus) or $100 \mu\text{mol L}^{-1}$ (jejunum and colon) 8-bromoguanosine 3',5' cyclic monophosphate (8-Br-cGMP; 10 min contact time) was studied. In a third series, the influence of the NOS inhibitor N^G -nitro-L-arginine methyl ester (L-NAME; $300 \mu\text{mol L}^{-1}$) was tested against the relaxation evoked by EFS. In colonic tissues, the $\text{PGF}_{2\alpha}$ -EFS cycle was repeated a third time to test the combination of L-NAME ($300 \mu\text{mol L}^{-1}$) plus the small conductance Ca^{2+} -dependent K^{+} channel blocker apamin (500 nmol L^{-1}). In a fourth series of experiments, the relaxing effect of CO ($300 \mu\text{mol L}^{-1}$; 10-min contact time) was studied in fundus and colonic strips.

In all series, the reproducibility of the relaxant responses was evaluated by running time-control vehicle-treated strips in parallel. At the end of each experiment, the tissue wet weight was determined (mg wet weight, see data analysis). The drug application protocol for fundus, jejunum, and colon strips is added as a supplementary figure (Figure S1).

Protocol in pyloric rings—In a first set of experiments, 6 min after adding $\text{PGF}_{2\alpha}$ ($3 \mu\text{mol L}^{-1}$), when the contractile response was stable for at least 2 min, relaxations were induced by application of EFS (40 V, 0.1 ms, 1–2–4 Hz for 10 s at 5-min intervals). The pyloric rings were then washed for 30 min and the relaxant responses were studied again in the presence of the NOS inhibitor L-NAME ($300 \mu\text{mol L}^{-1}$) or its solvent. In a second set of experiments, the relaxation by exogenous NO ($100 \mu\text{mol L}^{-1}$; 5-min contact time) was studied before and in the presence of the sGC inhibitor ODQ ($10 \mu\text{mol L}^{-1}$) or its solvent.

The drug application protocol for the pyloric rings was added as a supplementary figure (Figure S2).

Data analysis (Data S1)—In the fundic strips and pyloric rings that show tonic responses, the amplitude of the contractile and relaxant responses can be determined, and the relaxant responses were then expressed as % of the contraction evoked by $\text{PGF}_{2\alpha}$. As jejunal and colonic strips show phasic activity, the area above the curve for the relaxant responses was determined. EC_{50} values of concentration-response curves were calculated by linear interpolation.

Gastric emptying

Gastric emptying was measured 15 min after gavaging 250 μL of a phenol red meal; details are given in the Supplementary Methods (Data S1).

Transit and small intestinal contractility

Intestinal transit and small intestinal contractility—Intestinal transit and small intestinal contractility were measured 90 min after gavaging 200 μL of non-absorbable fluorescein-labeled dextran, by fluorescence imaging and spatiotemporal motility mapping as described previously.²⁸ Details are given in the Supplementary Methods (Data S1).

Whole-gut transit time (carmine method)—As adapted from Friebe *et al.*,¹⁸ 200 μL carmine (6% w v⁻¹ dissolved in 0.5% methylcellulose) was administered by gavage. Mice were then returned to individual cages, without food deprivation. The time taken for excretion of the first red-colored feces was determined at 30-min intervals.

Distal colonic transit—Distal colonic transit was measured according to previously described methods.^{29,30} After overnight fasting, a single 2-mm plastic bead was inserted 2 cm into the distal colon of each mouse using a custom-made polished metal applicator. The applicator and bead were preheated to 37 °C. After the bead insertion, mice were immediately placed in their cages with a white paper on the bottom, to help visualization of bead expulsion. The time for expulsion of the bead was determined for each animal.

Histology

Specimens of fundic, pyloric, jejunal, and colonic tissues were harvested from WT and apo-sGC mice. The tissues were fixed in 4% neutral buffered formalin, dehydrated through a graded series of ethanol and embedded in paraffin wax. Serial transverse sections of 5- μm thickness were cut at 500- μm intervals, using a rotary microtome (SLEE CUT 4060, Mainz, Germany) and stained with hematoxylin and eosin for morphological observation.

sGC enzyme activity

sGC enzyme activity was measured as described³¹; full details are given in the Supplementary Methods (Data S1).

Drugs used

The following drugs were used: apamin (Alomone Labs, Jerusalem, Israel), atropine sulfate, 8-Br-cGMP sodium salt, carmine, guanethidine sulfate, L-NAME, phenol red, PGF_{2α} tris salt, VIP (all obtained from Sigma-Aldrich, Diegem, Belgium), DETA-NO (Alexis Biochemicals, Antwerp, Belgium), carbachol (Fluka AG, Diegem, Belgium), fluorescein-labeled dextran (70 kDa, FD70; Invitrogen, Ghent, Belgium), ODQ (Tocris Cookson, Bristol, UK). All drugs were dissolved in de-ionized water except for the following: ODQ, which was dissolved in 100% ethanol and DETA-NO, which was dissolved in sGC enzyme activity buffer (Data S1). Saturated NO (2 mmol L⁻¹) and CO (1 mmol L⁻¹) solutions were prepared by bubbling oxygenated Krebs solution with, respectively, 99.9% NO or CO gas (Air Liquide, Herenthout, Belgium) as described by Kelm and Schrader.³² The CO-saturated Krebs solution contained PGF_{2α} (300 nmol L⁻¹ for the experiments in the fundus; 3 μmol L⁻¹ for the experiments in the colon) and was maintained at 37 °C.

