Constitutive and permissive roles of nitric oxide activity in embryonic ciliary cells

Shandra A. Doran, Cam Ha Tran, Cagla Eskicioglu,
Tev Stachniak, Kee-Chan Ahn, and Jeffrey I. Goldberg

Constitutive and permissive roles of nitric oxide activity in embryonic ciliary cells. Am J Physiol Regul Integr Comp Physiol 285: R348–R355, 2003. First published April 3, 2003; 10.1152/ajpregu.00634.2002.—Embryos of Helisoma trivolvis exhibit cilia-driven rotation within the egg capsule during development. In this study we examined whether nitric oxide (NO) is a physiological regulator of ciliary beating in cultured ciliary cells. The NO donor S-nitroso-N-acetylpenicillamine (SNAP; 1–1,000 μM) produced a dose-dependent increase in ciliary beat frequency (CBF). In contrast, the nitric oxide synthase (NOS) inhibitor 7-nitroindazole (100 μM) inhibited the basal CBF and blocked the stimulatory effects of serotonin (100 μM). NO production in response to serotonin was investigated with 4,5-diaminofluorescein diacetate imaging. Although SNAP (100 μM) produced a rise in NO levels in all cells, only 22% of cells responded to serotonin with a moderate increase. The cGMP analog 8-bromo-cGMP (8-Br-cGMP; 0.2 and 2 mM) increased CBF, and the soluble guanylate cyclase inhibitor LY-83583 (10 μM) blocked the cilioexcitatory effects of SNAP and serotonin. These data suggest that NO has a constitutive cilioexcitatory effect in Helisoma embryos and that the stimulatory effects of serotonin and NO work through a cGMP pathway. It appears that in Helisoma cilia, NO activity is necessary, but not sufficient, to fully mediate the cilioexcitatory action of serotonin.

Helisoma; mollusk; ciliary beat frequency; 4,5-diaminofluorescein; serotonin

Cilia, cellular organelles that appear as hairlike extensions from the cell membrane, are ubiquitous throughout biology, excluding insects and nematodes (36). They exhibit a conserved microtubular ultrastructure and are responsible for the generation of fluid currents over the surface of the cell (reviewed in Ref. 39). Studies on a wide variety of organisms have generated little consensus over the regulation of ciliary beating. It appears that ciliary beat frequency (CBF) is normally controlled by phosphorylation of specific ciliary proteins and changes in intracellular Ca2+ concentration ([Ca2+]i). A CAMP-protein kinase A (PKA)-dependent pathway has been implicated in the stimulation of CBF in human nasal epithelium (13), hamster oviductal epithelium (33), frog esophagus (5), and gill epithelium (33), frog esophagus (5), and gill epithelium (33), frog esophagus (5), and gill

Nitric oxide (NO) is a gaseous signaling molecule that has recently been implicated in the regulation of ciliary function. It is produced in the conversion of L-arginine into L-citrulline by the enzyme NO synthase (NOS) and may function as both a neurotransmitter and an intracellular signal transduction molecule in vertebrates and invertebrates (2, 3, 24, 34). In rabbit airway epithelium, a β-adrenoceptor-mediated pathway causes NO synthesis, which in turn increases CBF (44). Likewise in human airway epithelium, muscarinic receptor activation stimulates an increase in CBF through NO production (48). Furthermore, ethanol stimulates NO production and a subsequent increase in CBF in bovine airway epithelium (40). In a variety of mammalian systems examined, the cilioexcitatory actions of NO are mediated by the cGMP second messenger system (32, 45, 47).

Although there is mounting evidence supporting the role of NO in mammalian CBF regulation, there has been little examination of the role of NO within invertebrate ciliated cells, with the exception of Paramecium (reviewed in Ref. 4). This is surprising given the apparent conservation of this signaling molecule throughout the animal kingdom (reviewed in Ref. 11). Before the development of the central nervous system in Helisoma trivolvis, a bilateral pair of serotonergic sensory-motor neurons, embryonic neurons C1 (ENC1s), innervates the paired dorsolateral ciliary...
bands and a single pedal ciliary band (14, 18, 26, 28). These simple neural circuits regulate the first embryonic behavior, cilia-driven embryo rotation within the egg capsule (27). The rate of embryonic rotation is regulated according to the oxygen content within the egg capsule, whereby hypoxia causes a faster rotation that stirs the capsular fluid and promotes oxygen diffusion to the embryo (27). This response is mediated in part by the release of serotonin onto pedal and dorsolateral ciliary cells, which stimulates CBF through a novel PKC isoform and Ca\(^{2+}\) influx (9, 10). In the present study, we examined whether NO may also contribute to the regulation of ciliary activity.

