Nitric oxide inhalation transiently elevates pulmonary levels of cGMP, iNOS mRNA, and TNF-α

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Nitric oxide inhalation transiently elevates pulmonary levels of cGMP, iNOS mRNA, and TNF-α. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L509-L515, 1998.—The initial pulmonary vasodilation that occurs during nitric oxide (NO) inhalation does not appear to be maintained chronically in many cases. NO may acutely relax vascular smooth muscle by increasing levels of guanosine 3',5'-cyclic monophosphate (cGMP), tumor necrosis factor (TNF-α), and inducible nitric oxide synthase (iNOS) while decreasing levels of lipid peroxidation. It was hypothesized that the acute NO-induced changes in cGMP, TNF-α, iNOS, and lipid peroxidation, all of which may mediate vasodilation, are transient rather than sustained. Lungs from rats kept in chambers containing 6 parts/million NO for 1 h, 1 day, or 1 wk were analyzed for levels of NO-induced vasodilatory mediators. Pulmonary cGMP, iNOS mRNA, and TNF-α were increased 1 h after NO exposure but decreased to control values at later times. Levels of malonaldehyde, an indicator of lipid peroxidation, were decreased at all times during NO inhalation. As a whole, the data suggest that in lungs the vasodilatory mediators cGMP, iNOS, and TNF-α are only acutely and transiently elevated during inhalation of NO, consistent with the initially positive clinical response to inhaled NO that deteriorates over time.

Lung; pulmonary hypertension; critical care medicine; interferon-γ; superoxide dismutase

Nitric oxide (NO) is an ubiquitous radical gas that is produced within many biological systems for a variety of functions, including muscle relaxation and neurotransmission. With varied success, inhaled NO has been used therapeutically for conditions such as pulmonary hypertension (19, 28). Although NO rapidly relaxes vascular smooth muscle, resulting in acute vasodilation, the long-term effects of inhaled NO therapy have not been well studied. Clinical outcomes from multiple-week trials suggest that the beneficial effects of acute NO treatment may not persist in chronic therapy. A sustained benefit from inhaled NO in neonates with persistent pulmonary hypertension, for instance, is observed in only 60% of patients (23). The reasons why the initial NO-mediated reduction in pulmonary vascular tone is not sustained during chronic exposure are not well understood. Elucidation of potential mechanisms behind this phenomenon may suggest more effective protocols for the use of NO as a therapeutic agent. In this study, temporal changes in the predominant cellular and intracellular effects of NO that directly affect vascular smooth muscle relaxation were measured because such changes may indicate potential mechanisms for the differences in the clinical response to NO between acute and chronic exposures.

There are several characterized mediators of NO-induced vasodilation. NO upregulates the production of guanosine 3',5'-cyclic monophosphate (cGMP) (2), which then relaxes vascular smooth muscle (27). Exposure to NO stimulates the release of tumor necrosis factor (TNF-α) (36), an inflammatory cytokine that causes acute vasodilation (3, 33). In environments where extracellular levels of the inflammatory radical superoxide are low, NO reduces levels of lipid peroxidation (30). Recent evidence suggests that diminished lipid peroxidation enhances relaxation of vascular smooth muscle (14). Finally, although there is some evidence that NO exposure limits endogenous NO production by downregulating expression of the inducible form of nitric oxide synthase (iNOS) in certain settings (e.g., Ref. 7), NO may actually stimulate iNOS expression in other settings (1), thereby increasing production of NO and amplifying stimulation of all the above vasodilatory mechanisms. Despite the clinical relevance to NO therapy, no studies have described the effect of inhaled NO on pulmonary levels of TNF-α, iNOS, or lipid peroxidation. Furthermore, the temporal changes in lung cGMP levels after acute and chronic NO inhalation have not been reported.

