Biochemical Pharmacology, Vol. 45, No. 2, pp. 367–374, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

INHIBITION OF NITRIC OXIDE SYNTHESIS BY METHYLENE BLUE

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(*Received* 29 *July* 1992; *accepted* 15 *October* 1992)

Abstract—Methylene blue appears to inhibit nitric oxide-stimulated soluble guanylyl cyclase and has been widely used for inhibition of cGMP-mediated processes. We report here that endothelium-dependent relaxation of isolated blood vessels and NO synthase-dependent cGMP formation in cultured endothelial cells were both markedly more sensitive to inhibition by methylene blue than effects induced by direct activation of soluble guanylyl cyclase. These discrepancies were also observed when superoxide dismutase (SOD) was present to protect NO from inactivation by superoxide anion. Subsequent experiments showed that formation of L-citrulline by purified NO synthase was completely inhibited by 30 μM methylene blue (IC_{50} = 5.3 and 9.2 μM in the absence and presence of SOD, respectively), whereas guanylyl cyclase stimulated by *S*-nitrosoglutathione was far less sensitive to the drug (50% inhibition at ~60 μM, and maximal inhibition of 72% at 1 mM methylene blue). Experimental evidence indicated that oxidation of NADPH, tetrahydrobiopterin or reduced flavins does not account for the inhibitory effects of methylene blue. Our data suggest that methylene blue acts as a direct inhibitor of NO synthase and is a much less specific and potent inhibitor of guanylyl cyclase than hitherto assumed.

Endothelium-dependent smooth muscle relaxation is mediated by L-arginine-derived nitric oxide, the endothelium-derived relaxing factor (EDRF), which is released from vascular endothelial cells in response to stimulation by hormones and neurotransmitters elevating intracellular Ca^{2+} concentrations [1]. Ca^{2+} is required for the activation of calmodulin-dependent NO synthase isozymes which synthesize NO together with L-citrulline from L-arginine and are expressed as both soluble and membrane-associated forms in endothelial cells [2–4]. NO apparently exerts its vasodilatory actions via activation of soluble guanylyl cyclase in smooth muscle cells adjacent to the endothelial cell layer. The molecular mechanism of this NO-induced stimulation of cGMP formation has been the subject of detailed investigations, and it has been suggested that NO interacts with the heme group bound to soluble guanylyl cyclase [5–8]. This hypothesis was supported by results obtained with methylene blue, which is generally accepted to oxidize protein-bound heme and non-heme ferrous iron [9–14]. Methylene blue was found to inhibit the stimulation of soluble guanylyl cyclase by NO and nitrovasodilators in cell-free systems [15, 16] as well as to block smooth muscle relaxation induced by nitrovasodilators or acetylcholine [17–19]. The compound has been extensively used as a "selective" inhibitor of soluble guanylyl cyclase to demonstrate the involvement of cGMP accumulation in vascular relaxation, platelet aggregation and neurotransmission [20–23].

Nevertheless, some studies indicated that methylene blue was more potent in inhibiting endothelium-dependent than nitrovasodilator-induced relaxation [24–26]. Moreover, methylene blue was shown to inhibit prostacyclin synthesis by endothelial cells and isolated arteries independent of its inhibitory effects on cGMP accumulation [27, 28]. Also, the pronounced inhibitory effects of 5 μM methylene blue on acetylcholine-induced vasodilation in cerebral and skeletal arterioles were completely prevented by simultaneous application of superoxide dismutase (SOD†) [29, 30]. Under aerobic conditions, reduced forms of methylene blue are readily reoxidized by molecular oxygen thereby generating superoxide anion [9]. Thus, superoxide-mediated inactivation of NO [31] may provide an explanation for effects of methylene blue unrelated to guanylyl cyclase inhibition, although endogenous SOD activity normally protects and stabilizes NO.

