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Molecular Actions of a Mn(III)Porphyrin Superoxide Dismutase Mimetic and Peroxynitrite Scavenger: Reaction with Nitric Oxide and Direct Inhibition of NO Synthase and Soluble Guanylyl Cyclase

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ABSTRACT

Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP), described as a superoxide dismutase mimetic and peroxynitrite scavenger, has been used previously to investigate the cytotoxic potential of superoxide and peroxynitrite in several pathological models. Here we report on the interference of MnTMPyP with NO/cGMP signaling using cultured endothelial cells as well as purified soluble guanylyl cyclase (sGC) either activated by the NO donor 2,2-diethyl-1-nitroso-oxyhydrazine sodium salt (DEA/NO) or reconstituted with nitric oxide synthase (NOS). MnTMPyP inhibited endothelial cGMP accumulation induced by A23187 (0.3 μM) with an IC_{50} of 75.0 $\pm$ 10.4 μM but had no significant effect on the potency of the Ca^{2+} ionophore. Purified NOS was inhibited by MnTMPyP (IC_{50} = 5.5 $\pm$ 0.8 μM) because of an interference of the Mn-porphyrin with the reductase domain of the enzyme. The most pronounced actions of MnTMPyP were direct inhibition of sGC and scavenging of NO. Purified sGC stimulated with either Ca^{2+}/calmodulin-activated NOS (in the presence of GSH) or DEA/NO (in the absence of GSH) was inhibited with IC_{50} values of 0.8 $\pm$ 0.09 μM and 0.6 $\pm$ 0.2 μM, respectively. In the presence of GSH, MnTMPyP was reduced to the Mn(II) complex, resulting in efficient scavenging of NO under these conditions. Our data demonstrate that MnTMPyP (i) interferes with the reductase domain of NOS, (ii) scavenges NO in the presence of GSH, and (iii) is a potent direct inhibitor of sGC. These results cast doubt on the usefulness of MnTMPyP and related Mn-porphyrin complexes as probes to study the involvement of peroxynitrite/superoxide in biological systems.

Nitric oxide is a widespread messenger molecule regulating biological processes as diverse as blood vessel relaxation, neuronal cell-to-cell communication and immune function (Mayer and Hemmens, 1997). The major physiological target of NO is sGC (GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2), which catalyzes the formation of cGMP from GTP. The enzyme contains a regulatory heme group that binds NO at diffusion-controlled rates and confers the pronounced NO stimulation of the enzyme (Ignarro, 1991; Wedel *et al.*, 1994).

NO is synthesized by three NOS isoforms, which all catalyze an NADPH- and O_2-dependent oxidation of L-arginine to form L-citrulline and NO (Hemmens and Mayer, 1997; Stuehr, 1997). Two NOS isoforms constitutively expressed in cells such as neurons (nNOS) and endothelium (eNOS) are activated by Ca^{2+}-dependent calmodulin binding, whereas expression of a third, Ca^{2+}-independent isoform is induced by cytokines (iNOS). All three NOS isoforms are homodimeric proteins whose subunits are comprised of an amino-terminal oxygenase domain that binds heme, L-arginine, and H_4biopterin and a carboxyl-terminal reductase domain that binds calmodulin, FMN, FAD, and NADPH. The reductase domain shuttles electrons from NADPH via the flavins to the oxygenase domain, which is the site of heme iron reduction, O_2 activation and NO synthesis. At low concentrations of L-arginine or in its absence, the enzymatic reduction of O_2 uncouples from substrate oxidation leading to the production of O_2^- and H_2O_2 instead of NO (Heinzel *et al.*, 1992; Pou *et al.*, 1992). In addition to the amino acid substrate, the pteridine cofactor H_4biopterin is also required for the tight

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ABBREVIATIONS: DEA/NO, 2,2-diethyl-1-nitroso-oxyhydrazine sodium salt; H_4biopterin, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; MnTMPyP, Mn(III)-tetrakis-(1-methyl-4-pyridyl)porphyrin; NOS, nitric oxide synthase; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; iNOS, inducible nitric oxide synthase; sGC, soluble guanylyl cyclase; SOD, superoxide dismutase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

coupling of O_2 reduction to L-arginine oxidation (Mayer and Werner, 1995).

