

The Soluble Guanylyl Cyclase Inhibitor 1*H*-[1,2,4]Oxadiazolo-[4,3,-*a*]quinoxalin-1-one Is a Nonselective Heme Protein Inhibitor of Nitric Oxide Synthase and Other Cytochrome P-450 Enzymes Involved in Nitric Oxide Donor Bioactivation¹

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ABSTRACT

Soluble guanylyl cyclase (sGC) is an important effector for nitric oxide (NO). It acts by increasing intracellular cyclic GMP (cGMP) levels to mediate numerous biological functions. Recently, 1*H*-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one (ODQ) was identified as a novel and selective inhibitor of this enzyme. Therefore, ODQ may represent an important pharmacological tool for differentiating cGMP-mediated from cGMP-independent effects of NO. In the present study, we examined the inhibitory action of ODQ both functionally and biochemically. In phenylephrine-precontracted, endothelium-intact, isolated aortic rings from the rat, ODQ, in a concentration-dependent manner, increased contractile tone and inhibited relaxations to authentic NO with maximal effects at 3 μ M. Pretreatment of vascular rings with ODQ induced a parallel, 2-log-order shift to the right of the concentration-response curves (CRCs) to histamine, ATP, NO, the NO-donors S-nitrosoglutathione, S-nitroso-*N*-acetyl-D,L-penicillamine, and spermine NONOate [*N*-[4-[1-(3-amino propyl)-2-hydroxy-2-nitroso hydrazino]butyl]-1,3-propane diamine], and the direct sGC-stimulant [3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole] YC-1 but did not affect relaxations induced by papaverine and atropine-

tin II. Moreover, the rightward shift of the CRCs to Angeli's salt, peroxynitrite, and linsidomine was similar to that of NO. These results suggested that ODQ is specific for sGC. Furthermore, they indicate that NO can cause vasorelaxation independent of cGMP. Three interesting exceptions were observed to the otherwise rather uniform inhibitory effect of ODQ: the responses to acetylcholine, glycerol trinitrate, and sodium nitroprusside. The latter two agents are known to require metabolic activation, possibly by cytochrome P-450-type proteins. The 3- to 5-log-order rightward shift of their CRCs suggests that, in addition to sGC, ODQ may interfere with heme proteins involved in the bioactivation of these NO donors and the mechanism of vasorelaxation mediated by acetylcholine. In support of this notion, ODQ inhibited hepatic microsomal NO production from both glycerol trinitrate and sodium nitroprusside as well as NO synthase activity in aortic homogenates. The latter effect seemed to require biotransformation of ODQ. Collectively, these data reveal that ODQ interferes with various heme protein-dependent processes in vascular and hepatic tissue and lacks specificity for sGC.

Nitric oxide (NO) has emerged as a key intercellular and intracellular messenger of a number of cellular functions in physiology and pathophysiology (Moncada et al., 1991; Schmidt and Walter, 1994). The unpaired electron in the

outer electron shell of NO not only confers radical character to this effector molecule but also accounts for its high affinity toward other free radicals, thiols, and transition metals such as heme iron (Stamler, 1994; Beckman and Koppenol, 1996). This latter observation explains why iron- and copper-containing proteins, such as hemoglobin and soluble guanylyl cyclase (sGC; Arnold et al., 1977; Böhme et al., 1978), are among the most important cellular targets of NO in a biological setting. Indeed, the NO/sGC signaling pathway (Schmidt et al., 1993) has been demonstrated to mediate a variety of biological re-

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ABBREVIATIONS: NO, nitric oxide; sGC, soluble guanylyl cyclase; NOS, nitric oxide synthase; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one; cGMP, cyclic GMP; SNP, sodium nitroprusside; YC-1, 3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole; L-NAME, *N*^ω-nitro-L-arginine methyl ester; AP II, atropine II; Sper-NO, spermine NONOate [*N*-[4-[1-(3-amino propyl)-2-hydroxy-2-nitroso hydrazino]butyl]-1,3-propane diamine]; GSNO, S-nitrosoglutathione; SNAP, S-nitroso-*N*-acetyl-D,L-penicillamine; ONOO⁻, peroxynitrite; GTN, glycerol trinitrate; SIN-1, linsidomine; DMSO, dimethyl sulfoxide; ACh, acetylcholine; CHAPSO, 3-[(3-cholamidopropyl)dimethyl-ammonio]-2-hydroxy-1-propanesulfonate; CRC, concentration response curve; NO₂⁻, nitrite.

sponses, including vasodilation, inhibition of platelet aggregation, and neuronal signaling (Moncada et al., 1991; Schmidt and Walter, 1994).

In addition to the well described biological actions of the NO/cyclic GMP(cGMP)-mediated signaling pathway, NO has other direct effects, including interactions with cellular and extracellular proteins (Stamler et al., 1992), nitrosylation of receptors (Lipton et al., 1993), and activation of ion channels (Bolotina et al., 1994; Koh et al., 1995). Importantly, there may be other NO signaling pathways independent of sGC activation that have not been identified and yet may be potentially targeted in the development of novel therapeutic strategies. Thus, to understand NO signaling and for therapeutic reasons, there is a need to discriminate between direct NO-mediated and cGMP-mediated effects. Previous attempts have largely focused on inhibiting the activity of sGC. However, experiments with the prototypical sGC inhibitor, methylene blue, often revealed conflicting results, mainly because of an inability of this compound to discriminate between sGC and NO synthase (NOS; Liu et al., 1993; Mayer et al., 1993). Moreover, other putative sGC inhibitors, such as LY83583, probably decrease the effective concentration of NO by generating superoxide anions rather than lowering sGC activity directly (Gryglewski et al., 1986; Mülsch et al., 1988; Wolin et al., 1990). Conceivably, this would influence both cGMP-dependent and -independent actions of NO. Similarly, pharmacological intervention of endogenous NO synthesis with inhibitors of NOS does not allow any discrimination between primary and secondary effector molecules because both NO and cGMP formation are decreased. Recently, however, ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; Fig. 1) was introduced as a specific inhibitor of sGC (Garthwaite et al., 1995). This compound has since been used widely to probe for the involvement of cGMP in a given pharmacological response (e.g., Brunner et al., 1995; Moro et al., 1996) and to differentiate between cGMP-dependent and -independent effects of NO (e.g., Boulton et al., 1995; Fedele et al., 1996; Franck et al., 1997).

In the present study, we examined a possible interference of ODQ with both endogenous (from NOS) and exogenous (from NO donor compounds) NO formation. In particular, we investigated the specificity of ODQ both biochemically, by measuring the direct effects of ODQ on NOS and cytochrome P-450 activity, and in functional studies using different NO donors and stimulants of endogenous NO production in vascular smooth muscle. Furthermore, its effects on the direct stimulation of sGC by 3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole (YC-1) were investigated. Here we show that al-

though ODQ is a potent inhibitor of sGC, it also affects organic nitrate- and sodium nitroprusside (SNP)-mediated vasorelaxation by inhibiting their bioactivation via one or more different cytochrome P-450 enzyme systems. Moreover, ODQ was found to inhibit endothelium-dependent relaxation, presumably by virtue of metabolic conversion to an NOS inhibitor, and vasorelaxations elicited by YC-1. The implications of the present findings for the experimental analysis of NO-signaling pathways are discussed.