We speculated about an additional, as yet unrecognized, mechanism being responsible for the powerful effects of methylene blue on NO-mediated cellular signalling. We have recently shown that purified NO synthase, in addition to reduced flavins and tetrahydrobiopterin, also contains tightly bound iron [32], but a catalytic role for this iron has not yet been demonstrated. Provided that iron has an essential function in NO synthesis, methylene blue should act as artificial electron acceptor and inhibit NO synthase. It was the aim of the present work to address this question and to investigate the effects of methylene blue on the synthesis and the biological actions of NO.

MATERIALS AND METHODS

Materials. NO synthase was isolated from pig cerebellum as described previously [33]. Soluble guanylyl cyclase purified from bovine lung [34] was a kind gift of Dr E. Böhme, Berlin, Germany. Stock

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† Abbreviations: GSNO, *S*-nitrosoglutathione; SNP, sodium nitroprusside; SOD, superoxide dismutase.

solutions of *S*-nitrosoglutathione (GSNO) were prepared freshly each day by mixing equimolar amounts of glutathione (dissolved in 0.02 M hydrochloric acid) and aqueous sodium nitrite. SOD from bovine erythrocytes and methylene blue were from Sigma (Deisenhofen, Germany), all other reagents were obtained from sources described previously [32, 33, 35–37].

Relaxation of bovine coronary arteries. Circular strips of bovine coronary arteries were prepared, mounted in organ baths, equilibrated and precontracted with a combination of the thromboxane receptor agonist U46619 plus serotonin as described previously [36]. Indomethacin (3 μM) was added prior to precontraction, and methylene blue (30 μM) once a stable muscle length had been reached (approximately 30 min after onset of precontraction). SOD (30 U/mL) and N^{ω}-nitro-L-arginine (0.1 and 0.5 mM, respectively) were added 20 min later than methylene blue, and the A 23187 concentration–response curve was started another 15 min later. Following the highest concentration of A 23187 (10 μM), 0.1 mM GSNO was added, and finally 0.3 mM papaverine.

The following number of tissues (strips) were used (number of hearts in parentheses): A 23187 concentration–response curves in the absence and presence of methylene blue: 16 (6) each; in the presence of SOD: 6 (2); in the combined presence of SOD and methylene blue: 8 (2).

Changes in muscle length were recorded isotonically [36]. Whereas contracted strips to which no relaxant agents had been added were remarkably stable throughout the experiment (2–2.5 hr), methylene blue induced an initial transient increase (contraction) and later slow decrease (relaxation) of muscle length. N^{ω}-Nitro-L-arginine rapidly increased muscle length to a stable level. SOD had no influence on muscle length. The results are expressed as per cent of control relaxation obtained with papaverine. The base line was the stable length resulting from precontraction (controls in absence and presence of SOD), or (in the presence of methylene blue or N^{ω}-nitro-L-arginine) the length at the beginning of the A 23187 concentration–response curve. The gradual loss of muscle length induced by methylene blue was subtracted for each A 23187 concentration.

Cell culture techniques and cGMP measurements. Intracellular cGMP levels were measured in cultured porcine aortic endothelial cells as described previously [37]. Briefly, endothelial cells were isolated by enzymic treatment (0.1% collagenase) and cultured up to three passages in Opti-MEM (GIBCO/BRL GmbH, Eggenstein, Germany) containing 3% foetal calf serum and antibiotics. Prior to the experiments, endothelial cells were subcultured in 6-well plastic plates. After reaching confluence (~10^6 cells/dish) the culture medium was removed and the cells were preincubated at 37° with isotonic HEPES buffer (10 mM, pH 7.4) containing 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM 3-isobutyl-1-methylxanthine and 1 μM indomethacin. Methylene blue and SOD were present as indicated. After 15 min, A 23187, GSNO or sodium nitroprusside (SNP) was added and the reaction was stopped 4 min later by removal of the incubation medium and addition of 1 mL of 0.01 N hydrochloric acid. Intracellular cGMP was completely released into the supernatant within 1 hr and measured by radioimmunoassay.

