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# YC-1 Stimulates the Expression of Gaseous Monoxide-Generating Enzymes in Vascular Smooth Muscle Cells

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## ABSTRACT

The benzylindazole derivative 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1) is an allosteric stimulator of soluble guanylate cyclase (sGC) that sensitizes the enzyme to the gaseous ligands carbon monoxide (CO) and nitric oxide (NO). In this study, we examined whether YC-1 also promotes the production of these gaseous monoxides by stimulating the expression of the inducible isoforms of heme oxygenase (HO-1) and NO synthase (iNOS) in vascular smooth muscle cells (SMCs). YC-1 increased HO-1 mRNA, protein, and promoter activity and potentiated cytokine-mediated expression of iNOS protein and NO synthesis by SMCs. The induction of HO-1 by YC-1 was unchanged by the sGC inhibitor, 1H-(1,2,4)oxadiazolo[4,3-$\alpha$]quinozalin-1-one (ODQ) or by the protein kinase G inhibitors (8*R*,9*S*,11*S*)-(−)-2-methyl-9-methoxyl-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11*a*-triazadibenzo(*a*,*g*)cyclocta9(*cde*)trinen-1-one (KT 5823) and YGRKKRRQRRRPPLRKKKKKH-amide (DT-2) and was not duplicated by 8-bromo-cGMP or the NO-independent sGC stimulator 5-cyclopropyl-2[1-(2-fluorobenzyl)-1H-pyrazolo [3,4-b] pyridine-3-yl] pyrimidin-4-ylamine (BAY 41-2272). However, the YC-1-mediated induction of HO-1 was inhibited by the phosphatidylinositol-3-kinase (PI3K) inhibitors wortmannin and 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride (LY294002). In contrast, the enhancement of cytokine-stimulated iNOS expression and NO production by YC-1 was prevented by ODQ and the protein kinase A inhibitor (9*S*,10*S*,12*R*)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo(1,2,3-*fg*:3′,2′,1′-kl)pyrrolo(3,4-*i*)(1,6)-benzodiazocine-10-carboxylic acid hexyl ester (KT 5720) and was mimicked by 8-bromo-cGMP and BAY 41-2272. In conclusion, these studies demonstrate that YC-1 stimulates the expression of HO-1 and iNOS in vascular SMCs via the PI3K and sGC-cGMP-protein kinase A pathway, respectively. The ability of YC-1 to sensitize sGC to gaseous monoxides and simultaneously stimulate their production through the induction of HO-1 and iNOS provides a potent mechanism by which the cGMP-dependent and -independent biological actions of this agent are amplified.

The benzylindazole derivative 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1) is a nitric oxide (NO)-independent allosteric stimulator of soluble guanylate cyclase (sGC) that elevates intracellular cGMP levels in diverse tissues, including platelets, blood vessels, and vascular endothelial and smooth muscle cells (SMCs) (Ko et al., 1994; Friebe et al., 1996; Mulsch et al., 1997; Friebe and Koesling, 1998; Tulis et al., 2000; Schmidt et al., 2001; Tulis et al., 2002). YC-1 activates sGC by stabilizing the active configuration of the

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**ABBREVIATIONS:** YC-1, 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole; sGC, soluble guanylate cyclase; HO-1, heme oxygenase; iNOS, inducible nitric-oxide synthase; SMC, smooth muscle cell; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; BAY 41-2272, 5-cyclopropyl-2[1-(2-fluorobenzyl)-1H-pyrazolo [3,4-b] pyridine-3-yl] pyrimidin-4-ylamine; ODQ, 1*H*-(1,2,4)oxadiazolo[4,3-$\alpha$]quinozalin-1-one; PKG, protein kinase G; PKA, protein kinase A; 8-Br-cGMP, 8-bromo-guanosine 3′,5′-cyclic monophosphate; KT 5823, (8*R*,9S,11*S*)-(−)-2-methyl-9-methoxyl-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7*b*,11*a*-triazadibenzo(*a*,*g*)cyclocta9(*cde*)trinen-1-one; KT 5720, (9*S*,10*S*,12*R*)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo(1,2,3-*fg*:3′,2′,1′-kl)pyrrolo(3,4-*i*)(1,6)-benzodiazocine-10-carboxylic acid hexyl ester; DT-2 peptide, YGRKKRRQRRRPPLRKKKKKH-amide; PI3K, phosphatidylinositol-3-kinase; MAPK, mitogen-activated protein kinase; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; U0125, 1,4-diamino-2,3-dicyano-1,4-*bis*(phenylthio)butadiene; PD98059, 2′-amino-3′-methoxyflavone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; SP600125, anthra(1,9-*cd*)pyrazol-6(2H)-one; ERK1/2, extracellular signal-regulated kinases 1/2; JNK, c-Jun $NH_2$-terminal kinase; PKG, protein kinase G; PKA, protein kinase A.

enzyme and increasing the affinity of sGC for substrate GTP (Friebe et al., 1996; Friebe and Koesling, 1998; Lee et al., 2000). Activation of sGC by YC-1 is crucially dependent on the presence of the reduced prosthetic heme moiety of the enzyme because its removal or oxidation virtually abolishes YC-1-induced sGC activation (Freibe and Koesling, 1998). It is interesting that YC-1 enhances sGC activation by the ligands NO and carbon monoxide (CO) through stabilization of the active enzyme configuration and by decreasing the dissociation rates of these gases from the activated enzyme (Friebe and Koesling, 1998; Russwurm et al., 2002). It is remarkable that YC-1 converts the relatively weak sGC activator CO to a potent activator whose effectiveness is comparable with that of NO (Friebe et al., 1996; Stone and Marletta, 1998).

Vascular SMCs generate CO and NO via the heme oxygenase (HO) and NO synthase enzymes, respectively. HO oxidatively degrades heme to yield equimolar amounts of CO, biliverdin, and iron. Two distinct isoforms of HO, HO-1 and HO-2, have been identified in vascular SMCs (Wu and Wang, 2005; Durante et al., 2006). Although HO-2 is primarily expressed constitutively, HO-1 is a highly inducible isoform that is up-regulated by a number of biochemical and biophysical stimuli. Induction of HO-1 elicits potent antiproliferative, anti-inflammatory, antithrombotic, and antioxidant effects in the circulation via the generation of CO and biliverdin. On the other hand, the predominant source of NO formation by vascular SMCs is via the metabolism of L-arginine by inducible NO synthase (iNOS). The iNOS isoform is expressed after stimulation with inflammatory mediators and generates large amounts of NO over a prolonged period of time. In addition to reducing vasomotor tone and platelet aggregation, the generation of NO by vascular SMCs limits intimal thickening at sites of vascular injury by blocking SMC proliferation and migration (Kibbe et al., 1999).

YC-1 represents the prototype of an important novel class of NO-independent stimulators of sGC that exhibit therapeutic potential for the treatment of a range of vascular diseases, including hypertension, thrombosis, erectile dysfunction, and postangioplasty restenosis (Tulis, 2004; Evgenov et al., 2006). In this respect, YC-1 inhibits platelet aggregation and thrombosis in various experimental models (Teng et al., 1997). In addition, YC-1 lowers blood pressure and dilates blood vessels in normotensive and hypertensive animals (Rothermund et al., 2000). Furthermore, studies from our laboratories and others found that YC-1 inhibits vascular SMC proliferation and neointima formation after balloon injury of rat carotid arteries (Tulis et al., 2000, 2002; Wu et al., 2004; Liu et al., 2006). Moreover, YC-1 markedly enhances the antiaggregatory and vasodilator potency of NO and CO (Freibe et al., 1998; McLaughlin et al., 2000; Rothermund et al., 2000; Motterlini et al., 2005).

