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Biological Studies of a Nitroso Compound that Releases Nitric Oxide upon Illumination

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SUMMARY

2-Methyl-2-nitrosopropane (MNP) has long been known to undergo photochemical and thermal decomposition, generating di-*tert*-butyl nitroxide, in organic solvent. The present study was undertaken to demonstrate that MNP can be used as a caged-nitric oxide (NO), which can liberate NO upon illumination. Photolysis of MNP leads to the generation of *tert*-butyl radical and NO, as detected by spin-trapping/ESR spectroscopy and by oxyhemoglobin/visible spectroscopy, respectively. Using soluble guanylate cyclase in neuroblastoma N1E-115 cells as an NO target, we found that MNP in the presence of light caused a dose- and time-dependent increase in cGMP. Finally, illumination of a solution of MNP was also found to induce relaxation of preconstricted isolated rat pulmonary artery rings. These studies demonstrated that MNP can be a useful biochemical research tool for delivering NO in a controlled manner, by using light.

The discovery that the free radical NO is the species responsible for the pharmacological action attributed to the endothelium-derived relaxation factor (1–4) reveals a new and exciting aspect of the biological role of free radicals. NO has been implicated in the regulation of various important biological processes (for reviews, see Refs. 5 and 6), including physiological control of blood pressure (7), inhibition of platelet aggregation (8), macrophage-mediated tumoricidal action (9–11), stimulation of auto-ADP-ribosylation of glyceraldehyde (12), and neurotransmission (13). Because of these diverse and important pharmacological roles, there has been interest in developing agents that can release NO. Several classes of compounds, including sydnoeimine (14), nitroglycerin (14), and *S*-nitroso derivatives (15, 16), have been synthesized and studied for their abilities to release NO. All of these compounds require redox activation to generate NO. Recently, Maragos *et al.* (17) have shown that *N*-nitroso compounds with the structure XN(O-)N=O, where X is a nucleophile, can also serve as vehicles to deliver NO in a biological system. The ability to rapidly regulate the concentration of NO in a localized manner would be a powerful tool for studying the physiological and biochemical roles of this free radical in intact cells. One approach to the rapid release of NO is photolysis of a "caged-NO" compound. Flash photolysis has been used successfully to regulate several important intracellular biological mediators (for review, see Ref. 18), including ATP (19), cyclic nucleotides (20), cholinergic agonists (21), inositol-1,4,5-triphosphate (22), and calcium (23). The organometallic compound sodium nitroprusside has long been known to generate NO upon light activation (24). Recently, Bettache *et al.* (25) reported that another organometallic compound, ruthenium nitrosyl trichloride, generated NO after illumination with a laser pulse. Studies conducted by Makings and Tsien (26) suggested that, whereas light exposure of ruthenium nitrosyl trichloride generated NO, the compound failed to inhibit platelet aggregation.

Our interest in this area of research is to develop caged compounds that can release NO upon light activation. It is well established that some nitroso-containing compounds, including MNP, undergo photochemical decomposition, generating *tert*-butyl radical, and by implication NO, in organic solvent (27). Based on these studies, we postulated that MNP can serve as a caged-NO prototype that can liberate NO on demand upon light activation for the studies of biological systems. If our hypothesis is correct, such caged-NO compounds might serve as promising biomedical research tools for delivering NO in a controlled manner.

In the present report, the extent of NO release from light-activated MNP and its biological effects on cGMP formation and on the vasorelaxant properties of isolated rat PA rings

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ABBREVIATIONS: NO, nitric oxide; MNP, 2-methyl-2-nitrosopropane; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ACh, acetylcholine; PE, phenylephrine; PA, pulmonary artery.

were studied. We demonstrate that MNP is a caged-NO that can be activated to generate NO by illumination.

Materials and Methods

Reagents. Dulbecco's modified Eagle's medium and calf serum were purchased from GIBCO Laboratories (Grand Island, NY). HEPES, hemoglobin from human blood, meclofenamic acid sodium salt, N^{ω}-nitro-L-arginine, PE hydrochloride, ACh, and MNP, which was used without further purification, were obtained from Sigma Chemical Co. (St. Louis, MO). [8-^{3}H]Guanosine was purchased from ICN Radiochemicals (Irvine, CA). The cGMP radioimmunoassay kit was obtained from Amersham (Arlington Heights, IL). Sodium hydrosulfite was from Aldrich Chemical Co. (Milwaukee, WI). A regular 150-W General Electric light bulb was used as the light source in ESR experiments and guanylate cyclase assays.

