Endothelium-dependent vasoconstrictions in isolated vessel grafts: a novel mechanism of vasospasms?

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Abstract

Background: YC-1 (3-(5'-hydroxymethyl-2'furyl)-1-benzyl-indazole) is an allosteric activator of soluble guanylyl cyclase (sGC) and a vasodilator. This study describes a paradox action of YC-1 in isolated vessels of coronary artery disease (CAD) patients which appears to trigger an endothelium-dependent vasoconstrictor pathway present in vessels with endothelial dysfunction. Methods: Effects of YC-1 on isolated vessel tensions were investigated in an organ bath. Vasoconstrictors released from vessels were quantified by ELISA. Results: YC-1 elicited long-lasting constrictions in saphenous veins and radial arteries from CAD patients, but not in human umbilical veins. Half-maximal effective dose was 1.0 µmol L^{-1}. Constrictions were attenuated by nifedipine (L-type Ca channel blocker), bosentan (ET_A/ET_B inhibitor), BQ-788 (ET_B inhibitor), and by denuding, but not by ODQ (sGC inhibitor), BQ-123 (ET_A inhibitor), and phosphoramidon (endothelin converting enzyme inhibitor). Indometacin (cyclooxygenase-1/2 inhibitor) and SQ 29,548 (TP receptor antagonist) suppressed YC-1 induced constrictions, whereas 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone (DFU, cyclooxygenase-2 inhibitor) had no effect. Saphenous vein rings released significantly more endothelin-1 in the presence of YC-1. Conclusions: YC-1 induced vasoconstrictions demonstrate the existence of an endothelium-dependent vasoconstrictor pathway in vessels of CAD patients which to date has only been described in animal models of hypertension. CAD patients with elevated endothelin-1 plasma levels are thus prone to endothelium-dependent vasoconstrictions which may also play a role in graft vasospasms.

Introduction

Coronary vasospasm is one of the major causes of ischemic heart conditions and may lead to stable and unstable angina, myocardial infarction, and sudden death. Endothelial dysfunction, elevated plasma levels of endothelin-1 (ET-1), and reactive oxygen species play a crucial role in the pathogenesis of vasospasm [1].

Vasospasm also affects the patency rates of coronary artery bypass grafts. The risk of vasospasm can be reduced by an appropriate choice of the graft source [2]. Venous grafts are commonly distended, although this may cause structural damages [3]. Arterial grafts are often treated with vasodilators [4] to suppress vasospasm. The nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) system [5] is one of the targets of vasospasm prevention. YC-1 (3-(5'-hydroxymethyl-2'furyl)-1-benzyl-indazole) was found to activate soluble guanylyl cyclase (sGC), a key enzyme of this system, by a different mechanism than NO-releasing agents [6], which initiated the development of related vasodilator drugs for arterial and pulmonary hypertension as well as angina pectoris [7]. YC-1 and related compounds activate sGC in a synergistic fashion with NO and carbon monoxide [8,9]. In contrast to organic nitrates they do not cause tolerance.

YC-1 and related drugs may therefore be useful tools in vasospasm prevention. However, we noticed a paradox vasoconstrictor action of YC-1 in addition to the well-known vasodilator action in vessel segments from CAD patients. This study investigated key aspects of the mechanism of this vasoconstrictor action and its relationship to endothelial dysfunction.

Methods

Study subjects

Vessel segments were obtained from 157 patients (138 male, 19 female) who underwent elective aortocoronary bypass surgery (Table 1). The patients' mean age was 68.3±7.9 years (range: 48-87). Risk factors included hypertension in 132 patients, hyperlipidemia (99), and type 2 diabetes (49), with only 12 patients not diagnosed with any of these conditions.

Harvesting of blood vessels

All experiments were approved by the local ethics committee. Undistended segments of human saphenous vein (HSV) and radial artery (HRA) were harvested after obtaining written informed consent from the patients. Human umbilical cords were collected from term pregnancies after obtaining written informed consent from the expectant mothers, and umbilical veins (HUV) were dissected.