Recently manganese complexes of substituted mesoporphyrins have been described as a new class of SOD mimetics which are cell permeable and stable in the presence of metal ion chelators. These Mn-porphyrins were shown to protect *Escherichia coli* against paraquat-induced oxidative stress (Liochev and Fridovich, 1995) and to facilitate the growth of SOD-deficient *E. coli* strains (Faulkner *et al.*, 1994; Batinic-Haberle *et al.*, 1997). Substituted metalloporphyrins also potently inhibit peroxynitrite-induced oxidation of dihydrorhodamine-123 (Zingarelli *et al.*, 1997). It has been demonstrated that peroxynitrite reacts rapidly with MnTMPyP in a 1:1 stoichiometry, thereby generating an oxomanganese intermediate, which catalyzes plasmid DNA strand breaking under physiological conditions (Groves and Marla, 1995). By the use of Mn-porphyrins, the involvement of peroxynitrite has been demonstrated in the depression of cellular respiration (Szabo *et al.*, 1996) and in the oxidation of mitochondrial as well as nuclear proteins in immunostimulated macrophages (Szabo *et al.*, 1997). In addition, Mn-porphyrins were reported to ameliorate vascular contractile and cellular energetic failure in endotoxin-treated rats (Zingarelli *et al.*, 1997).

The present study was designed to investigate the interference of MnTMPyP with the NO/cGMP signaling pathway. Our results confirm that the Mn-porphyrin acts as an efficient scavenger of peroxynitrite but provide no evidence for a SOD-mimetic effect of this compound in cells. Instead, MnTMPyP was found to be a potent NO scavenger in the presence of GSH and to directly inhibit both NOS and sGC.

Experimental Procedures

Materials. MnTMPyP was purchased from Alexis (Läufelfingen, Switzerland). Stock solutions (0.1 M) were prepared with Nano-pure water (Barnstead ultrafiltered type I, resistance $> 18\ M\Omega\ cm^{-1}$) and kept at −20° before use. DEA/NO was from NCI Chemical Carcinogen Repository (Kansas City, MO). Tenfold concentrated stock solutions of the NO donor were prepared daily in 10 mM NaOH. L-[^{3}H]Arginine was from Amersham, supplied by MedPro (Vienna, Austria). NO solutions were prepared by dissolving NO gas (99% pure; Linde München, Germany) in deoxygenated water as described previously (Kukovetz and Holzmann, 1989). All other chemicals were obtained from Sigma (Vienna, Austria).

Culture of endothelial cells and determination of endothelial cGMP and L-citrulline formation. Porcine aortic endothelial cells were cultured as previously described (Mayer *et al.*, 1993). Before experiments, the cells were subcultured in 24-well plastic plates and grown to confluence ($\sim 2 \times 10^5$ cells/well). The culture medium was removed, and the cells were washed once and equilibrated in buffer A (isotonic phosphate buffer, pH 7.4, containing 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM 3-isobutyl-1-methylxanthine, and 1 μM indomethacin) in the absence or presence of MnTMPyP at the concentrations indicated. After 15 min, Ca^{2+} ionophore A23187 or DEA/NO was added to give initial final concentrations of 0.3 and 1 μM, respectively. Reactions were terminated 4 min later by removal of buffer and addition of 1 ml of 0.01 N HCl. After incubating for 1 hr, intracellular cGMP was measured in the supernatants of the lysed cells by radioimmunoassay.

For determination of endothelial L-citrulline formation, the culture medium was removed, and the cells ($\sim 10^6$ cells/well) were washed once and equilibrated in buffer A in the absence or presence of MnTMPyP at concentrations as indicated. After 15 min, the cells were washed twice with 1 ml of prewarmed buffer B (50 mM HEPES, pH 7.4, containing 100 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, and 1 mM $MgCl_2$) and incubated for 10 min at 37° in 1 ml of buffer B containing L-[^{3}H]arginine ($\sim 1 \times 10^6$ dpm) and 0.3 μM Ca^{2+}-ionophore A23187. The cells were then washed twice with 1 ml of ice-cold buffer B containing 5 mM EDTA and lysed with 1 ml of 0.01 N HCl. After 1 hr, an aliquot of 0.1 ml was analyzed for incorporated radioactivity by liquid scintillation counting. To the remaining sample (0.9 ml), 0.1 ml of 0.2 M sodium acetate buffer (pH 13.0) containing 10 mM L-citrulline, was added (final pH ~ 5.0), and L-[^{3}H]citrulline was separated from L-[^{3}H]arginine by cation exchange chromatography as described previously (Mayer *et al.*, 1994). Values are expressed as percent conversion of the incorporated L-[^{3}H]arginine to L-[^{3}H]citrulline.