Preparation and handling of MNP solutions. A stock solution of MNP (10^{-2} M) in HEPES, pH 7.4, was wrapped in aluminum foil to avoid exposure to light and was prepared by stirring with moderate heating (60–70°) until the solution became light blue. This was determined by briefly checking the color of the aluminum foil-wrapped solution. The blue color of the solution is indicative of MNP monomer. The solution was then transferred to a 37° water bath. When MNP solutions were kept at 37° for 1 hr, 80% of the efficacy in inducing cGMP production in neuroblastoma N1E-115 cells was lost. This is presumably due to the dimerization of MNP, which caused MNP to lose its ability to generate NO (27). Therefore, in all experiments, MNP solutions were prepared and used within 10 min of preparation. MNP in solution was very sensitive to photolysis, such that either incandescent or fluorescent lighting in the laboratory could lead to the trapping of di-*tert*-butyl radical, as detected by ESR (data not shown). Therefore, the laboratory lights were turned off during experiments involving the use of MNP solutions.

Detection of *tert*-butyl radical by ESR. A solution of MNP (10^{-3} M) in HEPES, pH 7.4, was transferred to a quartz ESR flat cell fitted into the cavity of the spectrometer (Century Line model E109; Varian Associates, Palo Alto, CA) and a scan was obtained before illumination. The ESR cell was then removed from the spectrometer, placed 10 cm in front of the light source, and photolyzed, and spectra were recorded at specific time intervals, as described in the figure legends.

Preparation of oxyhemoglobin. Oxyhemoglobin was obtained according to the procedure of Salvemini *et al.* (28). Briefly, hemoglobin (final concentration, 10^{-3} M) was reduced with excess sodium hydrosulfite (final concentration, 10^{-2} M), followed by gel filtration using a prepacked Sephadex G-25M disposable column (PD10; Pharmacia) previously equilibrated and eluted with HEPES buffer, pH 7.4. The concentration of oxyhemoglobin was determined spectrophotometrically using the characteristic absorbance at 576 nm, with an extinction coefficient of 14.6 mM^{-1} cm^{-1} (29).

Detection of NO with oxyhemoglobin. A solution of MNP (10^{-3} M) and oxyhemoglobin (7.9×10^{-6} M) in a quartz cuvette was placed 10 cm in front of a 150-W halogen light source (Transillumator model OS 3000; Medical Instrument Research Associates, Waltham, MA) and spectra were recorded at specific time intervals after continuous irradiation. The change in absorbance at 576 nm was used to estimate the concentration of NO.

Cell culture. Murine neuroblastoma clone N1E-115 cells were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) calf serum, as described previously (30). Confluent cells from passages 10–16 were obtained after 15–20 days of subculture and were utilized in experiments. Primary vascular smooth muscle cells from Sprague-Dawley rats were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) calf serum, as described previously (31). Confluent cells were obtained after 20 days and were used in experiments.

Assay of guanylate cyclase activity in cell suspensions. The assay was performed as described previously (30). Briefly, N1E-115 cells were harvested and labeled at 37° for 1 hr with 10^{-6} Ci/ml [^{3}H] guanosine (12 Ci/mmol) in HEPES buffer (110 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM glucose, 20 mM HEPES, supplemented with sucrose to adjust the osmolarity of the buffer to 340 mOsM, pH 7.4). Cells were washed, distributed into 24-well trays ($0.1–0.2 \times 10^6$ cells/well), and incubated for 15 min at 37°. MNP (10^{-3} M) was then added and cells were illuminated for the indicated time. The temperature of the solutions did not change during this brief exposure to light. Control cells were kept in the dark under the same conditions. The reaction was terminated by addition of 5% trichloroacetic acid, and [^{3}H]cGMP was isolated by ion exchange chromatography and counted as described previously (30). cGMP production in vascular smooth muscle cells was measured by radioimmunoassay, according to the procedure described in the kit.