Experimental Procedures

Materials. FAD, FMN, L-arginine hydrochloride, calmodulin, *N*^ω-nitro-L-arginine methyl ester (L-NAME) and atriopeptin II (AP II; rat atrial natriuretic peptide fragment 5–27) were obtained from Sigma Chemical Co. (Deisenhofen, Germany); histamine hydrochloride was obtained from Serva (Heidelberg, Germany). (6*R*)-5,6,7,8-tetrahydro-L-biopterin was obtained from Dr. Schircks Laboratories (Jona, Switzerland); [2,3,4,5-³H]L-arginine hydrochloride was obtained from Amersham (Braunschweig, Germany); NADPH was obtained from AppliChem (Darmstadt, Germany). GSH was obtained from Boehringer-Mannheim (Mannheim, Germany), ODQ was obtained from Biomol (Hamburg, Germany), and spermine NONOate [Sper-NO; *N*-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]-1,3-propane diamine] was obtained from Alexis (Grünberg, Germany). Angeli's salt (sodium trioxodinitrate, Na₂N₂O₃) was synthesised as described previously (Zamora and Feelisch, 1994) and stored dry and protected from light under argon. *S*-nitrosoglutathione (GSNO) and *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) were synthesized as described previously (Field et al., 1978; Hart, 1985). Stock solutions of peroxynitrite (ONOO[−]) were prepared as described previously (Beckman et al., 1994). Dilutions of glyceryl trinitrate (GTN) were prepared directly from a commercially available preparation in 5% glucose (perlinganit; Schwarz Pharma AG, Monheim, Germany). Hoechst Marion Roussel (Frankfurt, Germany) kindly provided linsidomine (SIN-1) and YC-1. All other chemicals and solvents were of the highest analytical grade available from either Merck AG (Darmstadt, Germany) or Sigma. Water was deionized to 18 MΩ (Milli-Q; Millipore, Eschborn, Germany). All test agents were dissolved in degassed, argon-equilibrated water, except the nitrosothiols, which were prepared in citrate/HCl buffer (pH 2.0) and Angeli's salt, which was prepared in argon-gassed 0.1 M NaOH. All stock solutions were kept for a maximum of 1 h on ice in the dark; dilutions were made up immediately before use. Both YC-1 and ODQ were initially prepared as stock solutions (10 mM) in dimethyl sulfoxide (DMSO) and stored in aliquots at −20°C. On the day of the use, these compounds were further diluted in deionized Milli-Q water. The highest concentration of DMSO used in the bioassay experiments was 0.3% (v/v); in all other test systems, it was 3%. Isotonic aqueous solutions of NO were prepared as described previously (Feelisch, 1991) using argon-gassed saline for saturation with NO.

Assessment of Vasodilator Responses. Male Wistar rats weighing 275 to 325 g were anesthetized with diethyl ether and sacrificed. The descending thoracic aorta was cannulated and flushed with 10 ml of saline (0.9% NaCl) to prevent intravascular clot formation. After surgical removal, the vessel was placed in ice-cold, oxygenated Krebs-Henseleit solution, carefully dissected free of adipose and connective tissue, and cut into 4- to 5-mm rings. In some experiments, the endothelium was removed by in situ perfusion of the aorta with 1 ml of saline containing 0.2% desoxycholate directly after the initial flushing with saline. The vascular rings were then mounted on stainless steel hooks and suspended in water-jacketed, 20-ml organ baths containing oxygenated (95% O₂/5% CO₂) Krebs-Henseleit buffer (pH 7.4; 126.8 mM NaCl, 5.9 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄, 30 mM NaHCO₃, and 5 mM D-glucose). In some experiments, the bathing medium was additionally supplemented with the cyclooxygenase inhibitor indo-

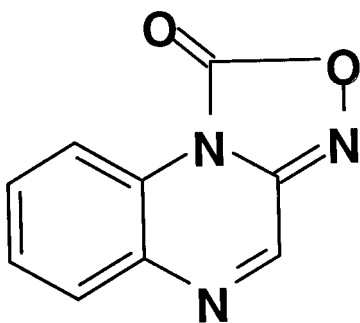


Fig. 1. Structure of ODQ

methacin (1 μ M). The tissues were allowed to equilibrate for 90 min under a resting tension of 2.0 g at 37°C. During this period, the bathing medium was exchanged every 15 min. After final adjustment of the passive resting tension to 2.0 g, vascular segments were contracted submaximally with 0.2 μ M L-phenylephrine. The developed force of contraction using this concentration of vasoconstrictor amounted to 4.19 ± 0.04 g ($n = 64$). The endothelial integrity of each vascular preparation was routinely checked in one representative aortic segment by reaching a >60% relaxation in response to the addition of 1 μ M acetylcholine (ACh). Preparations revealing a much-reduced contraction to phenylephrine or an impaired endothelium-dependent response were excluded from the study. Once a stable contractile tone was reached, either ODQ (at a final concentration of 3 μ M) or the vehicle (0.3% DMSO) was added to the organ bath and present throughout the entire experiment. A cumulative concentration-response curve (CRC) to either NO, an NO donor, or an NO-independent vasodilator was then constructed 30 to 45 min later. Changes in isometric tension were measured by means of force displacement transducers (F30 type 372; Hugo Sachs Elektrouik KG, March, Germany) and documented on a six-channel recorder (Graph-ec Linearorder Mark VII, WR 3310 with Bridge couplers type 570; Hugo Sachs Elektrouik KG). Relaxant responses were expressed as a percentage of the initial contraction achieved with phenylephrine. Each vascular segment was used only for a single test agent. In a few cases, no full CRCs could be obtained either because of limitations in compound solubility (NO, ONOO⁻, GTN, SNP, YC-1) or opposing mode of action (vasoconstriction versus endothelium-dependent relaxation with A23187). The pH of the bathing solution was routinely checked after the addition of the highest concentration of stock solution to ensure that the buffer capacity of the bathing medium was sufficient to prevent pH-dependent vasomotor artifacts. Reported values represent the final bath concentration. In some experiments, the L-arginine-based NOS inhibitor L-NAME (100 μ M), was added to the organ bath instead of ODQ.

Cytochrome P-450 Studies. We investigated a possible interaction of ODQ with cytochrome P-450-related enzyme activity by measuring NO and nitrite (NO₂⁻) formation during the reductive biotransformation of GTN in rat and human liver microsomes under aerobic conditions. Rat hepatic microsomes were prepared from livers of 10 untreated male Wistar rats as described previously (Clement et al., 1993). Human hepatic microsomes were obtained from pooled liver fragments of eight patients undergoing abdominal surgery. All incubations were carried out at 37°C under aerobic conditions. To measure NO₂⁻ formation, microsomes (0.5 nmol of cytochrome P-450/ml) were incubated in the presence of 0.5 mM NADPH and 0.44 mM GTN in a total volume of 110 μ l of phosphate buffer (50 mM; pH 7.4). ODQ (0.75–3 mM) was preincubated with the microsomes for 10 min, and reactions were started by the addition of NADPH. The reactions were stopped 20 min later with 25 U/ml lactate dehydrogenase and 1.2 mM pyruvate to oxidize all remaining NADPH. Proteins were removed by centrifugation after the addition of 165 μ l of acetonitrile, and the NO₂⁻ concentration in the supernatant was then determined by the Griess reaction (Griess, 1864).

To determine NO formation, microsomes (0.8 and 0.6 nmol of cytochrome P-450/ml for rat and human microsomes, respectively) were incubated in the presence of 0.5 mM NADPH, 1 mM GTN or SNP, 10 μ M oxyhemoglobin, 500 U of superoxide dismutase, and 100 U of catalase in a total volume of 500 μ l of phosphate buffer (100 mM; pH 7.4). NO formation was measured using the oxyhemoglobin method in the dual wavelength mode (577 versus 523 nm) with a spectrophotometer (Beckman DU7500; Beckman Instruments GmbH, Munich, Germany) as described previously (Feelisch et al., 1996). Incubations were performed for 20 min and the initial rates of NO formation in the presence of ODQ were compared with those in the absence of the inhibitor. The respective blanks contained the same components except GTN or SNP, which were replaced by phosphate buffer. In separate experiments aimed at discriminating between direct and indirect effects of ODQ, GTN (1 mM) was added

after preincubation of ODQ with microsomes. Conditions were the same as described before, except that a fixed concentration of ODQ (25 μ M) was used and preincubation times were varied between 5 and 40 min (5, 10, 20, 30, or 40 min). In these experiments, NADPH (0.5 mM) was present already at the start of the preincubation period to allow a possible flavin-dependent metabolism of ODQ to take place. Immediately after the addition of GTN, a second amount of NADPH was added to a final concentration of 0.5 mM to ensure sufficient cofactor availability for GTN metabolism.