In a recent study, NADPH diaphorase staining suggested the presence of NOS in the dorsolateral ciliary cells and ENC1's sensory apparatus at stage E25, with additional expression in the pedal ciliary cells and ENC1's soma and descending neurite at later stages (stage E35–E40) (12). Additionally, pharmacological manipulations of NO content altered embryonic behavior. Whereas the application of NO donors increased the rate of embryonic rotation, NOS inhibitors decreased the rate of embryonic rotation (12). Furthermore, the behavioral data suggested that NO had effects on both ENC1 and ciliary cells. Taken together these results prompted us to test the hypothesis that embryonic ciliary cells are major sites of NO activity. In the present study on cultured dissociated ciliary cells, we now report that endogenous NO constitutively increased the rate of embryonic rotation (12). Further-

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### MATERIALS AND METHODS

**Animals.** *Helisoma trivolvis* embryos were collected from a laboratory-reared albino colony raised at the University of Alberta. Snails were raised in flow-through aquaria containing dechlorinated water at ~25°C, an oyster shell substrate, and maintained on a 12:12-h light-dark cycle. Snails were fed Romaine lettuce and trout pellets (NU-WAY, United Feeds, Calgary, Canada). Egg masses were collected daily, as previously described, from large plastic petri plates placed in the aquaria for the purpose of egg mass deposition (14). These egg masses were transferred to a 35-mm petri dish containing artificial pond water (APW; 0.025% Instant Ocean, Aquarium Systems, Mentor, OH) and maintained at room temperature through development. Embryos between stages E20 and E40 were used in this study (15, 20).

**Chemicals and solutions.** Embryonic cells were cultured in *Helisoma* defined medium (HDM; 50% Liebovitz-15 (GIBCO, Burlington, ON), 40.0 mM NaCl, 1.7 mM KCl, 4.1 mM CaCl\(_2\), 1.5 mM MgCl\(_2\), 5.0 mM HEPES, 50 μg/ml gentamicin, 150 μM-glutamine μg/ml (Sigma, St. Louis, MO), pH 7.3–7.35). Serotonin (5-hydroxytryptamine creatine sulfate complex; 100 μM; Sigma) was dissolved in *Helisoma* saline (HS: 51.3 mM NaCl, 1.7 mM KCl, 4.1 mM CaCl\(_2\), 1.5 mM MgCl\(_2\), 5.0 mM HEPES; pH 7.35). S-nitroso-N-acetylpenicillamine (SNAP; 1–1,000 μM; Tocris, Ellsville, MO), 7-nitroindazole (7-NI; 10 and 100 μM; Tocris, Ellsville, MO) and 8-bromo-cGMP (8-Br-cGMP; 0.2 and 2 mM; Sigma) were dissolved in DMSO (Sigma) and then diluted to working concentrations in HS so that the DMSO level did not exceed 0.1%. This DMSO concentration has previously been demonstrated to have no effect on CBF (10). LY-83583 (LY; 10 μM; Tocris, Ellisville, MO) was dissolved in ethanol (EtOH) and diluted in HS so that the EtOH concentration did not exceed 0.1%. N\(^{-}\)-nitro-L-arginine methyl ester hydrochloride (l-NNAME; 0.1 and 1 mM; Sigma) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO; 300 μM; Calbiochem, San Diego, CA) were dissolved in water and then diluted to working concentrations in HS. 4,5-Diaminofluorescein diacetate (DAF-2 DA; Calbiochem, San Diego, CA) was dissolved in DMSO. All drugs were prepared the day of use.