Determination of guanylyl cyclase activity. Purified soluble guanylyl cyclase (0.1–0.2 μg) was incubated at 37° for 10 min in a total volume of 0.1 mL of a triethanolamine–HCl buffer (50 mM, pH 7.4) containing 0.1 mM [α-^{32}P]GTP (200,000–300,000 cpm), 1 mM cGMP, 1 mM 3-isobutyl-1-methylxanthine, 3 mM Mg^{2+}, 10 μM NADPH, 3 μM tetrahydrobiopterin and 0.1 mM GSNO in the presence of increasing concentrations of methylene blue. Reactions were stopped by $ZnCO_3$ precipitation, and [^{32}P]cyclic GMP was isolated by column chromatography [38].

Determination of NO *synthase activity.* NO synthase activity was determined as formation of [^{3}H]citrulline from [^{3}H]arginine as described previously [39]. Unless otherwise indicated, purified NO synthase (0.2–0.4 μg) was incubated at 37° in a total volume of 0.1 mL of a triethanolamine–HCl buffer (50 mM, pH 7.0) containing 0.1 mM [^{3}H]-arginine (50,000–70,000 cpm), 0.1 mM NADPH, 10 μM tetrahydrobiopterin, 10 μg/mL calmodulin and 3 μM free Ca^{2+} in the presence of increasing concentrations of methylene blue.

Co-incubation of NO *synthase and soluble guanylyl cyclase.* Purified NO synthase and soluble guanylyl cyclase (0.1 μg each) were co-incubated at 37° for 10 min in a total volume of 0.1 mL of a triethanolamine–HCl buffer (50 mM, pH 7.4) containing 0.1 mM [α-^{32}P]GTP (200,000–300,000 cpm), 0.1 mM L-arginine, 1 mM cGMP, 1 mM 3-isobutyl-1-methylxanthine, 1 mM free Mg^{2+}, 3 μM free Ca^{2+}, 10 μg/mL calmodulin, 30 μM NADPH and 0.3 μM tetrahydrobiopterin in the presence of increasing concentrations of methylene blue. Guanylyl cyclase activities were determined in triplicates as described above.

Cytochrome c *reduction.* Reduced forms of methylene blue directly reduce cytochrome *c* [9]. We used this assay to determine to what extent the dye reacts with the NO synthase cofactors NADPH (0.1 mM), FAD (10 μM) and tetrahydrobiopterin (10 μM). Reduction of cytochrome *c* (0.1 mM) was determined in the presence of 10 U/mL SOD and 10 μM methylene blue by continuously monitoring the increase in absorbance at 550 nm against appropriate blank samples as described previously [35].

Presentation of data. Data points were averaged and concentration–response curves fitted by non-linear least squares regression using the Hill equation. In relaxation studies, the EC_{50} represents the concentration of A 23187 producing half maximal effects irrespective of the absence or presence of additional compounds. In all other experiments, IC_{50} refers to that concentration of methylene blue which reduced the formation of cGMP or L-citrulline by 50%.

RESULTS

Methylene blue (30 μM) antagonized the relaxation of bovine coronary arteries induced by A 23187. In

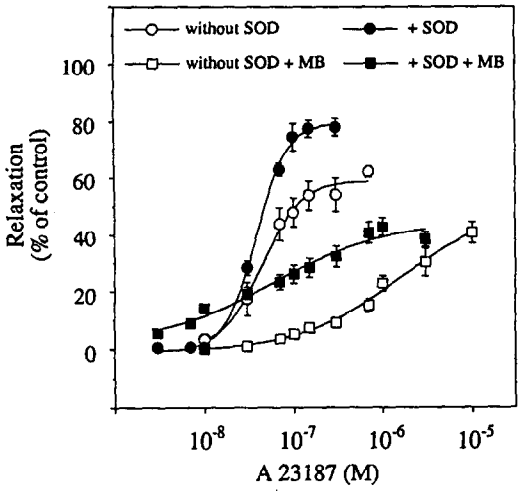

Fig. 1. Inhibition of A 23187-induced relaxation of bovine coronary artery by methylene blue (30 μM) and effect of SOD (30 U/mL). Endothelium-preserved tissues were precontracted, A 23187 added cumulatively (30 nM–10 μM) and the relaxant effect determined as per cent of maximum relaxation (=100%) induced by papaverine (see Materials and Methods). The symbols show mean values ± SEM for the number of tissues given in Materials and Methods.

