Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 14848–14853, December 1997
Neurobiology

Targeted gene deletion of heme oxygenase 2 reveals neural role for carbon monoxide

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Contributed by Solomon H. Snyder, October 15, 1997

ABSTRACT **Neuronal nitric oxide synthase (nNOS) generates NO in neurons, and heme-oxygenase-2 (HO-2) synthesizes carbon monoxide (CO). We have evaluated the roles of NO and CO in intestinal neurotransmission using mice with targeted deletions of nNOS or HO-2. Immunohistochemical analysis demonstrated colocalization of nNOS and HO-2 in myenteric ganglia. Nonadrenergic noncholinergic relaxation and cyclic guanosine 3′,5′ monophosphate elevations evoked by electrical field stimulation were diminished markedly in both nNOS$^{\Delta/\Delta}$ and HO-2$^{\Delta/\Delta}$ mice. In wild-type mice, NOS inhibitors and HO inhibitors partially inhibited nonadrenergic noncholinergic relaxation. In nNOS$^{\Delta/\Delta}$ animals, NOS inhibitors selectively lost their efficacy, and HO inhibitors were inactive in HO-2$^{\Delta/\Delta}$ animals.**

Nitric oxide synthase (NOS) and heme oxygenase (HO) display numerous similarities. Inducible and constitutive isoforms reflect multiple distinct genes encoding either NOS or HO (1). Both endothelial NOS and HO-2, which is most concentrated in brain and testes (1), occur in the endothelial layers of blood vessels and mediate vasorelaxation (1, 2). Neuronal NOS (nNOS) and HO-2 are colocalized within adventitial neurons of blood vessels (1) and in autonomic ganglia (1) with NO being a likely transmitter in the autonomic nervous system (3). Both enzymes give rise to more than one product: NO and citrulline from NOS and carbon monoxide and biliverdin from HO (1, 2). NO and CO stimulate soluble guanylyl cyclase activity (1), and inhibitors of NOS (1, 2) or HO (4) lower cyclic guanosine 3′,5′ monophosphate (cGMP) levels in certain tissues (1, 4).

Intestinal myenteric plexus neurons express nNOS (5) and HO-2 (6, 7), the respective biosynthetic enzymes for NO and CO in the nervous system (1). NOS inhibitors (8) and HO inhibitors (9) partially reverse nonadrenergic noncholinergic (NANC) relaxation of various portions of the gastrointestinal pathway. Studies investigating NO and CO functions with inhibitors of NOS or HO-2 are confounded by potential nonspecificity of these agents. For example, concentrations of metalloporphyrins that inhibit HO also can inhibit soluble guanylyl cyclase (7, 10, 11) and NOS (7, 12).

To elucidate a potential neural role for HO-2 products, we have used mice with targeted deletions of HO-2 (13) or nNOS (14). In the present study, we report diminished neurally evoked intestinal relaxation and depressed cGMP levels in nNOS$^{\Delta/\Delta}$ and HO-2$^{\Delta/\Delta}$ mice. Furthermore, HO-2 and nNOS were colocalized within neurons associated with myenteric ganglia of wild-type mice.

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MATERIALS AND METHODS

Immunohistochemistry. HO-2 antibody was prepared and used as described (7).

Double-label immunofluorescence. Intestinal segments from male Sprague–Dawley rats were placed into an oxygenated organ chamber containing Krebs buffer (see organ bath methods) and held at 37°C. Colchicine was added directly into the organ chamber, and tissue was fixed in 4% paraformaldehyde after a 12-h incubation in 95% O_2/5% CO_2 at 37°C. Tissue was fixed and sectioned as described (7). Sequential double-labeling was performed as described (15). Sections were incubated in excess unlabeled rabbit Ig before application of the second primary antisera to prevent cross-reactivity of secondary antibodies with the inappropriate antigen. To assess validity of staining, the order of incubation of the primary antibody was reversed with similar results. Omission of either primary antibodies resulted in singly labeled cells. For confirmation of neuronal staining and for quantitative determinations of neurons expressing nNOS or HO-2, sections were incubated in either nNOS or HO-2 antisera and neurofilament antibodies (Sigma) and peroxidase-linked secondary antibodies (data not shown). After development with peroxidase substrates, sections were counterstained with cresyl violet before viewing. Rat primary cortical cultures were prepared as described (16) and labeled as above.