Although YC-1 has been shown to potentiate the biological actions of NO and CO by sensitizing sGC to these gaseous monoxides, the ability of YC-1 to influence the production of these gases has not been fully investigated. In the present study, we investigated the role of YC-1 in regulating HO and iNOS expression in vascular SMCs. In addition, we identified signaling pathways by which YC-1 modulates the expression of these gas-generating enzymes. The ability of YC-1 to coordinately stimulate the expression of enzymes responsible for generating diatomic gases and sensitize sGC to these gaseous ligands may provide a novel mechanism by which this agent is able to amplify increases in tissue cGMP and promote its beneficial effects in the circulation. In addition, the induction of HO-1 and iNOS by YC-1 may contribute to the versatile pharmacological profile of this promising therapeutic compound.

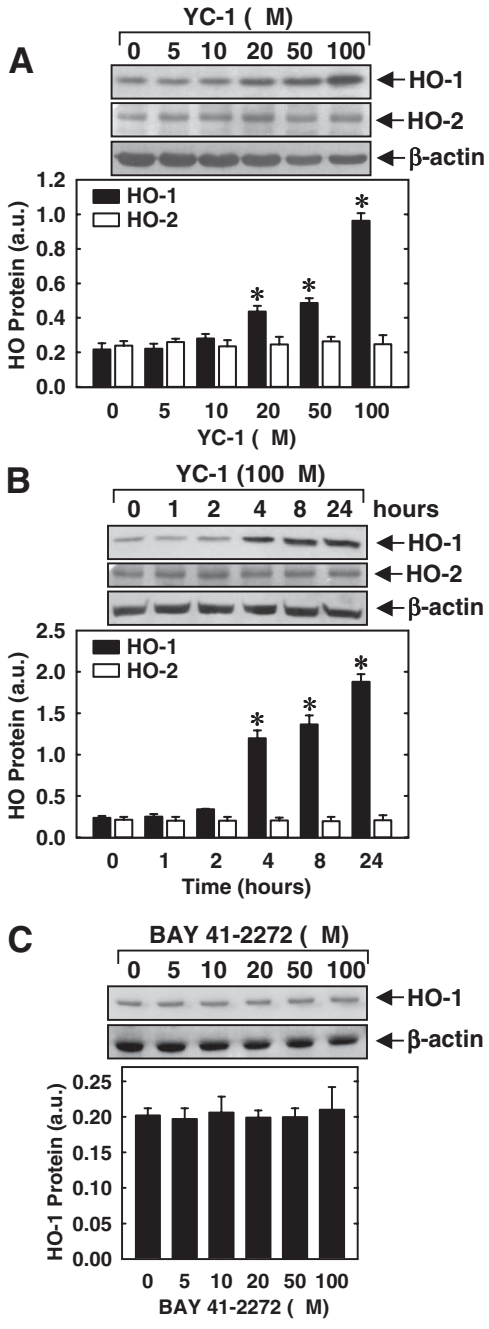


**Fig. 1.** YC-1 stimulates HO-1 protein expression in vascular SMCs. A, concentration-dependent effect of YC-1 (5–100 $\mu$M for 24 h) on HO-1 and HO-2 protein expression. B, time course of HO-1 and HO-2 protein expression after the administration of YC-1 (100 $\mu$M). C, BAY 41-2272 (5–100 $\mu$M for 24 h) fails to stimulate HO-1 protein expression. HO-1 and HO-2 protein levels are expressed in arbitrary units (a.u.). Results are means ± S.E.M. of three to four experiments. *, statistically significant effect of YC-1.

## Materials and Methods

**Materials.** Streptomycin, penicillin SDS, cycloheximide, actinomycin D, trypsin-EDTA, TES, HEPES, collagenase, elastase, glutamine, glycerol, bromphenol blue, 8-bromo-cGMP, BAY 41-2272, DT-2, 3-isobutyl-1-methylxanthine, naphthyl ethylenediamine dihydrochloride, curcumin, sulfanilamide, and minimum essential medium were from Sigma-Aldrich (St. Louis, MO); 1H-(1,2,4)oxadiazolo[4,3-$\alpha$]quinozalin-1-one (ODQ), KT-5823, KT-5270, wortmannin, LY294002, U0125, PD98059, SB203580, and SP600125 were from Calbiochem-Novabiochem (San Diego, CA); interleukin-1$\beta$ was from R&D Systems (Minneapolis, MN); tumor necrosis factor-$\alpha$ was from Genzyme (Boston, MA); polyclonal HO-1 and HO-2 antibodies were from Nventa Biopharmaceuticals (Victoria, BC, Canada); a polyclonal iNOS antibody was from BD Biosciences (San Jose, CA); a polyclonal antibody against $\beta$-actin was from Santa Cruz Biotechnologies (Santa Cruz, CA); antibodies against phospho-ERK1/2, phospho-p38 mitogen-activated protein kinase (MAPK), phospho-JNK, and phospho-Akt were from Cell Signaling Technology (Danvers, MA); and [$^{32}$P]dCTP (3000 Ci/mmol) was from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK).

**Cell Culture.** Vascular SMCs were isolated by collagenase and elastase digestion of rat thoracic aortas and characterized by morphological and immunological criteria, as we described previously (Durante et al., 1993). Cells were cultured serially (passage 3–12) in minimum essential media supplemented with 10% bovine serum, 2 mM L-glutamine, 5 mM TES, 5 mM HEPES, 100U/ml penicillin, and 100U/ml streptomycin and incubated in an atmosphere of 95% air/5% $CO_2$.

**Western Blotting.** Cells were lysed in sample buffer (125 mM Tris, pH 6.8, 12.5% glycerol, 2% SDS, 50 mM sodium fluoride, and trace bromphenol blue), and proteins were separated by SDS-polyacrylamide gel electrophoresis. After transfer to nitrocellulose membrane, blots were blocked with phosphate-buffered saline and nonfat milk (5%) and then incubated with antibodies directed against HO-1 (1:1500), HO-2 (1:500), iNOS (1:2000), phospho-ERK1/2 (1:1000), phospho-p38 MAPK (1:500), phospho-JNK (1:1000), phospho-Akt (1:1000), or $\beta$-actin (1:2000). Membranes were washed in phosphate-buffered saline, incubated with horseradish peroxidase-conjugated goat anti-rabbit, rabbit anti-mouse, or donkey anti-goat antibody, and developed with commercial chemoluminescence reagents (GE Healthcare). The expression of HO-1 and HO-2 protein was quantified by scanning densitometry and normalized with respect to $\beta$-actin.

**HO-1 mRNA Expression.** HO-1 mRNA levels were determined by Northern blotting. Total RNA (30 $\mu$g) was loaded onto 1.2% agarose gels, fractionated by electrophoresis, and blots were transferred to Gene Screen Plus membranes (PerkinElmer Life and Analytical Sciences, Waltham, MA). Membranes were prehybridized for 4 h at 68°C at in rapid hybridization buffer (GE Healthcare) and then incubated overnight at 68°C in hybridization buffer containing [$^{32}$P]DNA probes ($1 \times 10^8$ cpm) for HO-1 or 18S rRNA. DNA probes were generated by reverse-transcriptase polymerase chain reaction and labeled with [$^{32}$P]dCTP using a random priming kit (GE Healthcare). After hybridization, membranes were washed, exposed to X-ray film at −70°C, and HO-1 expression was quantified by scanning densitometry and normalized with respect to 18S rRNA.