Isolated PA rings. PA rings were obtained and prepared as described by Anderson *et al.* (32). Segments of PA (length, 2.5–3 mm; o.d., 2–2.5 mm) were dissected from sacrificed adult male Sprague-Dawley rats and suspended by stainless steel hooks in 5- or 10-ml tissue baths filled with modified Krebs-Henseleit buffer (138 mM NaCl, 1.2 mM $MgSO_4$, 4.7 mM KCl, 1.2 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 5 mM HEPES, 10 mM glucose, adjusted to pH 7.4 with Tris buffer). Solutions were maintained at 37° and aerated by continuous bubbling with air. The upper hook was connected to a force transducer (Harvard Apparatus, Natick, MA, or Grass Instruments, Quincy, MA) and continuous recordings were made with a multichannel chart recorder (Linseis, Princeton, NJ, or Grass Instruments). After 1 hr of equilibration at a resting tension of 650 mg, PA rings were challenged with Krebs-Henseleit buffer containing 40 mM KCl (exchanged 1:1 with NaCl). Rings were constricted by the addition of 10^{-6} M PE, and the presence or absence of functional endothelium was confirmed by relaxation with 10^{-6} M ACh. After the assessment of endothelial function, all vessels were treated throughout the experiments with N^{ω}-nitro-L-arginine (10^{-4} M) and meclofenamate (10^{-6} M) to inhibit endogenous NO synthase and prostaglandin formation, respectively. The status of the endothelium made no difference in the response to MNP.

Unless otherwise noted, the following procedures were performed in darkness. Rings were contracted with 10^{-6} M PE. In separate experiments a series of dose-response curves for MNP were measured in complete darkness, to determine the non-photolysis–dependent component of the relaxation response and to determine the highest dose that failed to induce significant relaxation. In other experiments, the preconstricted vessel was exposed to a white-light source (model 375A; Dyonics, Andover, MA) at 5-min intervals until the light response was consistent. In the dark a dose of 10^{-5} M MNP was then added to the bath and any relaxation was noted. After tension had stabilized the light was turned on again and the response was recorded. The temperature of the solutions did not change during this brief exposure to light. The effect of MNP with and without light was compared by repeated-measures analysis of variance, with Fisher's least-significant difference test for *post hoc* comparison of means ± standard errors.

Results and Discussion

Ideal caged-NO substances should be inactive in the dark and able to liberate NO instantaneously upon illumination. In principle the delivery of active substances such as NO by photolysis can generate concentration changes that are rapid and spatially more controllable than with rapid mixing. Several years ago Wajer *et al.* (27), using ESR spectroscopy, demonstrated that nitroxide can be detected after photochemical or thermal decomposition of nitroso compounds, including MNP, in organic solvents. MNP is a nitroso compound that exists as a dimer in the solid form and as a blue monomer in solution. Studies conducted by Wajer *et al.* (27) suggested that only MNP monomer can be converted into a nitroxide. These results implied that MNP can generate NO under proper conditions.

Fig. 1 depicts how light-activated MNP produces *tert*-butyl radical and NO, as well as showing our strategy to detect these two radicals. Our initial experiments were designed to demonstrate that the carbon-nitrogen bond of MNP can be homolytically cleaved by light to generate *tert*-butyl radical and NO in a biological buffer. ESR spectroscopy, combined with spin trapping, was used to detect the *tert*-butyl radical. This technique consists of using a nitrone or a nitroso compound to trap the initial unstable free radical as a long-lived nitroxide, which can be observed by ESR spectroscopy at room temperature (33, 34). Because MNP is a nitroso compound, it can serve as a spin trap. Fig. 2 shows that irradiation of MNP (10^{-3} M) caused a time-dependent increase in the amplitude of a nitroxide ESR signal with a hyperfine splitting of a_N = 16.5 G, characteristic of di-*tert*-butyl nitroxide (35). In the dark only a very small ESR signal was observed. The finding that a solution of MNP gave a detectable ESR signal characteristic of di-*tert*-butyl nitroxide in the presence of light suggested that *tert*-butyl radical was formed. Therefore, in this process NO must also be produced. However, no NO-spin-trapped adduct was detected by ESR spectroscopy. This is not surprising, because we found that commonly used nitrone and nitroso spin traps could not be used to detect NO by ESR spectroscopy (36). Another analytical method must be used to establish the generation of NO from MNP. For our studies oxyhemoglobin, which is known to react specifically with NO (37–39), was used to spectrophotometrically characterize the generation of NO from light-activated MNP. Fig. 3 shows that, as the time of photolysis of MNP increased, a decrease in the characteristic absorbances of oxyhemoglobin at 576 nm and 540 nm was observed. Furthermore, photolysis of MNP (10^{-3} M) produced NO linearly for at least 20 min (data not shown). The generation of NO is instantaneous upon illumination. In the absence of MNP, illumination of a solution of oxyhemoglobin under identical conditions resulted in no appreciable change in the absorbance spectra (data not shown). Consistent with previous reports (27), these data established that photolysis of MNP leads to the generation of NO. While this work neared completion, Chamulitrat *et al.* (40) reported that UV irradiation (for 20 min) of another spin trap, phenyl-*N*-*tert*-butyl nitrone (0.14 M), generated NO. Because of the duration of UV irradiation and the concentration required to generate NO from phenyl-*N*-*tert*-butyl nitrone, this compound would not be useful as a caged-NO. More recently, Makings and Tsien (26) have synthesized and tested a series of several, very interesting, caged-NO compounds. MNP is different from the compounds reported by Makings and Tsien, in that it can be activated to generate NO by visible light, whereas caged-NO compounds require UV light to produce NO.