Organ Bath Experiments. Ileal segments were prepared as described (17) in Ca^{2+}-free Krebs buffer (6). Strips 2 cm in length were mounted between two L-shaped hooks in temperature-controlled (37°C), 25-ml tissue baths containing Krebs buffer (119 mM NaCl/4.6 mM KCl/15 mM $NaHCO_3$/1.2 mM $MgCl_2$/1.5 mM $CaCl_2$/1.2 mM NaH_2PO_4/11 mM glucose) and continuously bubbled with 95% O_2/5% CO_2. Tension was measured with an isotonic force transducer that was attached to one of the L-shaped hooks. After equilibration for 1 h under 0.5 g of resting tension, only strips that developed spontaneous tone were retained for experiments. Strips were pretreated with atropine (10^{-6} M), propranolol (10^{-6} M), and indomethacin (10^{-5} M) for 20 min to eliminate cholinergic, adrenergic, and prostaglandin-mediated responses, respectively, before addition of 1,1-dimethyl-4 phenylpiperazinium (DMPP) or electrical field stimulation (EFS). Tetrodotoxin (10^{-6} M) was from Research Biochemicals (Natick, MA). Porphyrins (Porphyrin Products, Logan, UT) were prepared as described (7). All other reagents were from Sigma. Hemoglobin and CO stock solutions were prepared as described (18). Aliquots from

Abbreviations: NOS, NO synthase; HO, heme oxygenase; nNOS, neuronal NOS; cGMP, cyclic guanosine 3′,5′ monophosphate; NANC, nonadrenergic noncholinergic; DMPP, 1,1-dimethyl-4 phenylpiperazinium; EFS, electrical field stimulation; SNP, sodium nitroprusside; CMV, cytomegalovirus; SnPP-IX, tin protoporphyrin-IX; L-NNA, N^G-nitro-L-arginine; LTP, long-term potentiation.

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these stock solutions were added directly to the organ baths. In principle studies with knockout mice, experimenters were blinded as to the preparation being tested. In each experiment of Fig. 2 *b–g*, control strips were treated with sodium nitroprusside (SNP; 10^{-4} M) as in Fig. 2*a*, and in all cases, maximal relaxations were similar to those in Fig. 2*a*. At the completion of each experiment, strips were treated with SNP 10^{-4} M to confirm the integrity of smooth muscle responses. cGMP was determined as described (7).

HO-2 Constructs and Transfections. pBS-HO-2 (rat) was the generous gift of Barney E. Dwyer (19). A *Sal*I–*Not*I fragment comprising the entire translated sequence of rat HO-2 (20) was generated by PCR and subcloned into the *Sal*I–*Not*I site of the cytomegalovirus (CMV)-driven eukaryotic expression vector pCMV-myc to generate a fusion protein consisting of an NH2-terminal Myc tag followed by a pentaglycine linker and the HO-2 insert. HEK-293 cells (American Type Culture Collection) were transfected with either pCMV-mycHO-2 or empty vector using the calcium phosphate method. Cells were harvested 48 h after transfection. For detection of recombinant HO-2, SDS/PAGE and Western blotting with anti-myc antibody (Calbiochem) were performed as described (7). cGMP was determined as described (7).

HO Assay. HO activity was measured by monitoring the conversion of [^{3}H]iron mesoporphyrin to [^{3}H]bilirubin using a modified version of a previously described protocol (21). Twenty microliters of 14,000 × *g* supernatant was added to a vial containing 6 μl of PBS. The reaction mix consisted of 2 mM NADPH and 15 μM [^{3}H]iron mesoporphyrin (NEN) and the supernatant. [^{3}H]iron mesoporphyrin was added to begin the reaction. After incubation for 20 min at 37°C, reaction tubes were placed on ice, and 200-μl of ice-cold PBS containing 1 mM hemin chloride and 1 mM bilirubin (Sigma) was added immediately. [^{3}H]bilirubin was recovered by extraction into toluene (toluene-1% isoamyl alcohol). The aqueous phase was frozen on dry ice, the organic phase was removed, and radioactivity was quantified in a scintillation counter. Using this method, extraction efficiency of bilirubin was determined to be 45%, compared with 15% of a previously published method (21). Typical signal from cells was ≈12,000 cpm. Omission of NADPH typically yielded a blank of 3,000 cpm. Boiled lysates gave a background of 2,000 cpm. All experiments were performed in the dark.

In some experiments, we used an assay monitoring the release of free $^{55}Fe^{2+}$ from [$^{55}Fe^{2+}$]iron mesoporphyrin (NEN). Incubations were the same as in assays with [^{3}H]iron mesoporphyrin with the following changes. The reaction was stopped with 1 ml of ice-cold 20 mM Tris·HCl (pH 7.4). The reaction mixture then was added to an anion exchange resin, 0.5-m column bed of Dowex AG1X-8 (Bio-Rad) and equilibrated in Tris·HCl (pH 7.4). Biliverdin and [$^{55}Fe^{2+}$]iron mesoporphyrin adhered to the column, and free $^{55}Fe^{2+}$ was eluted with 20 mM Tris·HCl (pH 7.4) - 1 M NaCl.