Determination of NOS Activity in Aortic Homogenates. The descending thoracic aorta was removed from anesthetized and exsanguinated male Wistar rats (0.3–0.4 kg b. wt.) or New Zealand white rabbits (1.5–2.5 kg b. wt.). The aortae were cleaned carefully of fat and connective tissue, weighed, and frozen in liquid nitrogen. The tissues were then homogenized in a shell mortar followed by a second treatment with a Potter-Elvehjem glass homogenizer in 50 mM triethanolamine/HCl buffer (pH 7.5) containing 0.5 mM Na₂EDTA, 7 mM GSH, and the protease inhibitors phenylmethylsulfonyl fluoride (0.2 mM), pepstatin A (1 μ M), and leupeptin (1 μ M). NOS activity was assayed by the conversion of L-arginine to L-citrulline (Bredt and Snyder, 1990; Schmidt et al., 1991). Briefly, crude aortic homogenate (50 μ l) was incubated for 15 min at 37°C in a total volume of 100 μ l at pH 7.2 in the presence of 50 nM calmodulin, 1 mM CaCl₂, 250 μ M 3-[(3-cholamidopropyl)dimethyl-ammonio]-2-hydroxy-1-propanesulfonate (CHAPSO), 5 μ M FAD, 5 μ M FMN, 1 mM NADPH, 7 mM GSH, 10 μ M L-arginine, 3 μ M (6R)-5,6,7,8-tetrahydro-L-biopterin, ODQ (0–300 μ M), or vehicle (DMSO, 3% v/v in rabbit and 1% v/v in rat studies). For activity assays, 5.55 kBq [2,3,4,5-³H]L-arginine was added to the reaction mixture and the L-citrulline formed was subsequently separated by cation-exchange chromatography and measured by liquid scintillation counting. In some experiments, we examined the effects of preincubating aortic homogenates with ODQ on the subsequent NOS activity. In these preincubation experiments, aortic homogenate (50 μ l) was incubated in a total volume of 80 μ l with 1.25 mM NADPH, 8.75 mM GSH, and ODQ (0–375 μ M) or vehicle (DMSO) for 15 min at 37°C. NOS activity was determined subsequently over the next 15 min as described above after the addition of 20 μ l of buffer (pH 7.2) containing calmodulin, CaCl₂, CHAPSO, FAD, FMN, L-arginine, [2,3,4,5-³H]L-arginine and (6R)-5,6,7,8-tetrahydro-L-biopterin to give the same final concentration as described above in the assay mixture.

Calculations and Statistics. Unless stated otherwise, all results described in the text and shown in the figures and tables represent means \pm S.E.M. from n independent experiments performed in duplicate (bioassay with paired rings, NO, and NO₂⁻ measurements) or triplicate (citrulline assay). Statistical analysis was performed by Student's unpaired t test (two-tailed) followed by Bonferroni correction for comparisons of multiple means. A p value of <.05 was taken to indicate statistical significance. For calculation of the concentrations required to relax vascular tissue by 25% (EC₂₅) and 50% (EC₅₀), respectively, of the initial contraction produced by phenylephrine, the data were fitted to the Boltzmann equation using the data analysis and graphics program Origin (version 4.1; Microcal, Inc., Northampton, MA).

Results

NO-Mediated and NO-Independent Vasorelaxation. Endothelium-intact vascular segments of rat thoracic aorta were precontracted submaximally with the α_1 -adrenoceptor agonist phenylephrine (0.2 μ M). After establishment of a stable contraction, the addition to the organ bath of ODQ (0.01–10 μ M) produced a concentration-dependent increase in tone that was maximal at 3 μ M. This increase in contractile tone was comparable in magnitude with that observed with 100 μ M L-NAME and 10 μ M oxyhemoglobin, respec-

tively ($n = 4$), and was absent in endothelium-denuded tissue, which indicates that the effect of ODQ was secondary to inhibition of the sGC-stimulating effect on basally released NO from the endothelium. The effect of ODQ on NO-mediated vascular responses was investigated using authentic NO as a test stimulus. In preliminary experiments, it was found that endothelium-denuded tissue exhibited an ~ 10 -fold higher sensitivity to NO than endothelium-intact tissue. For the sake of comparability of the results with authentic NO and endothelium-dependent vasodilators, all further experiments were carried out in endothelium-intact aortic rings. Single-bolus additions to the organ baths of an aqueous solution of NO at a final concentration of $3 \mu\text{M}$ produced a transient, $>50\%$ relaxation of phenylephrine-precontracted, endothelium-intact rat aortic rings. The vasorelaxation to NO was inhibited by ODQ in a concentration-dependent manner with maximal effects at $3 \mu\text{M}$ (93 ± 3 versus $92 \pm 4\%$ inhibition at 3 and $10 \mu\text{M}$ ODQ, respectively; $n = 6$; not significant). Therefore, this concentration of ODQ was used in all subsequent studies on NO-related and -unrelated vasodilators.

When a full CRC for authentic NO (0.01 – $100 \mu\text{M}$) was constructed, ODQ produced a parallel rightward shift of this curve. EC_{50} values for NO corresponded to $2.6 \mu\text{M}$ in the absence of ODQ and $87 \mu\text{M}$ in the presence of $3 \mu\text{M}$ ODQ (Fig. 2A). In contrast, the same concentration of ODQ did not affect the relaxation responses to either papaverine (0.01 – $300 \mu\text{M}$; Fig. 2B) or AP II (0.01 – 100 nM ; Fig. 2C), which suggests that the inhibitory action of ODQ was specific for the soluble isoform of guanylyl cyclase (i.e., sGC). Unexpectedly, ODQ produced a small but significant rightward shift of the CRC for 8-bromo-cGMP (0.1 – $1000 \mu\text{M}$; see Table 1).

Endothelium-Dependent Vasorelaxation. ODQ ($3 \mu\text{M}$) completely abolished the relaxation responses to the Ca^{2+} ionophore A23187 (0.001 – $10 \mu\text{M}$; Fig. 3). Therefore, the effect of ODQ was investigated on the relaxation responses to three other endothelium-dependent vasodilators (ATP and histamine, 0.01 – $1000 \mu\text{M}$; ACh, 1 nM – 30 mM) and compared with that of NOS-inhibition with an L-arginine-based inhibitor

under the same conditions. In the case of all three vasodilators tested, the extent of the rightward shift of the CRCs by ODQ ($3 \mu\text{M}$) was identical in magnitude to that produced by preincubation of vascular rings with the NOS-inhibitor L-NAME ($100 \mu\text{M}$; Fig. 4, A–C). Interestingly, the extent of the rightward shift differed largely between these vasoactive agents. ACh exceeded the expected shift as observed for authentic NO by almost 3 orders of magnitude (see Table 1; compare Figs. 2A and 4C). Virtually the same results were obtained when experiments were carried out in the presence of $10 \mu\text{M}$ indomethacin ($n = 2$ each; data not shown).

NO Donor-Mediated Vasorelaxation. The degree of inhibition by ODQ of the vasorelaxing responses to a number of structurally different NO donor compounds and ONOO^- was compared with that of authentic NO. The extent of the rightward shift of the respective CRCs for the two *S*-nitrosothiols, SNAP (0.001 – $1000 \mu\text{M}$) and GSNO (0.1 nM – $300 \mu\text{M}$), and Sper-NO (0.001 – $1000 \mu\text{M}$), did not differ considerably from that seen with authentic NO (compare Figs. 2A and 5). In general, there was a 2- to 6-fold greater shift to the right with the NO donors than with NO (Table 1). Interestingly, ODQ was more potent than oxyhemoglobin ($10 \mu\text{M}$) in inhibiting relaxation induced by these agents, and the addition of the latter to the organ bath before the addition of the NO donor did not lead to a further rightward shift of its CRC compared with ODQ alone. About 3-fold higher concentrations of ODQ were required to fully reverse maximal vasorelaxation to a given NO donor compared with inhibiting the effect of the same concentration of NO donor in ODQ-preincubated vascular tissue (data not shown).

Whereas the effect of ODQ on the relaxation responses to authentic ONOO^- (0.01 – $3000 \mu\text{M}$) was comparable with that of NO, the CRC for the NO/O_2^- -cogenerating compound SIN-1 (0.001 – $3000 \mu\text{M}$) exhibited a significantly larger rightward shift (about 10-fold) than those of either NO or ONOO^- (compare Figs. 2A and 6; also see Table 1). The ODQ-induced shift observed with the nitroxyl (HNO/NO^-) donor Angeli's salt (0.001 – $1000 \mu\text{M}$) is consistent with the idea that in vascular tissue, this compound acts as a donor of NO (Fig. 6A,