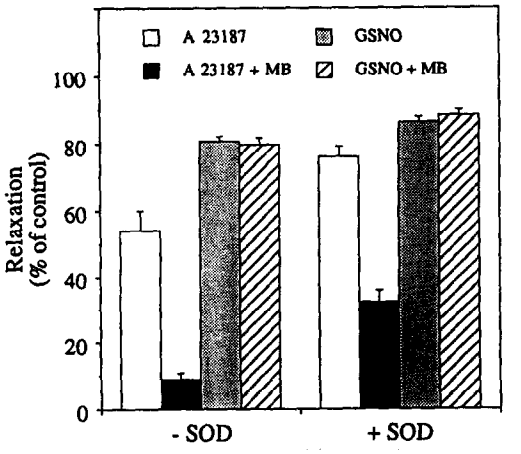

Fig. 2. Effects of methylene blue (MB) and SOD on the relaxation of bovine coronary artery induced by 0.3 μM A 23187 or 0.1 mM GSNO. The A 23187 data are the same as in Fig. 1. The columns show mean values ± SEM (of the number of experiments given in Materials and Methods).

the absence of the dye, the mean EC_{50} for the calcium ionophore was 45 nM, and the maximum relaxation was 59% of that induced by papaverine (open circles in Fig. 1). In the presence of methylene blue (open squares), the EC_{50} was 30-fold higher (1.7 μM) and E_{max} reduced. In the presence of SOD control relaxation was increased (79%, solid circles in Fig. 1) and the relaxation curve in the presence of methylene blue (solid squares) was shifted leftward.

The potency of A 23187 to relax bovine coronary artery strips was compared with that of the nitric oxide donor GSNO (Fig. 2). At 0.3 μM, A 23187-induced relaxation was 54 ± 6% in the absence and 9 ± 2% in the presence of methylene blue. In the presence of 30 U/mL SOD, both control and methylene blue-inhibited relaxation was increased to a similar extent, indicating that the effect of methylene blue was not attenuated by SOD. GSNO (0.1 mM) relaxed the tissues almost as potently as papaverine both in the absence (81 ± 1%) and presence (80 ± 2%) of 30 μM methylene blue, and SOD only slightly increased GSNO-induced relaxation (Fig. 2).

The concentration-dependent effects of methylene blue on the NO/cGMP system were studied by determination of intracellular cGMP levels in cultured porcine aortic endothelial cells which had been stimulated with NO synthase-dependent (A 23187) and -independent (SNP and GSNO) activators of soluble guanylyl cyclase. The ionophore A 23187 (1 μM) raised endothelial cGMP levels from 2.1 ± 0.2 to 30.0 ± 1.4 pmol/10^6 cells, and methylene blue completely blocked this response with half-maximally effective concentrations of 0.4 and 5.8 μM in the absence and presence of SOD, respectively (Fig. 3A). The drug inhibited the actions of direct guanylyl cyclase activators less effectively: in the presence of SOD, cGMP levels raised by SNP (Fig. 3B) and GSNO (Fig. 3C) were reduced to 50% of controls at 1 mM and 13 μM methylene blue, respectively. As further shown in Fig. 3B and C, both concentration–response curves were markedly shifted leftward when SOD had been omitted (open symbols), and, in contrast to A 23187-induced responses, the effects of the direct NO donors were not fully inhibited by methylene blue.

