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Molecular Mechanisms Involved in the Synergistic Activation of Soluble Guanylyl Cyclase by YC-1 and Nitric Oxide in Endothelial Cells

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ABSTRACT

YC-1 is a direct activator of soluble guanylyl cyclase (sGC) and sensitizes the enzyme for activation by nitric oxide (NO) and CO. Because the potentiating effect of YC-1 on NO-induced cGMP formation in platelets and smooth muscle cells has been shown to be substantially higher than observed with the purified enzyme, the synergism between heme ligands and YC-1 is apparently more pronounced in intact cells than in cell-free systems. Here, we investigated the mechanisms underlying the synergistic activation of sGC by YC-1 and NO in endothelial cells. Stimulation of the cells with YC-1 enhanced cGMP accumulation up to ~100-fold. The maximal effect of YC-1 was more pronounced than that of the NO donor DEA/NO (~20-fold increase in cGMP accumulation) and markedly diminished in the presence of L-N^G-nitroarginine, EGTA, or oxyhemoglobin. Because YC-1 did not activate endothelial NO synthase, the pronounced effect of YC-1 on cGMP accumulation was apparently caused by a synergistic activation of sGC by YC-1 and basal NO. The effect of YC-1 was further enhanced by addition of DEA/NO, resulting in a ~160-fold stimulation of cGMP accumulation. Thus, YC-1 increased the NO-induced accumulation of cGMP in intact cells by ~8-fold. Addition of endothelial cell homogenate increased the stimulatory effect of YC-1 on NO-activated purified sGC from 1.2- to 3.7-fold. This effect was not observed with heat-denatured homogenates, suggesting that a heat-labile factor present in endothelial cells potentiates the effect of YC-1 on NO-activated sGC.

sGC [GTP pyrophosphate-lyase (cyclizing), E.C. 4.6.1.2] catalyzes the conversion of GTP to cGMP, a second messenger that modulates a variety of physiological processes, such as smooth muscle relaxation, platelet aggregation, and neurotransmitter release in the brain (Garthwaite and Boulton, 1995; Moncada et al., 1991). The enzyme, which represents one of the most important physiological targets of NO, is an α/β heterodimer with an overall molecular mass of 150 kDa (Koesling et al., 1991), containing stoichiometric amounts of ferroprotoporphyrin-IX bound to His-105 of the β-subunit (Wedel et al., 1994). NO binds with high affinity to the heme iron, resulting in a change in heme geometry that confers enzyme activation (Ignarro, 1992).

In 1995, the benzylindazole derivative YC-1 was described as a novel, apparently NO-independent activator of platelet sGC (Wu et al., 1995). Subsequent work with sGC purified from bovine lung showed that YC-1 causes a pronounced sensitization of the enzyme for stimulation by NO and CO as

well as a slight increase in maximal enzyme activity (Friebe et al., 1996; Mülsch et al., 1997; Stone and Marletta, 1998) These effects are probably caused by a conformational change of the heme pocket, resulting in both ligand-independent enzyme activation and reduced rates of NO and CO dissociation from the heme iron (Friebe and Koesling, 1998; Sharma et al., 1999; Stone and Marletta, 1998). In accordance with the results obtained with purified sGC, YC-1 potentiates the vascular (Galle et al., 1999; Hwang et al., 1999; Mülsch et al., 1997) and antiplatelet (Friebe et al., 1998) effects of NO. In intact cells, the synergistic action of NO and YC-1 was reported to result in enormous increases in cGMP levels. In platelets, for instance, the combination of DEA/NO and YC-1 was about 100-fold more effective than the individual compounds, resulting in an increase of more than 1300-fold in cGMP (Friebe et al., 1998). Because the maximal activity of NO-activated purified sGC is increased only marginally by YC-1 (Friebe et al., 1996; Mülsch et al., 1997; Stone and Marletta, 1998), these results suggested that additional mechanisms may be involved in the pronounced synergistic interaction of NO and YC-1 in intact cells. Indeed,