Transit Analysis. Animals were fasted 24 h before experiments with *ad libitum* access to water. Polystyrene rings were removed from Sitzmark capsules and cut into 1-mM pieces. Animals were fed 10–15 markers and radiographed after the first 30 min after marker ingestion and then at multiple intervals over the next 30 h.

Statistical Analysis. Data were analyzed with a one-way ANOVA and Student's *t* test for paired and unpaired observations. All analysis was performed with STATVIEW software (Abacus Concepts, Berkeley, CA).

RESULTS

Enteric Localization of nNOS and HO-2. In the enteric nervous system, nNOS is expressed predominantly in the myenteric plexus (5). Mice lacking the gene for nNOS develop enlarged stomachs with hypertrophied circular muscle layers (14), and inhibitory membrane potentials in circular smooth muscle cells are reduced in nNOS$^{\Delta/\Delta}$ mice (22). Like nNOS, HO-2 is localized to discrete neuronal populations in the brain and in the periphery (1, 4, 7). HO-2 localization in the intestine previously was described by using a commercial antibody generated against recombinant HO-2 (6). This commercial antibody under some conditions recognizes HO1, another form of HO expressed most highly in the spleen, liver, and other nonneural tissues (R.Z., unpublished data). By using, an HO-2-specific antibody generated by our laboratory, we found HO-2 immunoreactivity predominantly within cell bodies of myenteric ganglia and nerve fibers coursing in parallel with inner circular muscle (Fig. 1). No staining was observed in HO-2$^{\Delta/\Delta}$ mice. Gross and histochemical analysis revealed no obvious differences between wild-type and knockout mice.

In doubly labeled sections from colchicine-treated murine ilea, both HO-2 and nNOS were expressed within the same subpopulations of myenteric neurons, representing ≈60–70% of ganglionic neurons (Fig. 1). In contrast, HO-2 and nNOS did not colocalize in primary cultures of rat cortical neurons.

Nonadrenergic, Noncholinergic Relaxation in Knockout Mice. NANC nerves associated with the myenteric plexus are thought to mediate relaxation of enteric smooth muscle that cannot be abolished by anticholinergic or anti-adrenergic agents (23). We used both DMPP and EFS to elicit NANC relaxation in wild-type, HO-2$^{\Delta/\Delta}$, and nNOS$^{\Delta/\Delta}$ mice (Figs. 2 and 3). Segments from wild-type mice relaxed substantially more than segments from either HO-2$^{\Delta/\Delta}$ or nNOS$^{\Delta/\Delta}$ mice. Upon stimulation with DMPP (3×10^{-5} M) or electrical stimulation (16 Hz, 2 ms), relaxation in tissues from HO-2$^{\Delta/\Delta}$ and nNOS$^{\Delta/\Delta}$ were approximately half that of wild-type animals. All relaxations were abolished by tetrodotoxin, establishing that these methods of neuronal stimulation reflected neuronal depolarization rather than activation of smooth muscle or other nonneuronal cells. Moreover, hemoglobin, which scavenges both CO and NO, eliminated NANC responses. Responses of intestinal smooth muscle to CO and SNP, an NO donor, were similar in preparations from wild-type and mutant mice.

Effect of NOS and HO Inhibitors on NANC Relaxation. We tested the influence of NOS and HO inhibitors on NANC relaxation in wild-type and mutant mice (Fig. 2). Tin protoporphyrin-IX (SnPP-IX; 10^{-5} M), a potent HO inhibitor (24), markedly reduced intestinal relaxations in both wild-type and nNOS$^{\Delta/\Delta}$ mice but had no effect in preparations from HO-2$^{\Delta/\Delta}$ mice. By contrast, the NOS inhibitor N^{G}-nitro-L-arginine (L-NNA; 10^{-4} M) markedly reduced relaxation in wild-type mice and HO-2$^{\Delta/\Delta}$ mice with no effect in nNOS$^{\Delta/\Delta}$ mice. HO and NOS inhibitors together nearly abolished relaxation in wild-type preparations. Protoporphyrin-IX, which does not inhibit HO, had no effect on relaxations in preparations from wild-type or mutant mice (data not shown). Addition of CO or SNP restored maximal relaxation in mutant preparations.

HO-2 and cGMP. Like NO, CO binds to the iron of the heme moiety in soluble guanylyl cyclase to stimulate the production of cGMP (25). However, the potency of CO appears to be less than that of NO in many systems (26, 27) although a potentially physiologic biophysical mechanism that sensitizes guanylyl cyclase to endogenous CO has been demonstrated (28). Exogenous CO increases cGMP levels in a variety of cell types (4, 29), but because it is difficult to approximate physiologic concentrations of CO, a physiologic role for CO in regulating cGMP levels has been difficult to establish conclusively. To examine the effect of HO-2 on cGMP levels, we transfected HEK-293 cells with a plasmid containing the full length cDNA encoding rat HO-2 (20) (Fig. 3). cGMP levels were elevated significantly in cells transfected with HO-2, and these cGMP elevations were abolished by SnPP-IX and 1H-[1,2,4] oxadiazol [4,3-a]quinoxalin-1-one (ODQ), an inhibitor of NO-sensitive soluble guanylyl cyclase (30). Levels of HO-2 activity in