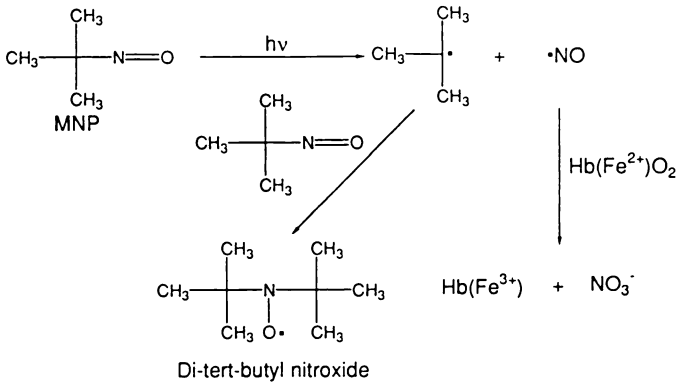

Fig. 1. Generation of *tert*-butyl radical and NO by light-activated MNP. *tert*-Butyl radical reacts with MNP to give di-*tert*-butyl nitroxide, which can be detected by ESR spectroscopy, whereas NO reacts with oxyhemoglobin to give methemoglobin, which can be monitored by visible absorption.

Our next objective was to determine the biological effects of MNP under light. One of the targets of NO in biological systems is soluble guanylate cyclase (5, 6). We and others have previously shown that the activation of soluble guanylate cyclase in neuroblastoma N1E-115 cells represents a convenient system for the study of the physiological role of NO (30, 41). Therefore, the formation of cGMP in intact neuroblastoma N1E-115 cells was selected for our investigation. In the presence of MNP (10^{-3} M), light caused a time-dependent change in the production of cGMP in N1E-115 cells (Fig. 4). The time course of light-mediated cGMP formation in the presence of MNP was rapid, with a maximum between 1 and 2 min, followed by a rapid decrease between 3 and 5 min and a plateau after 5 min. In the dark no increase in cGMP was observed. Because NO is released by light-activated MNP linearly over a period of 20 min, it is surprising to observe the shape of the time course of light-mediated cGMP formation, as shown in Fig. 4. There is no obvious explanation as to why cGMP formation should decrease while NO is being released. For instance, we showed that in the presence of *N*-hydroxylamine, a compound known to be metabolized to generate NO, cGMP formation in intact neuroblastoma N1E-115 cells increased and reached a plateau without a large decrease, as observed in Fig. 4 (30). Furthermore, the efficacy of MNP solution kept at 37° for 10 min after its preparation was similar to that of freshly prepared MNP solution. Therefore, it is possible that either MNP or a breakdown product of MNP inhibits cGMP production. Another possibility is the desensitization of guanylate cyclase to NO. To test these hypotheses, first the cells were treated with MNP (10^{-3} M) for 5 min in the dark and then the light was turned on for 1 min. Under these conditions no increase in cGMP formation was observed (Fig. 5, *column C*). However, after pretreatment of the cells with MNP (10^{-3} M) for 5 min in the dark and for 1 min in the light, further addition of either fresh MNP (10^{-3} M), followed by exposure of the cells to light for 1 min, or sodium nitroprusside (5×10^{-5} M) for 5 min caused an increase in cGMP formation similar to that produced by treatment with either MNP (10^{-3} M)/light (1 min) or sodium nitroprusside (5×10^{-5} M) alone (Fig. 5, *columns D, E, F,* and *G*). Light without MNP had no effect on cGMP production in neuroblastoma N1E-115 cells (Fig. 5, *column A*). These data implied that MNP was not active after 5 min in the presence of neuroblastoma N1E-115 cells. Furthermore, there was neither inhibition of cGMP formation nor desensitization of guanylate cyclase by MNP or a breakdown product of MNP. Because only the monomer of MNP can be induced by light to generate NO (27), we suggest that the cells catalyze the dimerization of MNP and render it inactive to light.