ABBREVIATIONS: sGC, soluble guanylyl cyclase; NO, nitric oxide; NOS, nitric-oxide synthase; eNOS, endothelial nitric-oxide synthase; PDE, phosphodiesterase; DMSO, dimethyl sulfoxide; IBMX, 3-isobutyl-1-methylxanthine; L-NNA, L-N^G-nitroarginine; OxyHb; oxyhemoglobin; DEA/NO, 2,2-diethyl-1-nitroso-oxyhydrazine.

it has been reported that the drug inhibits PDE-catalyzed cGMP hydrolysis in platelets (Friebe et al., 1998) and smooth muscle cells (Galle et al., 1999). Inhibition of cGMP breakdown certainly contributes to the efficacy of YC-1 in tissues, but pronounced increases in maximal intracellular cGMP levels have been observed even in the presence of PDE inhibitors (Friebe and Koesling, 1998; Hwang et al., 1999; Mülsch et al., 1997), indicating that PDE inhibition does not fully explain the effects of the drug in intact cells.

Recently, Wohlfahrt et al. (1999) and coworkers reported on another intriguing effect of YC-1. These authors claimed that the drug promotes a transient, Ca^{2+}-dependent NO release from endothelial cells, suggesting that stimulation of endogenous NO formation might contribute to cGMP accumulation in tissues. However, the electrochemical NO signal produced by YC-1 was not very pronounced and disappeared within seconds. Moreover, the authors did not provide any additional evidence for eNOS activation.

Taken together, the data available so far do not appear to sufficiently explain the mechanisms involved in YC-1-triggered cellular cGMP accumulation. In the present study, we have addressed this issue by measuring cGMP accumulation and NOS activity in intact endothelial cells treated with various combinations of YC-1, NO donors, and NOS inhibitors. Our results suggest that basal formation of endogenous NO and an as-yet-unidentified heat-labile factor enhancing YC-1 activation of sGC mediate YC-1-triggered cGMP accumulation.

Experimental Procedures

Materials. Cell culture media, antibiotics and fetal calf serum were purchased from PAA Laboratories GmbH (Linz, Austria). $[\alpha\text{-}^{32}P]GTP$ (400 Ci/mmol) and L-$[2,3,4,5\text{-}^{3}H]$arginine (57 Ci/mmol) were from American Radiolabeled Chemicals Inc., purchased through Humos Diagnostica GmbH (Maria Enzersdorf, Austria). DEA/NO was obtained from Alexis Corporation (Läufelfingen, Switzerland), YC-1 was a generous gift from Dr. K. Schönafinger (Hoechst Marion Roussel, Frankfurt, Germany) and sGC was purified from bovine lung as described previously (Humbert et al., 1990). All other chemicals were purchased from Sigma (Vienna, Austria). A stock solution of YC-1 (100 mM) was prepared in DMSO and diluted with buffer containing 20% DMSO to yield a YC-1 concentration of 2 mM. All further dilutions were made with the buffer. The solvent (final concentration of DMSO in the assay, ≤ 2%) affected neither the cGMP accumulation in intact cells nor the activity of purified sGC.

Cell Culture. Porcine aortic endothelial cells were isolated as described previously (Schmidt et al., 1989) and cultured at 37°C, 5% CO_2, for up to three passages in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 1.25 μg/ml amphotericin B.

Preparation of Endothelial Cell Homogenates. Endothelial cells from 50 Petri dishes (diameter, 90 mm) were harvested, washed twice with prewarmed PBS and resuspended in 1 ml of ice-cold 50 mM triethanolamine buffer, pH 7.4, containing 0.5 mM EDTA and 12 mM 2-mercaptoethanol. Cells were disrupted by sonication, and homogenates were stored in 0.2 ml-aliquots at −70°C. Protein was determined with the Bradford (1976) method using bovine serum albumin as standard.