We used NO synthase purified from porcine cerebellum (sp. act. ~400 nmol L-citrulline/mg/min) to investigate the effects of methylene blue on NO synthesis. Enzyme activity was assayed both as conversion of radiolabelled L-arginine into L-citrulline and NO formation by co-incubation of NO synthase with purified guanylyl cyclase. Figure 4 (open symbols) shows that methylene blue completely inhibited L-citrulline formation with an IC_{50} value of 5.3 μM and maximal effects at about 30 μM. In the presence of 10 U/mL SOD, the inhibition curve was only slightly shifted rightward (IC_{50} = 9.2 μM; Fig. 2, solid symbols).

Under the conditions used for the co-incubation with NO synthase, the basal activity of purified soluble guanylyl cyclase was ~0.005 μmol cGMP/mg/min, and addition of 0.1 μg of purified NO synthase increased this activity to 1.8–2.2 μmol cGMP/mg/min. Methylene blue completely blocked the NO synthase-induced stimulation of cGMP formation (IC_{50} value ~70 nM; Fig. 5A, open circles). In the presence of SOD (solid circles), the methylene blue concentration–response curve was markedly shifted rightward (50% inhibition at 2.1 μM).

To ascertain whether guanylyl cyclase inhibition was also involved in the inhibitory actions of methylene blue, we determined the effects of the dye on purified guanylyl cyclase which had been

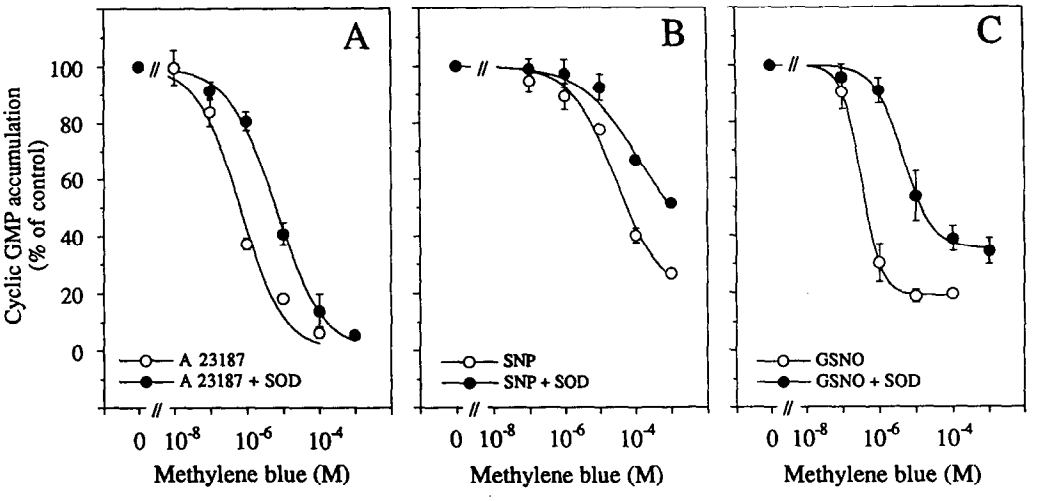

Fig. 3. Effects of methylene blue on endothelial cGMP levels raised by A 23187 (A), SNP (B) and GSNO (C). Endothelial cells were preincubated as described in Materials and Methods with various concentrations of methylene blue in the absence (○) or presence (●) of 10 U/mL SOD. After 15 min, maximally active concentrations of A 23187 (1 μM), SNP (1 mM) or GSNO (100 μM) were added and the reaction was terminated 4 min later. Results are expressed as per cent of control activities obtained in the absence of methylene blue. Mean values ± SEM of four separate experiments are shown.

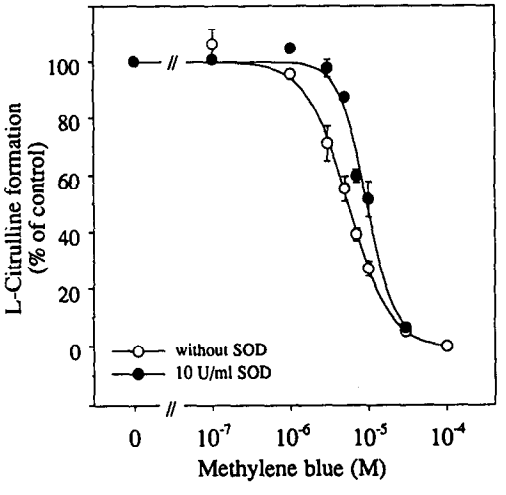

Fig. 4. Inhibition of purified NO synthase by methylene blue in the absence (○) and presence (●) of 10 U/mL SOD. Assay conditions were as described in Methods. Results are expressed as per cent of control activities obtained in the absence of methylene blue. Mean values ± SEM of three separate experiments are shown.