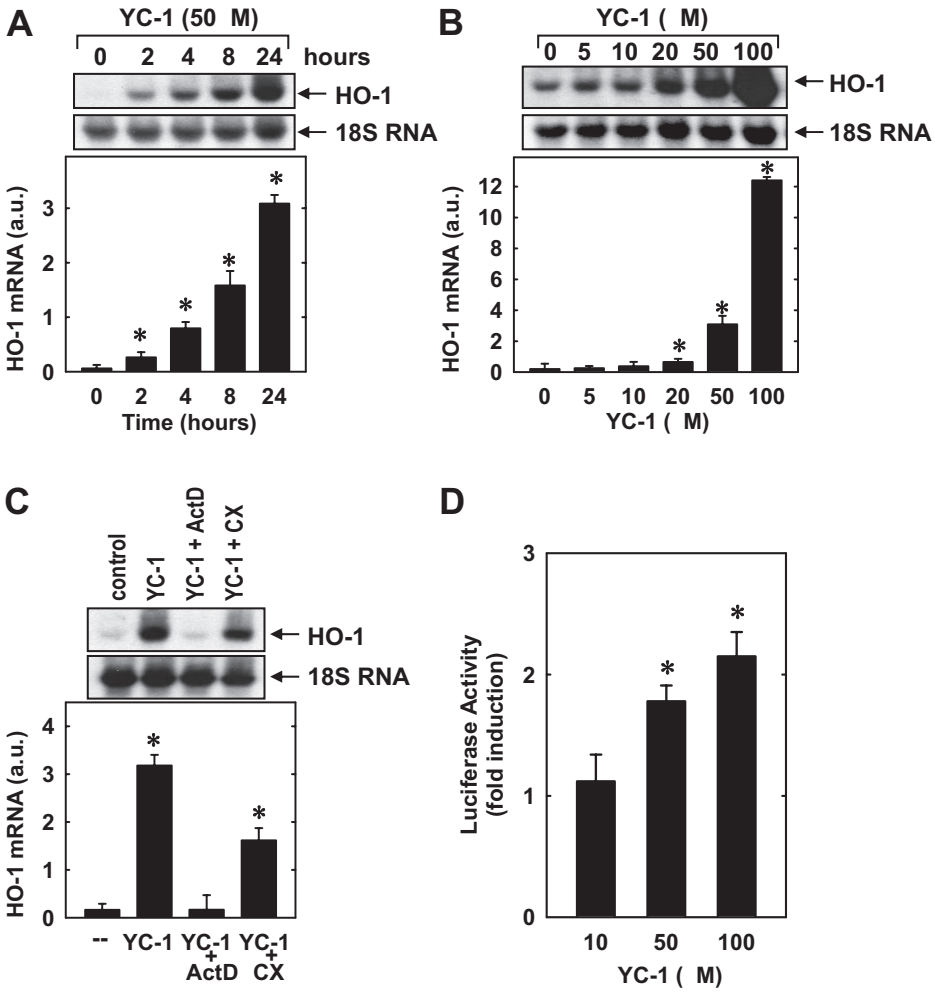


**Fig. 2.** YC-1 stimulates HO-1 mRNA expression and promoter activity in vascular SMCs. A, time course of HO-1 mRNA expression after the administration of YC-1 (50 $\mu$M). B, concentration-dependent increase in HO-1 mRNA after the administration of YC-1 (5–100 $\mu$M for 24 h). C, effect of actinomycin D (ActD; 2 $\mu$g/ml) and cycloheximide (CX; 5 $\mu$g/ml) on YC-1 (50 $\mu$M for 8 h)-mediated increase in HO-1 mRNA. D, effect of YC-1 (10–100 $\mu$M for 24 h) on HO-1 promoter activity. HO-1 mRNA expression is expressed in arbitrary units (a.u.). Results are means ± S.E.M. of three to five experiments. *, statistically significant effect of YC-1.

**HO-1 Promoter Analysis.** HO-1 promoter activity was determined using a full-length murine HO-1 promoter/firefly luciferase construct that was generously provided by Dr. Jawed Alam (Ochsner Clinic Foundation, New Orleans, LA). The HO-1 promoter (1 $\mu$g/ml) and pCMV$\beta$gal (1 $\mu$g/ml) were transfected into SMCs using Lipofectamine, cultured for 24 h, and then treated with YC-1. Cells were collected, lysed, and luciferase activity was measured using a luciferase assay system and a GloMax luminometer (Promega, Madison, WI). Luciferase activity was normalized with respect to $\beta$-galactosidase activity (Liu et al., 2007).

**MAPK and PI3K Activation.** MAPK and PI3K activity were determined by Western blotting using phospho-specific antibodies, as we described previously (Liu et al., 2007).

**cGMP Assay.** Vascular SMCs were pretreated with 3-isobutyl-1-methylxanthine (0.5 mM) for 30 min and then treated with YC-1 or BAY 41-2272. In some instances, cells were also pretreated with ODQ (30 $\mu$M) for 30 min before the addition of YC-1. Cells were lysed using 0.1 M HCl, and cGMP concentrations were measured using a commercially available kit (Molecular Devices, Sunnyvale, CA).

**NO Synthesis.** NO synthesis was assessed by measuring the accumulation of nitrite in the culture media, as we described previously (Durante et al., 1993). Because NO breaks down to both nitrite and nitrate, the levels of nitrite in the culture media were determined after incubation of samples with nitrate reductase to convert nitrate to nitrite. Samples were then mixed with an equal volume of Griess reagent (0.1% naphthyl ethylenediamine dihydrochloride and 1% sulfanilamide in 3% $H_3PO_4$), incubated at room temperature for 10 min, and absorbance was measured at 540 nm. Concentrations were determined relative to a standard curve obtained using aqueous solutions of nitrite.

**Statistics.** Results were expressed as the means ± S.E.M. Statistical analyses were performed using a Student's two-tailed $t$ test or with an analysis of variance with post hoc Bonferroni testing when multiple groups were compared. $P < 0.05$ was considered statistically significant.

## Results

Treatment of vascular SMCs with YC-1 (5–100 $\mu$M) stimulated a time- and concentration-dependent increase in HO-1 protein. An increase in HO-1 protein was detected after 24 h with 20 $\mu$M YC-1, and higher concentrations of YC-1 resulted in a progressive increase in HO-1 protein (Fig. 1A). A significant increase in HO-1 protein was first detected 4 h after YC-1 exposure and levels remained elevated after 24 h of treatment (Fig. 1B). In contrast, YC-1 had no effect on HO-2 protein expression (Fig. 1, A and B). It is interesting that another structurally similar NO-independent sGC stimulator, BAY 41-2272 (5–100 $\mu$M), failed to stimulate HO-1 protein expression (Fig. 1C).