**Ciliated cell culture.** Embryonic ciliated cells were cultured as previously described (9, 10). Briefly, intact egg masses (stage E20–E35) were disinfected in 35% ethanol, and the embryos were removed. Isolated embryos were treated with 0.2% trypsin (Sigma) for 30 min and then mass dissociated by repeatedly passing them through a 63-μm nylon mesh (Small Parts, Miami, FL). Dissociated cells were cultured in poly-l-lysine-coated (hydrobromide, mol wt 4,000–15,000, 1 μg/ml, Sigma) culture dishes (Falcon 3001, Becton Dickinson, Franklin Lakes, NJ) containing arti-

...and maintained at room temperature (20–22°C) for 18–24 h to allow for cell adhesion to the substrate.

**Time-lapse videomicroscopy.** Ciliary beating was monitored with a CCD video camera (JVC, TK-860U) mounted on a compound microscope (Nikon, Diaphot) with phase-con-

...acters (Omega Optical, Brattle-

...lders (Hamamatsu, Japan). Emission fluorescence, at 515 nm, was collected using an intensi-

...tative and control cultures.** In each experiment included a parallel control culture that received only the vehicle solution. Error bars represent the SE of the mean. Statistical significance was determined by ANOVA followed by Fisher's protected least significant difference test. Each bar graph contains an inset that indicates the statistical comparison between all treatment groups. Statistically significant differences occurred between treatment groups that do not share the same underscore (P < 0.05). Within each inset, the treatment groups are arranged in decreasing amplitude of the response.

**NO imaging.** Embryonic ciliated cells were loaded with 5 μM DAF-2 DA in HS for 45 min at room temperature. Culture dishes were rinsed with HS three times before imaging. In the presence of NO, DAF-2 is converted to the fluorescent product DAF-2 triazole (25). Given that this fluorescent product accumulates within the cell, no attempts were made to wash out the serotonin-induced fluorescence. However, in every experiment SNAP (100 μM) was applied after serotonin to ensure that the indicator was responding to NO. Ciliated cells were imaged with a ×100 oil-immersion objective (1.3 NA; Fluor) on an inverted microscope (Zeiss, Axiovert 135) with excitation at 495 nm from an Hg-Xe arc lamp (Hamamatsu, Hamamatsu, Japan). Emission fluorescence, at 515 nm, was collected using an intensified charge-coupled device (ICCD) video camera (Paultek Imaging, Grass Valley, CA). Neutral density filters (Omega Optical, Brattleboro, VT) were used to ensure that fluorescent images were within the sensitivity range of the camera. Data were collected using custom software courtesy of Dr. S. Kater (Univ. of Utah) as eight-bit images. Captured images were digitized...
through a QuickCapture frame grabber board (Data Translation, Mississauga, ON, Canada) and saved to a computer for offline analysis (Macintosh Quadra 950). Images were analyzed for fluorescence intensity on a Macintosh G4 computer using the public domain program NIH Image (version 1.61) and a macro for DAF imaging custom written by C. J. H. Wong. Fluorescence was thresholded in a region of interest that included most of the surface of the cell, and fluorescence was measured as the average pixel intensity and background subtracted. Fluorescence was measured as changes in arbitrary fluorescence units (ΔF) relative to the average of fluorescence measured 30 and 60 s before the start of the experiment. A response was defined as a sustained increase in fluorescence of 3 or more fluorescence units within five time points after the addition of a drug. In no cases did perfusion with HS stimulate an increase in fluorescence. Given that DAF-2 is a nonratiometric indicator, no attempts were made to quantify NO.

RESULTS

Effect of NO on ciliary beating. Evidence from a variety of systems suggests that NO has cilioexcitatory actions. To determine if NO produces similar effects on *Helisoma* ciliated cells, the NO donor SNAP was applied to cultured ciliated cells, and CBF was measured with time-lapse videomicroscopy. Addition of SNAP for 10 min increased CBF in a dose-dependent manner (Fig. 1A). The 100 μM SNAP concentration produced a maximal increase of 118.1 ± 4.1% (P < 0.05 vs. HS control, n = 10), whereas application of 1,000 μM SNAP had no effect (Fig. 1A). The SNAP-induced increase in CBF was compared with a serotonin treatment, a known endogenous cilioexcitatory factor in *Helisoma* embryos (Fig. 1B). A 10-min application of 100 μM serotonin, a dose known to maximally increase CBF (9), stimulated a significant increase in CBF to 132.5 ± 3.2% (P < 0.05 vs. HS control, n = 11; Fig. 1B). Coapplication of 100 μM SNAP and 100 μM serotonin produced a significant increase in CBF to 125.7 ± 5.6% (P < 0.05, n = 14), not statistically different from the independent treatments of either SNAP or serotonin alone (Fig. 1B). These data suggest that there was no additivity between serotonin and SNAP responses.