Statistics

All results are expressed as means ± SEM. *n* refers to tissues obtained from different animals unless otherwise indicated. Comparison between apo-sGC and WT tissues was done with an unpaired Student's *t*-test. Comparison within tissues of either WT or apo-sGC was done by a paired Student's *t*-test. When more than two sets of results within the same tissue had to be compared, repeated measures ANOVA followed by a Bonferroni-corrected *t*-test was applied. A *P*-value less than 0.05 was considered to be statistically significant (Graphpad, San Diego, CA, USA).

RESULTS

All *in vivo* and *in vitro* experiments were performed in mice of both sexes, except for the measurement of sGC activity, distal colonic transit, and the experiments with pyloric rings, which were investigated in male mice only. No systematic differences between the sexes were observed; results are therefore presented for male mice only.

General observations and histology

The body weight of apo-sGC mice was significantly smaller than that of WT mice. The stomach of apo-sGC mice was significantly enlarged. The mean empty stomach weight of apo-sGC mice was significantly larger than this of WT mice. The length of the small intestine and colon in apo-sGC mice was significantly higher than in WT mice (Table 1). Smooth muscle layers of the muscularis externa were markedly thicker in the fundus of apo-sGC mice than in WT mice (Fig. 1). In addition, the muscularis externa of the pylorus was thicker in apo-sGC mice, although the difference with WT mice was less pronounced than for the fundus. No histologic differences between WT and apo-sGC mouse at the level of the jejunum and the colon were observed.

sGC enzyme activity

Baseline sGC activity was slightly higher in the colon of apo-sGC mice than in WT mice; (Fig. 2). DETA-NO significantly increased sGC activity in WT tissues, but not in apo-sGC

tissues. The level of sGC activity in the presence of DETA-NO was as a result significantly lower in apo-sGC vs WT tissues.

Muscle tension experiments

Tissue weight—When comparing strips or rings of the same dimensions, the fundic strips (2×11 mm) and the pyloric rings (width: 2 mm) of apo-sGC mice weighed significantly more than these prepared from WT mice (Table 1). On the other hand, the colonic strips (4×5 mm) of apo-sGC mice were slightly lighter than these of WT mice (Table 1).

Contractile responses to carbachol and $\text{PGF}_{2\alpha}$ —The EC_{50} and E_{max} of the cumulative concentration–response curves of carbachol and $\text{PGF}_{2\alpha}$ (1 nmol L^{-1} – $30 \text{ }\mu\text{mol L}^{-1}$) did not significantly differ in fundic, jejunal, or colonic strips from apo-sGC and WT mice (Table S1). $\text{PGF}_{2\alpha}$ at a concentration of 300 nmol L^{-1} was chosen to precontract fundic and jejunal strips to investigate the relaxant responses to EFS, NO, VIP, and 8-br-cGMP (see below); in the pylorus and colonic strips, $3 \text{ }\mu\text{mol L}^{-1}$ $\text{PGF}_{2\alpha}$ was used.

Fundus— $\text{PGF}_{2\alpha}$ (300 nmol L^{-1}) induced an increase in the tone in fundus strips. In WT fundus strips, application of EFS (1–8 Hz) induced frequency-dependent relaxations, consisting of a progressive decline in tone, which recovered upon ending stimulation (see Fig. 3A for the response at 8 Hz and Fig. 3C for mean responses); these relaxations were abolished by ODQ ($10 \text{ }\mu\text{mol L}^{-1}$; Fig. 3C) and L-NAME ($300 \text{ }\mu\text{mol L}^{-1}$; data not shown). In apo-sGC fundus strips, the relaxant responses to EFS at 1–4 Hz were totally abolished; EFS at 8 Hz induced a variable effect: relaxation was abolished in four of six strips (see Fig. 3A for an example where EFS at 8 Hz even induced a small contractile response), but in two of six strips, a relaxant response (10 and 53%) was obtained, no longer occurring when EFS at 8 Hz was repeated in the presence of ODQ. In an additional series with fundic strips from five apo-sGC mice, EFS at 8 Hz again induced relaxation in two tissues (Figure S3A and B), which was abolished by ODQ. In the series where L-NAME was tested, two of seven fundic strips of apo-sGC mice showed a relaxation in response to EFS at 8 Hz; this relaxation was not influenced by L-NAME (Figure S3C).