Determination of Endothelial cGMP Formation. Accumulation of intracellular cGMP was determined as described previously (Schmidt et al., 1999). Briefly, endothelial cells grown in 24-well plates were washed and preincubated for 15 min at 37°C in 50 mM Tris buffer, pH 7.4, containing 100 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1 mM IBMX, and 1 μM indomethacin. Where

indicated, preincubation was performed in the presence of 0.3 mM L-NNA, 0.1 mM OxyHb or 0.1 mM EGTA (instead of $CaCl_2$). Reactions were started by addition of the compounds to be tested and terminated after 4 min by removal of the incubation medium and addition of 0.01 N HCl. Within 1 h, intracellular cGMP was completely released into the supernatant and measured by radioimmunoassay.

Determination of sGC Activity. Purified sGC (50 ng) was incubated for 10 min at 37°C in a total volume of 0.1 ml of a 50 triethanolamine buffer, pH 7.4, containing 0.5 mM $[\alpha\text{-}^{32}P]GTP$ (~300,000 cpm), 3 mM $MgCl_2$, 1 mM cGMP, 1 mM IBMX, 1 mM EGTA, 2 mM dithiothreitol, 5 mM creatine phosphate, 15 mU creatine phosphokinase, and drugs as indicated. Incubations were terminated by $ZnCO_3$ precipitation, and $[\alpha\text{-}^{32}P]cGMP$ was isolated by column chromatography as described previously (Schultz and Böhme, 1984).

Determination of eNOS Activity. NOS activity in intact cells was determined by monitoring the conversion of L-$[^{3}H]$arginine into L-$[^{3}H]$citrulline as described previously (Schmidt and Mayer, 1999). Briefly, endothelial cells grown in six-well plates were washed and equilibrated for 15 min at 37°C in 50 mM Tris buffer, pH 7.4, containing 100 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 2.5 mM $CaCl_2$. Reactions were started by addition of L-$[2,3,4,5\text{-}^{3}H]$arginine (~ 10^6 dpm) and drugs as indicated and terminated after 4 min by washing the cells with chilled incubation buffer. After lysis of the cells with 0.01 N HCl, an aliquot was removed for determination of incorporated radioactivity. Sodium acetate buffer (200 mM; pH 13.0) containing 10 mM L-citrulline was added (final pH ~5.0) to the remaining sample, and L-$[^{3}H]$citrulline was separated from L-$[^{3}H]$arginine by cation exchange chromatography.

Results

Incubation of endothelial cells with YC-1 led to an increase in intracellular cGMP from a basal level of 3.9 ± 1.3 up to 425 ± 59 pmol cGMP/10^6 cells (Fig. 1). The effect of 0.2 mM YC-1 was much more pronounced than that produced by a maximal active concentration of the NO donor DEA/NO (85 ± 8 pmol cGMP/10^6 cells). Addition of OxyHb (0.1 mM) reduced basal cGMP formation to 1.2 ± 0.5 pmol/10^6 cells
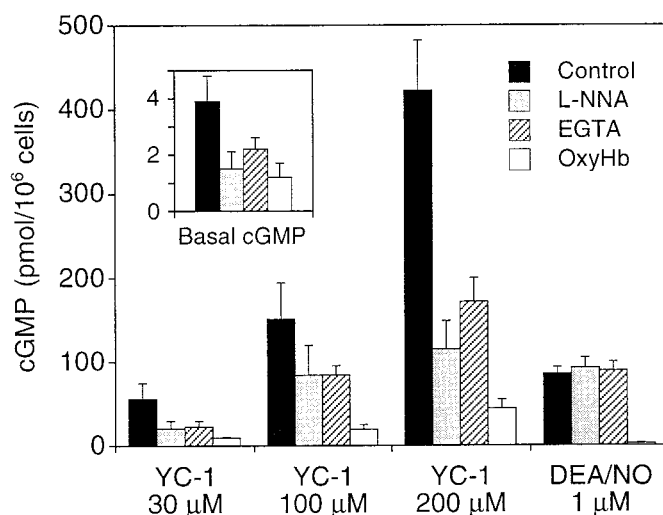