Likewise, the induction of HO-1 protein by YC-1 was associated with a time- and concentration-dependent increase in HO-1 mRNA that preceded the increase in HO-1 protein (Fig. 2, A and B). In addition, incubation of vascular SMCs with the transcriptional inhibitor actinomycin D (2 $\mu$g/ml) completely blocked the induction of HO-1 mRNA, whereas the protein synthesis inhibitor cycloheximide (5 $\mu$g/ml) only partially inhibited the increase in HO-1 message (Fig. 2C). In the absence of YC-1, actinomycin D or cycloheximide minimally affected HO-1 mRNA expression (data not shown). Furthermore, treatment of vascular SMCs with YC-1 (10–100 $\mu$M) for 24 h stimulated a concentration-dependent increase in HO-1 promoter activity (Fig. 2D). To evaluate whether stabilization of HO-1 mRNA or protein also contributed to the induction of HO-1, YC-1 (100 $\mu$M)-pretreated SMCs were incubated with actinomycin D (2 $\mu$g/ml) or cycloheximide (5 $\mu$g/ml) either in the presence or absence of YC-1 (100 $\mu$M). However, YC-1 had no effect on the degradation of HO-1 mRNA or protein (Fig. 3, A and B).

In subsequent experiments we determined the upstream signaling molecules that mediate the induction of HO-1 by YC-1. Because YC-1 activates sGC, we first examined whether cGMP was responsible for triggering HO-1 expression. Treatment of vascular SMCs with YC-1 stimulated the intracellular accumulation of cGMP, and this was completely blocked by the sGC inhibitor ODQ (30 $\mu$M) (Fig. 4A). Bay 41-2272 (5 $\mu$M) also resulted in a substantial 7-fold increase in intracellular cGMP, increasing levels from 12 ± 4 to 88 ± 10 fmol/well. However, incubation of SMCs with ODQ or the protein kinase G (PKG) inhibitors KT 5823 (5 $\mu$M) or DT-2 (5 $\mu$M) failed to prevent the YC-1-mediated induction of HO-1 protein or mRNA (Fig. 4, B–D). Moreover, exposure of SMCs to the cGMP lipophilic analog 8-Br-cGMP (5–500 $\mu$M) had no effect on HO-1 protein expression (Fig. 4E).

Treatment of vascular SMCs with YC-1 resulted in the rapid activation of ERK1/2, JNK1, p38 MAPK, and PI3K, as reflected by the phosphorylation of Akt (Fig. 5A). Activation of these proteins occurred within 5 min and persisted for 30

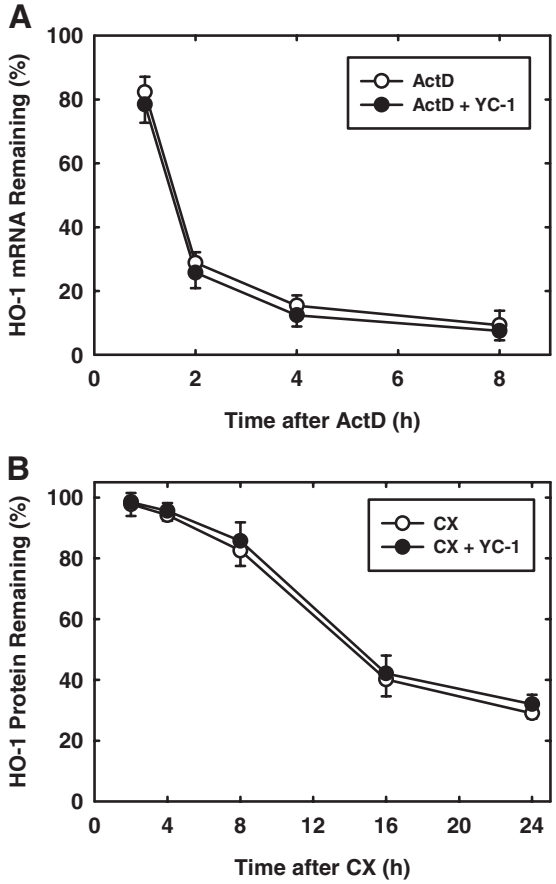


**Fig. 3.** YC-1 has no effect on HO-1 mRNA or protein stability in vascular SMCs. A, effect of YC-1 on HO-1 mRNA stability. SMCs were pretreated with YC-1 (100 $\mu$M for 24 h), washed, and then exposed to actinomycin D (ActD; 2 $\mu$g/ml) in the presence or absence of YC-1 (100 $\mu$M), and the decay of HO-1 mRNA was followed over 8 h. B, the effect of YC-1 on HO-1 protein stability. SMCs were pretreated with YC-1 (100 $\mu$M for 24 h), washed, and then exposed to cycloheximide (CX; 5 $\mu$g/ml) in the presence or absence of YC-1 (100 $\mu$M), and the decay of HO-1 protein was followed over 24 h. Results are means ± S.E.M. of three to four experiments.

min after YC-1 exposure. It is interesting that the sGC inhibitor ODQ (30 $\mu$M), potentiated the YC-1-mediated induction of ERK1/2 and JNK1 but had no effect on the activation of p38 MAPK (Fig. 5B), suggesting that YC-1 activates the three arms of MAPK via different signaling pathways. In addition, pretreatment of vascular SMCs with the ERK inhibitors U0126 (20 $\mu$M) or PD98059 (30 $\mu$M), the p38 MAPK inhibitor SB203580 (30 $\mu$M), or the JNK inhibitor SP600125 (20 $\mu$M) had no effect on the induction of HO-1 protein by YC-1 (Fig. 5C). In contrast, the PI3K inhibitors wortmannin (100 nM) and LY294002 (10 $\mu$M) blocked the induction of HO-1 protein and mRNA (Fig. 5, D and E). Control experiments confirmed the effectiveness of each inhibitor for its respective enzyme (data not shown). Finally, we also examined the interaction between YC-1 and curcumin, another known inducer of PI3K activation and HO-1 gene expression (Pugazhenthi et al., 2007). Although YC-1 and curcumin were both able to stimulate PI3K activation and HO-1 protein expression, the combined addition of these two agents did not lead to further increases in either PI3K activation or HO-1 protein (Fig. 6, A and B).

Thereafter, we determined whether YC-1 also regulates the expression of an NO-generating enzyme. Control, untreated SMCs produce minimal amounts of nitrite; however, treatment of cells with a cytokine mixture consisting of interleukin-1$\beta$ (5 ng/ml) and tumor necrosis factor-$\alpha$ (20 ng/ml) resulted in a significant accumulation of nitrite in the culture medium (Fig. 7A). It is interesting that the simultaneous administration of YC-1 (10–50 $\mu$M) potentiated the cytokine-mediated production of NO from vascular SMCs (Fig. 7A). The cytokine-mediated production of nitrite was accompanied by the induction of iNOS protein, and iNOS protein levels were further increased in the presence of YC-1 (Fig. 7B). In the absence of inflammatory cytokines, YC-1 failed to stimulate nitrite production or iNOS expression (Fig. 7, A and B). The ability of YC-1 to enhance cytokine-mediated nitrite synthesis and iNOS protein expression was dependent on sGC activity because it was prevented by ODQ (Fig. 8, A and B). In addition, the lipophilic analog of cGMP, 8-bromo-cGMP (100–500 $\mu$M), and BAY 41-2272 (5–20 $\mu$M) potentiated the cytokine-mediated increase in nitrite production (Fig. 9, A and B). Moreover, 8-bromo-cGMP and BAY 41-2272 augmented the induction of iNOS protein by cytokines (Fig. 9C). In the absence of cytokines, 8-bromo-cGMP or BAY 41-2272 minimally affected nitrite production (Fig. 9, A and B) and failed to induce iNOS protein (data not shown).