Exogenous applied NO (1 – 10 – $100 \text{ }\mu\text{mol L}^{-1}$) induced concentration-dependent relaxations in WT strips, consisting of a quick and transient decline in tone (see Fig. 3A for the response to $10 \text{ }\mu\text{mol L}^{-1}$ NO and Fig. 3C for mean responses). ODQ abolished the relaxant response to $1 \text{ }\mu\text{mol L}^{-1}$ NO and reduced those to 10 – $100 \text{ }\mu\text{mol L}^{-1}$ NO (Fig 3C). In apo-sGC strips, the relaxant responses to NO were totally abolished (Fig. 3A and C). In a small series, CO ($300 \text{ }\mu\text{mol L}^{-1}$) was tested. In WT fundus strips, CO induced a quick and transient decline in tone ($n = 4$), which was abolished in apo-sGC strips ($n = 4$; Fig. 3B).

8-Br-cGMP ($10 \text{ }\mu\text{mol L}^{-1}$) and VIP (100 nmol L^{-1}) induced a sustained decrease in tone. Mean responses to 8-Br-cGMP and VIP were not different between WT and apo-sGC strips (Fig. 3D). ODQ ($10 \text{ }\mu\text{mol L}^{-1}$) did not significantly decrease the responses to 8-Br-cGMP and VIP in either WT strips or apo-sGC strips (results not shown).

Pyloric rings—PGF_{2α} (3 μmol L⁻¹) induced an increase in tone in pyloric rings. In WT pyloric rings, EFS (1–4 Hz) induced a quick decline in tone recovering immediately upon the end of stimulation followed by a rebound contraction (Fig. 4A). The amplitude of relaxation increased by stimulation at 2 Hz compared with that at 1 Hz, but did not further increase by EFS at 4 Hz (Fig. 4A and C). L-NAME (300 μmol L⁻¹) abolished the EFS-induced responses at 1 Hz, and reduced the responses at 2 and 4 Hz (Fig. 4C). In apo-sGC pyloric rings, the relaxant response to EFS was reduced at all stimulation frequencies, with the response at 1 Hz being nearly abolished; relaxation still occurring with EFS at 2 and 4 Hz was not influenced by L-NAME (Fig. 4A and C).

The response to NO (100 μmol L⁻¹) in WT pyloric rings consisted of a quick and transient decline in tone (Fig. 4B). ODQ (10 μmol L⁻¹) decreased the relaxant response to NO in WT pyloric rings (109.8 ± 10.9%) to 33.3 ± 13.2% (*n* = 5). In apo-sGC mice, the relaxant response to NO was totally abolished (Fig. 4B).

Jejunum—In jejunal strips, PGF_{2α} (300 nmol L⁻¹) induced a combined tonic and phasic response. The tone decreased back to baseline within maximally 3 min after addition of PGF_{2α}, but the increase in phasic activity remained. In WT strips, EFS induced suppression of phasic activity; upon ending stimulation at 8 Hz, a rebound contraction was observed (Fig. 5A). The EFS-induced relaxations had a similar size at the different stimulation frequencies (Fig. 5A and B) and were abolished by ODQ (10 μmol L⁻¹; Fig. 5B) and L-NAME (300 μmol L⁻¹; data not shown). In apo-sGC strips, no relaxant response to EFS was obtained at 1–4 Hz. At 8 Hz, the phasic activity was initially suppressed. However, near the end of the stimulation train, an increase in phasic activity was systematically observed, which further progressed when the stimulation train was ended (Fig. 5A).

In jejunal strips of WT mice, NO (1–10–100 μmol L⁻¹) suppressed preimposed phasic activity with a concentration-dependent duration (Fig. 5A and B). ODQ abolished the relaxant response to 1 μmol L⁻¹ NO and reduced that to 10 and 100 μmol L⁻¹ NO (Fig. 5B). In jejunal strips of apo-sGC mice, the relaxant responses to NO were totally abolished (Fig. 5A and B).

In both WT and apo-sGC strips, 8-Br-cGMP (100 μmol L⁻¹) and VIP (100 nmol L⁻¹) induced a sustained suppression of phasic activity. Mean responses to 8-Br-cGMP and VIP were not significantly different between WT and apo-sGC strips (Fig. 5C). ODQ (10 μmol L⁻¹) did not significantly decrease the responses to 8-Br-cGMP and VIP in either WT strips or apo-sGC strips (results not shown).