Once the time course of light-mediated cGMP formation was known, we next established a dose-response curve for light-activated MNP. Fig. 6 shows the photoactivated MNP concentration-dependent increase in the formation of cGMP in N1E-115 cells, with a half-maximal response of about 5×10^{-4} M MNP. To confirm the involvement of NO in the activation of guanylate cyclase, the photolysis of MNP (10^{-3} M) was per-

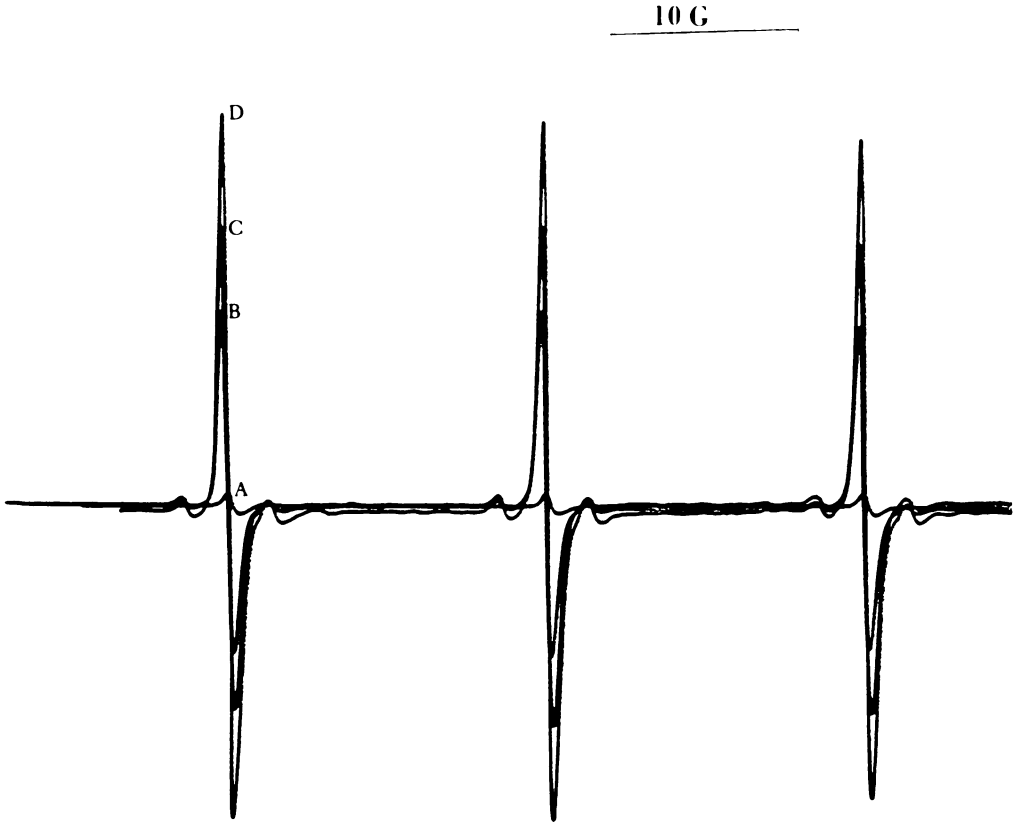

Fig. 2. ESR spectra obtained after irradiation of MNP (10^{-3} M). *Spectrum A*, no irradiation; *spectrum B*, 5-min irradiation; *spectrum C*, 15-min irradiation; *spectrum D*, 30-min irradiation. Instrument settings were as follows: microwave power, 20 mW; modulation amplitude, 1.0 G; sweep time, 12.5 G/min; response time, 1 sec; receiver gain, 6.3×10^3.

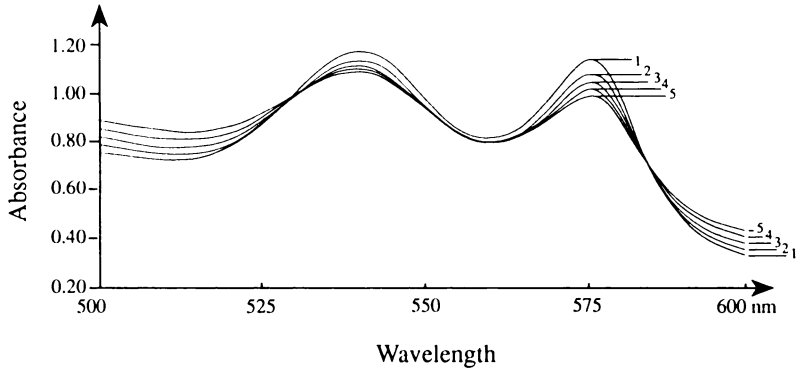

Fig. 3. Visible spectra of oxyhemoglobin (7.9×10^{-6} M) in the presence of MNP (10^{-3} M), obtained after irradiation. *Spectrum 1*, no irradiation; *spectrum 2*, 5-min irradiation; *spectrum 3*, 10-min irradiation; *spectrum 4*, 15-min irradiation; *spectrum 5*, 20-min irradiation.

formed in the presence of oxyhemoglobin. Fig. 7 shows that oxyhemoglobin inhibited the production of cGMP in response to light in a dose-dependent manner. Under these conditions, complete inhibition of cGMP formation was observed at 5×10^{-6} M oxyhemoglobin. Because it is not expected that oxyhemoglobin penetrates the cells, the complete inhibition of cGMP production in the presence of this compound is indicative of the extracellular generation of NO by light-activated MNP.