cGMP, TNF-α, lipid peroxidation, and iNOS may all be means by which NO induces vasodilation. Because clinical evidence indicates that the initial vasodilatory response to inhaled NO is often not maintained chronically, it was hypothesized that levels of cGMP, TNF-α, lipid peroxidation, and iNOS present during acute NO inhalation are significantly different from such levels during chronic inhalation. Specifically, it seems reasonable to hypothesize that cGMP, TNF-α, and iNOS are elevated and lipid peroxidation is diminished during acute exposure to inhaled NO. Because no study has examined levels of cGMP, TNF-α, lipid peroxidation, or iNOS during both brief and long-term inhalation of NO, these parameters were analyzed in the lungs of rats after acute (1 h), intermediate (1 day), or chronic (1 wk) exposure to inhaled NO. Consistent with the transient vasodilatory response to NO inhalation observed in many cases, evidence is presented describing acute elevations in cGMP, TNF-α, and iNOS levels that decrease with time of NO exposure. These
observations may offer a mechanism for the deterioration of the vasodilatory response to inhaled ·NO.

METHODS

Animal exposures. Pathogen-free male Fischer 344 rats (CDF 9F-34401CR 1 BR, n = 5 per condition) were exposed at 7 wk of age. A 12-h on-and-off daily light cycle was used. Food (Purina rat chow, St. Louis, MO) and water were provided ad libitum. For exposure to ·NO, animals were placed in a standard housing container that was sealed in a gastight glove bag (approximate volume of 26 liters) that was flushed with O2 and appropriate mixtures of ·NO and N2 using metering valves. Exposures to ·NO were for 1 h, 24 h, or 7 days. The 7-day exposure was temporarily halted (15 min) twice daily to relocate the rats to a container with fresh bedding. The ·NO exposure group received ·NO from a tank containing 1,056 parts/million (ppm) ·NO in N2 (11.4 ml/min, National Welders Gas, Raleigh, NC) plus 420 ml/min from a pure O2 tank and a balance of N2 (1,568 ml/min) to achieve a total flow of 2 l/min, resulting in a final normoxic ·NO concentration of 6 ppm. This concentration was used because it approximates the average of the lowest levels of ·NO reported to manifest clinically relevant vasodilatory effects. ·NO therapy is generally used in the range of 6–80 ppm, depending on the age of the patient, to produce general pulmonary vasodilation in cases of hypoxic pulmonary vasoconstriction and pulmonary hypertension (12, 28). A concentration of 3 ppm ·NO has been reported to produce maximal effects on platelet aggregation and agglutination (31). In a duplicate exposure setup, the clean-air control group breathed a mixture of 420 ml/min of O2 balanced with N2 to 2 l/min.

The concentration of ·NO and of potentially contaminating ·NO2 was measured using a chemiluminescence analyzer (model 8101-C; Bendix, Ronceverte, WV) calibrated according to Environmental Protection Agency reference method RFNA-0777-022. The average residual ·NO2 level was 0.03 ppm for the ·NO-exposed groups. Normoxic conditions in the exposure bags were verified by measurement with a Clark electrode. Accuracy of the flow rate settings was verified with the use of a bubble flowmeter.

To harvest tissues for biochemical assays, rats were removed from the chambers and killed individually while the remaining rats continued to be exposed. On removal from the exposure chamber, rats were quickly anesthetized with pentobarbital sodium (60 mg/kg) by intraperitoneal injection. After being weighed, rats were killed by severing the abdominal aorta. The lungs were rapidly excised, weighed, and processed for individual assays. The lungs of rats from additional control and 1-wk exposure groups were fixed for histopathological examination by intratracheal instillation of 10% Formalin. Samples from the preserved lungs were prepared for both light and transmission electron microscopic evaluation using standard methods previously described (24).

Cyclic guanosine monophosphate and cyclic AMP measurement. Determination of cGMP and cAMP levels in lung tissues was based on a standardized radioimmunoassay (8) using Amersham (Arlington Heights, IL) cyclic nucleotide assay kits. Lung homogenate was prepared in a small tissue blender by the addition of 200 mg of liquid nitrogen-powdered lung tissue to 0.5 ml of ice-cold isotonic saline containing 2 mM theophylline. TCA (0.5 ml; 20% wt/vol) was added to the homogenate. Precipitated proteins were removed by centrifugation for 10 min at 2,500 g, and the TCA in the supernatant was removed by five successive extractions with 4 ml of ether-saturated water. After removal of TCA, 1 ml of 50 mM Tris·HCl-8 mM EDTA (pH 7.5) buffer was added to each sample and aliquots were taken for cGMP assay (TRK 500, Amersham) and cAMP assay (TRK 432, Amersham). The efficiency of recovery of cGMP and cAMP was determined by the addition of tritiated cGMP or cAMP to duplicate tissue samples. Because no significant differences in recovery of cGMP or cAMP were found between clean-air and ·NO-exposed groups, the average values of 83% for recovery of cGMP and 90% for recovery of cAMP were used to correct the reported values for losses during preparation of all samples. Data are expressed as picomoles per 100 milligrams of lung.