Organ bath experiments

Vessel tensions were measured in an organ bath as described previously [10,11]. Vessel rings were equilibrated for at least 2h. Resting tensions were adjusted repeatedly to 25 mN. A stable baseline was confirmed by adding KCl (150 mmol L^{-1}) followed by a washout. Receptor-dependent contractions were measured using either norepinephrine or 5-hydroxytryptamine (for HUV).

Antagonist actions were investigated by recording cumulative YC-1 dose-response

curves with 45 min incubation time per dose. Antagonists were added 15 min before the first YC-1 dose. To record vasodilator dose-response curves, vessels were constricted by norepinephrine to 80% of the previously established maximum. Concentration series of vasodilators were added to all rings but the time controls.

Some vessels were endothelium-denuded by rubbing the luminal surface with a wooden toothpick for approx. 60 s. Endothelium removal was ascertained in representative samples by histology and scanning electron microscopy as described previously [10], and YC-1 dose-response curves were constructed. As denuding may cause partial damage of the smooth muscle layer, tension data of experiments involving denuded vessels were normalized to their responses to 150 mmol L^{-1} KCl.

Determination of endothelin release

Vessel rings were mounted in organ baths. YC-1 or DMSO as solvent control were added to the baths. After 60 min, bath contents were concentrated approx. 20-fold at 4°C using centrifugal devices (Pall, Dreieich, Germany). Concentrates were stored at -80°C, and analyzed using an endothelin-1 ELISA kit (Enzo).

Data analysis and statistical procedures

Data are presented as mean±standard deviation. n refers to the number of patients. Four to eight rings were analyzed per subject for each type of experiment. Multiple-dose substance effects were compared to the vehicle control using analysis of variance (ANOVA) followed by Dunnett's post-test. Dose-response curves were compared by a two-way repeated measurements ANOVA, followed by Holm-Sidak post-test. Differences were assumed to be significant if the error probability p was less

than 0.05. Half maximal effective concentrations (EC_{50}) were calculated by fitting Hill functions.

Drugs, chemicals, and reagents

Sodium nitroprusside, 1H-(1,2,4)oxadiazole(4,3-a)quinoxalin-1-one (ODQ), N^G-nitro-L-arginine-methyl ester (L-NAME), indometacin, N-(α-rhamnopyranosyloxyhydroxyphosphinyl)-L-leucyl-L-tryptophan (phosphoramidon), BQ-123, BQ-788, and SQ 29,548 (1S-[1α,2α(Z),3α,4α]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptanoic acid) were obtained from Alexis (Läufelfingen, Switzerland). 5-Hydroxytryptamine (serotonin) and sodium nitroprusside were purchased from Sigma (Taufkirchen, Germany). Norepinephrine was from Aventis (Frankfurt/Main, Germany) and nifedipine was obtained from Bayer (Leverkusen, Germany). 4-tert-Butyl-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2'-bipyrimidin-4-yl]-benzenesulfonamide (bosentan) sodium salt and 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone (DFU) were generous gifts from Actelion (Allschwil, Switzerland) and from MSD Sharp & Dohme (Haar, Germany), respectively. To exclude artifacts due to potential impurities, different batches of YC-1 from two unrelated manufacturers (Alexis and Sigma) were used in this study with identical results.

Results

YC-1 constricted human saphenous veins

HSV rings treated with YC-1 (3 μmol L^{-1}) developed tonic contractions after a lag

phase and did not return to their resting tensions even after repeated wash cycles (Figure 1A). Rings precontracted with YC-1 still responded to norepinephrine, and the NO donor sodium nitroprusside (50 µmol L^{-1}) relaxed them completely. Contractions persisted for at least 2 h (not shown). Some vessels developed low frequency oscillations (Figure 1B). The median lag phase between YC-1 administration and the onset of contractions was 16.1 min (6.0 to 24.8 min). The intensity of contractions showed a large inter-patient variability (Figure 1C).

Differentiation of relaxing and constricting effects of YC-1

Preconstricted vessel rings were relaxed by a concentration series of YC-1 (Figure 2). The relaxing effect was noticeable at concentrations as low as 100 nmol L^{-1} YC-1, although the highest dose did not relax the rings completely. A single dose of the NO donor SNP (10 nmol L^{-1}) prior to the YC-1 concentration series sensitized the vessels towards YC-1 and increased the relaxing effect. SNP relaxed the vessels completely, with visible effects starting at 10 nmol L^{-1}.