Colon—PGF_{2α} (3 μmol L⁻¹) induced a combined tonic and phasic response. In WT strips, EFS induced suppression of phasic activity as well as a decrease in tone during the stimulation train, followed by a rebound contraction (Fig. 6A). The size of the relaxant response was similar at all stimulation frequencies. Only the response to 1 Hz was reduced by ODQ (10 μmol L⁻¹; Fig. 6B); the combination of L-NAME (300 μmol L⁻¹) plus apamin (500 nmol L⁻¹) was able to decrease the response at all frequencies in these strips, but did not abolish them (Fig. 6C). In apo-sGC strips, EFS at 1 Hz only induced a short decrease in tone at the beginning of the stimulation train. At higher frequencies, a pronounced relaxant

response was present (Fig. 6A). ODQ (Fig. 6B), L-NAME, or L-NAME plus apamin (see Fig. 6C) did not influence the remaining EFS-induced responses at 2–8 Hz in apo-sGC strips.

Nitric oxide ($1\text{--}10\text{--}100\ \mu\text{mol L}^{-1}$) suppressed preimposed phasic activity in WT strips with a concentration-dependent duration (Fig. 6A). ODQ abolished the relaxant response to $1\ \mu\text{mol L}^{-1}$ NO and reduced the response to 10 and $100\ \mu\text{mol L}^{-1}$ NO (Fig. 6B). In apo-sGC mice, the relaxant responses to NO were totally abolished (Fig. 6A and B). In a small series of experiments, CO ($300\ \mu\text{mol L}^{-1}$) was tested. In WT strips, CO induced a transient inhibition of phasic activity ($n = 4$); in apo-sGC strips, the ability of CO to inhibit phasic activity was abolished ($n = 6$; results not shown).

In both WT and apo-sGC strips, 8-Br-cGMP ($100\ \mu\text{mol L}^{-1}$) and VIP ($100\ \text{nmol L}^{-1}$) induced a sustained suppression of phasic activity, sometimes accompanied by a decrease in tone. Mean responses to 8-Br-cGMP and VIP were not significantly different between WT and apo-sGC strips (Fig. 6D). ODQ ($10\ \mu\text{mol L}^{-1}$) did not significantly decrease the responses to 8-Br-cGMP and VIP in either WT strips or apo-sGC strips (results not shown).

Gastric emptying, small intestinal transit, and whole-gut transit time

Fifteen minutes after gavage, gastric emptying of a phenol red solution was significantly lower in apo-sGC mice ($51 \pm 10\%$, $n = 8$) than in WT mice ($74 \pm 3\%$, $n = 10$) (Fig. 7A). In three apo-sGC mice, gastric emptying was measured with a test meal volume of $500\ \mu\text{L}$, yielding $37 \pm 8\%$ gastric emptying, excluding the possibility that the observed delay of gastric emptying in apo-sGC mice was related to using the same test meal volume for the clearly greater stomach in apo-sGC mice.

Ninety minutes after gavage, we observed a delayed intestinal transit of a fluorescein-labeled dextran solution in apo-sGC vs WT mice as manifested from the significant decrease in geometric center (Fig. 7D). Small intestinal contractility at that time point was, however, not different between WT and apo-sGC mice (% contraction amplitude in apo-sGC: $23 \pm 3\%$ vs WT: $22 \pm 3\%$, $n = 5\text{--}6$; Fig. 7E). The whole-gut transit time of a carmine solution was between 120 and 180 min in WT mice. In apo-sGC mice, the whole-gut transit time was more variable and the mean value was significantly increased (apo-sGC: 320 ± 25 min vs WT: 146 ± 10 min, $n = 6\text{--}7$; $P < 0.001$; Fig. 7B). Distal colonic transit time did not differ between apo-sGC (22 ± 3 min, $n = 8$) and WT mice (20 ± 3 min, $n = 9$; Fig. 7C).

DISCUSSION

The consequence of switching sGC to the heme-deficient state on nitrergic signaling and motility in the gut was studied in apo-sGC mice. The NO-donor DETA-NO failed to increase sGC activity in the gastrointestinal tissues of apo-sGC mice. In addition, exogenous and endogenous NO was not able to induce *in vitro* relaxation in the fundic, jejunal, and colonic smooth muscle strips of apo-sGC mice. In view of the redundant role of sGC in ICC and smooth muscle cells in inducing gastrointestinal nitrergic relaxation,^{20,21} the lack of responsiveness to NO in gastrointestinal smooth muscle strips of apo-sGC mice must also be related to the heme-deficient state of sGC β_1 in both ICC and smooth muscle cells. The

observation that the cGMP analog 8-Br-cGMP relaxes gastrointestinal smooth muscle strips equally in WT and apo-sGC mice indicates that the relaxant pathway downstream of sGC is intact. Furthermore, the similar relaxant response to VIP that acts through adenylate cyclase coupled VIP receptors in WT and apo-sGC mice argues against compensatory increase in cAMP-induced relaxation in apo-sGC mice. Furthermore, contractile mechanisms are not influenced by sGC β_1 ^{H105F} mutation as contractions to carbachol and PGF_{2 α} did not consistently differ between apo-sGC and WT mice. *In vivo*, the observed gastrointestinal phenotype of the apo-sGC mice included delayed gastric emptying and intestinal transit and increased whole-gut transit time.