stimulated 200–300-fold by GSNO. NADPH (30 μM) and tetrahydrobiopterin (0.3 μM), which were present in some experiments to ensure conditions comparable with the reconstituted system described above, had no significant effect (not shown). As shown in Fig. 5B, methylene blue inhibited GSNO-stimulated guanylyl cyclase in a concentration-dependent manner (50% inhibition at ~60 μM), and the inhibitory effects of the dye were not significantly affected by SOD. As in the experiments with intact cells, methylene blue failed to block cGMP formation completely. Even in the presence of 1 mM, the highest concentration of the dye used in the present study, GSNO still produced about 80-fold increases in cGMP generation, so that approximately 30% of maximal enzyme activity was not affected by methylene blue.

As it seemed possible that the apparent inhibition of NO synthase was due to a chemical reaction of methylene blue with one of the redox-active compounds required for full enzyme activity, redox-cycling of 10 μM methylene blue was assayed as reduction of cytochrome *c* [9] in the presence of NADPH, FAD and tetrahydrobiopterin. In the absence of these compounds, we observed no reduction of cytochrome *c*, and FAD (10 μM) failed to induce such a reaction. In the presence of NADPH (0.1 mM) and tetrahydrobiopterin (10 μM) we observed moderate methylene blue-dependent reaction rates of 1.0 and 0.40 μM cytochrome *c* reduced/min, respectively. Considering that the concentrations of the NO synthase cofactors used saturate the enzyme at least 10-fold [33], these reaction rates could hardly account for the observed effects of methylene blue. Moreover, we failed to overcome methylene blue-induced enzyme inhibition in the presence of 10-fold increased concentrations of tetrahydrobiopterin, FAD or NADPH (not shown).

IC_{50} values and maximal effects of methylene blue, as calculated from Figs 3–5, are summarized in Table 1. Only NO synthase-mediated processes seemed to be fully inhibited by methylene blue, whereas soluble guanylyl cyclase remained considerably sensitive to stimulating compounds even at methylene blue