Fig. 1. Stimulation of endothelial cGMP accumulation by YC-1 and DEA/NO. Endothelial cells were preincubated for 15 min at 37°C in the absence and presence of 0.3 mM L-NNA, 0.1 mM EGTA (instead of 2.5 mM $CaCl_2$), or 0.1 mM OxyHb and stimulated for 4 min with YC-1 or DEA/NO. After lysis of the cells, intracellular cGMP was measured by radioimmunoassay. *Inset*, effect of L-NNA, EGTA, and OxyHb on basal cGMP accumulation. Data are mean values ± S.E.M. of four experiments, with individual measurements performed in triplicate.

and markedly diminished the effect of YC-1 (43 ± 11 pmol cGMP/10^6 cells at 0.2 mM YC-1). A similar, albeit less pronounced effect on both basal and YC-1-stimulated cGMP accumulation was observed upon pretreatment of the cells for 15 min with 0.3 mM L-NNA (1.5 ± 0.6 and 115 ± 34 pmol cGMP/10^6 cells, respectively) or chelating of extracellular Ca^{2+} with 0.1 mM EGTA (2.2 ± 0.4 and 171 ± 28 pmol cGMP/10^6 cells, respectively). As expected, DEA/NO-induced cGMP accumulation was not affected by inhibition of endogenous NO formation (91 ± 12 and 89 ± 10 pmol cGMP/10^6 cells in the presence of L-NNA and EGTA, respectively) and abolished by OxyHb (2.0 ± 0.7 pmol cGMP/10^6 cells).

The inhibition of YC-1-induced cGMP accumulation by L-NNA or Ca^{2+} chelation suggested that the effect of YC-1 was partially mediated by endogenous NO. Therefore, we recorded concentration-response curves with the NO donor DEA/NO in the absence and presence of a fixed concentration of YC-1 (0.2 mM). Figure 2 shows that exogenously applied NO increased cGMP levels in YC-1-treated cells from 417 ± 23 to 563 ± 44 pmol/10^6 cells. The latter level was 7.8-fold higher than the maximal cGMP levels obtained with DEA/NO alone (72 ± 5 pmol/10^6 cells). In the absence or at low concentrations of DEA/NO, L-NNA significantly inhibited the effect of YC-1, but this inhibition was fully antagonized by ≥ 30 nM NO donor.

These data indicated that, despite a direct (i.e., NO-independent) effect of YC-1 on endothelial sGC, the major component of YC-1-triggered cGMP accumulation was mediated by endogenous NO. Although low amounts of NO may be formed by eNOS under basal (i.e., nonstimulated conditions) (Gold et al., 1990), it has been proposed that YC-1 stimulates endogenous NO formation through activation of eNOS (Wohlfart et al., 1999). To test this hypothesis, we used the arginine-to-citrulline conversion assay to measure NOS activity in intact cells (Teubl et al., 1999). As shown in Fig. 3, stimulation of endothelial cells with bradykinin, ATP, or A 23187 enhanced the conversion of incorporated L-[^{3}H]argin-ine into L-[^{3}H]citrulline from $2.8 \pm 0.3\%$ to $18.1 \pm 3.2\%$, $18.3 \pm 3.7\%$, and $29.8 \pm 2.9\%$, respectively. The corresponding endothelial cGMP levels were 3.9 ± 1.3 under control conditions and increased to 18.5 ± 2.8, 19.5 ± 3.6, and 31.0 ± 4.0 pmol/10^6 cells after stimulation with bradykinin, ATP, and A 23187, respectively. In contrast, YC-1 did not cause a detectable increase in arginine-to-citrulline conversion, suggesting that the pronounced increases in cGMP accumulation observed with this drug are caused by a synergistic interaction with basal NO produced by eNOS in nonstimulated endothelial cells.