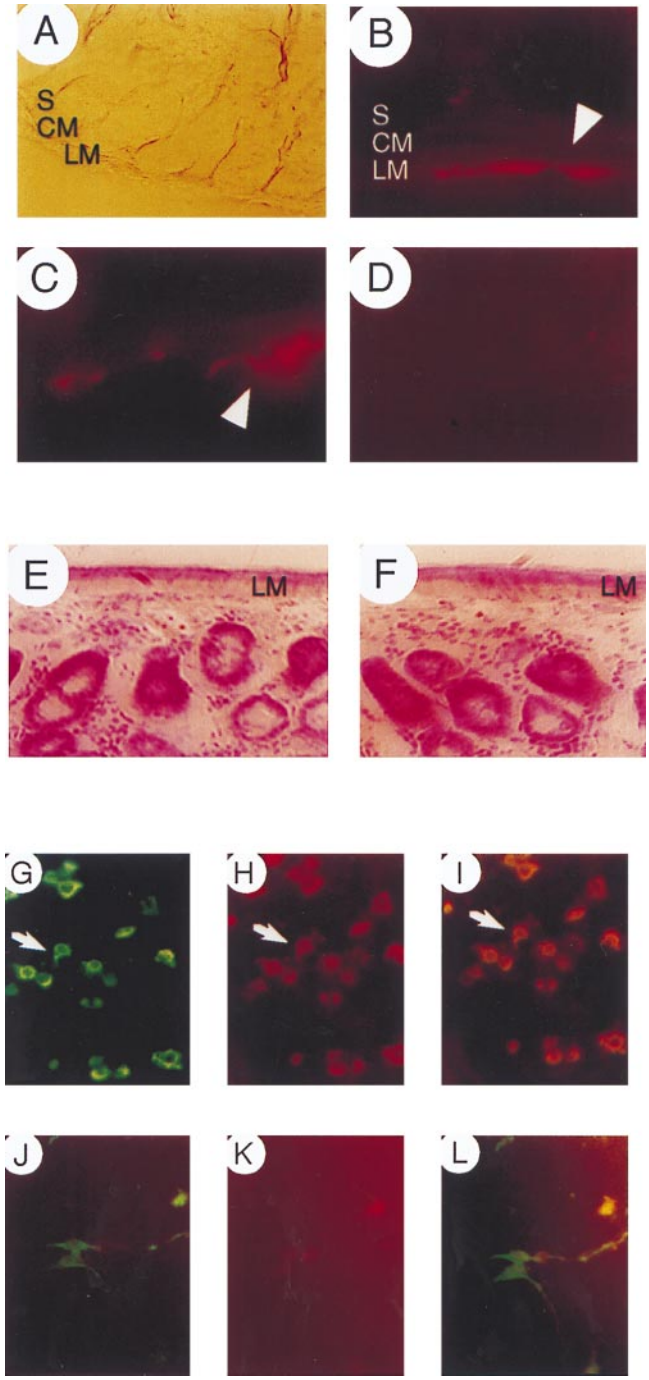

FIG. 1. HO-2 and nNOS expression in the enteric nervous system. (*A*) Nomarski view of ileum from wild-type mouse. S, submucosa; CM, circular muscle; LM, longitudinal muscle. (*B* and *C*) HO-2 expression in myenteric ganglia (arrowheads) in wild-type mice. (*B*) Low power magnification. (*C*) High power magnification. (*D*) No HO-2 immunoreactivity is observed in HO-2 $^{\Delta/\Delta}$ mice. (*E* and *F*) Cross-sections of ilea from (*E*) wild-type and (*F*) HO-2 mutants stained with nuclear red for histochemical analysis. (*Bottom*) Colocalization of HO-2 and nNOS. Doubly labeled myenteric ganglia (arrows denote example) from colchicine-treated ilea of wild-type mice with (*G*) anti-HO-2 and (*H*) anti-nNOS, double exposure (*I*). Rat primary cortical neurons doubly labeled with (*J*) anti-HO-2 and (*K*) anti-nNOS, double exposure (*L*). For additional controls, see *Materials and Methods*. Double labeling with two different NOS antibodies yielded similar results.

transfected cells were comparable to or lower than those found in most mammalian tissues (31), so cGMP formation is not simply a reflection of nonphysiologic overexpression of HO-2. Total heme (free and protein-bound) levels were similar in