Cytokine measurement. For measurement of cytokines, a 10% (wt/vol) solution of liquid nitrogen-powdered lung tissue in 50 mM Tris·HCl (pH 7.5) was analyzed. Levels of rat interferon (IFN)-γ and rat TNF-α were quantitated by ELISA (Biosource, Camarillo, CA) according to the manufacturer’s instructions.

RNA isolation and RT-PCR. Immediately after excision, 100 mg of lung tissue were homogenized in 1 ml of guanidium thiocyanate lysis solution (Biotex, Houston, TX). Samples were quickly frozen and stored at −70°C. Pure RNA was isolated by phenol extraction according to the manufacturer’s instructions. RT-PCR was performed using recombinant Tth polymerase with magnesium and manganese acetate buffers (Perkin-Elmer, Foster City, CA). Novel primers were created for rat iNOS (5'-AGC ACA TGC AGA ATG AGT ACC, 3'-TGA TGC TCC CGG ACA CCG GA), rat extracellular superoxide dismutase (SOD) (5'-TAG CCT AGC TGC TGC CGC CAT A, 3'-GGG CGC ACA GAG GCG ATT GA), rat Cu,ZnSOD (5'-STC GTC TCC TTC TTG TTT, 3'-WCG TGG ACC ACC ATA GTA), and rat MnSOD (5'-CGC CTC AGC AAT GTT GTG T, 3'-AGG CGG CAA CTA TCT GTA AGC GA). RT-PCR for all primers was executed under the same thermocycling protocol: 30 min at 60°C × 1 cycle, 1 min at 94°C × 1 cycle, 30 s at 94°C followed by 1 min at 65°C × 40 cycles, 7 min at 65°C × 1 cycle. Samples were run in 3% agarose and stained with ethidium bromide. RT-PCR for iNOS mRNA was performed on all groups; RT-PCR for SOD mRNA was performed on control, 1-h exposure, and 1-day exposure groups. Control RT-PCR reactions for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were run separately using novel primers (5'-GTT GTC AAC GGA TTT GGG CCT GTT, 3'-CAT GCC AGT GAG GCC TTT CCC GGT CA).

Western blotting. Lung tissue was homogenized in a_near-bolling lysis solution (50 mM Tris·HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM NaVO4, and 1 mM NaF). Lung homogenate was centrifuged at 10,000 g for 10 min at 4°C, and the supernatant was stored at −70°C. Approximately 50 µg of protein were loaded on a 12% polyacrylamide gel and electrophoresed on a minigel apparatus (Hoefer, San Francisco, CA). Western blotting for iNOS was performed using a polyclonal rabbit antibody against murine iNOS (Transduction Laboratories, Lexington, KY). Chemiluminescence detection was performed using a horse-radish peroxidase-conjugated secondary antibody (ECL System; Amersham, Arlington Heights, IL).

Malonyl dialdehyde measurement. A qualitative measure of the level of lipid peroxidation was assessed using the thiobarbituric acid assay for animal tissues (26). Lung homogenate was prepared by adding 0.18 ml of a 50 mM Tris·HCl (pH 7.5)-0.9% NaCl-1 mM EDTA solution to 20 mg of liquid nitrogen-powdered lung tissue and by homogenizing with a tissue blender. Sodium dodecyl sulfate (0.2 ml at 8.1%), 1.5 ml of 20% acetic acid (pH 3.5), 1.5 ml of 0.8% aqueous thiobarbituric acid, and 0.6 ml of deionized water were added. After heating to 95°C for 1 h, 5 ml of n-butanol was added. The mixture was centrifuged at 3,000 g for 15 min, and the
absorbance of the upper organic layer was determined at 532 nm. A standard curve using tetraethyoxpropane was used to express the data in terms of malonyl dialdehyde (MDA) equivalents.