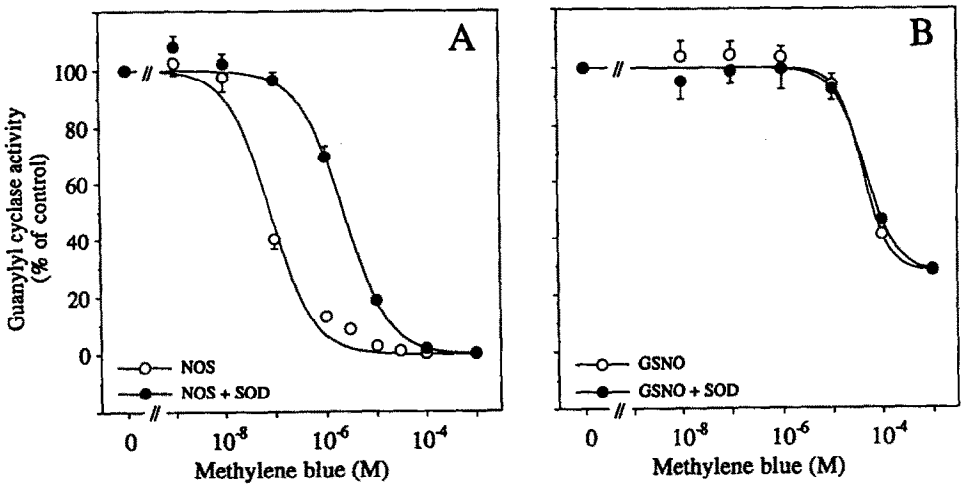

Fig. 5. Effects of methylene blue on purified soluble guanylyl cyclase activities. (A) Purified NO synthase (NOS) and soluble guanylyl cyclase were co-incubated as described in Materials and Methods in the absence (○) and presence (●) of 10 U/mL SOD. (B) Purified soluble guanylyl cyclase was incubated in the presence of 0.1 mM GSNO with (●) and without (○) 10 U/mL SOD. Results are expressed as per cent of control activities obtained in the absence of methylene blue. Mean values ± SEM of three separate experiments are shown.

Table 1. Effects of methylene blue (MB) and SOD on cGMP accumulation in endothelial cells (EC), NO and L-citrulline (L-Cit) formation by purified brain NO synthase (NOS), and on the generation of cGMP by GSNO-stimulated purified soluble guanylyl cyclase (sGC)

		IC_{50} (μM MB)		Inhibition at 1 mM MB (% of control)	
System	Activator or product	−SOD	+SOD	−SOD	+SOD
cGMP in EC	A 23187	0.4	5.8	100	100
	SNP	56	>1000	69	47
	GSNO	0.5	12.8	83	65
NOS activity	L-Cit	5.3	9.2	100	100
	NO	0.07	2.1	100	100
sGC activity	GSNO	60	60	72	72

concentrations as high as 1 mM. Interestingly, endothelial responses to SNP differed markedly from those to GSNO with respect to the inhibitory potency of methylene blue, which blocked the actions of GSNO at ~100-fold lower concentrations than those of SNP.

DISCUSSION

Methylene blue (30 μM) markedly inhibited A 23187-induced endothelium-dependent relaxation of bovine coronary arteries, but small relaxant effects of the ionophore were still observed in the presence of the dye. Since these methylene blue-insensitive relaxations were also largely insensitive to inhibition by N^{ω}-nitro-L-arginine (not shown), a mechanism unrelated to the L-arginine/NO pathway may additionally be involved in A 23187-induced relaxations of bovine coronary arteries. In contrast to endothelium-dependent relaxations, the relaxant responses of the vessels to GSNO were not inhibited by 30 μM methylene blue, indicating that smooth muscle guanylyl cyclase was not affected by the drug. Moreover, it seemed unlikely that methylene blue acted via superoxide anion-mediated inactivation of NO, since SOD failed to prevent the inhibitory effects of the dye on endothelium-dependent relaxation.

Similarly, we observed striking differences in the inhibitory action profiles of methylene blue on endothelial cGMP levels raised by either direct or indirect activators of soluble guanylyl cyclase (see Fig. 3). It is remarkable that the effects of both SNP and GSNO were not completely inhibited by methylene blue. As we obtained similar results using purified guanylyl cyclase (see Fig. 5B), it appears

that NO and/or NO-containing compounds activate the enzyme by two different mechanisms. Accordingly, binding of NO to the heme moiety of guanylyl cyclase may be inhibited by methylene blue, and S-nitrosylation of regulatory sulphydryl groups [40, 41] may account for the methylene blue-insensitive component of NO-induced enzyme stimulation.

In assays with endothelial cells, methylene blue was considerably less potent when SOD was present, i.e. IC_{50} values were increased, whereas the maximal inhibitory effect of the drug was decreased by exogenously added SOD. Given that SOD predominantly acts by increasing the steady state concentrations of extracellular NO, these data clearly suggest that SNP and GSNO, at least partially, elevate endothelial cGMP levels via release of free NO.