Determination of NOS activity. Rat nNOS and bovine eNOS were purified from recombinant baculovirus-infected Sf9 cells as described previously (Harteneck *et al.*, 1994; List *et al.*, 1996). Formation of L-[^{3}H]citrulline from L-[^{3}H]arginine was determined by incubation of 0.2–0.3 μg of enzyme at 37° for 10 min in a 50 mM triethanolamine/HCl buffer (pH 7.0), containing 0.1 mM L-[^{3}H]arginine (~50,000 cpm), 0.2 mM NADPH, 5 μM flavin adenine dinucleotide, 10 μM H_4biopterin, 0.5 mM $CaCl_2$, and 10 μg/ml calmodulin, followed by isolation of L-[^{3}H]citrulline by cation exchange chromatography (Mayer *et al.*, 1994). Uncoupled reductive activation of oxygen was determined as Ca^{2+}/calmodulin-dependent oxidation of NADPH in the absence of L-arginine and H_4biopterin by continuously monitoring the decrease in absorbance at 340 nm (Mayer *et al.*, 1991). The cytochrome P450 reductase activity of NOS was assayed as reduction of cytochrome *c* (0.2 mM) by continuously monitoring the increase in absorbance at 550 nm against calmodulin-deficient blanks (Klatt *et al.*, 1992). MnTMPyP was added in 10-fold concentrated stock solutions.

Determination of sGC activity. For direct activation with the NO donor DEA/NO, purified sGC (50 ng; V_{max} ~6–8 μmol/mg/min) was incubated at 37° for 10 min in a total volume of 0.1 ml of a 50 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.4, containing 0.5 mM [α-^{32}P]GTP (200,000–300,000 cpm), 3 mM $MgCl_2$, 1 mM cGMP, and 0.05 mg/ml bovine serum albumin. Reactions were started by adding DEA/NO (1 μM final concentration) and 10-fold concentrated stock solutions of MnTMPyP or vehicle to the assay mixtures and transferring the samples from 4 to 37°. For reconstitution with nNOS, purified sGC (50 ng) was incubated at 37° for 10 min in a total volume of 0.1 ml of a 50 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.4, containing 0.5 mM [α-^{32}P]GTP (200,000–300,000 cpm), 3 mM $MgCl_2$, 1 mM cGMP, 0.05 mg/ml bovine serum albumin, 0.1 mM L-arginine, 10 μM $CaCl_2$, 10 μg/ml calmodulin, 50 μM NADPH, 40 μM CHAPS, and 1 mM GSH. Reactions were started by adding nNOS (2 μg/ml; specific activity 0.06–0.08 μmol/mg/min under these conditions; V_{max} ~0.8 μmol/mg/min) and 10-fold concentrated stock solutions of MnTMPyP or vehicle to the assay mixtures, followed by transferring the samples from 4° to 37°. Reactions were terminated by $ZnCO_3$ precipitation, followed by isolation of [α-^{32}P]cGMP as described (Schultz and Böhme, 1984). Results were corrected for enzyme-deficient blanks and recovery of cGMP.

Electrochemical detection of NO. NO was measured with a Clark-type electrode (Iso-NO, World Precision Instruments, Berlin, Germany), which was connected to an Apple Macintosh computer *via* an analog to digital converter (MacLab, World Precision Instruments). The output current was recorded at 0.6 Hz under constant stirring at 37°. Two-point calibration of the electrode was performed daily according to the procedure recommended by the manufacturer. Solutions to be tested were applied by injection into 1.8-ml glass vials completely filled with 50 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.4, containing additives as indicated and sealed with a septum.

Light absorbance spectroscopy of MnTMPyP. Light absorbance spectra were recorded at ambient temperature with a Hewlett-Packard 8452A diode array spectrophotometer in 50 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.4, containing additives as indicated. Tenfold

concentrated stock solutions of MnTMPyP were added to give the final concentrations indicated in the text and figures. The Mn(II)-NO-TMPyP complex (Soret band at 434 nm) was prepared by addition of sodium dithionite to convert Mn(III)TMPyP (Soret band at 462 nm) to Mn(II)TMPyP (Soret band at 450 nm) (Faulkner *et al.*, 1994) and addition of 10 μl of a 2 mM aqueous NO solution.