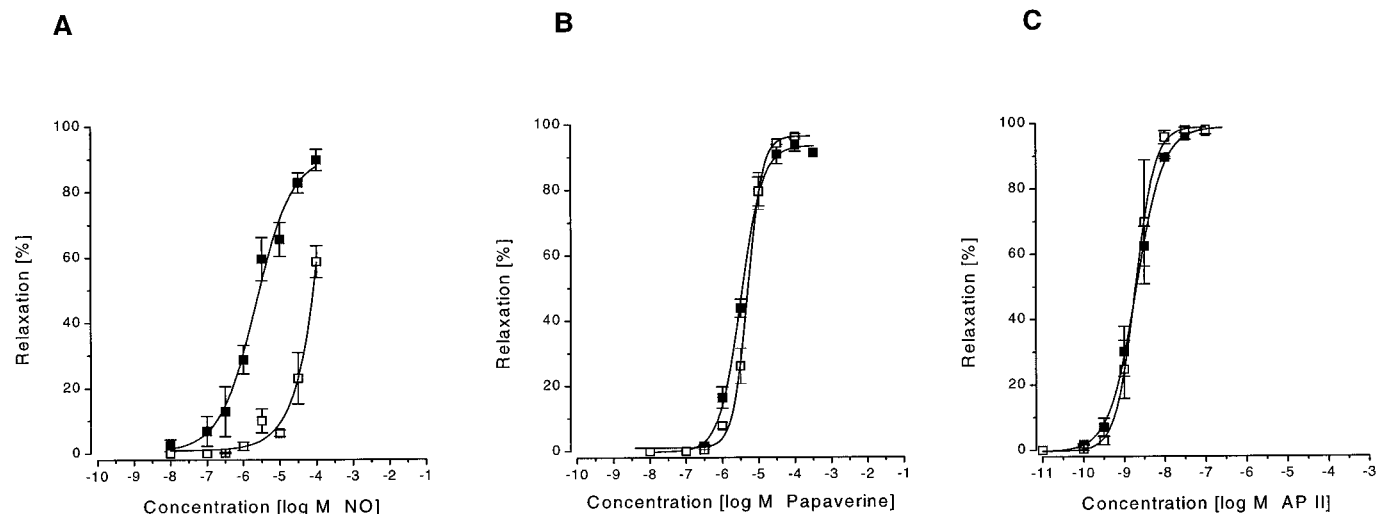


Fig. 2. Effects of ODQ on the relaxation responses elicited by various vasodilators in endothelium-intact aortic rings. Tissues were precontracted with phenylephrine ($0.2 \mu\text{M}$) and then subsequently exposed to increasing concentrations of either authentic NO (A), the cGMP-independent relaxant papaverine (B), or the activator of particulate guanylyl cyclase AP II (C). The corresponding EC_{50} values for vasorelaxation under control conditions were 2.88, 3.80, and $0.002 \mu\text{M}$ for NO, papaverine, and AP II, respectively. Closed symbols indicate CRCs to a vasodilator in the absence of ODQ (control) and open symbols indicate CRCs to a vasodilator in the presence of $3 \mu\text{M}$ ODQ; $n = 3$.

Table 1). Unexpectedly, ODQ inhibited the relaxant effect of GTN (0.1 nM–1 mM) and SNP (0.1 nM–30 mM) to a far greater extent than expected for NO (compare Figs. 2A and 7; see Table 1), which suggests an additional mechanism of inhibition by ODQ. This may involve an interference with enzymatic processes responsible for the metabolic activation of these compounds.

Direct Activation of sGC with YC-1. In phenylephrine-precontracted rat aortic rings, YC-1 (0.01–30 μ M), a recently described direct activator of sGC (Ko et al., 1994), was found to elicit concentration-dependent relaxations with an \sim 10-fold higher potency in endothelium-intact (EC_{50} , 0.3 μ M) compared with endothelium-denuded tissue (EC_{50} , 4.0 μ M). Only a 3- to 4-fold difference in potency of YC-1 was seen in endothelium-intact rings between control and L-NAME (100 μ M)-pretreated tissue (Fig. 8). In contrast, the addition of ODQ (3 μ M) to the organ bath led to a significantly greater rightward shift of the CRC in response to YC-1 (see Fig. 8 and Table 1). In endothelium-denuded aortic rings, the addition to the organ bath of 2 μ M YC-1, a concentration that failed per se to cause any relaxation, led to an increase in tissue responsiveness to NO, as evidenced by a parallel leftward shift of the CRCs for GSNO, Sper-NO, and GTN by factors of 3.3, 5.8, and 10.0, respectively ($n = 3$ each). These results are consistent with a mixed mechanism of action of YC-1: in addition to direct sGC stimulation, YC-1 potentiates exogenously and endogenously formed NO (compare Fig. 2A with Fig. 8). Collectively, these data suggest that ODQ inhibits both the NO-dependent and the direct sGC-stimulating action of YC-1.

Effects of ODQ on Microsomal Biotransformation of GTN and SNP. The effect of ODQ on the biotransformation of GTN and SNP was examined in rat and human hepatic microsomes. GTN metabolism was found to be strictly dependent on the cofactor NADPH and occurred in a protein- and concentration-dependent manner. NO_2^- was the main metabolic product. The rate of NO and NO_2^- formation from 1 mM GTN corresponded to 0.11 ± 0.01 and 0.63 ± 0.02 nmol/min/mg of protein, respectively. Preincubation of rat liver microsomes with ODQ (5–250 μ M) led to a concentration-

dependent inhibition of NO formation (Fig. 9) but did not affect NO_2^- formation at concentrations up to 0.75 mM (data not shown). Significant inhibition of NO_2^- formation from GTN was seen only at considerably higher concentrations (22 and 39% inhibition compared with control at 1.5 and 3 mM ODQ, respectively; $n = 2$) and considered unspecific. Virtually the same results were obtained with human hepatic microsomes ($n = 2$; data not shown). Data from a time-course study in which preincubation times were varied between 5 and 40 min (see *Experimental Procedures* for details) revealed that the degree of inhibition of NO formation from GTN did not increase on prolonged microsomal preincubation of ODQ. This suggests that ODQ itself, rather than a metabolite, accounts for the inhibition of GTN biotransformation. In addition, the microsomal metabolism of SNP was investigated with regard to its susceptibility for inhibition by ODQ. As with GTN, ODQ was found to effectively inhibit NO formation from SNP (1.70 nmol/min/mg of protein at 1 mM SNP in the presence of 25 μ M ODQ versus 2.32 nmol/min/mg of protein in the absence of ODQ; $n = 2$). NO formation rates from SNP measured by the oxyhemoglobin assay have to be interpreted with caution because of the possible interference of SNP metabolites causing opposite spectral changes. These data may thus underestimate the true inhibitory potency of ODQ. Notwithstanding these limitations, a clear inhibition by ODQ was observed with both GTN and SNP.

Effects of ODQ on NOS Activity in Aortic Homogenate. To investigate the possible influence of ODQ on NOS activity, L-citrulline formation from L-arginine was examined in aortic homogenates. L-Citrulline formation in aortic homogenates was completely prevented in the presence of L-NAME (100 μ M), which confirms the specific involvement of NOS in this process (Fig. 10B). The inhibitory effect of L-NAME on NOS activity was not significantly altered by tissue preincubation (for 15 min at 37°C) with the compound (Fig. 10B, filled column) in the absence of various cofactors and substrates (see *Experimental Procedures*). In rabbit aortic homogenate, ODQ was found to significantly inhibit NOS enzyme activity (Fig. 10B) only at the highest concentration used (300 μ M). Interestingly, in rat aortic homogenate, a

TABLE 1

Effects of ODQ (3 μ M) on the potency of authentic NO, endothelium-dependent and -independent vasodilators, and nitrogen oxide-donating compounds in phenylephrine-precontracted rat aortic rings in organ baths (for details and n numbers, see *Experimental Procedures* and *Results* sections as well as figure legends).

Class	Compound	EC ₂₅		Shift in EC ₂₅
		Control	ODQ	
		μ M		fold
Endothelium-Independent Dilators	NO	0.72	35.5	49
	AP II	0.001	0.001	—
	Papaverine	1.74	3.16	1.8
	8-bromo-cGMP	3.05	42.5	14
Endothelium-Dependent Dilators	A23187	0.51	>100	n.d.
	ATP	3.02	132	44
	Histamine	2.75	2630	956
	ACh	0.13	5888	45292
	SNAP	0.033	7.76	235
NO Donors	GSNO	0.061	6.03	99
	Sper-NO	0.29	75.8	261
	SIN-1	0.12	61.7	514
	ONOO ⁻	38.0	3548	93
	Angeli's salt	0.22	14.1	64
	GTN	0.023	151	6580
	SNP	0.005	603	120520
	YC-1	0.17	7.59	45
Direct sGC Stimulators				

lower concentration of ODQ (100 μM) produced a comparable degree of enzyme inhibition (compare Figs. 10A and 10B), revealing possible species differences in enzyme sensitivity to ODQ.