In a final series of experiments, we examined the role of downstream protein kinases in mediating the augmentation

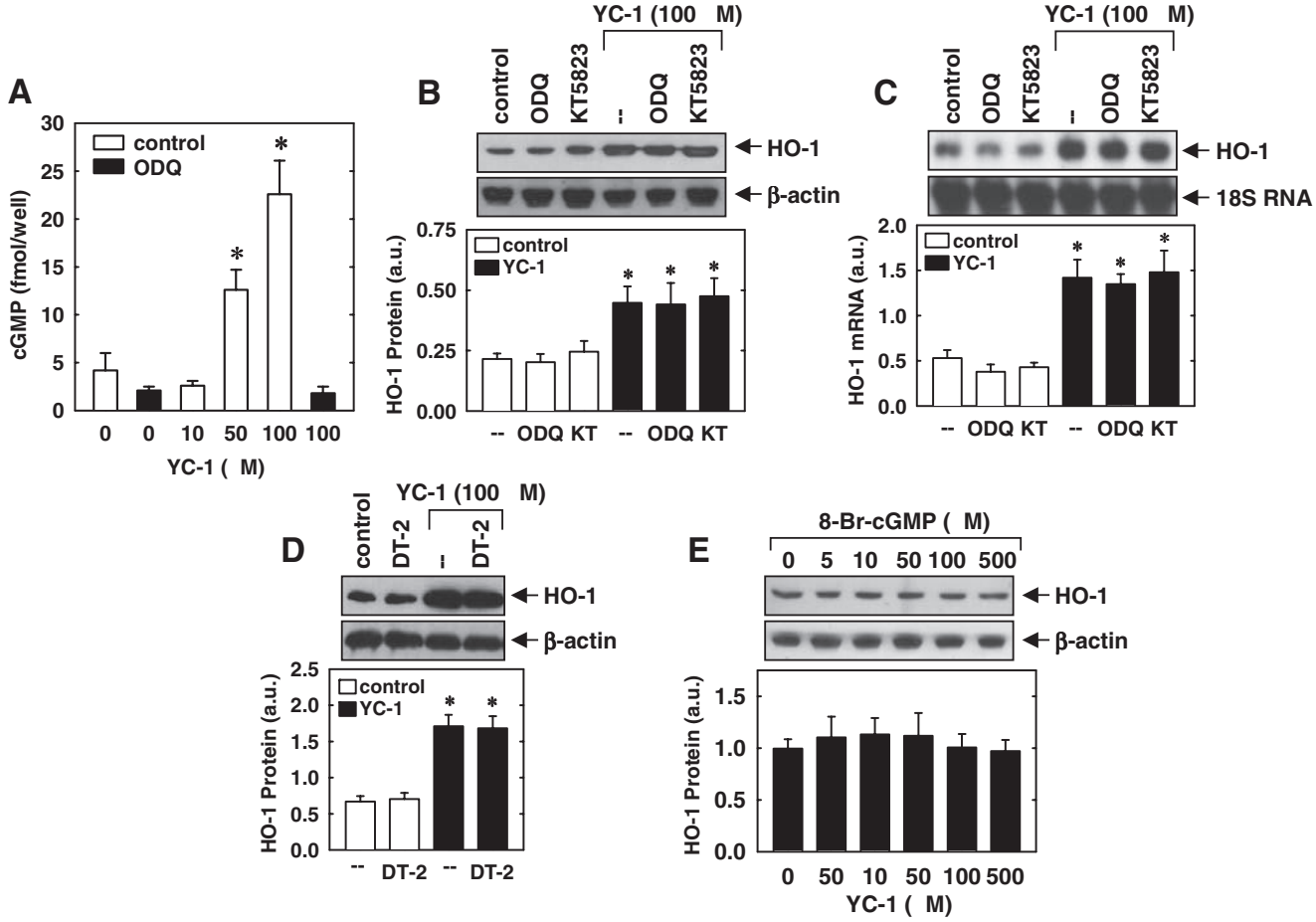


**Fig. 4.** YC-1 stimulates HO-1 gene expression in an sGC- and PKG-independent manner. A, effect of the sGC inhibitor ODQ (30 $\mu$M) on YC-1 (10–100 $\mu$M for 10 min)-mediated increases in intracellular cGMP concentration. B, effect of the sGC inhibitor ODQ (30 $\mu$M) or the PKG inhibitor KT 5823 (5 $\mu$M) on YC-1 (100 $\mu$M for 24 h)-mediated increases in HO-1 protein. C, effect of the sGC inhibitor ODQ (30 $\mu$M) or the PKG inhibitor KT 5823 (5 $\mu$M) on YC-1 (100 $\mu$M for 24 h)-mediated increases in HO-1 mRNA. D, effect of the sGC inhibitor DT-2 (5 $\mu$M) on YC-1 (100 $\mu$M for 24 h)-mediated increases in HO-1 protein. E, 8-Br-cGMP (0–500 $\mu$M for 24 h) fails to stimulate HO-1 protein expression. HO-1 protein and mRNA are expressed in arbitrary units (a.u.). Results are means ± S.E.M. of three to four experiments. *, statistically significant effect of YC-1.

of nitrite production by YC-1. Treatment of SMCs with the PKG inhibitor KT 5823 (5 $\mu$M) or DT-2 (5 $\mu$M) had no effect on the ability of cytokines to stimulate nitrite formation and did not prevent the increase in nitrite synthesis by YC-1 (Fig. 10, A and B). In contrast, the protein kinase A (PKA) inhibitor KT 5720 (5 $\mu$M) blunted the cytokine-mediated increase in nitrite formation and reversed the ability of YC-1 to elevate cytokine-mediated nitrite production (Fig. 10C). In the absence of cytokines, KT 5838, DT-2, or KT 5720 failed to influence basal nitrite synthesis.

## Discussion

In the present study, we identified YC-1 as a novel inducer of HO-1 and iNOS protein expression in vascular SMCs. It is interesting that the induction of these two enzymes by YC-1 occurs via distinct signaling pathways. Although YC-1 stimulates the expression of HO-1 via the PI3K signaling pathway, the augmentation of iNOS expression occurs through the activation of the sGC-cGMP-PKA pathway. This newly discovered capacity of YC-1 to stimulate the expression of the enzymes that generate CO and NO in addition to its documented sGC-sensitizing ability may provide an important amplification mechanism by which YC-1 is able to elevate cellular cGMP and regulate SMC function. Moreover, the induction of the NO and CO systems by YC-1 may also contribute to its cGMP-independent actions in vascular tissues.

Treatment of vascular SMCs with YC-1 results in time- and concentration-dependent increases in HO-1 mRNA and protein. The induction of HO-1 by YC-1 does not involve mRNA or protein stabilization and is probably due to the transcriptional activation of the gene because actinomycin D completely blocks HO-1 transcript expression. Moreover, transient luciferase reporter assays demonstrate that YC-1 directly stimulates HO-1 promoter activity. It is interesting that the degree of the YC-1-mediated induction of HO-1 mRNA or protein is greater than the increase in HO-1 promoter activity. Although the cause for this discrepancy is not known, it is important to note that promoter activity was monitored using a murine HO-1 promoter construct, whereas HO-1 expression was assessed in rat vascular SMCs. Thus, the variation in magnitude between YC-1-mediated increases in HO-1 promoter activity and expression may reflect differences in HO-1 promoter regulation between species (Sikorski et al., 2004).

