BASIC AND TRANSLATIONAL—ALIMENTARY TRACT

Cell-Specific Deletion of Nitric Oxide–Sensitive Guanylyl Cyclase Reveals a Dual Pathway for Nitrergic Neuromuscular Transmission in the Murine Fundus

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BACKGROUND & AIMS: It is not clear how nitric oxide (NO) released from enteric neurons relaxes gastrointestinal (GI) smooth muscle. In analogy to the vascular system, NO might directly induce relaxation of smooth muscle cells (SMCs) by acting on its receptor, NO-sensitive guanylyl cyclase (NO-GC). Alternatively, intermediate cells, such as the interstitial cells of Cajal (ICCs), might detect nitrergic signals to indirectly regulate smooth muscle tone, and thereby regulate the motor function of the GI tract. We investigated the role of ICCs and SMCs in nitrergic relaxation using mice with cell-specific disruption of the gene encoding the β_1 subunit of NO-GC (*GUCY1B3*). **METHODS:** We created mice that lack NO-GC specifically in SMCs (SM-guanylyl cyclase knockout [GCKO]), ICCs (ICC-GCKO), or both (SM/ICC-GCKO). We investigated the effects of exogenous and endogenous NO on murine fundus using isometric force studies. Total gut transit time was measured to monitor the functional consequences of NO-GC deletion on GI motility in vivo. **RESULTS:** NO-GC is expressed in ICC and SMC. Deletion of the NO receptor from SMCs incompletely reduced NO-induced fundus relaxation, which was hardly affected after ICC-specific deletion. Gut transit time did not change in SM-GCKO or ICC-GCKO mice compared with control mice. However, nitrergic relaxation was not observed in SM/ICC-GCKO mice, which had increased gut transit time compared with controls. **CONCLUSIONS: In mice, NO-GC is the only NO receptor to relax the fundus; deletion of NO-GC from the combination of SMCs and ICCs blocks nitrergic signaling. Therefore, ICCs and SMCs jointly mediate the relaxant effect of enteric NO.**

Keywords: cGMP; Knockout Mice; Electrical Field Stimulation; Mouse Model.

The exact mechanism of nitrergic relaxation of gastrointestinal (GI) smooth muscle has been disputed for many years. This controversy is based on the question of whether smooth muscle cells (SMCs) or interstitial cells of Cajal (ICCs) act as primary targets of nitric oxide (NO) released from enteric neurons.[1–11] Normal GI function depends on the controlled contraction and relaxation of smooth muscle in different sections of the GI tract. GI contraction is controlled by cholinergic and adrenergic neurons, and relaxation is mediated by nonadrenergic noncholinergic (NANC) neurons that release NO,[12–15] as well as adenosine triphosphate and vasoactive intestinal polypeptide[16–18] as neurotransmitters.

ICCs have been postulated to play an important role in the regulation of GI motility. ICCs reside in GI smooth muscle; they act as physiological pacemakers of the gut and have been postulated to mediate enteric neurotransmission. Innervation of ICCs occurs through both cholinergic and nitrergic nerve fibers and ICCs are thought to regulate both contractile and relaxant smooth muscle responses.[1,3,19] NO-sensitive guanylyl cyclase (NO-GC) is expressed in ICCs; surprisingly, the expression levels in ICC appear to be higher than those in GI SMCs.[4,20]

Investigation of nitrergic relaxation has been hampered by the lack of suitable animal models. The W/W^v mouse strain in which ICCs are deleted in some parts of the GI tract has been a valuable tool to dissect NO-mediated signaling.[11] However, this model suffers from the fact that ICCs are deleted only locally and that this leads to a reduction in both relaxant and contractile responses.

Abbreviations used in this paper: CCh, carbachol; DEA-NO, 2-(N,N-diethylamino)-diazenolate-2-oxide diethylammonium salt; cGMP, guanosine 3′,5′-cyclic monophosphate; EFS, electric field stimulation; GCKO, guanylyl cyclase knockout; ICC, interstitial cell of Cajal; ICC-GCKO, ICC-specific guanylyl cyclase knockout; NANC, nonadrenergic noncholinergic; NO, nitric oxide; NO-GC, nitric oxide-sensitive guanylyl cyclase; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; SMC, smooth muscle cell; SM-GCKO, smooth muscle-specific guanylyl cyclase knockout; SM/ICC-GCKO, smooth muscle and interstitial cells of Cajal–specific guanylyl cyclase knockout.

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We recently generated a mouse line that lacks NO-GC (GC knockout [GCKO]).[21] Neither pharmacologically applied NO nor endogenous NO release led to relaxation of GI smooth muscle from these mice,[20] which indicates that NO only acts via the NO-GC/guanosine 3′,5′-cyclic monophosphate (cGMP) pathway. In addition, NO-GC in SMCs is dispensable for nitrergic relaxation as NO-induced relaxation is retained in mice lacking NO-GC specifically in SMCs (SM-GCKO).[20] From these data, we conclude that there has to be an additional, non-SMC–based mechanism mediating nitrergic relaxation.