Next, the activation by MNP of guanylate cyclase in cultured vascular smooth muscle cells was investigated. Treatment of vascular smooth muscle cells with MNP (10^{-3} M) in the dark caused a 5-fold increase in cGMP formation over basal levels in cells in the dark without MNP, as measured by radioimmunoassay. However, when MNP (10^{-3} M) was exposed to light for 1 min, an 80-fold increase in cGMP production over basal levels was observed. In the absence of MNP, light had no effect on the production of cGMP. As in the case of neuroblastoma N1E-115 cells, oxyhemoglobin (5×10^{-6} M) inhibited cGMP production stimulated by light-activated MNP. These results demonstrate the usefulness of light-activated MNP as an NO-generating system to stimulate soluble guanylate cyclase.

Finally, the vasorelaxant effect of light-activated MNP was investigated using a standard isolated PA ring preparation. Fig. 8 represents the strip chart recording of typical experiments from two individual vessel segments. As shown in the last part of the strip chart recording in Fig. 8A, MNP in the dark induced PA relaxation in a dose-dependent manner, with no further relaxation with 10^{-3} M MNP upon exposure to light. This result is consistent with our previous report (32), as well as with the

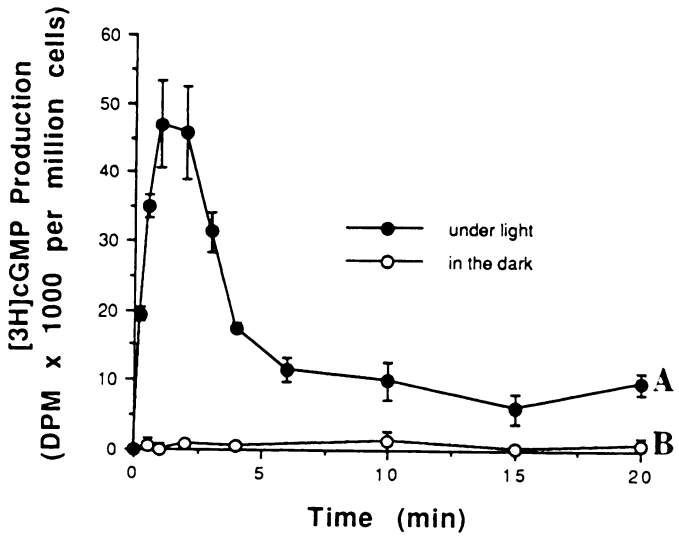

Fig. 4. Time course of cGMP accumulation in N1E-115 cells. *Curve A*, [^{3}H]guanosine-labeled cells were irradiated in the presence of MNP (10^{-3} M); *curve B*, [^{3}H]guanosine-labeled cells were kept in the dark. Data shown are representative of three independent experiments; *error bars*, standard deviations.