The inhibitory effect of ODQ on NOS activity was potentiated in studies where tissue homogenates were preincubated with ODQ and NADPH for 15 min at 37°C (see *Experimental Procedures*) before the assay of NOS activity (Fig. 10, A and B, filled columns). Under these conditions, a lower concen-

tration of ODQ (30 μM) was now effective at inhibiting NOS enzyme activity from both rat and rabbit tissue (Fig. 10, A and B, filled versus open columns), indicating that ODQ may have undergone metabolic activation. In the case of the rat homogenate, preincubation with a lower concentration of 10 μM ODQ also showed a tendency for inhibition, although this failed to reach statistical significance (Fig. 10B, open and hatched columns). Because of the instability of NOS in vascular homogenates, preincubation times longer than 15 min led to a significant decrease of basal enzyme activity, precluding reliable testing at extended incubation periods.

Discussion

Despite the wide spectrum of physiological and pathophysiological actions of NO, the functional relevance of its interaction with the key target enzyme, sGC, is poorly understood. This is largely because previously used inhibitors of sGC suffered from a lack of specificity. Recently, ODQ has been introduced as a potent and, importantly, selective heme-site inhibitor of sGC (Garthwaite et al., 1995; Schrammel et al., 1996) and is used increasingly as a pharmacological tool for discrimination between cGMP-dependent and -independent actions of NO. However, no study has yet addressed the interaction of ODQ with heme-containing enzymes other than sGC. Moreover, no comparative study of its effects on endogenous and exogenous NO production has been reported. Therefore, we examined, both functionally and biochemically, the actions of ODQ in vascular tissue and hepatic microsomes.

In the present study, ODQ was found to inhibit the vasorelaxing effects of NO, NO donors, endothelium-dependent vasodilators, and the direct sGC-activator YC-1, with no cross-reactivity to either stimulation of particulate guanylyl cyclase or inhibition of phosphodiesterase activity (as evidenced by the lack of effect on papaverine and atriopeptin II-mediated relaxations, respectively). Thus, these results confirm and extend previous reports on the apparent speci-

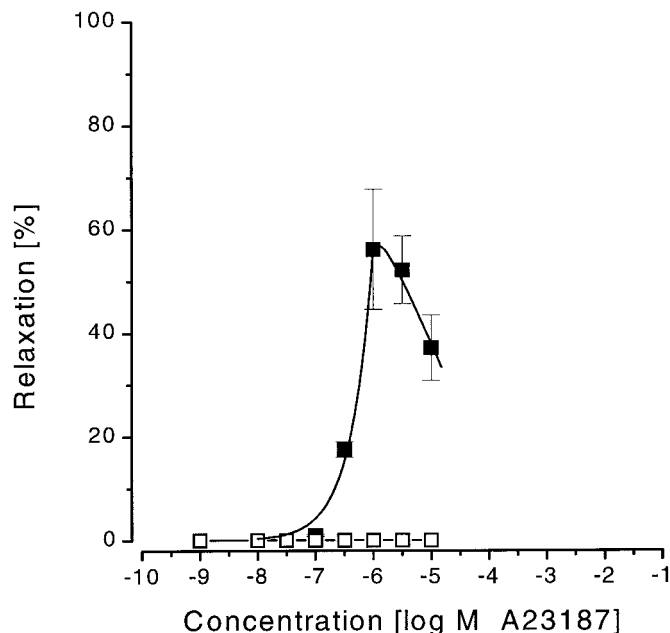


Fig. 3. Effects of ODQ on the relaxation responses elicited by the Ca^{2+} -ionophore A23187 in endothelium-intact aortic rings. Tissues were precontracted with phenylephrine (0.2 μM) and then subsequently exposed to increasing concentrations of A23187 in the absence (■) or presence of 3 μM ODQ (□). The EC_{50} for A23187 under control conditions was 0.93 μM . Vasorelaxing responses to the Ca^{2+} -ionophore were completely abolished by ODQ; $n = 3$.

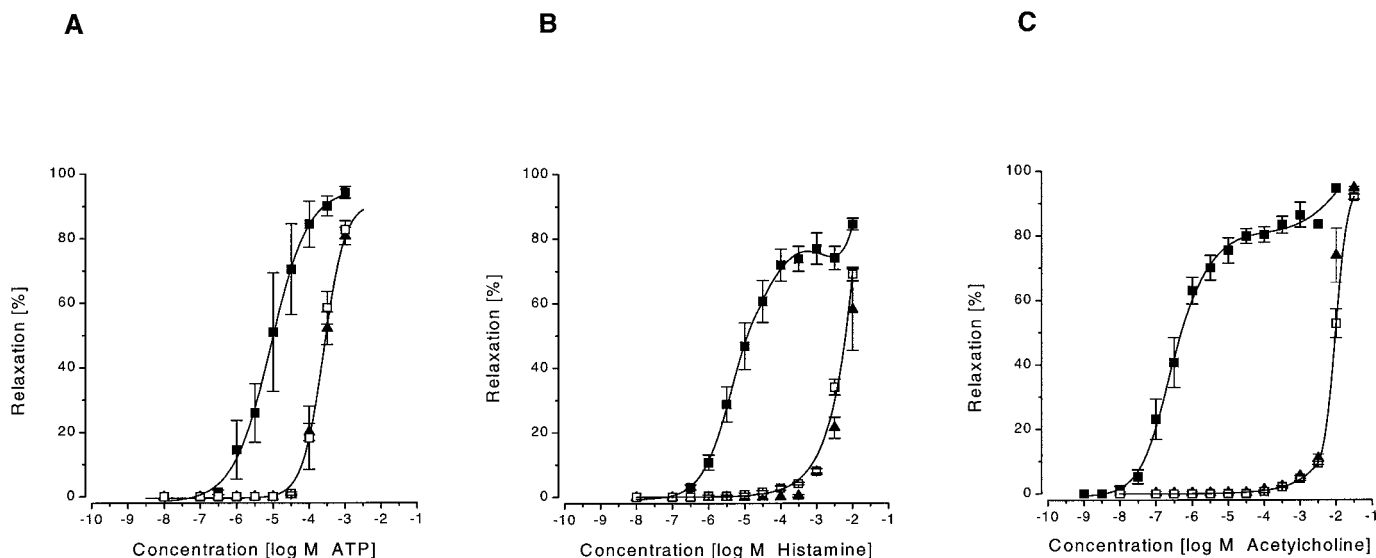


Fig. 4. Effects of ODQ and L-NAME on the relaxation responses elicited by ATP, histamine, and ACh in endothelium-intact rat aortic rings. Tissues were precontracted with phenylephrine (0.2 μM) and then subsequently exposed to increasing concentrations of either ATP (A), histamine (B), or ACh (C). The EC_{50} values for vasorelaxation under control conditions corresponded to 10.2, 12.0, and 0.54 μM for ATP, histamine, and ACh, respectively. Symbols indicate CRCs to the indicated vasodilator in the absence of ODQ (■, control), in the presence of 3 μM ODQ (□), and in the presence of 100 μM L-NAME (▲); $n = 3$ (L-NAME) and 4 (control, ODQ), respectively.

ficacy of ODQ for sGC. However, the small inhibition of vasorelaxation to the membrane-permeable cGMP-analog 8-bromo-cGMP suggested that ODQ might have an additional component of action distal to its effect on sGC. Alternatively, this interference with the cGMP analog may reflect a change in sensitivity of the signaling cascade (i.e., cGMP-dependent protein kinases and phosphodiesterases) involved in cGMP-mediated vasorelaxation.