Data evaluation. Unless indicated otherwise, data are mean values ± standard errors of three experiments performed in duplicate. Parameters of the concentration-response curves were calculated according to the Hill equation.

Results

To test for a possible SOD-mimetic effect of MnTMPyP, we studied the effect of this compound on endothelial cGMP accumulation induced by the Ca^{2+} ionophore A23187. Basal cGMP levels of unstimulated cells were 2.0 ± 0.3 pmol/10^6 cells. Increasing concentrations of A23187 led to a pronounced stimulation of cGMP formation (up to 19.2 ± 1.1 pmol/10^6 cells) with an EC_{50} of 51.0 ± 13.2 nM (Fig. 1A, *filled symbols*). This effect of A23187 was completely inhibited by the NOS inhibitor N^G-nitro-L-arginine (10 μM; not shown). MnTMPyP (10 μM) did not potentiate the effect of the Ca^{2+} ionophore (EC_{50} = 69.0 ± 9.0 nM) but significantly decreased its maximal effect (15.1 ± 2.1 pmol of cGMP/10^6 cells) (*open symbols*). Similar data were obtained when the NO donor DEA/NO was used to directly activate endothelial sGC (data not shown). Fig. 1B (*filled symbols*) shows that MnTMPyP inhibited A23187-induced cGMP accumulation in a concentration-dependent manner with an IC_{50} of 75.0 ± 10.4 μM. The effect of the porphyrin was virtually complete at 0.3 mM (2.52 ± 0.2 pmol cGMP/10^6 cells). This pronounced inhibition was not explained by inhibition of NOS, as evident by the much lower sensitivity to MnTMPyP of endothelial arginine-to-citrulline conversion (~30% inhibition at 0.3 mM; Fig. 1*B*, *open symbols*).

Although NOS inhibition was not apparent in the experiments with intact cells, MnTMPyP was a fairly potent inhibitor of purified recombinant nNOS. As shown in Fig. 2A, MnTMPyP inhibited formation of L-citrulline in a concentration-dependent manner with an IC_{50} of 5.5 ± 0.8 μM. The porphyrin inhibited L-citrulline formation of the inducible isoform with a similar potency (IC_{50} = 9.0 ± 1.4 μM), but was a slightly less potent inhibitor of the endothelial enzyme (IC_{50} = 23 ± 1.1 μM; data not shown). In the light of our

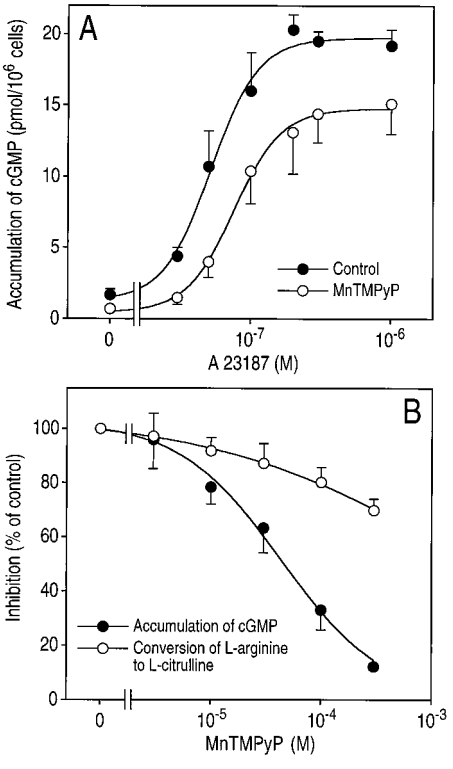

Fig. 1. Effect of MnTMPyP on A23187-induced cGMP accumulation and L-citrulline formation in endothelial cells. A, Endothelial cells were preincubated in the absence (*filled symbols*) or presence (*open symbols*) of MnTMPyP (10 μM) for 15 min, followed by incubation with A23187 at the indicated concentrations for 4 min and determination of cellular cGMP levels by radioimmunoassay. Data are mean values ± standard error of three experiments performed in duplicate. B, Endothelial cells were preincubated in the presence of increasing concentrations of MnTMPyP for 15 min, followed by incubation with 0.3 μM A23187 for 10 min and determination of cellular cGMP levels by radioimmunoassay (*filled symbols*) or conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline (*open symbols*; see Experimental Procedures for details). Results are expressed as percent inhibition (100% = formation of cGMP or conversion of incorporated L-[^{3}H]arginine to L-[^{3}H]citrulline in the absence of MnTMPyP). Data are mean values ± standard error of three experiments performed in duplicate.