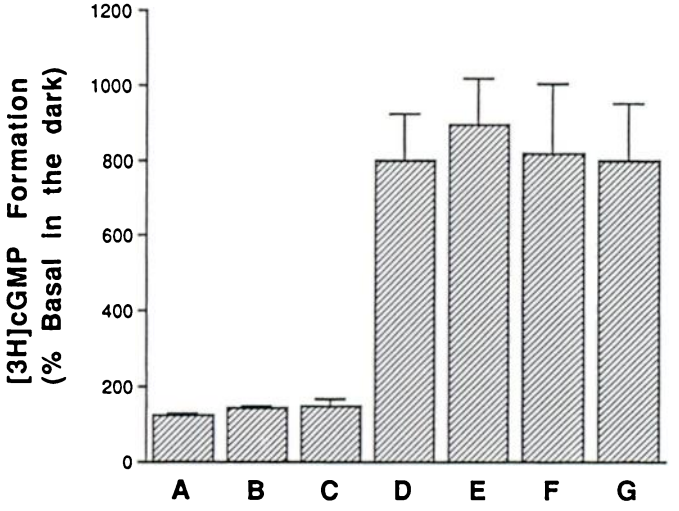

Fig. 5. Effect of different treatments on cGMP accumulation in N1E-115 cells, expressed as percentage of basal levels in the dark. *Column A*, cells and light for 1 min; *column B*, cells and MNP (10^{-3} M) in the dark; *column C*, cells and MNP (10^{-3} M) in the dark for 5 min and then in the light for 1 min; *column D*, cells and MNP (10^{-3} M) in the light for 1 min; *column E*, cells and MNP (10^{-3} M) in the dark for 5 min and in the light for 1 min, addition of fresh MNP (10^{-3} M), and then light for 1 min; *column F*, cells and MNP (10^{-3} M) in the dark for 5 min and in the light for 1 min and addition of fresh sodium nitroprusside (5×10^{-5} M) for 5 min; *column G*, cells and sodium nitroprusside (5×10^{-5} M) for 5 min.

data obtained by Konorev *et al.* (42) demonstrating that nitrone and nitroso spin traps, including MNP, can cause vasorelaxation. In the dark at this concentration, MNP had no effect on cGMP production in neuroblastoma N1E-115 cells but caused a modest increase in cGMP formation in cultured vascular smooth muscle cells (see above). Presumably, in the case of vascular smooth muscle cells and in PA, MNP released NO via some reaction with the cell surface, in the manner of organic nitrates (15).

We found that at concentrations below 10^{-5} M MNP had no relaxant effect on PA rings in the dark. However, in the

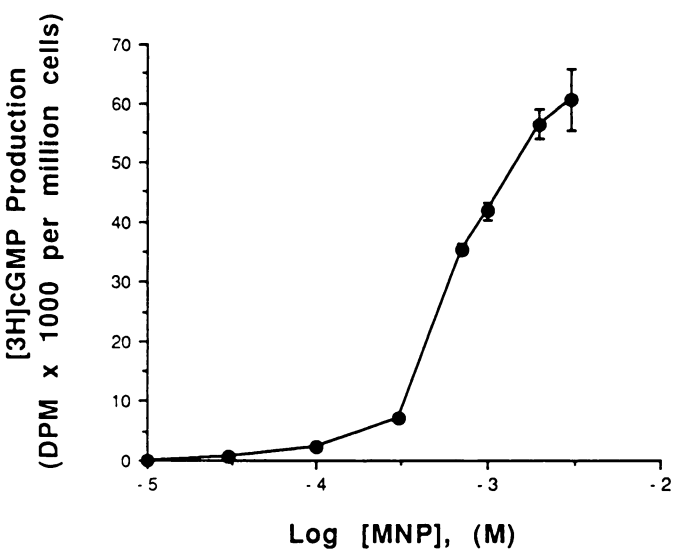

Fig. 6. cGMP accumulation in N1E-115 cells in the presence of increasing doses of light-activated MNP. The light was turned on for 1 min. Data shown are representative of three independent experiments; *error bars*, standard deviations.

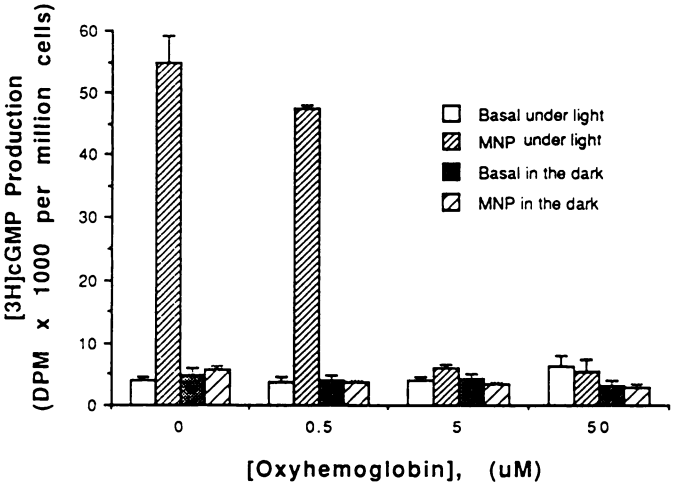

Fig. 7. Effect of oxyhemoglobin on cGMP accumulation in N1E-115 cells. Four conditions were tested as labeled, 1) light and cells, 2) light, cells, and MNP (10^{-3} M), 3) dark and cells, and 4) dark, cells, and MNP (10^{-3} M). The light was turned on for 1 min. Data shown are representative of three independent experiments; *error bars*, standard deviations.