Most functional studies have examined the effect of ODQ in vascular tissue using endothelium-denuded preparations, precluding investigations on the possible effects of endogenously produced NO and endothelium-dependent vasodilators. In the present study, in endothelium-intact aortic rings, we demonstrate that ODQ inhibits both basal and stimulated endothelial NO production. The degree of inhibition observed

with 3 μM ODQ was virtually identical with that observed with maximally effective concentrations of the NO-scavenger oxyhemoglobin or the NOS-inhibitor L-NAME. Interestingly, we found marked differences in the degree of the ODQ-induced rightward shift of the CRCs of four endothelium-dependent vasodilators. Whereas the vasorelaxing action of the receptor-independent agonist A23187, the mechanism of which involves activation of endothelial NOS secondary to an increase in intracellular Ca^{2+} , was completely abolished in the presence of ODQ, that of ATP, histamine, and ACh was inhibited to increasing degrees. The most prominent effect was seen with ACh, exceeding the shift observed for NO by almost 3 orders of magnitude. This suggests that either the coupling efficiency between NO and cGMP formation may vary among different endothelium-dependent agonists or

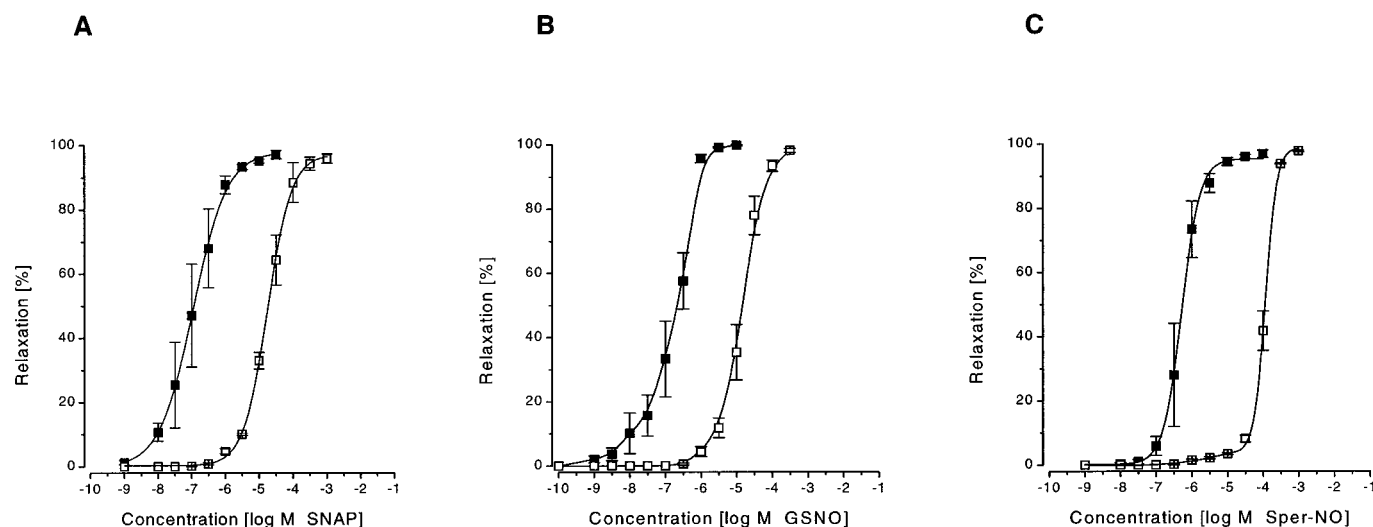


Fig. 5. Effects of ODQ on the relaxation responses elicited by SNAP, GSNO, and Sper-NO in endothelium-intact aortic rings. Tissues were precontracted with phenylephrine (0.2 μM) and then subsequently exposed to increasing concentrations of either SNAP (A), GSNO (B), or Sper-NO (C). The EC_{50} values for vasorelaxation under control conditions corresponded to 0.12, 0.22, and 0.56 μM for SNAP, GSNO, and Sper-NO, respectively. Closed symbols indicate CRCs to a vasodilator in the absence of ODQ (control) and open symbols indicate CRCs to a vasodilator in the presence of 3 μM ODQ; $n = 3$ to 5.

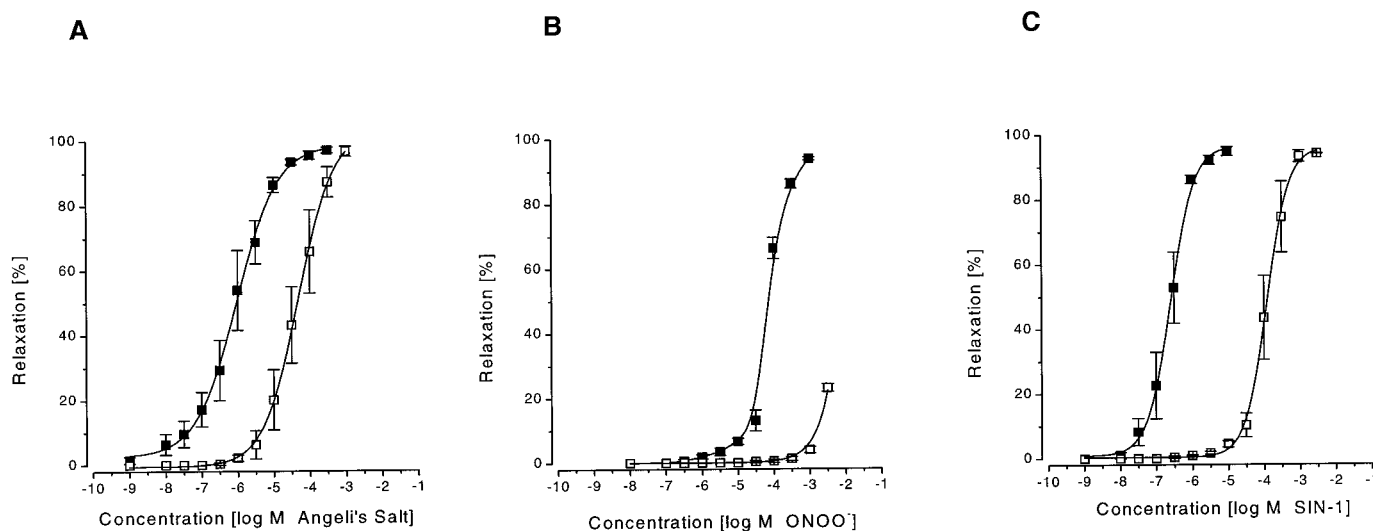


Fig. 6. Effects of ODQ on the relaxation responses elicited by Angeli's salt, authentic ONOO^- , and the ONOO^- -generating compound SIN-1 in endothelium-intact aortic rings. Tissues were precontracted with phenylephrine (0.2 μM) and then subsequently exposed to increasing concentrations of either Angeli's salt (A), ONOO^- (B), or SIN-1 (C). The EC_{50} values for vasorelaxation under control conditions corresponded to 0.93, 74.1, and 0.28 μM for Angeli's salt, ONOO^- , and SIN-1, respectively. Closed symbols indicate CRCs to a vasodilator in the absence of ODQ (control) and open symbols indicate CRCs to a vasodilator in the presence of 3 μM ODQ; $n = 3$ to 5.

that the chemical composition of endothelium-derived relaxing factors may differ, depending on the nature of the stimulus used to trigger their release. The latter issue was addressed by comparing the effects of ODQ with those of NOS inhibition under identical conditions. With all three receptor-dependent agonists, the observed rightward shift of the CRC in the presence of ODQ was identical in shape and magnitude with that in the presence of the substrate-based NOS inhibitor L-NAME. These results rule out the possibility that major differences exist between the vasoactive entity released in response to endothelial stimulation via adenosine, histamine, or muscarinic receptor occupation and is compatible with the view that the NO/cGMP pathway largely accounts for the vasorelaxation by these agonists. The reason for the markedly more pronounced rightward shift of the CRC for ACh secondary to inhibition of either sGC or NOS remains unclear. Functional muscarinic antagonism could be ruled out as possible explanation for this effect of ODQ (H. Kilbinger, personal communication), although an interference with muscarinic receptor coupling and intracellular signal transduction cannot be excluded.

Although there is sufficient evidence in the literature to show that, in the concentration range used to block sGC activity, ODQ does not directly inhibit NOS (Garthwaite et al., 1995; Moro et al., 1996; Olson et al., 1997), little is known about its metabolic fate in the cell and how this may affect its pharmacological properties. Using rat and rabbit aortic homogenates, we have addressed this issue and found that ODQ alone inhibited NOS only at relatively high concentrations, confirming previous observations. However, we also found that preincubation with ODQ markedly potentiated its NOS inhibitory effect, which suggests that the parent com-

pound may be metabolically converted to a more potent NOS inhibitor. The apparent difference in the effective concentration of ODQ to inhibit endothelium-dependent relaxation ($0.3\text{--}3\text{ }\mu\text{M}$) and NOS activity ($30\text{--}300\text{ }\mu\text{M}$), respectively, may be explained by the difference in preincubation times applied in the bioassay (30–45 min) and in the biochemical studies (15 min), respectively. Such time-dependence would be expected if a metabolite of ODQ rather than the parent compound was responsible for the inhibition, as metabolite formation would be time-dependent.