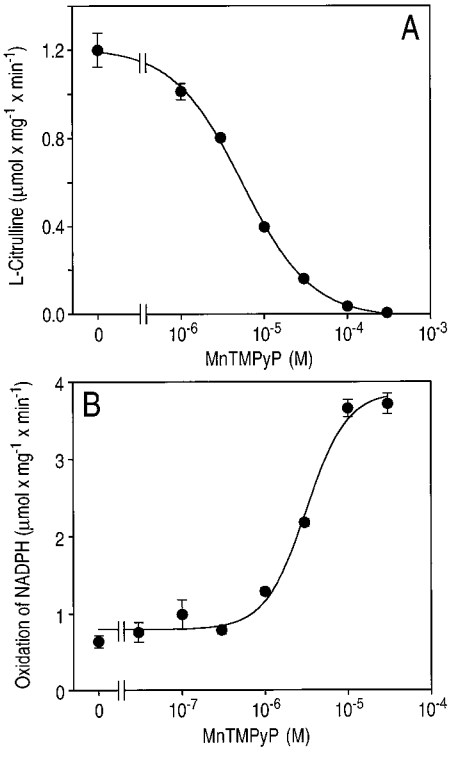

Fig. 2. Effect of MnTMPyP on L-citrulline formation (A) and NADPH (B) oxidation by purified nNOS. A, Purified rat nNOS (0.2–0.3 μg) was incubated for 10 min at 37° in 0.1 ml of a 50 mM triethanolamine/HCl buffer (pH 7.0) containing 0.1 mM L-[^{3}H]arginine (~50,000 cpm), 0.2 mM NADPH, 5 μM flavin adenine dinucleotide, 10 μM H_4biopterin, 0.5 mM $CaCl_2$, and 10 μg/ml calmodulin in the presence of increasing concentrations of MnTMPyP, followed by isolation of L-[^{3}H]citrulline by cation exchange chromatography. Data are mean values ± standard error of three experiments performed in duplicate. B, Purified rat nNOS (1 μg) was incubated at 37° in 0.2 ml of a 50 mM triethanolamine/HCl buffer (pH 7.0) containing 0.2 mM NADPH, 0.5 mM $CaCl_2$, and 10 μg/ml calmodulin in the presence of increasing concentrations of MnTMPyP. Oxidation of NADPH was measured by continuously monitoring the decrease in absorbance at 340 nm against calmodulin-deficient blanks. Enzyme activity was calculated using an extinction coefficient of 6.34/mM/cm. Data are mean values ± standard error of three experiments.

previous results with SOD mimetic copper complexes (Mayer *et al.*, 1996), it was likely that MnTMPyP behaved similarly and interfered as a parasitic electron acceptor with the cytochrome P_{450} reductase activity of NOS, which can be assayed as Ca^{2+}/calmodulin-dependent reduction of cytochrome *c* (Klatt *et al.*, 1992). The cytochrome *c* reductase activity of purified nNOS was 10.0 ± 0.3 and 5.9 ± 0.4 μmol/mg/min in the absence and presence of 50 μM MnTMPyP, respectively. Further, MnTMPyP led to a pronounced, approximately 4-fold stimulation of NOS-catalyzed NADPH oxidation (measured in the absence of L-arginine). As shown in Fig. 2B, MnTMPyP increased the NADPH oxidase activity of the enzyme in a concentration-dependent manner from 0.64 ± 0.08 μmol/mg/min to 2.7 ± 0.4 μM; the EC_{50} was 2.69 ± 0.2 μM, maximal effects were obtained with 0.1 mM of the porphyrin. Similar data were obtained with purified recombinant bovine eNOS and murine macrophage iNOS (data not shown).