To further investigate the involvement of an NO pathway in the regulation of ciliary activity, the effects of a NOS inhibitor on CBF were examined (Fig. 2). Application of 7-NI produced a dose-dependent decrease in CBF after 10 min (Fig. 2A). Application of 10 μM 7-NI significantly reduced CBF to 80.8 ± 3.3% of the pretreatment rate (P < 0.05, n = 10), and the 100 μM dose of 7-NI significantly decreased CBF to 75.4 ± 1.3% of the pretreatment rate (P < 0.05, n = 13). There was no significant difference between the 10 μM 7-NI and 100 μM 7-NI treatments. Application of an additional NOS inhibitor, l-NAME, also produced a significant decrease in the rate of ciliary beating at the concentrations tested, 0.1 mM (P < 0.05; n = 7) and 1 mM (P < 0.05; n = 7; data not shown). These findings suggest that endogenous NO may have a constitutive excitatory action on ciliary beating in *Helisoma* embryos.

To determine whether NOS inhibition affects the cilioexcitatory action of serotonin, 100 μM 7-NI was coapplied with 100 μM serotonin. Application of serotonin stimulated CBF to increase to 132.6 ± 3.0% when applied on its own, but no significant change in CBF when coapplied with 7-NI (Fig. 2B). As expected, 100 μM 7-NI alone inhibited CBF to 85.3 ± 3.5% (P < 0.05, ANOVA; Fig. 2B). Thus it appeared that the NOS inhibitor blocked the cilioexcitatory action of serotonin.

To determine if the site of NO activity was strictly intracellular, we examined the effects of the extracellular NO scavenger c-PTIO on the ciliary activity of isolated ciliary cells. Application of 300 μM c-PTIO had no effect on basal CBF (control vs. c-PTIO, P > 0.05) or the cilioexcitatory response to serotonin (serotonin vs. c-PTIO + serotonin, P > 0.05; Fig. 3A) As a positive control, we tested whether c-PTIO could attenuate the ciliary response to the NO donor SNAP, which should elevate NO extracellularly. Indeed, 300 μM c-PTIO completely blocked the cilioexcitatory response to serotonin, 100 μM 7-NI.
SNAP (SNAP vs. c-PTIO/H11001
P/H11021 0.05; Fig. 3B). These data suggest that the NO produced within ciliated cells acts intracellularly rather than in an autocrine or paracrine fashion.

**Effect of serotonin on NO production.** Because 7-NI inhibited ciliary beating when presented alone, its blockade of the serotonin response does not prove that NO participates in the signal transduction of serotonin’s effect. Therefore, we used the fluorescent NO indicator DAF-2 DA to determine if serotonin directly stimulates NO production. In most cells examined, the baseline fluorescence increased gradually under basal conditions in HS (Fig. 4). This increase of ~5–8 fluorescence units over 30 min represents basal NO production and is consistent with the idea of constitutive NO activity. To ensure that solution changes did not alter fluorescence, the HS was replaced with fresh HS before applying the drug treatment (Fig. 4). Application of 10 μM and 100 μM SNAP revealed that only the higher dose of the NO donor produced a detectable increase in DAF-2 fluorescence (Fig. 4A). Thus 100 μM SNAP was added after each serotonin treatment as a positive control for the effectiveness of DAF-2. Seventy-eight percent of the cells (28 of 36 cells) examined did not show an increase in NO in response to application of 100 μM serotonin for 15 min, as represented in Fig. 4B. In contrast, the remaining cells displayed a marginal steplike increase of 3–8 fluorescence units in response to 100 μM serotonin (Fig. 4C). In all cases, this response was smaller than that produced by 100 μM SNAP. Taken together, these results suggest that serotonin does not effectively stimulate NO production in ciliated cells.