Statistics. Results of cytokine (n = 4 data points), cGMP (n = 5 data points), and cAMP (n = 5 data points) analyses were compared by one-way ANOVA followed by one-tailed, one-degree-of-freedom a priori comparisons of means because only increases in NO-stimulated levels were to be considered. ANOVA followed by Duncan's multiple comparison test was used to evaluate the significance between average MDA levels (n = 5 data points). For all tests, α = 0.05. Data are given as means ± SE.

Cell experiments. Human thoracic aorta smooth muscle cells (HA-VSMC) were purchased from the American Type Culture Collection (Manassas, VA) and were grown in six-well plates (Falcon, Lincoln Park, N.) in Ham's F12-K (Kaighn's Modification) medium (GIBCO, Grand Island, NY) with 2 mM glutamine, 1.5 g/l sodium bicarbonate, 10 µg/ml insulin, 10 µg/ml transferrin, 10 ng/ml sodium selenite, 20 µg/ml endothelial cell growth supplement (GIBCO), 10 µl/ml penicillin (GIBCO), 10 µg/ml streptomycin (GIBCO), and 10% fetal bovine serum (GIBCO). At confluency, medium was replaced with Ham's F12-K supplemented only with 1% fetal bovine serum. After 24 h, cells were exposed to 0.1 mg/ml DETA NONOate ([Z]-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium,1,2-diolate) for 8 h or 3 days. DETA NONOate-treated medium was changed every 8 h in the 3-day exposure group. After exposures, cells were analyzed for protein content or were lysed for RNA extraction as described above. RT-PCR for guanylate cyclase was performed using novel primers for human guanylate cyclase mRNA (5'-GCA CCA GGT CAA GTT CCT AAC GA, 3'-GTC CAA CAT TCA GCC GTT CAA) under the following thermocycling protocol: 60 min at 61°C × 1 cycle; 1 min at 94°C × 1 cycle; 30 s at 94°C followed by 1 min at 63°C × 40 cycles; 7 min at 63°C × 1 cycle. Control RT-PCR reactions for human GAPDH mRNA were run separately using novel primers (5'-TCA GCC GCA TCT TCT TTT GCC G, 3'-GAC CAG GCC CCC AAT ACG A).

RESULTS

Animal and lung weights. As shown in Table 1, continuous inhalation of 6 ppm NO for 1 wk did not produce statistically significant changes in body weight or wet lung weights compared with those of control animals. All data are presented as means ± SE (n = 5 animals).

Table 1. Body and lung weights of rats exposed to 6 ppm NO for 1 week vs. control rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight, g</th>
<th>Wet Lung Weight, g</th>
</tr>
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<tbody>
<tr>
<td>Clean-air control</td>
<td>273 ± 9</td>
<td>1.46 ± 0.06</td>
</tr>
<tr>
<td>1-wk 6 ppm NO</td>
<td>287 ± 10</td>
<td>1.59 ± 0.10</td>
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Data are means ± SE; n = 5 animals per group. NO, nitric oxide.

higher than controls (P < 0.004). cGMP levels at 1 wk were statistically similar to controls. There were no significant differences in cAMP levels between control and NO exposure groups at any time point studied (Fig. 2A). Because habituation of the NO-mediated increase in cGMP could be caused by decreased transcription of guanylate cyclase, the enzyme that synthe-
sizes cGMP, smooth muscle cells were exposed to DETA NONOate, a long-acting ·NO donor (32, 34) for short (8 h) or long (3 days) periods. The ·NO donor increased cellular guanylate cyclase transcription in proportion to the time of exposure (Fig. 3).