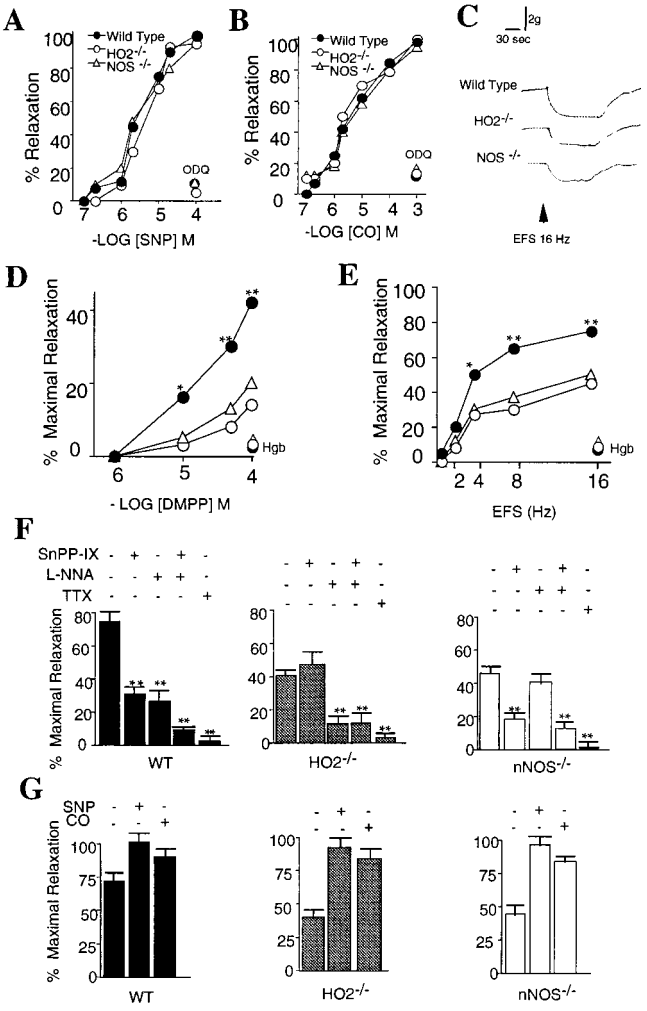

FIG. 2. Intestinal relaxation in mice lacking either HO-2 or neuronal NOS. (*A* and *B*) Concentration–response curves of enteric muscle from wild-type and mutant mice to SNP (10^{-7}–10^{-4} M) and CO (10^{-7}–10^{-3} M) and ODQ (10^{-5} M). Results are expressed as a percentage of the relaxation of wild-type muscle to SNP 10^{-4} M (100%). SE ranged between 5 and 11%, n = 5–7 (see *Materials and Methods*). Because the responses of congenic controls for either HO-2 or nNOS mutant mice were not statistically different, in all figures wild-type responses are expressed as an average of both HO-2$^{+/+}$ and nNOS$^{+/+}$ mice. (*C*) Typical traces from intestinal segments after EFS (16 Hz). (*D* and *E*) NANC relaxation in ileal segments after addition of DMPP (10^{-6}–10^{-4} M) or EFS (2–16 Hz). Results are expressed as a percentage of the mean maximal relaxation achieved with SNP 10^{-4} M in wild-type tissues. SE ranged between 4 and 13%. (*B–G*) In each experiment, control strips were treated with SNP (10^{-4} M) as in *A*, and in all cases, maximal relaxations were essentially the same as in *A*. At the completion of each experiment, strips were treated with SNP 10^{-4} M to confirm the integrity of smooth muscle responses. Asterisks denote significant differences between wild-type responses and those of both HO-2$^{\Delta/\Delta}$ and nNOS$^{\Delta/\Delta}$ mice. Note: Strips were stimulated only once, and values represent the mean of seven to nine independent experiments. (*, $P < 0.05$; **, $P < 0.01$ between wild-type mice and both nNOS and HO-2 mutants, ANOVA). Hgb, hemoglobin (10^{-3} M). (*F*) Effect of pharmacologic inhibition of NOS and HO on EFS-induced NANC relaxations in wild-type and mutant mice. Results are expressed as a percentage of the mean maximal wild-type relaxations to SNP 10^{-4} M in *A*. NANC relaxation was elicited with EFS (16 Hz, 2 ms) (n = 7). SnPP-IX, 10^{-5} M; L-NNA, 10^{-4} M; CO, 10^{-4} M; SNP, 10^{-4} M. SnPP-IX and L-NNA exerted no significant effects on baseline tensions. (*G*) Effect of CO and SNP on EFS-induced NANC relaxation. Results are expressed as a percentage of the mean maximal wild-type relaxations to SNP 10^{-4} M in *A*. (n = 7; **, $P < 0.01$, compared to untreated controls. Student's t test for unpaired observations.) Experiments with DMPP yielded similar results.