**Involvement of cGMP in excitatory ciliary responses.** One of the most recognized signal transduction pathways utilized by NO is the cGMP pathway (6, 21). We decided to use a membrane-permeable cGMP analog to determine if this pathway participates in ciliary regulation in *Helisoma* embryos (Fig. 5). Application of 0.2 and 2.0 mM 8-Br-cGMP caused significant increases in
CBF to 116.6 ± 1.5% (P < 0.05 vs. control, n = 18) and 125.5 ± 2.2% (P < 0.05, n = 14) of pretreatment values, respectively (Fig. 5). Neither dose of 8-Br-cGMP produced an increase in CBF that was significantly different from the serotonin-positive control (100 μM). These data demonstrate that activation of a cGMP pathway produces an increase in ciliary activity.

To examine if a cGMP pathway is involved in the cilioexcitatory effects of NO and serotonin, an inhibitor of the enzyme sGC was used (Fig. 6). Application of 10 μM LY alone produced no change in CBF after 10 min but significantly decreased CBF to 86.7 ± 4.8% of the pretreatment value when coapplied with 100 μM SNAP (Fig. 6A). Similarly, the stimulatory effect of serotonin was blocked by 10 μM LY when coapplied with LY (Fig. 6B). These findings suggest that a cGMP pathway may be necessary to mediate the cilioexcitatory effects of both NO and serotonin.
DISCUSSION

Evidence from mammalian systems suggests an important role for NO in the regulation of ciliary activity. These findings prompted us to examine embryos of the pond snail Helisoma trivolvis for the involvement of NO in ciliary regulation. We determined that the application of the NO donor SNAP produced an increase in CBF that was not additive to the stimulatory effects of serotonin. The NOS inhibitor 7-NI decreased the basal rate of ciliary beating and blocked the stimulatory effect of serotonin. Serotonin did not appear to stimulate a significant increase in NO production, as revealed through fluorescent imaging. Finally, a cGMP analog stimulated ciliary beating and an sGC inhibitor blocked the stimulatory effects of both serotonin and SNAP.

A novel result from this study is that 7-NI significantly reduces the rate of unstimulated ciliary beating. This result suggests that NO has a constitutive action in Helisoma ciliated cells. The rising baseline of fluorescence in the DAF-2 experiments supports this idea of basal NO production. Furthermore, constitutive NO activity is also supported by whole animal examinations of embryonic behavior, where both 7-NI and the arginine analog L-NAME caused nearly a 50% reduction in the embryonic rotation (12). Studies on other systems have not revealed a similar constitutive role for NO. In both human adenoid tissue and rat airway epithelium, application of NOS inhibitors did not result in a significant inhibition of CBF (32, 48). In both these mammalian systems, NO is recruited as a signal transduction element rather than being continually produced to drive basal ciliary activity. More ciliary systems need to be examined to determine whether the novel constitutive action of NO observed in the present study is unique or widespread among many species.

It is interesting to note that, in contrast to 7-NI, application of the sGC inhibitor LY did not significantly affect CBF when applied alone. This suggests that the constitutive NO activity may not act through the sGC-cGMP pathway. NO has been shown in other cellular responses to generate physiological activity through direct protein interactions (reviewed in Ref. 7). For example, S-nitrosylation, the transfer of an NO group to cysteine residues on proteins, has been proposed to be as prevalent a regulatory mechanism as phosphorylation events. Given that NO is a highly conserved signaling molecule that is being increasingly identified to modulate ciliary activity, it may be that direct interactions at the level of ciliary proteins is a common regulatory mechanism. Further experiments are required to determine whether the constitutive action of NO in embryonic ciliary cells from Helisoma operates through such a mechanism.

Serotonin, the endogenous cilioexcitatory neurotransmitter in Helisoma, is released from a pair of bilateral neurons that innervate two subpopulations of embryonic ciliary cells (26, 28). Previous studies have revealed that Ca2+ influx and activation of a unique PKC isoform are necessary to produce the serotonin-induced changes in CBF (9, 10). The cilioexcitatory actions of NO identified in the present study, together with the expression of NOS in both ENC1 neurons and ciliary cells (12), prompt the hypothesis that NO plays a role in the ciliary response to serotonin. Furthermore, the ability of the extracellular NO scavenger c-PTIO to block the SNAP-induced rise in CBF without affecting basal CBF or the response to serotonin suggests that NO acts intracellularly rather than in an autocrine or paracrine fashion.