presence of light MNP at 10^{-5} M induced relaxation of PA rings precontracted with PE (Fig. 8B). This tracing also demonstrates the typical photorelaxation (43) observed in all vessels in the absence of MNP. As illustrated, the relaxation induced by the initial light exposure was larger than that induced by subsequent exposures. The light exposures were repeated until a stable response was achieved; the tension produced in response to this final exposure to light was, although observable, only slightly decreased from the PE-induced contraction. In seven PA rings studied as in Fig. 8B, we found no significant effect of either light alone (453.6 ± 98.4 mg) or 10^{-5} M MNP in the dark (505.7 ± 106.7 mg) on PE-induced contraction (517.1 ± 105 mg). Exposure to light in the presence of MNP (10^{-5} M) caused a significant ($p < 0.001$) relaxation (217.9 ± 61.4 mg). Although the mechanism by which MNP induced relaxation of PE-induced contraction of PA rings was not investigated, the

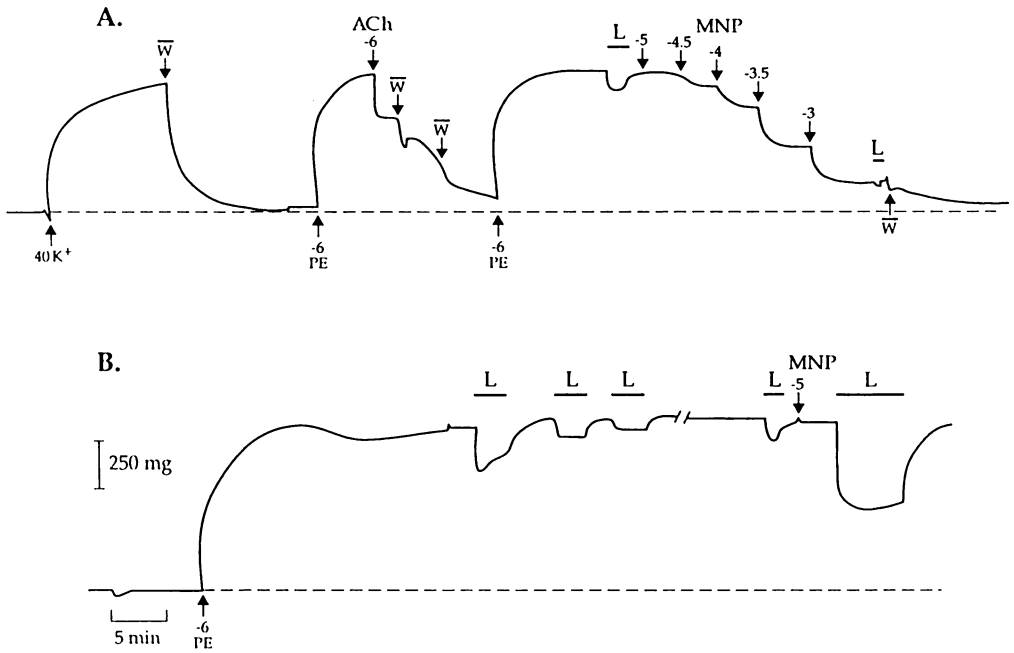

Fig. 8. Reproduction of strip chart recordings taken from a typical experiment. A, First, contraction induced by 40 mM K^+ indicates vessel viability. Second, contraction induced by PE (10^{-6} M), followed by relaxation in the presence of cumulative doses of ACh (10^{-6} M), indicates viable endothelium. After repeated contraction with PE and exposure to light (*L*), which caused a slight relaxation, brief cumulative doses of MNP (10^{-5} to 10^{-3} M) were added. B, Repeated exposures to light of a PE-precontracted vessel are shown. MNP (10^{-5} M, a dose that did not cause significant relaxation in the dark) was added in the dark. Exposure to light while MNP was present in the tissue bath elicited marked relaxation, significantly greater than that produced by light alone. $\overline{W}$, wash-out of the added drugs from the tissue bath.

results obtained in the studies with cultured cells suggested that relaxation caused by MNP was due to the release of NO, which activated guanylate cyclase, and was independent of endothelial cells.

In conclusion, we have demonstrated that MNP is a caged-NO that readily liberates NO upon illumination with visible light. Thus, MNP can serve as a useful tool in many applications requiring the controlled generation of NO. Although MNP itself might have some limitations, such as instability in the presence of cells and the inability to generate NO intracellularly, we believe that we can overcome these limitations through proper structure modifications. Structure-activity studies are in progress in our laboratory that will lead to the next generation of caged-NO compounds with improved properties.

Acknowledgments

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