Here, we further investigated the role of NO-GC in nitrergic relaxation. We used novel mouse strains lacking NO-GC specifically in ICCs or SMCs (ICC-GCKO and SM-GCKO, respectively) and compared them with animals carrying a double deletion (SMC + ICC; SM/ICC-GCKO). Neither of the single-knockout mice (ICC-GCKO and SM-GCKO) had an overt GI phenotype; the double knockout, however, showed absence of nitrergic relaxation and reduced gut motility.

We show here that nitrergic relaxation occurs exclusively through NO/cGMP signaling in two different cell types: cGMP generated in either SMC or ICC can mediate smooth muscle relaxation. Taken together, we provide conclusive, mechanistic evidence for a dual-pathway mechanism for nitrergic relaxation in the murine fundus. In addition, we show a novel principle of NO-induced smooth muscle relaxation that is based on an elevation of cGMP in an intermediary cell type rather than directly in SMCs.

Methods

All experiments were conducted in accordance with the German legislation on protection of animals and approved by the local animal care committee. Whole gut transit time, immunohistochemical analysis, and isometric force studies were performed as described previously.[20] Regarding the isometric force studies, novel substances not introduced in Groneberg et al[20] include U46619, a thromboxane A2 analogue used to precontract fundus smooth muscle; and Bay 41-2272, a heme-dependent activator of NO-GC used to augment NO-induced cGMP increases.

Generation of SM-GCKO, ICC-GCKO, and SM/ICC-GCKO Mice

SM-GCKO and ICC-GCKO mice carry a floxed exon (exon 10 of the β_1 subunit of NO-GC) and are transgenic for the Cre recombinase/mutant estrogen receptor fusion protein under the inducible smooth muscle myosin heavy chain or cKit promotor (smooth muscle myosin heavy chain-CreER^{T2} or cKIT-CreER^{T2}). cKIT-CreER^{T2} mice were generated as described by Klein et al.[22]

SM/ICC-GCKO mice were generated by crossing SM-GCKO with ICC-GCKO mice. Mice from all 3 knockout lines aged 6–8 weeks were injected with tamoxifen (1 mg intraperitoneally) on 5 consecutive days in order to remove the floxed exon. As shown previously,[20] deletion of NO-GC in GI smooth muscle was complete only after 50 days; therefore, all mice were analyzed at least 50 days after the last tamoxifen injection. In each experiment, wild-type or heterozygous littermates were used as controls wherever feasible. In control animals, about 33.3% ± 3.7% of cKit-positive fundus cells stained negative for NO-GC (N = 4; 100 cells per animal counted), whereas in ICC-GCKO animals, approximately 84.4% ± 0.6% of cKit-positive fundus cells were negative for NO-GC (N = 7; 100 cells per animal counted), indicating effective deletion of NO-GC in ICC. The efficacy of NO-GC deletion in the SM-GCKO has been shown previously.[20]

Electric Field Stimulation

To measure the relaxation by endogenously released NO under NANC conditions, electric field stimulation was applied to fundus strips precontracted with U46619 (0.1 μmol/L) in the presence of atropine and guanethidine (1 μmol/L, each). Electric field stimulation (EFS) was applied through 2 platinum wire electrodes (5 mm distance; maximal voltage 0.5 ms, 0.5–4 Hz, 3 s). In the case of NO-GC sensitization, strips were preincubated with Bay 41-2272 (0.1 μmol/L) for 20 min.

To measure the inhibition of endogenous contraction by endogenously released NO, fundus strips were mounted in the organ bath without NANC conditions and precontraction. EFS was applied through 2 platinum wire electrodes (5 mm distance; maximal voltage, 0.5 ms, 1–32 Hz, 10 s). The ratio of the 4-Hz peaks with and without 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was taken for analysis.

Materials

The 2-(N,N-diethylamino)-diazenolate-2-oxide diethylammonium salt (DEA-NO), ODQ, U46619, and nifedipine were purchased from Axxora (Lörrach, Germany). Atropine, Bay 41-2272, carmine, carbachol (CCh), 3-isobutyl-1-methylxanthine, methylcellulose, and tamoxifen were from Sigma (Taufkirchen, Germany). Guanethidine was from ABCR (Karlsruhe, Germany).

Statistical Analysis

For calculation of statistical tests, GraphPad Prism software (GraphPad Software Inc., La Jolla, CA) was used. For comparison of independent variables (different animals at different concentrations or stimulation frequencies), all groups were compared by Kruskal-Wallis test. If P was $\leq .05$ for the global test, 2 groups were compared by Mann-Whitney U test in a predefined sequence and comparison was stopped if P was $>.05$ to prevent alpha inflation. Comparisons of individual groups were only reported if global tests reached significance. The individual statistical analyses for each figure are given in the Supplementary Material.

Results

Using immunohistochemistry, we first determined which cells expressed NO-GC in the GI tract. Supplementary Figure 1*A* and *C* shows that, in addition to SMCs, ICCs express NO-GC.

NO-Induced Relaxation in Fundus From SM-GCKO, ICC-GCKO, and SM/ICC-GCKO Mice

To investigate the effect of NO on ICCs, we generated mice lacking NO-GC specifically in these cells (ICC-GCKO) using a mouse expressing cre recombinase under control of the cKit promoter. Efficient deletion of NO-GC in cKit-positive fundus cells of the ICC-GCKO is