Because YC-1 stimulates sGC activity in vascular SMCs (Tulis et al., 2000, 2002; Pan et al., 2004), we initially determined the involvement of this enzyme in mediating the induction of HO-1. Although treatment of vascular SMCs with the sGC inhibitor ODQ blocks the YC-1-mediated increase in cGMP production, ODQ does not prevent the induction of HO-1. Likewise, the PKG inhibitor KT 5823 failed to prevent the induction of HO-1 by YC-1. Because KT 5823 may not be effective in intact cells (Burkhardt et al., 2000), results obtained with this compound were corroborated using the cell-permeable PKG inhibitory peptide DT-2. However, DT-2 also

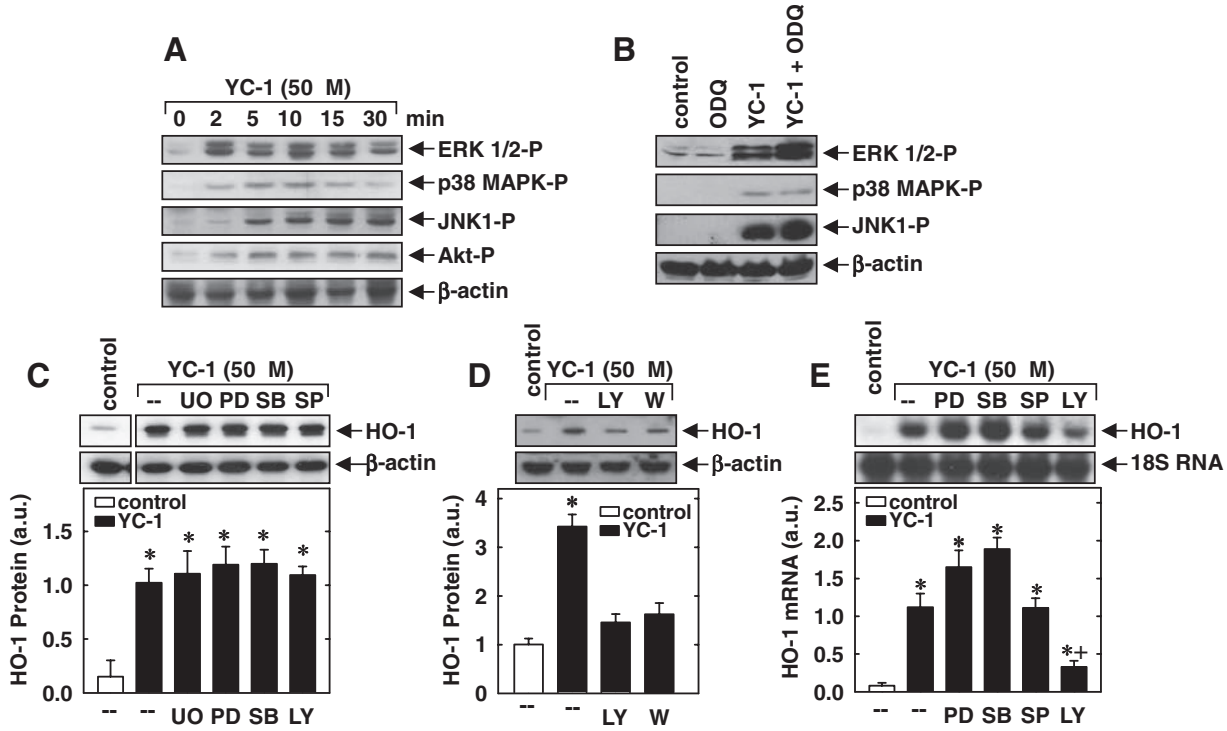


**Fig. 5.** YC-1 stimulates HO-1 gene expression in a PI3K-dependent manner. A, effect of YC-1 (50 $\mu$M for 0–30 min) treatment on ERK1/2, p38 MAPK, JNK, and Akt activity. B, effect of the sGC inhibitor ODQ on YC-1-mediated increases in MAPK. Cells were pretreated with ODQ (30 $\mu$M) for 30 min and then treated with YC-1 (50 $\mu$M for 5 min). C, effect of MAPK inhibitors on YC-1-mediated increases in HO-1 protein. Cells were pretreated with U0125 (U0; 20 $\mu$M), PD98059 (PD; 30 $\mu$M), SB203580 (SB; 30 $\mu$M), or SP600125 (SP; 20 $\mu$M) for 1 h and then treated with YC-1 (50 $\mu$M for 24 h). D, effect of PI3K inhibitors on YC-1-mediated increases in HO-1 protein. Cells were pretreated with LY294002 (LY; 10 $\mu$M) or wortmannin (W; 100 nM) for 1 h and then treated with YC-1 (50 $\mu$M for 24 h). E, effect of MAPK or PI3K inhibition on YC-1-mediated increases in HO-1 mRNA. Cells were pretreated with U0125 (U0; 20 $\mu$M), PD98059 (PD; 30 $\mu$M), SB203580 (SB; 30 $\mu$M), SP600125 (SP; 20 $\mu$M), or LY294002 (LY; 10 $\mu$M) for 1 h and then treated with YC-1 (50 $\mu$M for 24 h). HO-1 protein and mRNA are expressed in arbitrary units (a.u.). Results are means $\pm$ S.E.M. of three to five experiments. $*$, statistically significant effect of YC-1; †, statistically significant effect of LY.

failed to block YC-1-mediated HO-1 expression. In addition, administration of the cGMP analog 8-bromo-cGMP or the NO-independent sGC stimulator BAY 41-2272 had no effect on HO-1 expression. These findings are consistent with previous work from our laboratory showing that cGMP does not alter HO-1 gene transcription in vascular SMCs (Liu et al., 2007). Moreover, these results indicate that the induction of HO-1 is not a common property of allosteric sGC activators and may be a unique property of YC-1.

Because MAPK and PI3K have been implicated in the induction of HO-1 gene expression (Alam and Cook, 2007), we investigated the role of these kinases in mediating the induction of HO-1 by YC-1. Although YC-1 activates all three arms of the MAPK pathway, pharmacological inhibition of ERK, p38 MAPK, or JNK fails to block the induction of HO-1 by YC-1. Instead, we found that PI3K activity is necessary for the YC-1-mediated increase in HO-1 expression. YC-1 markedly increases PI3K activity in vascular SMCs, and pharmacological inhibition of PI3K activity blocks the YC-1-mediated induction of HO-1. This finding is in agreement with other studies demonstrating a key role for PI3K in stimulating the expression of HO-1 in several cell types, including vascular SMCs (Lee et al., 2004). It is interesting that YC-1 failed to augment PI3K activation or HO-1 protein expression by curcumin, suggesting that these two compounds may

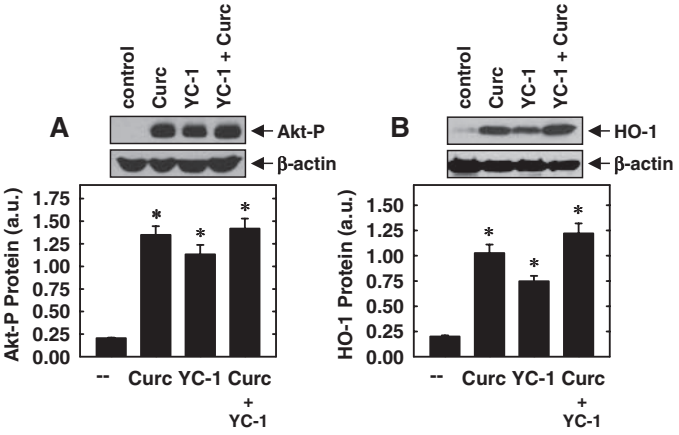


**Fig. 6.** Interaction between YC-1 and curcumin (Curc) in stimulating PI3K activity and HO-1 protein expression in vascular SMCs. A, effect of YC-1 and/or Curc on PI3K activity. Cells were treated with YC-1 (50 $\mu$M) and/or Curc (20 $\mu$M) for 5 min. B, effect of YC-1 and/or Curc on HO-1 protein expression. Cells were treated with YC-1 (50 $\mu$M) and/or Curc (20 $\mu$M) for 24 h. Results are means ± S.E.M. of three experiments. Akt phosphorylation and HO-1 protein are expressed in arbitrary units (a.u.). *, statistically significant from control.