TNF-α is elevated in rat lung after 1 h of ·NO inhalation. ELISA results indicated that pulmonary levels of TNF-α after 1 h of ·NO inhalation were higher than those of unexposed rats (Fig. 1B; \( P < 0.05 \)). TNF-α decreased to control values at later times after exposure. At all times after exposure, IFN-γ levels in the lungs of rats that inhaled ·NO were not increased above those of rats that breathed clean air (Fig. 2B).

iNOS mRNA is elevated in rat lung after 1 h of ·NO inhalation. By RT-PCR, small amounts of iNOS mRNA were detected in control rats not exposed to ·NO (Fig. 1C). iNOS mRNA was elevated above control levels after 1 h of ·NO inhalation but returned to basal levels in the day and week inhalation groups. Both GAPDH and Cu,ZnSOD mRNA levels, either of which may represent housekeeping gene expression, were unchanged throughout the exposures (data not shown). After a rigid isolation protocol with numerous proteinase inhibitors, no iNOS protein was detected in any group (data not shown).

MDA is decreased at all times after ·NO exposure. At all times after exposure, lungs excised from rats exposed to ·NO manifested lower (\( P < 0.05 \)) levels of MDA, a by-product and qualitative indicator of lipid peroxidation, than did rats not exposed to ·NO (Fig. 2C). This effect was not correlated with an upregulation of transcription of any of the three isoforms of the antioxidant enzyme SOD. RT-PCR of RNA isolated from lungs of rats \(( n = 4 \) ) exposed to clean air, 1 h of ·NO, or 1 day of ·NO was performed using primers specific for rat isoforms of Cu,ZnSOD, MnSOD, and extracellular SOD. There were no changes in SOD mRNA levels between control and ·NO-exposed rats after any time of exposure (data not shown).

Pulmonary histology is not influenced by ·NO exposure. Sections from ·NO-exposed lungs after 1 wk of exposure to 6 ppm ·NO were normal under light and electron microscope examination with no significant pathology. Alveolar macrophages were normal in ap-
pearence, distribution, and number in the air spaces. Isolated neutrophils and platelets were only occasionally identified in exposed and control lungs and in all cases were limited to the vascular space. Alveolar epithelial cells completely covered the basement membrane and were normal in appearance. As a whole, microscopic examination revealed no evidence for inflammation, cell injury, or ultrastructural alteration in the NO-exposed group.

**DISCUSSION**

This report presents data suggesting that the initial mechanisms by which inhaled NO might induce pulmonary vasodilation are not maintained during chronic exposure. cGMP, TNF-α, and iNOS are all rapidly but transiently upregulated in the lungs of rats immediately after inhalation of NO. This effect was not evident with cAMP or IFN-γ, neither of which is related to vasodilation. There was also no difference in lipid peroxidation between acute and chronic NO exposures. Based on data from this and other studies, Fig. 4 illustrates a putative mechanism underlying the reduction of vascular tone by acute NO inhalation. NO stimulates production of cGMP (2), which may then stimulate the release of TNF-α (17). NO alone might also stimulate the release of TNF-α (36), although it is not clear that this occurs independently of cGMP. Both cGMP (27) and TNF-α (3, 33) are capable of acting independently as vasodilators. TNF-α stimulates expression of iNOS (5), the enzyme that synthesizes NO. There is also evidence that NO alone upregulates iNOS expression (1). Such a positive feedback mechanism may enhance the acute vasodilatory effects of NO. Because the findings in this study indicate that cGMP, TNF-α, and iNOS are not elevated during chronic NO inhalation, the vasodilatory mechanism outlined in Fig. 4 ceases to function under long-term exposure to NO. Further evaluation of this scheme in both normal and inflamed lungs merits study.

Inhalation of NO rapidly increased pulmonary levels of cGMP, an effect that is consistent with previous reports (9) and is attributed to NO-mediated stimulation of guanylate cyclase (2), the enzyme that synthesizes cGMP. Data are now presented indicating that rats inhaling NO for as long as 1 wk do not manifest increased pulmonary levels of cGMP. A similar effect has been observed with plasma cGMP levels in infants after inhalation of NO for 3 days (6). Because recent work suggests that NO decreases the stability of mRNA encoding soluble guanylate cyclase in vascular smooth muscle cells within 4 h after exposure (10), it was hypothesized that habituation of the NO-mediated increase in cGMP levels is due to diminished levels of guanylate cyclase mRNA. However, the data presented here are not consistent with this hypothesis because levels of guanylate cyclase mRNA were higher in vascular smooth muscle cells chronically exposed (3 days) to a long-acting NO donor than in cells exposed for a significantly shorter amount of time (8 h).