Apo-sGC mice are hypertensive.²⁶ Although delayed gastric emptying was reported in spontaneously hypertensive rats,³³ it is unlikely that hypertension *per se* delayed gastric emptying and intestinal transit in apo-sGC mice: hypertension in mice with smooth muscle-specific deletion of sGC was not associated with abnormal gastrointestinal transit or impaired NO responsiveness in gastrointestinal smooth muscle.²⁰ The disturbed gastric emptying and intestinal transit in apo-sGC mice illustrate the importance of NO-sensitive sGC in gastrointestinal motility. Still, the gastrointestinal morphological consequences in apo-sGC mice, limited to the enlarged stomach, are less pronounced than in mice lacking sGC ubiquitously,¹⁸ suggesting that basal sGC activity, maintained in apo-sGC mice, plays a role in gastrointestinal motility. The disturbed gut motility in apo-sGC mice resembles that in mouse diabetic models.^{34,35} Diabetic gastroparesis has been related to oxidative stress-induced dysfunction of nitrergic nerves and ICCs,²⁴ but oxidative stress will also turn sGC to the heme-free state. The results presented here illustrate that the heme-free state of sGC in the diabetic stomach will also lead to disturbed gastrointestinal nitrergic signaling and might thus contribute to the pathogenesis of diabetic gastroparesis.

EFS-induced relaxations are abolished by L-NAME and ODQ in WT fundic strips, indicating that NO is the principal relaxant neurotransmitter acting through activation of sGC in the mouse gastric fundus. Still, EFS at 8 Hz induced relaxation, not sensitive to L-NAME, in some apo-sGC fundic strips suggesting that another neurotransmitter than NO can be released at stimulation frequencies from 8 Hz in the gastric fundus of apo-sGC mice. The identity of this neurotransmitter remains to be determined. As VIP is known to be released at higher stimulation frequencies in gastric fundus,^{36–39} it might already be released in apo-sGC mice at stimulation frequencies where this does not yet occur in WT mice to compensate for the loss in responsiveness to NO. Surprisingly, the relaxation by EFS at 8 Hz in gastric fundus of apo-sGC mice was inhibited by ODQ. VIP receptors are coupled to Gs proteins and adenylate cyclase, and ODQ is not expected to affect the activity of adenylate cyclase.⁴⁰ Non-specific effects of ODQ on excitation–contraction coupling with inhibition of contractile activity were previously described in canine colon.⁴¹ The mechanism responsible for the ability of ODQ to inhibit EFS-induced relaxation of apo-sGC fundus preparations remains to be determined.

Fundic nitrergic relaxation is essential for gastric accommodation⁴² and its deficiency in apo-sGC mice might be expected to speed up liquid gastric emptying as fundic storage of the liquids is impaired. However, similar to what was observed in nNOS KO mice⁶ and cGMP-dependent protein kinase (cGKI) KO mice,⁴³ liquid gastric emptying in apo-sGC

mice was delayed. In addition, the stomach of apo-sGC mice, nNOS^{5,6} and cGKI⁴³ KO mice, is markedly enlarged and characterized by hypertrophy of the muscularis externa of the fundus. Mashimo *et al.*⁶ suggested that this gastric smooth muscle thickening represents work hypertrophy secondary to functional pyloric obstruction. The muscular layer of the pylorus in apo-sGC mice was indeed enlarged as obvious from histology and from the higher pyloric weight of the pyloric rings, and the electrically induced relaxation in pyloric rings of apo-sGC mice was decreased. Impairment of pyloric relaxation will counteract the accelerating effect of deficient fundic relaxation on gastric emptying leading to delayed gastric emptying.^{6,44} Some electrically induced relaxation, not sensitive to L-NAME, was maintained in pyloric rings of apo-sGC mice. This illustrates that NO is not the sole inhibitory transmitter at the level of the pylorus, as was also reported for rat pylorus.^{45,46}

The disturbances in gastric emptying, delaying gavage liquid solution to enter the small intestine in apo-sGC mice, may contribute to the observed delay in small intestinal transit in apo-sGC mice. The complete inhibition of EFS-induced relaxations in WT jejunal strips by L-NAME, together with the absence of EFS-induced nitrergic relaxation in apo-sGC jejunal strips, identifies NO-sGC signaling as the principal inhibitory pathway in mouse jejunal smooth muscle. It seems therefore likely that an imbalance between inhibitory (nitrergic) and excitatory (cholinergic) input during peristalsis can contribute to the delay in intestinal transit observed in apo-sGC mice. Similarly, intestinal motility was impaired in cGKI KO mice, characterized by spastic contractions of long intestinal segments followed by scarce and slow relaxations.⁴³ Still, in apo-sGC mice, the spatiotemporal mapped contractility of a jejunal segment was not different from that in the WT mice; isolated small intestinal segments of total sGC knockout mice also maintained spontaneous rhythmic contractions.²⁰ These *in vitro* data appear to be in contrast to the observed delay in intestinal transit in apo-sGC mice and total sGC knockout mice. It is conceivable that the *in vitro* spontaneous oscillatory contractions are preserved in the jejunum of the apo-sGC mice, but that the coordinated interplay between ascending contractions and descending relaxations, essential for peristaltic propagation,⁴⁷ is disturbed in apo-sGC mice and contributes to the delay in small intestinal transit.