The YC-1-induced an increase of maximal endothelial cGMP levels of about 8-fold, measured in the presence of DEA/NO, is in striking contrast to the very moderate, 1.4-fold increase of maximal activity of purified sGC (Friebe et al., 1996). This finding was especially intriguing because the nonselective PDE inhibitor IBMX was present in all cell culture experiments. Therefore, we speculated that the effect of YC-1 may be mediated by unknown constituent(s) of the endothelial cells. This issue was addressed by measuring the effect of YC-1 on the activity of NO-stimulated purified sGC in the absence and presence of endothelial cell homogenates. The activity of endogenous (i.e., endothelial sGC) was not detectable under the assay conditions (0.5 mM GTP). As shown in Fig. 4, addition of homogenized endothelial cells (5 - 200 μg of protein/assay) slightly reduced the activity of purified sGC measured in the presence of 1 μM DEA/NO from 4.7 ± 0.5 to 3.5 ± 0.3 μmol cGMP/mg/min. In contrast to this slight inhibitory effect on NO stimulation of sGC, activation of NO-stimulated enzyme by YC-1 was markedly increased upon addition of the endothelial cell homogenate (6.1 ± 1.3 and 13.3 ± 2.1 μmol cGMP/mg/min in the absence and presence of 0.2 mg of endothelial protein, respectively). Thus, YC-1 produced a 1.2-fold increase in the activity of the purified enzyme stimulated maximally with 1 μM DEA/NO, but led to a 3.7-fold increase upon addition of endothelial homogenates containing 0.2 mg of protein. This effect was
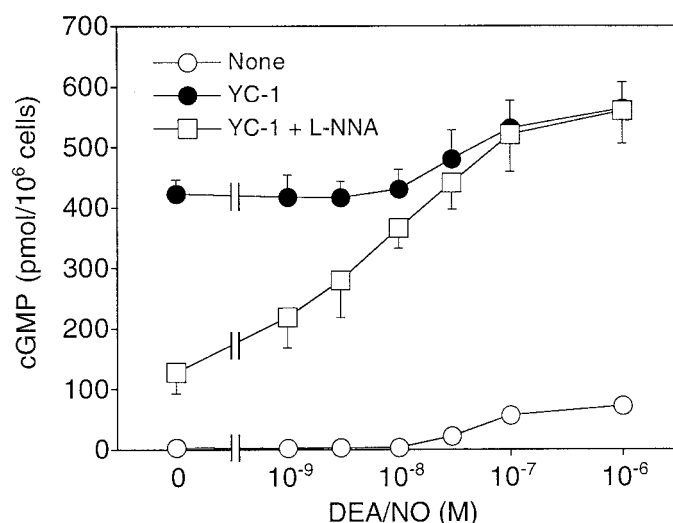

Fig. 2. Effect of YC-1 on NO-induced cGMP accumulation. Endothelial cells were preincubated for 15 min at 37°C in the absence and presence of 0.3 mM L-NNA and stimulated for 4 min with increasing concentrations of DEA/NO in the absence and presence of 0.2 mM YC-1. After lysis of the cells, intracellular cGMP was measured by radioimmunoassay. Data are mean values $\pm$ S.E.M. of three experiments, with individual measurements performed in triplicate.
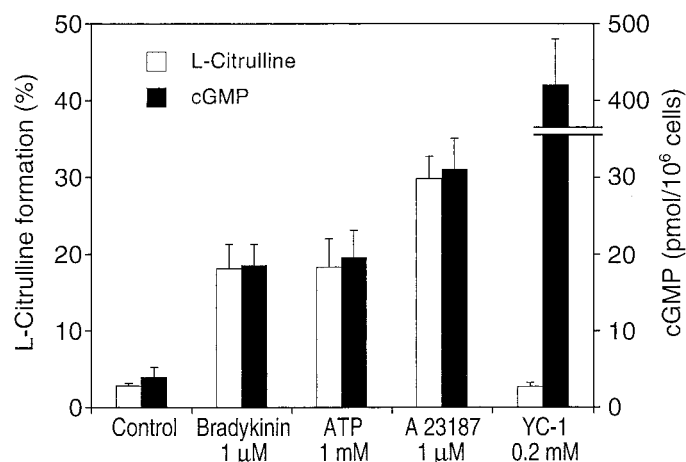