To demonstrate vasodilating and vasoconstricting effects of YC-1 in the same vessel, the sGC inhibitor ODQ (10 mmol L^{-1}) was added to HSV before recording YC-1 dose-response curves (Figure 3). In the presence of ODQ, the vessels responded significantly stronger to 10 and 30 µmol L^{-1} YC-1 ($p=0.012$, ANOVA, $n=6$), indicating that the dilating but not the constricting action of YC-1 can be suppressed by inhibiting cGMP synthesis.

Requirement of vascular endothelium

Denuding significantly attenuated, but did not abolish completely, HSV responses to

YC-1 (ANOVA, $p<0.001$, n=8, Figure 4).

Dependence on calcium influx

To assess the participation of voltage-gated calcium channels ($Ca_v1.2$), YC-1 dose-response curves were recorded in the presence and absence of nifedipine (1 µmol L^{-1}). In the absence of inhibitors, HSV tensions reached a maximum between 3 and 10 µmol L^{-1} YC-1 with an EC_{50} of 1.00 µmol L^{-1} ($\log(EC_{50}) = -6.00\pm0.08$). Nifedipine prevented constrictions by YC-1 of HSV completely (ANOVA, $p<0.001$, n=5, Figure 5). Vasoconstrictions induced by YC-1, but not those induced by norepinephrine, were also suppressed in the absence of external Ca^{2+} (not shown).

Involvement of endothelin

The ET_A receptor antagonist BQ-123 (3 µmol L^{-1}) and the endothelin-converting-enzyme inhibitor phosphoramidon (10 µmol L^{-1}) did not affect YC-1 dose-response curves, whereas the mixed ET_A/ET_B receptor antagonist bosentan (3 µmol L^{-1}) and the ET_B receptor antagonist BQ-788 (0.5 µmol L^{-1}) significantly lowered contractile responses towards YC-1 (Figure 5, ANOVA, $p<0.001$, n=6-7), resulting in maximum tensions of 14.6% and 32.0% of YC-1 without inhibitors, respectively.

HSV rings were incubated in the presence of different YC-1 concentrations. ET-1 accumulation in the baths increased with the YC-1 concentration (Figure 6, ANOVA, $p=0.032$, n=7).

Participation of COX-derived prostanoids

Constrictions induced by 3 µmol L^{-1} YC-1 were partially suppressed by 10 µmol L^{-1} of

the COX-1/COX-2 inhibitor indometacin and completely suppressed by 50 μmol L^{-1} indometacin (Table 2, ANOVA, $p<0.001$, n=4-6). In contrast, administration of 1 μmol L^{-1} of the COX-2 specific inhibitor DFU did not affect YC-1 induced contractions.

The TP receptor antagonist SQ 29,548 (0.3 μmol L^{-1}) suppressed YC-1-induced constrictions completely (Figure 5, ANOVA, $p<0.001$, n=6).

Evidence of endothelial dysfunction

L-NAME, an inhibitor of nitric oxide synthases, did not alter basal tones of HSV significantly (control: 1.01±2.12 mN vs. L-NAME 100 μmol L^{-1}: -0.43±2.17 mN) or their responses to 1 μmol L^{-1} norepinephrine (control: 51.78±15.78 mN vs L-NAME: 56.53±15.42 mN).

Other vessel types

HRA was used to determine whether YC-1 induced vasoconstrictions are a particular feature of venous vessels of CAD patients. HUV were used as readily available human control vessels. YC-1 was used at 3 μmol L^{-1} as there was no noticeable contribution of the relaxing effect at this dose (cf. Figure 3). YC-1 contracted HRA but not HUV (Table 3, ANOVA, $p<0.001$, n=3-6).

Comment

YC-1 has a variety of molecular targets other than sGC [12-16]. Nevertheless, it was prudent to determine whether sGC is involved in YC-1's vasoconstrictor action. The sGC inhibitor ODQ [17] allowed to separate the vasoconstrictor and vasodilator ac-

tions. The former is sGC-independent with a calculated EC_{50} of 1.0 µmol L^{-1} whereas the latter, sGC-dependent action is noticeable only at higher doses. This agrees well with published data of YC-1 induced relaxations of animal vessels [18], although HRA relaxation by YC-1 was reported to have an EC_{50} of approx. 5 nmol L^{-1} [19]. This may be due to a higher level of endogenous NO in HRA compared to HSV, which enhances sGC stimulation by YC-1 [9].