The increase in TNF-α levels in the lung after only 1 h of NO inhalation is likely explained by an NO-mediated release of stored cytokine (36), although de novo synthesis cannot be excluded. Rapid release of stored TNF-α has been reported elsewhere (16) and has been demonstrated to occur in lung via mast cells (13). Because cGMP may be intimately involved in the release of TNF-α (17), the lack of continued TNF-α release may be related in turn to the lack of elevated cGMP levels that is evident during chronic inhalation of NO. Microscopic analysis of the NO-exposed rat lungs in this study indicates that the increase in TNF-α cannot be accounted for by changes in the number or distribution of inflammatory cells such as macrophages or neutrophils.

Inhalation of NO induced a rapid increase in levels of iNOS mRNA after 1 h of exposure. Previous work has also demonstrated that iNOS transcription can be quickly upregulated. For example, an increase in iNOS mRNA was observed in pulmonary fibroblasts as soon as 2 h after stimulation with TNF-α and other cytokines [Willis et al. (35) did not test iNOS transcription at 1 h]. NO alone is thought to induce iNOS transcription by reducing iron availability (1), whereas NO-induced TNF-α activates both cis (TNF-α response element) and trans (nuclear factor (NF)-κB) transcription factors for iNOS (25). Data are now presented suggesting that long-term inhalation of NO does not continue to stimulate transcription of iNOS. NO has been reduced to increase iNOS transcription in a cell culture system, an effect linked to decreased activation of NF-κB (7). Lack of NF-κB activation might be explained by the finding in this study that TNF-α levels during chronic exposure to NO are not elevated. The presence of small amounts of iNOS mRNA in control rats not exposed to NO is consistent with the results of other reports (15, 22) and suggests that some cells in the lung may transcribe this enzyme constitutively (21). The finding that rat alveolar macrophages upregulate iNOS expression more readily than human alveolar macrophages in response to various stimuli (18) may suggest that elevated iNOS mRNA observed in rats after NO inhalation in this study may not be directly relevant to human therapy. Furthermore, although an increase in iNOS transcription was ob-

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**Fig. 4.** Putative mechanism by which acute NO exposure induces vasodilation. Acute inhalation of NO leads to increases in pulmonary cGMP, TNF-α, and iNOS. Other studies suggest that cGMP and TNF-α mediate vasodilation by distinct mechanisms. There is also evidence that cGMP induces expression of iNOS by increasing levels of TNF-α, which is a strong inducer of iNOS expression. Increased levels of iNOS in turn generate more NO. Data from this report suggest that NO-mediated stimulation of cGMP, TNF-α, and iNOS is only transient, which may partially explain why pulmonary vasodilation is not maintained in many cases during chronic inhalation of NO.
Inhalation of TNF-α, and iNOS were not maintained during chronic ports of acute but transient vasodilation after inhalation (14), does not correlate with clinical decrease in lipid peroxidation, which may promote radicals and tissue injury.

Because thiobarbituric acid assay precisely quantifies lipid peroxidation only in defined lipid systems (11), differences among MDA levels reported here were considered qualitative indicators of changes in lipid peroxidation. Application of techniques that can quantitatively detect and identify specific lipid peroxidation products will be necessary to support these data further. The NO-mediated and apparently sustained decrease in lipid peroxidation, which may promote vasodilation (14), does not correlate with clinical reports of acute but transient vasodilation after inhalation of NO (23).

The observation that acute elevations of cGMP, TNF-α, and iNOS were not maintained during chronic inhalation of NO is consistent with the rapid clinical response to inhaled NO that deteriorates over time. The transient elevation of NO-induced vasodilatory mediators may partially explain why the vasodilating effect of NO inhalation, in many cases, is also transient. Elucidation of the mechanisms behind the lack of long-term response to inhaled NO in both normal and inflamed lungs could indicate novel strategies to improve chronic treatment of conditions such as pulmonary hypertension. The data from this report suggest that these strategies may involve specific manipulation of cGMP, TNF-α, and iNOS levels.

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