Fig. 3. Effect of bradykinin, ATP, A 23187, and YC-1 on NOS activity in intact endothelial cells. Endothelial cells were equilibrated for 15 min at 37°C in buffer and stimulated for 4 min with 1 μM bradykinin, 1 mM ATP, 1 μM A 23187, or 0.2 mM YC-1 in the absence (determination of cGMP) or presence of L-[^{3}H]arginine (determination of L-citrulline). After lysis of the cells, the conversion of incorporated L-[^{3}H]arginine into L-[^{3}H]citrulline or the accumulation of cGMP was determined as described under *Materials and Methods*. Data are mean values $\pm$ S.E.M. of three experiments, with individual measurements performed in triplicate.

virtually abolished when the homogenates had been boiled for 5 min before incubations, suggesting that endothelial cells contain a heat-labile factor that supports activation of NO-stimulated sGC by YC-1.

Discussion

The novel activator of soluble guanylyl cyclase YC-1 has attracted much attention recently because of its unique action profile, making it and related drugs promising therapeutic tools for the treatment of endothelial dysfunction without the adverse effects of classical nitrovasodilators. The positive effects of YC-1 seem to result from multiple actions of the drug, including NO-independent activation of sGC, potentiation of cGMP formation at low NO concentrations, and inhibition of PDE-catalyzed cGMP hydrolysis. Recently, the stimulation of NO production through activation of eNOS has been described as another beneficial action of this multifaceted drug (Wohlfart et al., 1999). This conclusion was based on stimulation of endothelial cGMP accumulation of more than 100-fold by YC-1 that was 1) partially inhibited by pretreatment of the cells with L-NNA and 2) preceded by a transient, partially L-NNA-sensitive release of NO, detected with a porphyrinic microsensor. However, the time course of NO formation differed markedly from that of cGMP accumulation in that the maximal effect on cGMP levels was observed after 5 min, whereas the NO release reached its maximum within 2 to 3 s and then rapidly (within ~15 s) declined to basal levels. Unfortunately, Wohlfahrt et al. (1999) did not provide a reliable explanation to account for this discrepancy.

In the present study, we investigated the effect of YC-1 on eNOS activation by measuring the conversion of L-[³H]arginine to L-[³H]citrulline by intact endothelial cells. For comparison, experiments were performed with the receptor agonists bradykinin and ATP and the Ca^{2+} ionophore A 23187. We observed that all these Ca^{2+}-mobilizing compounds elic-

Fig. 4. Effect of endothelial cell homogenate on the activation of purified sGC by NO and YC-1. Purified sGC (50 ng) was incubated for 10 min at 37°C with 1 μM DEA/NO or 1 μM DEA/NO plus 0.2 mM YC-1 in the absence and presence of endothelial cell homogenate and assayed for cGMP formation as described under *Materials and Methods*. Data are mean values ± S.E.M. of four experiments, with individual measurements performed in triplicate.

ited a pronounced increase in L-citrulline formation that was associated with a moderate elevation in cGMP levels (5—8-fold), suggesting that the sensitivity of the arginine-to-citrulline conversion assay was sufficient to detect eNOS activation in intact cells. In contrast to these well-established agonists of eNOS activation, YC-1 (0.2 mM) enhanced cGMP formation by more than 100-fold but did not cause a detectable increase in L-citrulline formation. Nonetheless, the effect of YC-1 on cGMP accumulation was inhibited markedly by scavenging of NO with OxyHb, removal of free Ca^{2+} with EGTA, or inhibition of eNOS with L-NNA. Because all of these agents also significantly reduced basal cGMP levels, we propose that YC-1 potentiated the effect of endogenous NO, formed by partially active eNOS in nonstimulated endothelial cells. Our observation that the inhibitory effect of L-NNA was antagonized by exogenous NO delivered from DEA/NO agrees well with this proposal.