YC-1 induced relaxations were immediate, whereas constrictions started after approx. 15 minutes and developed fully within 60 min. These properties suggest a multistep signal transduction mechanism. As YC-1 induced constriction could not be washed out but were terminated by sodium nitroprusside, the participation of a long-lasting vasoconstrictor appears likely.

Denudation experiments indicated that vasoconstrictions were endothelium-dependent, although a minor contribution of an endothelium-independent mechanism cannot be excluded. Constrictions induced by YC-1, but not those induced by norepinephrine, were inhibited by blocking L-type voltage-gated calcium channels ($Ca_v1.2$). Agonist-induced calcium entry in non-excitable cells depends either on non-voltage-gated calcium channels, or on receptor-activated calcium entry [20]. Therefore YC-1 apparently triggers release or synthesis of an endothelium-derived vasoconstrictor which depolarizes smooth muscle via $Ca_v1.2$ channels.

ET-1 has unique properties that match the characteristics of YC-1 induced contractions. ET-1 is endothelium-derived and elicits contractions of vascular smooth muscle which persist long after the substance has been removed from the bath [21]. ET_A receptors of smooth muscle induce the phospholipase C pathway without requir-

ing calcium influx [22]. As YC-1 induced contractions were attenuated by nifedipine, but not by antagonizing ET-1_A, direct stimulation of smooth muscle by ET-1 appears unlikely. However, ET-1 release increased after YC-1 administration, and both bosentan (ET_A/ET_B inhibitor) and BQ-788 (ET_B inhibitor) attenuated contractile responses. This indicates that YC-1 triggers endothelial ET-1 release, which acts in a paracrine fashion on ET_B receptors. As the ECE antagonist phosphoramidon [23] did not affect constrictions, ET-1 is apparently released from storage vesicles. Saunders and Scheiner-Bobis [24] reported that the cardiac glycoside ouabain induces the release of ET-1 from endothelial cells within several minutes [25]. The "receptor", if any, which is responsible for transmitting this effect of ouabain has not been identified to date, but a similar receptor may be involved in YC-1 induced constrictions.

The non-specific COX inhibitor indometacin suppressed YC-1 induced contractions, in contrast to the COX-2 specific inhibitor DFU. Therefore, COX-1 is involved in the synthesis of constricting prostanoids. Prostanoids act on smooth muscle via prostanoid receptors [26]. YC-1 induced constrictions were completely abolished by the TP receptor antagonist SQ 29,548, further corroborating the notion that vasoconstrictor prostanoid release is essential. In rat and canine arteries thromboxane A_2 induced activation of TP receptors requires an influx of external Ca^{2+} and is therefore affected by blocking voltage gated calcium channels [27-29]. The sensitivity of YC-1 induced contractions to the calcium channel blocker nifedipine thus further supports the assumption that TP receptors are involved.

Although our data demonstrate the participation of the above mentioned signalling pathway components, further work is required to unequivocally prove the sequence

of events suggested in Figure 7. However, a strikingly similar mechanism has been suggested to explain the physiological abnormalities in spontaneously hypertensive rats [30] which serve as a model of human hypertension caused by endothelial dysfunction. Healthy vessels from several species were reported to contract to the NOS inhibitor L-NAME in vitro [31] due to basal NO release. In contrast, basal HSV tones in the present study were not affected by L-NAME, and these vessels responded only weakly to endothelium-dependent vasodilators (data not shown), demonstrating endothelial dysfunction. ET binding to ET-1_B receptors on endothelial cells induces vasorelaxation in healthy vessels [32], although a constricting mechanism was demonstrated in rat vessels [33]. This constricting mechanism becomes dominant under pathological conditions, with ET-1 causing endothelium-dependent vasoconstrictions that involve the release of endothelium-derived contracting factors (EDCF) [34]. Aging, diabetes, and hypertension contribute to the shift of the endothelium to this constricting role [35,36,30]. Both the patients' characteristics and the manifest endothelial dysfunction are in line with the presence of a constricting endothelial phenotype in our study subjects. COX-derived prostanoids constitute a major part of EDCF [37]. Prostanoids act on smooth muscle via prostanoid receptors [26]. TP receptors have been shown to mediate vasoconstrictions by ET-1 in spontaneously hypertensive rats but not in normotensive control animals [38]. There is in vivo evidence that the same mechanism operates in humans suffering from endothelial dysfunction [39]. The present study is the first to demonstrate this mechanism in isolated human vessels of CAD patients. Due to the elevated ET-1 plasma levels in CAD patients [40] this mechanism may contribute to vasospasm in coronary arteries and by-