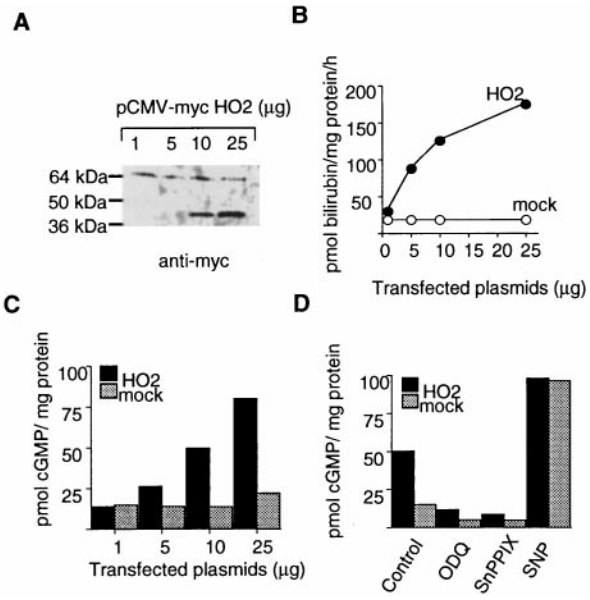

FIG. 3. Modulation of cGMP by HO-2. HEK-293 cells were transfected with cDNAs encoding either pCMV-mycHO-2 or parent vector. (*A*) Expression of pCMV-mycHO-2 was assessed by immunoblotting. (*B*) Activity was determined by measuring the conversion of [^{3}H]iron mesoporphyrin into [^{3}H]bilirubin. In *A*, the band at 64 kDa is an endogenous protein that is recognized by the anti-myc antibody; the band slightly >36 kDa is myc-tagged HO-2. (*C*) Increasing concentrations of HO-2 (1–25 μg) were transfected, and cGMP levels were determined. (*D*) Effects of inhibitors of HO and guanylyl cyclase on cGMP levels in cells transfected with 10 μg DNA. SE ranged between 7 and 15 pmol of cGMP/mg protein.

HO-2-transfected and mock-transfected cells, ruling out the possibility that transfection of HO-2 elevates cGMP by depleting endogenous heme that may normally act to scavenge CO and NO. In some systems, the catalase inhibitor aminotriazole has been found to decrease cGMP production (32). Pretreatment of HEK-293 cells with aminotriazole had no effect on cGMP levels (data not shown).

Intestinal relaxation is thought to involve cGMP-dependent mechanisms (33). We examined cGMP levels before and after treating enteric neurons with DMPP (10^{-5} M) (Table 1). Basal cGMP levels in HO-2$^{\Delta/\Delta}$ and nNOS$^{\Delta/\Delta}$ mice were only 55% and 74%, respectively, of wild-type controls (Table 1). Whereas DMPP stimulation more than doubled cGMP levels in wild-type intestine, it increased levels by only 34% and 54%, respectively, in HO-2$^{\Delta/\Delta}$ and nNOS$^{\Delta/\Delta}$ preparations. In DMPP-stimulated wild-type intestines, both SnPP-IX and L-NNA markedly reduced cGMP levels whereas both drugs had no effect in unstimulated preparations. In HO-2$^{\Delta/\Delta}$ intestine, L-NNA lowered cGMP, but SnPP-IX was ineffective whereas in nNOS$^{\Delta/\Delta}$ tissues SnPP-IX but not L-NNA lowered cGMP. As with intestinal relaxation, the lack of effect of SnPP-IX in HO-2$^{\Delta/\Delta}$ mice and of L-NNA in nNOS$^{\Delta/\Delta}$ mice establishes the specificity of these enzyme inhibitors. The soluble guanylyl cyclase inhibitor ODQ lowered cGMP levels of DMPP-stimulated samples in wild-type, HO-2$^{\Delta/\Delta}$, and nNOS$^{\Delta/\Delta}$ preparations.

Table 1. Modulation of cGMP levels in NANC relaxation

Treatment	cGMP, fmol/mg		
	Wild type	HO-2$^{\Delta/\Delta}$	nNOS$^{\Delta/\Delta}$
Unstimulated			
Control	817 ± 89.7	450 ± 49.2*	603 ± 51.7*
SnPP-IX 10^{-5} M	787 ± 56.3	472 ± 24.3	589 ± 47.6
L-NNA 10^{-4} M	840 ± 69.7	415 ± 32.9	615 ± 42.3
ODQ 10^{-5} M	553 ± 48.3	302 ± 21.8	438 ± 31.9
DMPP-stimulated			
Control	1785 ± 165	602 ± 62.7*	931 ± 73.8*
SnPP-IX 10^{-5} M	1271 ± 88.5†	588 ± 43.9	326 ± 38.9†
L-NNA 10^{-4} M	1006 ± 69.6‡	198 ± 21.6‡	822 ± 66.7
ODQ 10^{-5} M	603 ± 57.8	389 ± 27.3	467 ± 32.1
CO 10^{-3} M	1960 ± 143	2100 ± 187	2050 ± 225
SNP 10^{-4} M	2197 ± 178	2267 ± 209	2003 ± 126

Ileal muscle strips were treated with DMPP (3×10^{-5} M) for 30 s and instantaneously frozen. cGMP content was determined by radioimmunassay as described in *Materials and Methods*.
*Significant difference between knockout and control values. $P < 0.01$, Student's t test.
†Significant reduction by SnPP-IX, $P < 0.01$, Student's t test.
‡Significant reduction by L-NNA, $P < 0.01$, Student's t test.