The lack of additivity in the ciliary response to application of serotonin and SNAP, together with the effectiveness of the NOS inhibitor 7-NI in blocking the response to serotonin, suggest that NO and serotonin interact. However, our results do not suggest that NO acts as an independent signal transduction element in the serotonin response. The most effective dose of SNAP was unable to stimulate as large an increase in CBF as serotonin. This is consistent with evidence from whole animal studies that NO is less effective than serotonin in stimulating embryonic rotation (12). Furthermore, the partial effectiveness of NO is similar to our previous finding that activation of PKC is necessary, but not sufficient, to mediate the serotonin response (10). Perhaps NO and PKC operate as cofactors in the response pathway, whereas a rise in intracellular Ca2+ is able to fully mimic the serotonin response, represents a different step in the cascade.

The most recognized intracellular target for NO is sGC, which catalyzes the production of cGMP. NO, working through a cGMP pathway, has been shown to both increase CBF (45) and decrease CBF (44), depending on the species examined. In Helisoma ciliary cells, a cGMP analog produced an increase in CBF similar in magnitude to that of serotonin (100 μM) stimulation. Given that the most effective dose of SNAP did not maximally stimulate ciliary beating, this result suggests that NO only partially activates the cGMP second messenger system in Helisoma ciliated cells. The sGC inhibitor LY blocked the stimulatory effects of both exogenous NO and serotonin, further implicating this pathway in cilioexcitation.

Ciliary stimulation through the upregulation of NO production is supported by findings from rabbit trachea (44, 45), rat airway epithelium (32), bovine airway epithelium (40), and human adenoid explants (48). In contrast to these systems, NOS inhibitors reduced the CBF of Helisoma ciliated cells when presented alone. It is not known, therefore, whether constitutively expressed NO or NO generated as part of the serotonin signal transduction pathway is active in the serotonin response. To examine whether serotonin recruits NO, we used DAF-2 imaging, and our results indicate that serotonin does not stimulate a significant increase in NO production, suggesting that the serotonin stimulatory pathway functions in parallel to the NO stimulatory pathway. However, these experiments were performed on cultures of mixed cell populations, where the three known subpopulations of Helisoma ciliary cells could not be identified (28). It is possible that these different subpopulations exhibit different regulatory profiles and that the 22% of the weakly
responding cells are from one subpopulation in which serotonin does recruit NO. Alternatively, it is possible that serotonin stimulation results in a small, localized production of NO that is sufficient to stimulate an increase in CBF but is below the sensitivity of the dye. Concern for the specificity and accuracy of DAF-2 imaging has been raised, specifically that divalent cations in the medium were thought to alter detected fluorescence (8). However, these concerns were allayed when it was shown that gaseous NO reacted with DAF-2 to produce fluorescent product independent of Ca\(^{2+}\) and Mg\(^{2+}\) at physiological concentrations (41). In our hands, the indicator revealed that 100 \(\mu\)M serotonin and the lower dose of NO donor (10 \(\mu\)M SNAP) did not reliably produce a detectable change in fluorescence, whereas 100 \(\mu\)M SNAP did alter the fluorescent signal. It is possible that serotonin stimulates the production of an amount of NO that is below the amount liberated by 100 \(\mu\)M SNAP. This follows from the finding that the 10 \(\mu\)M dose of SNAP did not produce a detectable increase in NO with DAF-2 but did produce a slight increase in CBF. Thus, if serotonin does stimulate the generation of NO, it may be in physiological amounts below the lower limits of detection for the indicator.

NO has been identified to play a diverse number of roles within cells, including the regulation of invertebrate development (16, 17, 22). Although molluscan metamorphosis is incompletely understood, a picture is emerging that implicates both NO and serotonin (30). *Helisoma* undergoes direct development in the egg capsule without a metamorphosis event in its life cycle. However, the serotonergic and nitricergic systems remain very important in regulating embryonic development (18, 19). It may be that coordinated ciliary activity is central to normal embryonic development in *Helisoma* and that the function of both NO and serotonin are derived from marine ancestors that exhibited different life histories. The nitricergic system appears to regulate basal ciliary activity to support embryonic rotation in the egg capsule, which may facilitate development. In contrast, the serotonergic system may have developed to upregulate ciliary beating in response to new challenges faced by encapsulated embryos, such as metabolically or environmentally derived hypoxia (23, 27). Most importantly, these two systems interact, with NO playing a permissive role in the cilioexcitatory response to serotonin.

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**DISCLOSURES**

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