The delay in gastric emptying and intestinal transit most likely contributes to the increase in whole-gut transit time in apo-sGC mice. NOS inhibition was found to inhibit colonic propulsion of pellets in guinea pig colon⁴⁸ and to delay colonic transit in rats.⁴ However, because in mouse distal colon, NO – acting via sGC – is only the principal neurotransmitter at a stimulation frequency of 1 Hz and not at higher frequencies, the extent of delay in colonic transit was expected to be limited in apo-sGC mice. Distal colon expulsion of beads was indeed not delayed in apo-sGC mice.

We previously suggested that a redundant action of NO, acting at sGC, and another neurotransmitter, acting at small conductance Ca²⁺-dependent K⁺ channels, is responsible for the relaxant responses to EFS at 2–8 Hz in mouse distal colon. This hypothesis was based on the observations that L-NAME plus apamin, or ODQ plus apamin inhibited the relaxant responses to EFS at 2–8 Hz in mouse distal colon.⁴⁹ Gallego *et al.*⁵⁰ showed that the other neurotransmitter is ATP or a related purine. However, in apo-sGC mice, L-NAME plus apamin failed to influence the relaxant responses by EFS at 2–8 Hz. As well in WT

colonic strips, L-NAME plus apamin only partially attenuated the relaxations to EFS at 2–8 Hz. Together, these findings indicate the contribution of another neurotransmitter than NO and ATP to the relaxations at 2–8 Hz. The presence of this unidentified neurotransmitter could depend on the genetic background of the mice: in this study, mice on a mixed 129/SvJ-C57BL/6J background were used, whereas the previous study focused on mice on mixed Swiss-129 background.⁴⁹ The presence of a yet to be defined third neurotransmitter was also reported in rat distal colon.¹⁰

Because the relaxant effect of CO in gastrointestinal smooth muscle was abolished in apo-sGC mice, it is unlikely that CO, proposed as an inhibitory neurotransmitter in longitudinal muscle of C57BL/6J mouse distal colon,¹³ is the third neurotransmitter in mouse colon circular muscle. This result also definitely establishes that CO, although being a very weak activator of purified sGC, has a signaling pathway requiring sGC activation to induce relaxation in gastrointestinal tissue.

In conclusion, the gastrointestinal consequences of switching native sGC to heme-free sGC, which cannot be stimulated by NO, were most pronounced at the level of the stomach; the observed enlargement of the stomach with hypertrophy of the smooth muscle layers of the muscularis externa of the fundus and the pylorus and the delayed gastric emptying establish a pivotal role of the activation of sGC by NO in normal gastric functioning. In addition, the inability to stimulate sGC with NO induced delayed intestinal transit and increased whole-gut transit time.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

8-Br-cGMP	8-bromoguanosine 3',5' cyclic monophosphate
AUC	area under the curve
cGK (=PKG)	cGMP-dependent protein kinase
CO	carbon monoxide
DETA-NO	diethylenetriamine NONOate
EFS	electrical field stimulation

ICC	interstitial cells of Cajal
L-NAME	<i>N</i> ^ω -nitro-L-arginine methyl ester
<i>L</i>_o	optimal load
NANC	non-adrenergic non-cholinergic
nNOS (=NOS-1)	neuronal nitric oxide synthase
NO	nitric oxide
ODQ	1H[1,2,4,]oxadiazolo [4,3-a]quinoxalin-1-one
sGC	soluble guanylate cyclase
VIP	vasoactive intestinal polypeptide
WT	wild-type

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Figure 1.

Comparison of fundic, pyloric, jejunal, and colonic histologic transverse sections from a WT and an apo-sGC mouse. Microscopic view of a histologic transverse section of a WT (left panel) and an apo-sGC (right panel) mouse fundus (A, B), pylorus (C, D), jejunum (E, F), and colon (G, H). The transverse sections of 5 μm thickness were stained with hematoxylin and eosin for morphological observation.

Figure 2. sGC enzyme activity. Un-stimulated (baseline) and DETA-NO-stimulated sGC enzyme activity (expressed as picomoles cGMP produced per mg protein per min) in fundic (A), jejunal (B), and colonic (C) extracts of WT and apo-sGC mice. Means $\pm$ SEM of $n = 5-6$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$: apo-sGC vs WT (unpaired Student's t -test); ○ $P < 0.01$, ○○ $P < 0.001$: DETA-NO vs baseline (paired Student's t -test).

Figure 3.