As observed for NO, ODQ induced a rightward shift of the CRCs for all tested NO donors. The nature and extent of inhibition of the vasorelaxation to two *S*-nitrosothiols and Sper-NO was similar to that observed with authentic NO, which suggests that these agents largely exert their action by releasing NO. The CRC for Angeli's salt was shifted to the right to a comparable degree, indicating that in vascular tissue, nitroxyl anion (NO^-), the primary N-oxide released from this compound, is effectively converted to NO. Finally, the finding that the extent of the observed rightward shift of the CRC to SIN-1 was considerably greater than that for either NO or ONOO^- supports the notion that the vasorelaxing effect of SIN-1 is not mediated via ONOO^- (Feelisch, 1998). Rather, it may involve other NO-mediated and possibly cGMP-independent, ODQ-sensitive effects, such as those on potassium channels (Plane et al., 1996).

The present findings also reveal interesting new insights into the bioactivation mechanism of the two nitrovasodilators GTN and SNP. Whereas it is generally thought that their tissue bioactivation is mediated by different enzyme systems, the vasorelaxing effects of both of these compounds were markedly inhibited by ODQ. The observed rightward shift of

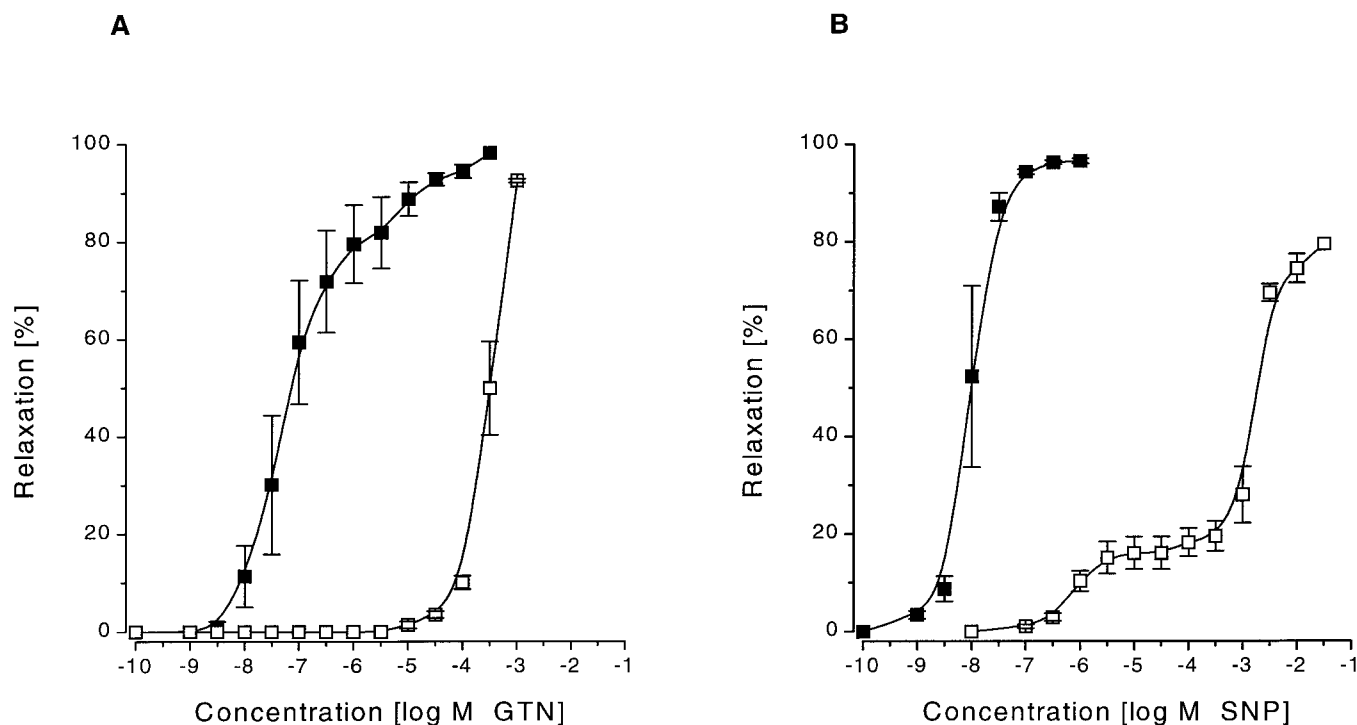


Fig. 7. Effects of ODQ on the relaxation responses elicited by the organic nitrate ester GTN and the inorganic NO-bearing complex SNP in endothelium-intact aortic rings. Tissues were precontracted with phenylephrine ($0.2\text{ }\mu\text{M}$) and then subsequently exposed to increasing concentrations of either GTN (A) or SNP (B). The EC_{50} values for vasorelaxation under control conditions were 0.074 and $0.009\text{ }\mu\text{M}$ for GTN and SNP, respectively. Closed symbols indicate CRCs to a vasodilator in the absence of ODQ (control) and open symbols indicate CRCs to a vasodilator in the presence of $3\text{ }\mu\text{M}$ ODQ; $n = 4$ (GTN) and 5 (SNP), respectively.

their CRCs in the presence of ODQ exceeded the expected shift for NO by 2 to 3 orders of magnitude. This suggests that, at least in rat aorta, NO formation from and subsequent vasorelaxation by these agents is brought about by a heme-dependent enzyme system. In agreement with this notion, ODQ was found to inhibit the microsomal biotransformation of GTN to NO in a concentration-dependent manner. Interestingly, under the same conditions, the formation of NO_2^- was not affected, which suggests that ODQ selectively inhibited the reductive biotransformation of GTN to NO. It was recently proposed that the bioactivation of GTN in vascular tissue is catalyzed by a cytochrome P-450-related enzyme system (McDonald and Bennett, 1993; Li and Rand, 1996). Unlike other cytochrome P-450 inhibitors, which uniformly affect GTN metabolism to NO and NO_2^- , ODQ selectively inhibits NO formation from organic nitrates (i.e., that process that is responsible for mediation of their vasorelaxing effect). Similarly, metabolic NO formation from SNP was inhibited by ODQ, giving support to the notion that a heme-dependent bioactivation step is involved in the tissue metabolism of these prodrugs. In contrast to NOS inhibition (see above), the extent of inhibition by ODQ did not increase with increasing preincubation time, which suggests that ODQ itself rather than a metabolite accounts for the inhibition of GTN biotransformation.

With only a few exceptions, full CRCs were recorded with all NO donors tested. In contrast to previous studies on ODQ and related compounds (Schrammel et al., 1996; Olson et al., 1997), in the present study, no evidence for a mixed competitive/noncompetitive type of inhibition of sGC by ODQ was obtained. In all but one case, ODQ caused parallel rightward shifts with no changes in either shape or slope of the respective CRCs. The attenuation of the maximal vasorelaxing

responses to NO, GTN, and SNP by ODQ (Brunner et al., 1996; Hussain et al., 1997; van der Zyp and Majewski, 1998) and the ODQ analog NS 2028 (Olesen et al., 1998) may be related to the inability of these investigators to construct full CRCs to these vasodilators. In the present study, no attempt was made to determine NO-induced tissue cGMP levels in the presence and absence of ODQ. However, it has been shown by other investigators that, despite effective prevention of any increase in cGMP by $>1 \mu\text{M}$ ODQ, higher concentrations of NO donors or authentic NO can still cause complete relaxation of vascular tissue (Onoue and Katusic, 1998; Weisbrod et al., 1998). This explains why, in the present study, ODQ was unable to fully abolish NO-mediated vasorelaxation but rather resulted in a rightward shift of the respective CRCs. It also indicates that mechanisms independent of cGMP production, such as direct activation of K_{Ca} channels (Bolotina et al., 1994) or Na^+/K^+ -ATPase activity (Gupta et al., 1994), may contribute to the smooth-muscle-relaxing effect of higher concentrations of NO and NO donors. A conjectural but intriguing possibility is that cGMP per se does not directly mediate vasorelaxation; rather, it may increase the sensitivity of some other vasodilatory mechanism to NO. However, before any conclusions can be drawn as to the relative contribution of either of these pathways for NO-mediated vasodilatation under physiological and therapeutic conditions, more information on the kinetics of cGMP production in tissues and the absolute amounts required to trigger relaxation is required. Interestingly, with SNP, the sigmoidal shape of the CRC under control conditions was transformed into a biphasic one when ODQ was present. This finding most likely reflects the two mechanisms of vasorelaxation exerted by SNP: At low concentrations (1–30 nM), NO release from SNP is probably largely nonenzymatic, possibly induced by ambient light or by interaction with tissue membrane thiols. This explains why the extent of the rightward shift of this part of the CRC (about 2 log orders) parallels that of other directly releasing NO donors and authentic NO, respectively. At higher concentrations, SNP seems to require