Gastrointestinal Transit Time in Knockout Mice. Regulated contractions of enteric smooth muscle ensure coordinated transit throughout the gastrointestinal tract. HO-2$^{\Delta/\Delta}$ mice showed significantly slower overall transit whereas neuronal NOS $^{\Delta/\Delta}$ mice exhibited delayed gastric emptying with nearly normal transit through the intestine and colon (Fig. 4). These observations indicate that mutant mice have gastrointestinal dysmotility without mechanical bowel obstruction.

Eliminating HO-2 might result in a deficiency of heme catabolism. Heme is critical for many proteins, especially in erythrocytes, but HO-2$^{\Delta/\Delta}$ mice have normal hematological profiles and are morphologically indistinguishable from wild-type mice (13). We found similar heme levels in total homogenates and in subcellular fractions prepared from multiple tissues of wild-type and HO-2$^{\Delta/\Delta}$ mice (data not shown). Also, activity of the heme containing enzymes soluble guanylyl cyclase and NOS in brain extracts was normal in HO-2$^{\Delta/\Delta}$ mice (data not shown). If excess heme was present in myenteric neurons of HO-2$^{\Delta/\Delta}$ mice, muscle relaxation or cGMP levels in response to sodium nitroprusside would be diminished in knockout mice because of scavenging of NO. Intestinal relaxation (Fig. 2) and cGMP levels (Table 1) in response to sodium nitroprusside were similar in wild-type and knockout mice at all concentrations tested.

DISCUSSION

Our results indicate that both HO-2 and nNOS play a role in NANC relaxation. The decrements in intestinal relaxation in HO-2$^{\Delta/\Delta}$ and nNOS$^{\Delta/\Delta}$ animals together can account for most of the NANC neurotransmission. However, other proposed NANC neurotransmitters (34), such as VIP, ATP, and substance P, also may play roles that require the presence of CO or NO. The relative roles of various NANC mediators in different tissues and species may differ. The impairment of relaxation in animals with targeted deletions of either HO-2 or nNOS after neuronal depolarization indicates that products of HO-2 and nNOS are physiologically released to influence intestinal relaxation and cGMP levels. Furthermore, the lack of influence of SnPP-IX on HO-2$^{\Delta/\Delta}$ tissues establishes that SnPP-IX blocks relaxation by inhibiting HO.

Previous studies using enzyme inhibitors have suggested that NO and CO might mediate synaptic events, including NANC transmission, in the intestine. However, the inherent nonspecificity of the drugs used has precluded any strong conclusions. For instance, long-term potentiation (LTP) in the hippocampus is inhibited by NOS inhibitors (35–37), but LTP is not diminished in nNOS$^{\Delta/\Delta}$ animals (38), and some studies suggest that the effects of L-NAME and other NOS inhibitors on LTP do not directly reflect nNOS inhibition (39, 40). Similarly, inhibition of LTP by HO inhibitors (41, 42) may derive from influences of the protoporphyrins used on guanylyl cyclase (7, 10, 11) or on NOS (7, 12) rather than HO because LTP is normal in HO-2$^{\Delta/\Delta}$ $_{\text{mice}}$ (13). Recent studies have shown that certain metalloporphyrins selectively inhibit HO at concentrations below 50 μM (7, 12). Results from early studies investigating roles for CO (9, 41, 42) may have reflected