Responses to EFS, NO, CO, 8-Br-cGMP, and VIP in fundus strips of WT and apo-sGC mice. (A, B) Representative traces showing the inhibitory responses to EFS (40 V; 0.1 ms; 8 Hz) and exogenously applied NO (10 $\mu\text{mol L}^{-1}$) or CO (300 $\mu\text{mol L}^{-1}$) in $\text{PGF}_{2\alpha}$ -precontracted circular muscle strips of gastric fundus from a WT mouse (upper trace) and an apo-sGC mouse (lower trace). (C) Frequency-response curves of EFS (40 V; 0.1 ms; 1–8 Hz) (left) and concentration-response curves of NO (1–100 $\mu\text{mol L}^{-1}$) (right) in WT ($\square$) and apo-sGC ($\circ$) strips of fundus before and after incubation with ODQ (10 $\mu\text{mol L}^{-1}$). Means $\pm$ SEM of $n = 7$ are shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$: apo-sGC before incubation vs WT before incubation (unpaired Student's t -test); $\circ$ $P < 0.05$, $\circ\circ$ $P < 0.01$, $\circ\circ\circ$ $P < 0.001$: WT after incubation with ODQ vs before (paired Student's t -test). (D) Relaxant responses to 8-Br-cGMP (left; 10 $\mu\text{mol L}^{-1}$) and VIP (right; 100 nmol L^{-1}) in fundus strips from WT and apo-sGC mice. Means $\pm$ SEM of $n = 12$ of six to seven animals are shown. An unpaired Student's t -test was applied, but no significance was found.

Figure 4.

Responses to EFS and NO in pyloric rings of WT and apo-sGC mice. (A) Representative traces showing the inhibitory responses to EFS (40 V; 0.1 ms; 1–4 Hz) in PGF_{2α}-precontracted pyloric rings from a WT mouse (upper trace) and an apo-sGC mouse (lower trace). (B) Representative traces showing the inhibitory responses to exogenously applied NO (100 μmol L⁻¹) in PGF_{2α}-precontracted pyloric rings from a WT mouse (upper trace) and an apo-sGC mouse (lower trace). (C) Frequency–response curves of EFS (40 V; 0.1 ms; 1–4 Hz) in WT (□) and apo-sGC (△) pyloric rings before and after incubation with L-NAME (300 μmol L⁻¹). Means ± SEM of *n* = 6–9 are shown. ***P* < 0.01, ****P* < 0.001: apo-sGC before incubation *vs* WT before incubation (unpaired Student's *t*-test); ○*P* < 0.05: WT after incubation with L-NAME *vs* before (paired Student's *t*-test).

Figure 5.

Responses to EFS, NO, 8-Br-cGMP, and VIP in jejunal strips of WT and apo-sGC mice. (A) Representative traces showing the inhibitory responses to EFS (40 V; 0.1 ms; 1–8 Hz) and exogenously applied NO (1–100 μmol L⁻¹) in PGF_{2α}-precontracted circular muscle strips of jejunum from a WT mouse (upper trace) and an apo-sGC mouse (lower trace). (B) Frequency–response curves of EFS (40 V; 0.1 ms; 1–8 Hz) (left) and concentration–response curves of NO (1–100 μmol L⁻¹) (right) in WT (□) and apo-sGC (△) strips of jejunum before and after incubation with ODQ (10 μmol L⁻¹). Negative values in the y-axis (left) indicate that a contractile response instead of a relaxation was obtained. Means ± SEM of *n* = 6–8 animals are shown. ***P* < 0.01, ****P* < 0.001: apo-sGC before incubation *vs* WT before incubation (unpaired Student's *t*-test); ○*P* < 0.05, ○○*P* < 0.01: WT after incubation with ODQ *vs* before (paired Student's *t*-test). (C) Relaxant responses to 8-Br-cGMP (left; 100 μmol L⁻¹) and VIP (right; 100 nmol L⁻¹) in jejunal strips from WT and apo-sGC mice. Means ± SEM of *n* = 12 of six to seven animals are shown. An unpaired Student's *t*-test was applied, but no significance was found.

Figure 6.

Responses to EFS, NO, 8-Br-cGMP, and VIP in colonic strips of WT and apo-sGC mice.

(A) Representative traces showing the inhibitory responses to EFS (40 V; 0.1 ms; 1–8 Hz) and exogenously applied NO (1–100 μmol L⁻¹) in PGF_{2α}-precontracted circular muscle strips of distal colon from a WT mouse (upper trace) and an apo-sGC mouse (lower trace).