Surprisingly, the actions of SNP and GSNO on endothelial cGMP levels were rather differently affected by methylene blue (Table 1), whereas no such discrepancies were observed in experiments with purified guanylyl cyclase (not shown). Thus, in the presence of SOD the effect of methylene blue on SNP-elevated endothelial cGMP levels was similar to the effects of the drug on purified guanylyl cyclase. Accumulation of endothelial cGMP induced by GSNO, however, was much more susceptible to inhibition by methylene blue. Presently, we have no conclusive explanation for these data, but it seems possible that an as yet unknown iron-containing enzyme may catalyse the liberation of NO from *S*-nitrosothiols in endothelial cells. This explanation is compatible with our finding that SOD enhanced the inhibitory effect of methylene blue on GSNO-induced cGMP accumulation in endothelial cells, while leaving GSNO-induced relaxation and guanylyl cyclase stimulation unaffected (see Figs 3C vs 2 and 5B).

The experiments using purified NO synthase further confirmed the results obtained with isolated blood vessels and cultured endothelial cells. When the enzyme was incubated together with purified soluble guanylyl cyclase in the presence of all required substrates, cosubstrates and enzyme activators, this reconstituted system produced cGMP with reaction rates even exceeding those observed by directly stimulating guanylyl cyclase with NO-containing compounds. Methylene blue inhibited this cGMP formation with half-maximal effects observed at 0.07 and 2 μM in the absence and presence of SOD, respectively (see Fig. 5A and Table 1). We have previously used NO chemiluminescence under non-reducing conditions to specifically demonstrate the generation of free NO by isolated NO synthase [33], making it highly likely that the pronounced effects of SOD in the reconstituted system were due to a stabilization of enzymatically formed free NO. The methylene blue concentrations required for a complete inhibition of cGMP generation by this system were about 30 μM in the presence of SOD, i.e. equal to those necessary for full inhibition of citrulline formation (see Fig. 4). Accordingly, NO synthase is completely inactive at a concentration of methylene blue which exhibits no great effects on direct activation of soluble guanylyl cyclase (see Fig. 5B). It was previously reported that the presence of 25 μM methylene blue reduced citrulline formation in homogenates of porcine aortic endothelial cells by 60% [42]. Differences in the enzyme assay conditions, such as a different substrate concentration, may account for the less pronounced effects of the dye described therein.

Methylene blue is known to affect iron-containing enzymes [9–14]. Stoichiometrical amounts of iron are present in both soluble guanylyl cyclase and NO synthase [6, 32]. The heme group of soluble guanylyl cyclase has been suggested as a target site of methylene blue, resulting in a reduced responsiveness of the enzyme to activation by NO [17, 18]. Consistently, heme-independent basal and arachidonic acid-stimulated cGMP formation were found to be insensitive to inhibition by methylene blue [43]. Xanthine oxidase, another iron-containing enzyme, is inhibited by methylene blue most likely due to a competition of the dye with molecular oxygen for xanthine-derived electrons [14]. Methylene blue apparently interacts with the iron–sulphur centre of xanthine oxidase, since its reduction was also catalysed by flavin- and molybdenum-free forms of the enzyme [10, 11]. Accordingly, methylene blue seems to act as a powerful oxidant of ferrous iron bound to various enzymes either in heme or non-heme forms. The present results show for the first time that this compound is a potent inhibitor of NO synthase and indicate a catalytic function of enzyme-bound iron in NO synthesis.

Experimental evidence suggests that enzymic liberation of NO accounts for the potent vasodilatory properties of organic nitrates [44–46]. We are presently investigating the effects of methylene blue on nitrate metabolism in smooth muscle tissues to determine if hemeproteins catalyse NO release from nitrovasodilators in vascular tissues, as has been suggested for the metabolism of glycerol trinitrate in liver microsomes [47, 48].

In conclusion, our data show that methylene blue, besides generation of superoxide anion, predominantly acts via a direct inhibition of NO synthase and other iron-containing enzymes and does not represent either a selective or a potent inhibitor of NO-stimulated soluble guanylyl cyclase as previously assumed.

Acknowledgements—We wish to thank Dr E. Böhme for providing purified guanylyl cyclase. The excellent technical assistance of Eva Leopold, Margit Rehn and Heike Stessel is gratefully acknowledged. This work was supported by grants from the Fonds zur Förderung der wissenschaftlichen Forschung in Österreich (P 8836 and P 8581).

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