The cell culture experiments indicated that effects unrelated to NOS inhibition may account for the potent interference of MnTMPyP with endothelial cGMP accumulation. Therefore, we tested the porphyrin for inhibition of purified sGC and scavenging of NO. Incubation of sGC with the NO donor DEA/NO (1 μM) or Ca^{2+}/calmodulin-activated NOS (0.2 μg/0.1 ml) led to a pronounced stimulation of cGMP formation (from 0.043 ± 0.009 to 7.89 ± 0.35 and 3.44 ± 0.01 μmol/mg/min, respectively). It should be pointed out that sGC stimulation by donors of pure NO, e.g., DEA/NO, is GSH-independent (Mayer *et al.*, 1995a), whereas stimulation of the enzyme by NOS or other NO/$O_2^{\cdot-}$-generating systems requires the presence of a thiol (Mayer *et al.*, 1998). Therefore, the experiments shown in Fig. 3 were performed either in the presence (NOS) or in the absence (DEA/NO) of GSH. MnTMPyP inhibited the formation of cGMP in a concentration-dependent manner with IC_{50} values of 0.8 ± 0.09 and 0.6 ± 0.2 μM when sGC was activated with NOS and DEA/NO, respectively. Full inhibition was observed with porphyrin concentrations as low as 10–100 μM. Identical results were obtained with DEA/NO-activated GC in the presence of GSH (data not shown). These results showed that MnTMPyP potently inhibited NO stimulation of sGC in a thiol-independent manner.

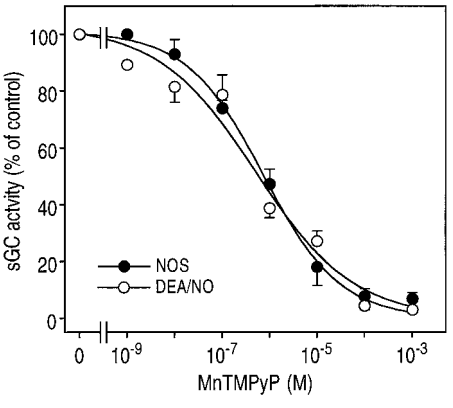

Fig. 3. Effect of MnTMPyP on sGC activity. Purified sGC (50 ng) was incubated with Ca^{2+}/calmodulin-activated nNOS (*filled symbols*) at 37° for 10 min in a total volume of 0.1 ml of a 50 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.4, containing 0.5 mM [α-^{32}P]GTP, 3 mM $MgCl_2$, 1 mM cGMP, 0.05 mg/ml bovine serum albumin, 0.1 mM L-arginine, 10 μM $CaCl_2$, 10 μg/ml calmodulin, 50 μM NADPH, 40 μM CHAPS, and 1 mM GSH. Reactions were started by adding nNOS (2 μg/ml) and 10-fold concentrated stock solutions of MnTMPyP. For direct activation with the NO donor DEA/NO (*open symbols*), purified sGC (50 ng) was incubated at 37° for 10 min in a total volume of 0.1 ml of a 50 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.4, containing 0.5 mM [α-^{32}P]GTP, 3 mM $MgCl_2$, 1 mM cGMP, and 0.05 mg/ml bovine serum albumin. Reactions were started by adding DEA/NO (1 μM final concentration) and 10-fold concentrated stock solutions of MnTMPyP to the assay mixtures. Incubations were terminated by $ZnCO_3$ precipitation, followed by isolation of [α-^{32}P]cGMP. Results were corrected for enzyme-deficient blanks and recovery of cGMP. Data are mean values ± standard error of three experiments performed in duplicate.

To study whether this inhibitory effect of MnTMPyP was the result of a direct interaction with sGC and/or scavenging of free NO, we measured NO autoxidation kinetics electrochemically in the absence and presence of the porphyrin under various conditions. Fig. 4 shows representative traces obtained with authentic NO in 50 mM phosphate buffer (pH 7.4) at 37°. Addition of 3.6 μl of an NO solution (~2 mM) to a total volume of 1.8 ml of buffer led to a pronounced response of the electrode, followed by a decrease of the signal with second order kinetics. The NO oxidation kinetics were identical in the absence (*a*) and presence (*b*) of 2 μM MnTMPyP, yielding third order rate constants of 2.39 ± 0.54 × 10^7/M^2/sec (n = 3) and 2.14 ± 0.20 × 10^7/M^2/sec (n = 4), respectively. However, the porphyrin was converted to an efficient NO scavenger under reducing conditions. The presence of 1 mM GSH did not appreciably affect the autoxidation of NO (k = 3.2 ± 0.1 × 10^7/M^2/sec; n = 3; *c*), but MnTMPyP (2 μM) led to a rapid disappearance of the NO signal with an apparent zero order rate constant of 145 ± 23 nM/sec under these conditions (n = 4; *d*). Thus, MnTMPyP scavenged NO in the presence but not in the absence of GSH. Identical data were obtained in the presence of the reducing compound NADPH (0.2 mM) (data not shown).