(B) Frequency–response curves of EFS (40 V; 0.1 ms; 1–8 Hz) (left) and concentration–response curves of NO (1–100 μmol L⁻¹) (right) in WT (□) and apo-sGC (◻) strips of colon before and after incubation with ODQ (10 μmol L⁻¹). The responses to EFS and NO in the apo-sGC colonic strips were multiplied with a factor ‘PGF_{2α} response in WT colonic strips/PGF_{2α} response in apo-sGC colonic strips’ to correct for the significantly smaller

PGF_{2α}-induced precontraction in the apo-sGC *vs* the WT colonic strips in these series (apo-sGC: 32.00 ± 4.94 (g.s) mg wet weight *vs* WT: 89.92 ± 17.94 (g.s) mg wet weight, *n* = 6, *P* < 0.05). Means ± SEM of *n* = 6–8 are shown. **P* < 0.05, ***P* < 0.01, ****P* < 0.001: apo-sGC before incubation *vs* WT before incubation (unpaired Student's *t*-test); ○*P* < 0.05, ○○*P* < 0.01: WT after incubation with ODQ *vs* before (paired Student's *t*-test). (C) Frequency–response curves of EFS (40 V; 0.1 ms; 1–8 Hz) in WT (left) and apo-sGC (right) colonic strips before (□) and after incubation with L-NAME (300 μmol L^{−1}; △), and L-NAME (300 μmol L^{−1}) plus apamin (500 nmol L^{−1}; ○). Means ± SEM of *n* = 6–7 animals are shown. ○*P* < 0.05: after incubation with L-NAME plus apamin *vs* after incubation with L-NAME (repeated measures ANOVA followed by a Bonferroni corrected *t*-test). (D) Relaxant responses to 8-Br-cGMP (left; 100 μmol L^{−1}) and VIP (right; 100 nmol L^{−1}) in colonic strips from WT and apo-sGC mice. Complementary to the EFS- and NO-induced relaxation, the responses to VIP in the apo-sGC colonic strips were multiplied with the correction factor 'PGF_{2α}response in WT colonic strips/PGF_{2α} response in apo-sGC colonic strips'. Means ± SEM of *n* = 9–12 of 6–7 animals are shown. An unpaired Student's *t*-test was applied, but no significance was found.

Figure 7.

In vivo measurements in WT and apo-sGC mice. (A) Gastric emptying 15 min after gavage of 250 μ L of a phenol red meal (0.1% w v^{-1} dissolved in water) in WT and apo-sGC mice. Values are means $\pm$ SEM of $n = 8$ –10 animals. (B) Scatter graph showing the whole-gut transit time of a carmine solution (6% w v^{-1} dissolved in 0.5% methylcellulose) in WT and apo-sGC mice. The mean value is represented by a solid line ($n = 6$ –7 animals). (C) Scatter graph showing the distal colon transit time in WT and apo-sGC mice. The mean value is represented by a solid line ($n = 8$ –9 animals). (D) Distribution of fluorescein-labeled dextran in 10 equal small bowel (sb) segments, cecum, and two equal colon (col) segments 90 min after gavage of 200 μ L fluorescein-labeled dextran (70 kDa; 2.5% w v^{-1} dissolved in water) and geometric center (GC) in WT and apo-sGC mice. Values are means $\pm$ SEM of $n = 5$ –6 animals. (E) Representative contractility traces showing spontaneous oscillatory contractions in a 10 mm jejunal segment (X -axis) as deviations in mm (Y -axis) for a period of 20 s (Z -axis); the intestinal diameter measured at $t = 20$ s was used as reference value. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$: unpaired Student's t -test (apo-sGC vs WT).

Table 1

Body weight, small/large intestine length, stomach weight, and weight of the gastrointestinal preparations

	WT	apo-sGC
Body weight (g)	30 ± 1 (<i>n</i> = 23)	22 ± 1 (<i>n</i> = 25) ***
Small intestine length (cm)	30.0 ± 0.8 (<i>n</i> = 23)	33.7 ± 0.7 (<i>n</i> = 25) ***
Colon length (cm)	5.4 ± 0.2 (<i>n</i> = 23)	5.9 ± 0.2 (<i>n</i> = 25) *
Stomach weight (mg)	209 ± 10 (<i>n</i> = 23)	379 ± 28 (<i>n</i> = 25) ***
Fundus strips (mg)	5.83 ± 0.27 (<i>n</i> = 46)	15.73 ± 1.55 (<i>n</i> = 50) ***
Pyloric rings (mg)	7.86 ± 0.37 (<i>n</i> = 26)	11.38 ± 0.75 (<i>n</i> = 21) ***
Jejunum strips (mg)	0.54 ± 0.05 (<i>n</i> = 47)	0.45 ± 0.05 (<i>n</i> = 45)
Colon strips (mg)	0.53 ± 0.03 (<i>n</i> = 41)	0.44 ± 0.03 (<i>n</i> = 41) *

Values are means ± SEM. *n* refers to separate animals for body weight, small intestinal length, colon length, stomach weight, and pyloric rings and to fundus, jejunum and colon strips taken per two from separate animals.

* $P < 0.05$,

*** $P < 0.001$: unpaired Student's *t*-test (apo-sGC vs WT).