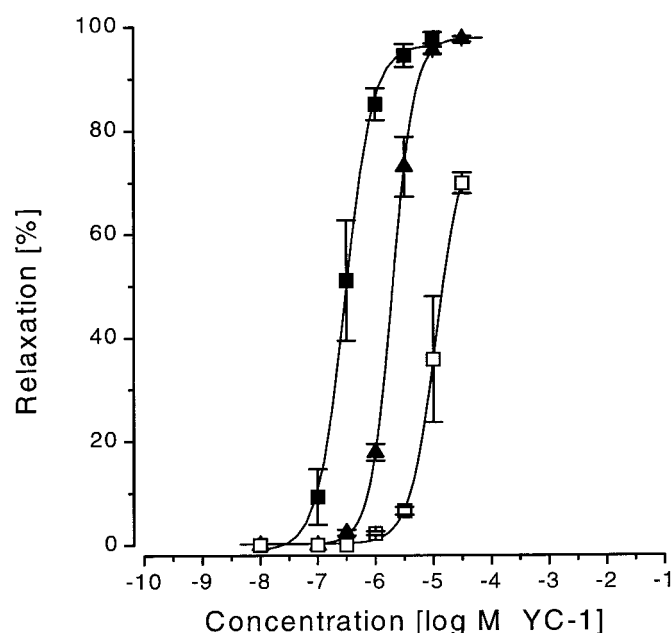


Fig. 8. Effects of ODQ and L-NAME on the relaxation responses elicited by the direct sGC activator YC-1 in endothelium-intact aortic rings. Tissue were precontracted with phenylephrine (0.2 μM) and then subsequently exposed to increasing concentrations of YC-1. The EC_{50} value for vasorelaxation to YC-1 under control conditions was 0.32 μM . Symbols indicate CRCs to YC-1 in the absence of ODQ (■, control), in the presence of 3 μM ODQ (□), and in the presence of 100 μM L-NAME (▲); $n = 3$.

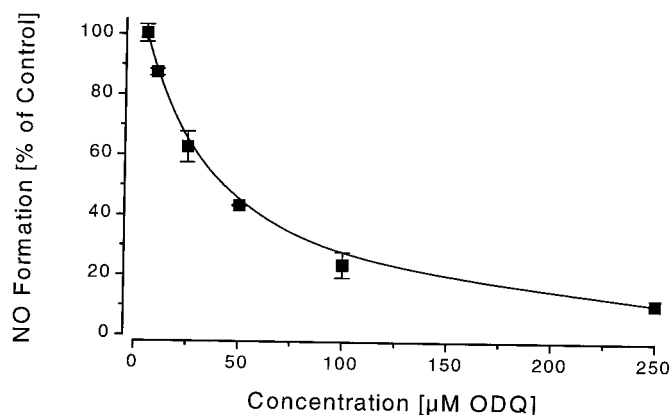


Fig. 9. Effects of ODQ on the formation of NO from GTN in rat liver microsomes. Hepatic microsomes were incubated in the absence or presence of ODQ (0–250 μM) and the rate of NO formation during biotransformation of GTN was determined by the oxyhemoglobin technique as described in *Experimental Procedures*. Results are expressed as a percentage of the initial rate of NO formation under control conditions, which corresponded to 107 ± 4 pmol of NO/min/mg of protein. The depicted results are means \pm S.E.M. from one experiment and are representative of data obtained in two additional experiments in rat and human microsomes.

intracellular bioactivation, and this process is susceptible to inhibition by ODQ.

In addition to the inhibition of endothelium-dependent and NO-mediated vasorelaxation, ODQ also induced a rightward shift of the CRC to the direct sGC stimulator YC-1. This observation is in agreement with the reported reversal of relaxation to and inhibition of the cGMP-stimulation by YC-1 in rat aortic tissue (Wegener et al., 1997) as well as with the marked attenuation of the antiaggregating effect of YC-1 by the nonspecific sGC-inhibitor methylene blue (Wu et al., 1995). These results imply that the vasorelaxing action of YC-1 is largely mediated by activation of the sGC/cGMP pathway. The difference in the extent of rightward shift of the CRC to YC-1 between L-NAME and ODQ pretreatment, respectively, reveals that YC-1 exerts a synergistic action on vascular tissue. It not only directly activates sGC in the smooth muscle but also potentiates the actions of endogenous NO released from the endothelium. Our data are in agreement with the finding that the ODQ-analog NS 2028 abolishes the activation by both NO (generated from SNP) and YC-1 (Mülsch et al., 1997) and suggest that ODQ inhibits both NO-mediated and direct sGC-stimulating effects. In endothelium-denuded tissue, the presence of YC-1, at concentrations that per se did not elicit a vasorelaxing effect, led

to an increased sensitivity to NO. This finding is in agreement with data obtained by Mülsch et al. (1997). However, in contrast to these authors, who reported a comparable (10-fold) shift to the left with the two NO donors GTN and SNP, we find that the extent of the leftward shift differs by a factor of 3 among GSNO, Sper-NO, and GTN. The reason for this discrepancy is unclear at present.

In conclusion, our results, obtained with an array of different endothelium-dependent and -independent vasorelaxing compounds and structurally distinct NO donors revealed that ODQ lacks specificity for sGC and interferes with other heme-dependent processes. In particular, we demonstrate that, besides its action on sGC, ODQ affects organic nitrate and SNP-mediated vasorelaxation by inhibiting their reductive bioactivation via the cytochrome P-450 enzyme system. Moreover, ODQ was found to inhibit endothelium-dependent relaxation by virtue of its metabolic conversion to a NOS inhibitor. Taken together, these results show that ODQ is of limited value as a pharmacological tool to discriminate between biological effects of NO mediated by cGMP and those unrelated to cGMP. The partial or full inhibition of a biological response by ODQ may easily be misinterpreted as evidence for the involvement of cGMP in a pathway mediating that particular response. However, should ODQ have no effect at all in a given biological system, this may be taken as an indication that neither cGMP nor an ODQ-sensitive cytochrome P-450 pathway is involved. Particular care should be taken not to misinterpret experimental results obtained with ODQ when working with endothelium-dependent vasodilators such as ACh or with NO donors requiring metabolic activation.

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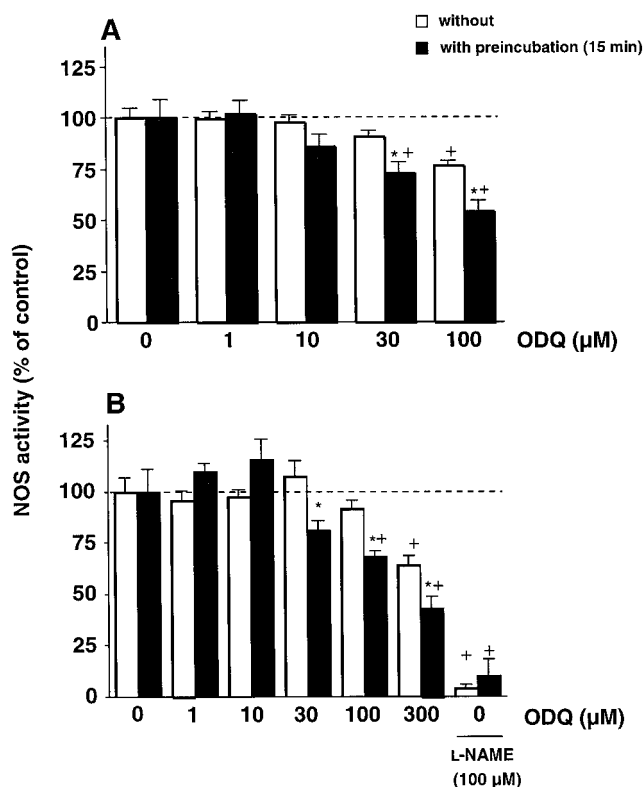


Fig. 10. Effects of ODQ on NOS activity from rat (A) and rabbit (B) aortic homogenate. NOS activity was determined as the formation of L-citrulline from L-arginine over 15 min at 37°C (see *Experimental Procedures*) in the absence or presence of ODQ (0–300 μM). In some experiments (filled columns), the effect of ODQ preincubation (at 37°C for 15 min; see *Experimental Procedures*) on NOS activity from aortic homogenate was also examined. Results are expressed as a percentage of control activity (=100%) from four to nine independent experiments, each performed in triplicate. *, statistical difference from control ($P < .05$; unpaired Student's t test with Bonferroni correction for multiple comparisons of means). +, statistical effect of preincubation from the corresponding experiment without preincubation ($P < .05$; unpaired Student's t test).

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