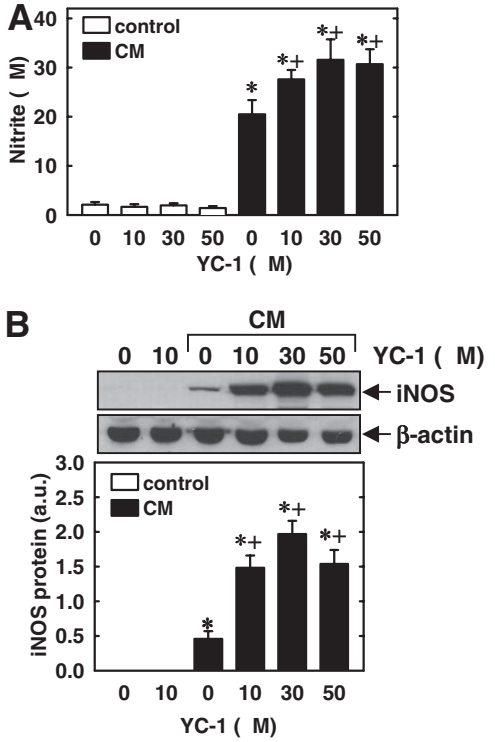


**Fig. 7.** YC-1 enhances cytokine-mediated NO synthesis and iNOS protein expression in vascular SMCs. A, effect of YC-1 (10–50 $\mu$M) on nitrite accumulation after the treatment of cells with a cytokine mixture (CM) consisting of interleukin-1$\beta$ (5 ng/ml) and tumor necrosis factor-$\alpha$ (20 ng/ml) for 24 h. B, effect of YC-1 (10–50 $\mu$M) on iNOS protein expression aftertreatment of cells with a CM consisting of interleukin-1$\beta$ (5 ng/ml) and tumor necrosis factor-$\alpha$ (20 ng/ml) for 24 h. iNOS protein is expressed in arbitrary units (a.u.). Results are means ± S.E.M. of four to six experiments. *, statistically significant effect of CM. †, statistically significant effect of YC-1.

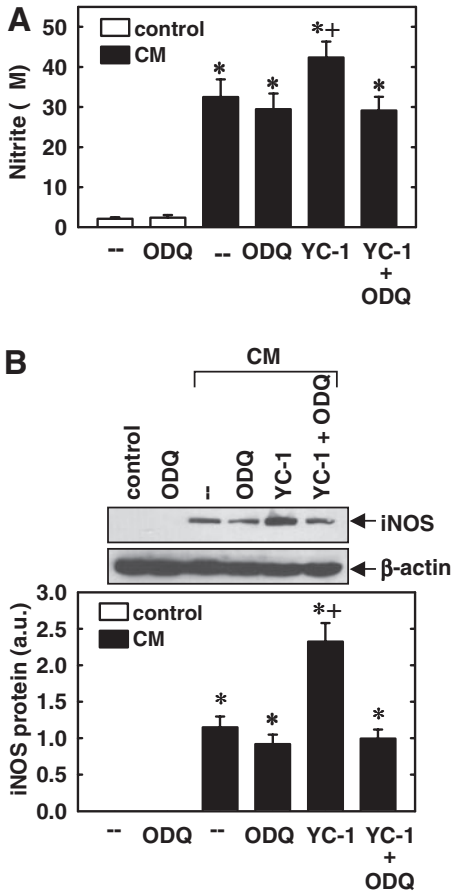


**Fig. 8.** YC-1 enhances cytokine-mediated NO synthesis and iNOS protein expression in an sGC-dependent manner. A, effect of the sGC inhibitor ODQ (30 $\mu$M) on the stimulatory effect of YC-1 (30 $\mu$M) on nitrite production after the treatment of cells with a cytokine mixture (CM) consisting of interleukin-1$\beta$ (5 ng/ml) and tumor necrosis factor-$\alpha$ (20 ng/ml) for 24 h. B, effect of the sGC inhibitor ODQ (30 $\mu$M) on the stimulatory effect of YC-1 (30 $\mu$M) on iNOS protein expression after the treatment of cells with a CM consisting of interleukin-1$\beta$ (5 ng/ml) and tumor necrosis factor-$\alpha$ (20 ng/ml) for 24 h. iNOS protein is expressed in arbitrary units (a.u.). Results are means ± S.E.M. of four to six experiments. *, statistically significant effect of CM. †, statistically significant effect of YC-1.

use a common signaling pathway in stimulating PI3K activity and HO-1 expression.

Aside from stimulating HO-1 expression, YC-1 influences the expression of an NO-generating enzyme in vascular SMCs. In particular, whereas YC-1 alone does not directly stimulate iNOS protein expression and NO synthesis, it significantly enhances the induction of iNOS protein and NO production by a mixture of inflammatory cytokines. In this case, the potentiation of iNOS expression and NO synthesis by YC-1 is mediated through the sGC-cGMP pathway because inhibition of sGC activity by ODQ abolishes the increase in both iNOS protein and NO synthesis. Moreover, a lipophilic analog of cGMP or sGC activation by BAY 41-2272 duplicates the stimulatory effects of YC-1 on cytokine-stimulated iNOS expression and NO formation. We were surprised to find that the YC-1-mediated increases in iNOS expression and NO synthesis are not mediated by PKG, because inhibition of this kinase by KT 5823 or DT-2 fails to block the stimulatory effect of YC-1 on NO production. Because YC-1 and cGMP can also activate PKA (Cornwell et al., 1994), the role of this kinase in promoting NO synthesis was also examined. Indeed, we found that the PKA inhibitor KT 5720 suppresses the YC-1-mediated increase in NO production in cytokine-treated cells. These findings are consistent with an earlier report showing that PKA is responsible for the enhancement of cytokine-mediated NO production by atrial natriuretic peptide in vascular SMCs (Iimura et al., 1998). Moreover, our finding that KT 5720 diminishes NO