An intriguing finding reported here, as well as in several previous articles (Mülsch et al., 1997; Friebe et al., 1998; Becker et al., 1999; Hwang et al., 1999) is the enormous increase in maximal cGMP levels in intact cells upon treatment with a combination of NO and YC-1. This effect is in striking contrast to data obtained with NO-stimulated purified sGC, the maximal activity of which is increased only marginally by YC-1 (Friebe et al., 1996; Mülsch et al., 1997; Stone and Marletta, 1998). This discrepancy clearly hints at additional mechanisms mediating the effect of YC-1 in cells. Inhibition of cGMP hydrolysis by PDE activity is certainly one of these mechanisms but does not seem to fully explain the efficacy of the drug, because all of the relevant data were obtained in the presence of the nonspecific PDE inhibitor IBMX. However, despite the fact that YC-1 did not cause inhibition of PDE in IBMX-treated platelets (Friebe et al., 1998), it cannot be excluded that endothelial cells express an IBMX-insensitive PDE isozyme that is inhibited by YC-1. We therefore measured PDE activity in endothelial cell homogenates but observed no effect of YC-1 on cGMP hydrolysis in the presence of IBMX (data not shown). To clarify the mechanism(s) involved in YC-1-triggered endothelial cGMP accumulation, we studied the effect of the drug on purified sGC in the absence and presence of endothelial homogenates. The experiments were carried out under substrate-saturated conditions (0.5 mM GTP, 5 mM Mg^{2+}) and with a GTP-regenerating system, to exclude that the observed effects of YC-1 are caused by a decrease in K_M value rather than an increase in V_{max} value (Denninger et al., 2000). We observed that addition of endothelial cell homogenates to purified NO-activated sGC slightly reduced the enzyme activity (apparently because of inactivation of NO by cellular components) but led to a pronounced increase in cGMP formation in the presence of YC-1. This stimulatory effect was dependent on the amount of added homogenate. Maximal enzyme stimulation by YC-1 (3.7-fold) was observed with ≥100 μg of endothelial protein. Because the effect of the homogenate was virtually abolished by brief boiling before incubation, these results suggest that endothelial cells contain a heat-labile factor that confers the effect of YC-1 on the activity of NO-activated sGC.

This heat-labile factor could be an as-yet-unknown sGC binding protein that cooperates with YC-1 to trigger a conformational change of sGC, resulting in increased maximal enzyme activity. However, it could also be possible that YC-1 is metabolized by the endothelial homogenate to a more
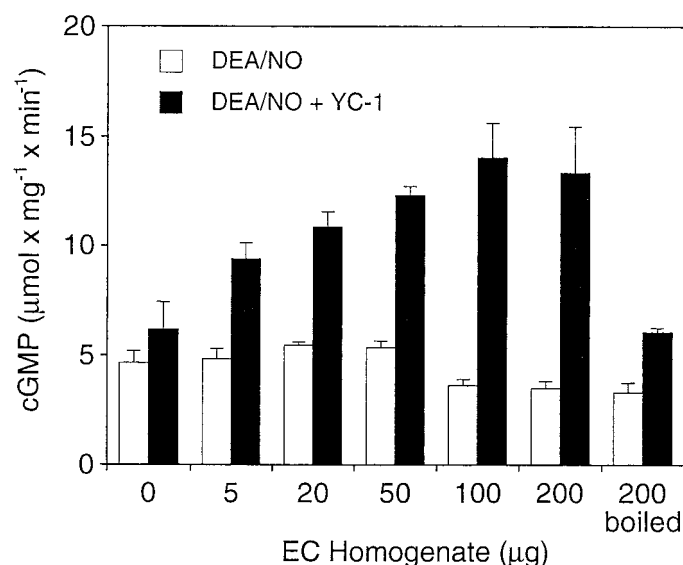
effective activator of sGC, which, in combination with NO, enhances the maximal catalytic activity of the enzyme by an as-yet-unknown mechanism. Thus, further work is clearly needed to identify and characterize this heat-labile factor that may be essentially involved in the regulation of the endothelial NO/cGMP signaling cascade.

Acknowledgments

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