The reactivity of MnTMPyP toward peroxynitrite and NO was studied by light absorbance spectroscopy. The parent complex exhibited a Soret absorbance maximum at 462 nm that was shifted to 420 nm upon addition of peroxynitrite (Fig. 5A). A previous study identified this 420 nm species as the corresponding oxo-Mn(IV) complex which rapidly decays back to the starting Mn(III)porphyrin (Groves and Marla, 1995). Treating MnTMPyP (2 μM) with a solution of NO (final concentration 0.1 mM) did not alter the absorbance spectrum (Fig. 5B; *solid line*). However, when the same experiment

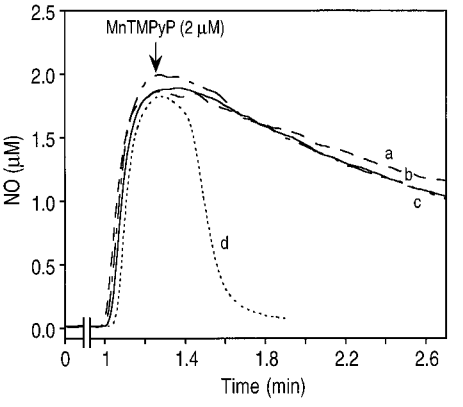

Fig. 4. Effect of MnTMPyP on NO autoxidation in the presence or absence of GSH. An aliquot (3.6 μl) of a saturated aqueous NO solution (~2 mM) was added to 1.8 ml of a 50 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.4 at 37°. Changes in NO concentrations were monitored over 5 min with a Clark-type NO electrode. Conditions were: buffer (*a*), 1 mM GSH (*b*), 2 μM MnTMPyP (*c*), and 1 mM GSH plus 2 μM MnTMPyP (*d*). MnTMPyP was injected at the peak level of the NO concentration (*arrow*). The traces shown are representative of four experiments.

was performed in the presence of 1 mM GSH, a pronounced shift of the Soret band from 462 to 434 nm was observed, indicative of the formation of the corresponding Mn(II)-nitrosyl complex (Yonetani *et al.*, 1972). Identical spectral changes were observed when NADPH (0.2 mM) was present as reducing agent instead of GSH (data not shown). As observed with the oxidized complex formed by peroxynitrite, the Mn(II)-NO species fairly rapidly decayed back to the Mn(III)porphyrin ($t_{1/2} \sim 2$ min). These experiments provided a reliable explanation for our electrochemical observations on scavenging of NO by MnTMPyP in the presence of GSH.

Discussion

The present data confirm previous reports on the rapid reaction of the cell permeable Mn-porphyrin MnTMPyP with peroxynitrite (Groves and Marla, 1995; Hunt *et al.*, 1997), but we question the usefulness of this compound and related drugs as specific tools to probe the involvement of peroxynitrite in biological processes. We obtained no evidence for a SOD-mimetic effect of the porphyrin. Scavenging of $O_2^{\cdot-}$ by SOD is known to prolong the half-life of NO (Gryglewski *et al.*, 1986), resulting in a potentiation of NO-mediated effects, including the accumulation of cGMP in vascular endothelial cells (Mayer *et al.*, 1993), but MnTMPyP inhibited cGMP formation in this cell culture system. Because there is solid evidence that MnTMPyP has SOD activity *in vitro* (Faulkner *et al.*, 1994; Liochev and Fridovich, 1995), our data suggest that the SOD-mimetic effect of MnTMPyP was overcome by inhibition of endothelial sGC at low concentrations of the drug.