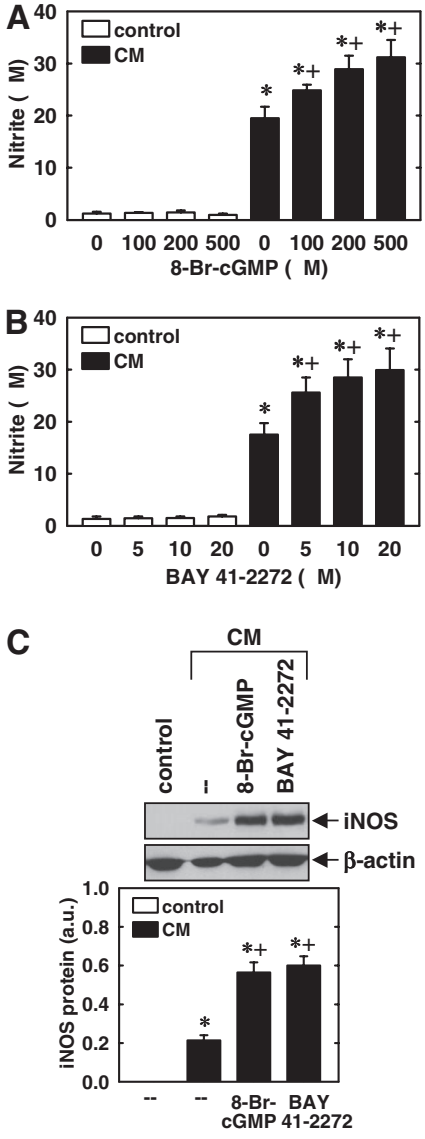


**Fig. 9.** 8-Br-cGMP and BAY 41-2272 enhance cytokine-mediated NO synthesis and iNOS protein expression in vascular SMCs. A, effect of 8-Br-cGMP (100–500 $\mu$M) on nitrite accumulation after the treatment of cells with a cytokine mixture (CM) consisting of interleukin-1$\beta$ (5 ng/ml) and tumor necrosis factor-$\alpha$ (20 ng/ml) for 24 h. B, effect of BAY 41-2272 (5–20 $\mu$M) on nitrite accumulation after the treatment of cells with a CM consisting of interleukin-1$\beta$ (5 ng/ml) and tumor necrosis factor-$\alpha$ (10 ng/ml) for 24 h. C, effect of 8-Br-cGMP (200 $\mu$M) or BAY 41-2272 (20 $\mu$M) on iNOS protein expression after treatment of cells with a CM consisting of interleukin-1$\beta$ (5 ng/ml) and tumor necrosis factor-$\alpha$ (20 ng/ml) for 24 h. iNOS protein is expressed in arbitrary units (a.u.). Results are means ± S.E.M. of four to six experiments. *, statistically significant effect of CM. †, statistically significant effect of 8-Br-cGMP or BAY41-2272.

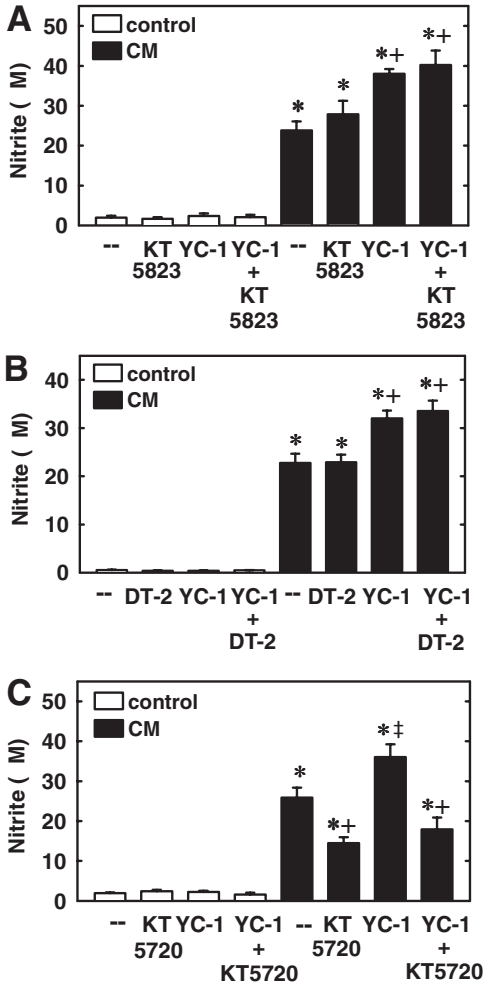


**Fig. 10.** YC-1 enhances cytokine-mediated NO synthesis in a PKA-dependent manner. A, effect of the PKG inhibitor KT 5823 (5 $\mu$M) on the stimulatory effect of YC-1 (30 $\mu$M) on nitrite production after the treatment of cells with a cytokine mixture (CM) consisting of interleukin-1$\beta$ (5 ng/ml) and tumor necrosis factor-$\alpha$ (20 ng/ml) for 24 h. B, effect of the PKG inhibitor DT-2 (5 $\mu$M) on the stimulatory effect of YC-1 (30 $\mu$M) on nitrite production after the treatment of cells with the CM for 24 h. C, effect of the PKA inhibitor KT 5720 (5 $\mu$M) on the stimulatory effect of YC-1 (30 $\mu$M) on nitrite production after the treatment of cells with the CM for 24 h. Results are means ± S.E.M. of five to seven experiments. *, statistically significant effect of CM. †, statistically significant effect of KT 5720. ‡, statistically significant effect of YC-1.

synthesis after cytokine stimulation is in accordance with previous work demonstrating a key role for PKA in mediating the induction of iNOS by cytokines (Boese et al., 1996). Our finding that YC-1 promotes the induction of iNOS and NO synthesis in vascular SMCs, complements a previous study showing that YC-1 stimulates the release of NO by endothelial NO synthase in endothelial cells (Wohlfart et al., 1999), further emphasizing the ability of YC-1 to up-regulate NO production in the vasculature. However, it contrasts with recent reports showing that YC-1 attenuates lipopolysaccharide-mediated iNOS expression in macrophages and microglia (Hsiao et al., 2004; Lu et al., 2007). The cause for these divergent results may reflect differences in cell type, culture conditions, and/or the nature of the inflammatory stimuli.

The ability of YC-1 to stimulate the production of gaseous monoxides through the induction of HO-1 and iNOS may be of pharmacological and therapeutic significance. In particular, up-regulation of CO and NO synthesis would provide an important long-term mechanism by which YC-1 elevates tissue cGMP levels. Thus, the induction of HO-1 and iNOS may amplify the many cGMP-dependent actions of YC-1, including its antihypertensive, antiaggregatory, antiproliferative, and antiapoptotic properties (Teng et al., 1997; Rothermund et al., 2000; Tulis et al., 2000, 2002; Pan et al., 2004; Tulis, 2004). It is interesting that the induction of HO-1 and iNOS by YC-1 may also underlie the versatile pharmacological profile of this agent. In this respect, YC-1 has been shown recently to elicit an antioxidant effect by inhibiting neutrophil superoxide production through a cGMP-independent pathway (Wang et al., 2002). Furthermore, YC-1 has been reported to exert a cGMP-independent, anti-inflammatory action in leukocytes and in endotoxemic mice (Pan et al., 2005). Given that HO-1 evokes potent antioxidant and anti-inflammatory effects through the generation of biliverdin and bilirubin (Wu and Wang, 2005; Durante et al., 2006), it is plausible that YC-1 may suppress both oxidative stress and inflammation through the induction of HO-1. Moreover, the ability of YC-1 to enhance iNOS-mediated NO synthesis may further intensify its anti-inflammatory actions, because the production of NO by iNOS has been shown to promote the nitrosylation and inactivation of NF-κB, a key transcription factor involved in the inflammatory cascade (Marshall and Stamler, 2001; Reynaert et al., 2004).

In conclusion, these studies demonstrate that YC-1 stimulates the expression of HO-1 and iNOS in vascular SMCs via the PI3K and sGC-cGMP-PKA pathways, respectively. The ability of YC-1 to stimulate CO and NO production through the induction of HO-1 and iNOS and to simultaneously sensitize sGC to these diatomic gases may serve to amplify the cGMP-dependent biological actions of this indazole derivative. In addition, the induction of HO-1 and iNOS by YC-1 may contribute to the pleiotropic beneficial effects of this agent in the circulation.

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