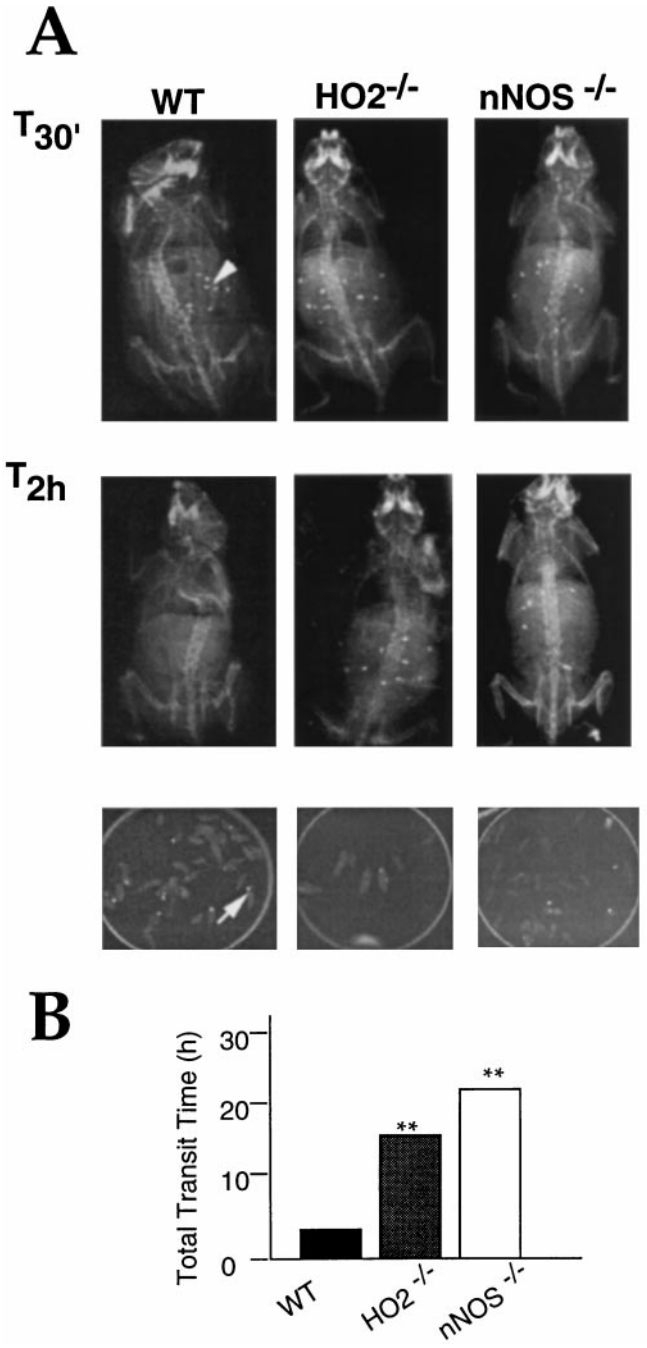

FIG. 4. Gastrointestinal transit in HO-$2^{\Delta/\Delta}$ and $NOS^{\Delta/\Delta}$ mice. (*A*) Ten to 15 radiopaque markers (arrow) were fed to mice and tracked by radiography at periodic intervals. Excretion of markers was also monitored by x-ray (*Bottom*). (*B*) Average time to excrete all markers. Results are expressed as number of hours from marker consumption to excretion. (**, $P < 0.01$, $n = 7$–11. Student's *t* test for unpaired observations.)

inhibition of guanylyl cyclase and/or NOS in addition to HO because metalloporphyrin concentrations of 1 mM were routinely used. Our studies showing that L-NAME loses its efficacy in $nNOS^{\Delta/\Delta}$ preparations and that SNPP-IX loses its efficacy in HO-$2^{\Delta/\Delta}$ intestine establish that, in these systems, the drugs are selectively inhibiting nNOS and HO-2.

The elimination of these relaxations in normal and mutant mice by the soluble guanylyl cyclase inhibitor ODQ favors a cGMP-dependent mechanism of relaxation in these experiments. Depressed cGMP levels in HO-$2^{\Delta/\Delta}$ mice reveal a role for HO-2 in modulation of cGMP levels. Because cGMP appears to be regulated by a product of HO activity, and CO stimulates guanylyl cyclase *in vitro* and *in vivo,* our data indicate that CO is the HO product that mediates this effect. The localization of HO-2 and nNOS to myenteric neurons and the obligatory involvement of these enzymes in ileal NANC neurotransmission establishes that CO, like NO, fulfills the major criteria for a neurotransmitter.

We thank members of the Snyder lab, J. R. Stone and L. Ny, for valuable comments and suggestions. We thank D. Ahearn and Rodney Seaforth from NEN for the generous gifts of radiolabeled hemes, T. Dawson for NOS knockouts and helpful discussions, B. Dwyer for pBSHO-2, P. Worley for the CMV expression vector, M. Schell for expert advice, C. Magee for animal radiography, and D. Dodson and N. Bruce for manuscript preparation. Critical reading of manuscript by A. Kolodkin, K. J. Hurt, and V. Velculscu is much appreciated. This work was supported by U.S. Public Health Service Grants DA-00266 and NS-32925 to S.T. and Research Scientist Award DA-00074 to S.H.S., a gift from the Shionogic Institute for Medical Science to S.T., Predoctoral fellowship AA05418 to R.Z., National Institutes of Health Grant NS32925 to S.T. and K.D.P, Training Grant GM-07309 to S.R.J., and a Howard Hughes Medical Institute Fellowship for Physicians to C.D.F.

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