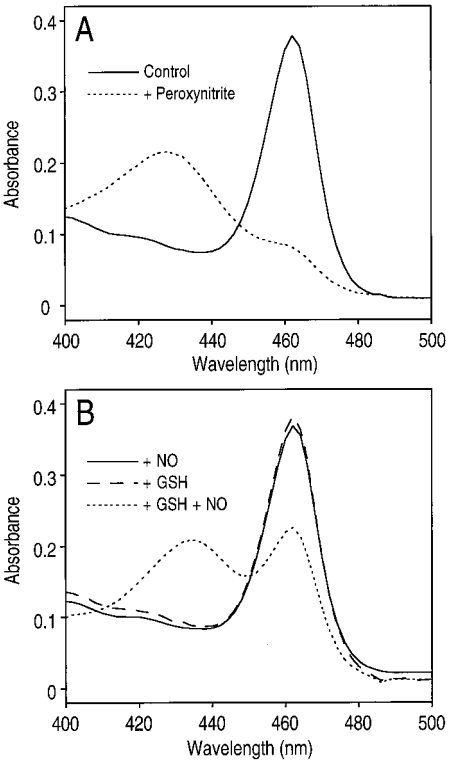

Fig. 5. Perturbations of the MnTMPyP light absorbance spectra induced by peroxynitrite (A) and NO/GSH (B). A, Light absorbance spectra of MnTMPyP (2 μM) were recorded before (*solid line*) and after (*dotted line*) addition of authentic peroxynitrite (0.1 mM final) in 50 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.4, at ambient temperature. The experiment shown is representative of three. B, Light absorbance spectra of MnTMPyP (2 μM) were recorded in the presence of 0.1 mM authentic NO (*solid line*), 1 mM GSH (*dashed line*), and in the presence of GSH plus NO (*dotted line*) at ambient temperature in 50 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.4. The experiments shown are representative of three.

In an earlier study, we found that the presence of SOD is essential for the detection of the formation of NO by purified NOS and concluded that, in the absence of SOD, NO is converted to peroxynitrite by enzymatically produced $O_2^{\cdot-}$ (Mayer *et al.*, 1995b). This conclusion was recently questioned in an article claiming that the effect of SOD is not a consequence of $O_2^{\cdot-}$ scavenging but results from oxidation of the postulated NOS product NO^- to NO (Schmidt *et al.*, 1996). Synthetic compounds with SOD-mimetic activity could be useful tools to unequivocally settle this issue. However, like the Cu(II) complexes studied previously (Mayer *et al.*, 1996), MnTMPyP turned out to be a potent inhibitor of purified NOS. The inhibition of enzymatic cytochrome *c* reduction, together with the pronounced stimulation of uncoupled NADPH oxidation, clearly demonstrates that the porphyrin interferes as a parasitic electron acceptor with the transfer of electrons from the flavin-containing reductase domain to the catalytic heme site of NOS. Inasmuch as we obtained virtually identical data with all three NOS isoforms, the low sensitivity of Ca^{2+}-activated arginine-to-citrulline conversion in the cell culture experiments hints at the existence of protective mechanisms that prevent the interaction of the Mn-porphyrin with NOS in intact cells. Alternatively, this compound may not so easily gain access to mammalian cells as to *E. coli* (Faulkner *et al.*, 1994; Liochev and Fridovich, 1995). It remains to be clarified whether the inhibition of NO_2^-/NO_3^- accumulation in the medium of endotoxin-treated macrophages by a related Mn-porphyrinic drug (Szabo *et al.*, 1996) was the result of radical scavenging or inhibition of iNOS.

It was surprising to find that MnTMPyP potently scavenged NO in the presence of reducing compounds such as GSH, the most abundant intracellular thiol in mammalian tissues (Meister, 1994). Together with light absorbance spectroscopy to monitor redox changes of MnTMPyP, the measurement of NO with a Clark-type electrode allowed us to elucidate the mechanism underlying this effect. Our data suggest that the parent Mn(III) complex does not bind NO but becomes reduced by GSH or NADPH to the Mn(II) species, the high NO binding affinity of which may have resulted in formation of the corresponding nitrosyl complex with a typical Soret band at 434 nm (Yonetani *et al.*, 1972; Dierks *et al.*, 1997). The scavenging of NO occurred at substoichiometric concentrations of MnTMPyP, pointing to redox cycling of the Mn-porphyrin. This may involve rapid dissociation of NO, followed by reoxidation of the Mn(II) complex by O_2 (Hoffman, 1979). Thus, although our results confirm that MnTMPyP does not scavenge NO in nonreducing buffers (Szabo *et al.*, 1996), this drug may act as a potent, catalytically active NO scavenger in the reducing environment of cells.

Finally, MnTMPyP proved to be a potent inhibitor of sGC, the major physiological target of NO. The inhibitory effect was most likely direct, i.e., not the result of scavenging of NO because MnTMPyP exhibited virtually identical potency in the absence and presence of GSH even though it did not scavenge NO in the absence of the thiol. Further, no scavenging of NO was observed in the sGC assay buffer in the

absence of GSH (electrochemical data; not shown). These findings indicate that MnTMPyP, like other metalloporphyrins such as Zn- and Sn-protoporphyrin IX (Ignarro, 1992; Luo and Vincent, 1994), is a potent inhibitor of sGC, presumably because of interference with heme-dependent NO stimulation of the enzyme.

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