pass grafts.

The current study has several limitations. Vasoactive medication used by the patients prior to surgery may influence contractile responses, although preliminary analysis did not reveal correlations with any of the patients' characteristics or medications. Specifically, the vessels obtained from the few patients which lacked the common risk factors of atherosclerosis responded to YC-1 as well. The current study was necessarily limited by the specificity of the inhibitors. The release of constricting prostanoids should be further investigated in cell culture models, but this was beyond the scope of this initial study.

In summary, this study has shown that the vasodilator YC-1 constricts saphenous veins and radial arteries from patients with severe CAD. These vasoconstrictions are mediated by a paracrine action of endothelium-derived ET-1 which causes synthesis of vasoconstrictor prostanoids via COX-1. These prostanoids contract vascular smooth muscle via TP receptors. YC-1 induced contractions in vessels with endothelial dysfunction and endothelium-dependent contractions observed in a rat model of hypertension share major parts of their signalling pathways which may constitute a novel mechanism of coronary artery and graft vasospasm.

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Conflicts of Interest

None

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Table 1: Patient characteristics

characteristic	**value**
age (yr) - mean±S.D (range)	68.3±7.9 (48 - 87)
male – No / %	138 / 87.9
female - No / %	19 / 12.1
body mass index - mean±S.D (range)	27.6±3.8 (19.0 - 36.7)
ACE inhibitors – No / %	101 / 64.3
beta-adrenergic antagonists – No / %	116 / 73.9
calcium antagonists – No / %	23 / 14.6
organic nitrates – No / %	18 / 11.5
NYHA class I – No / %	2 / 1.3
NYHA class II – No / %	38 / 24.2
NYHA class III – No / %	106 / 67.5
NYHA class IV – No / %	3 / 1.9
angina pectoris – No / %	83 / 52.9
acute myocardial infarction < 90 d before surgery – No / %	14 / 8.9
type 2 diabetes – No / %	49 / 31.2
hypertension – No / %	132 / 84.1
hyperlipidemia – No / %	99 / 63.1
current or previous smoking – No / %	79 / 50.3

Table 2: Effects of COX inhibition on YC-1 induced contractions of HSV

	YC-1 3 µmol L^{-1}	norepinephrine 1 µmol L^{-1}
control	31.52±15.39	83.59±31.41
indomethacin 10 µmol L^{-1}	19.81±26.01	86.04±27.89
indomethacin 50 µmol L^{-1}	-0.54±1.53[a]	75.97±39.97
DFU 1 µmol L^{-1}	18.79±17.37	76.48±25.77

[a]significantly different from control (ANOVA, $p < 0.001$, n=4-6)

Table 3: Comparison of vessel types

	YC-1 (% of KCl)	norepinephrine/serotonin (% of KCl)
HSV (n=6)	113.32±80.92 [a]	219.94±46.42 (norepinephrine)
HRA (n=3[b])	40.51±23.96	127.33±27.09 (norepinephrine)
HUV (n=6)	0.19±4.68	180.93±44.38 (serotonin)

Maximum responses of vessels to YC-1 (3 µmol L^{-1}) and to receptor-mediated vasoconstrictors (1 µ mol L^{-1}). Values are reported as percentage of the constrictions obtained with 150 mmol L^{-1} KCl. [a] significantly different from HUV (ANOVA, $p<0.001$). [b] not included in statistical tests due to low sample size. All three tested vessels contracted to YC-1.

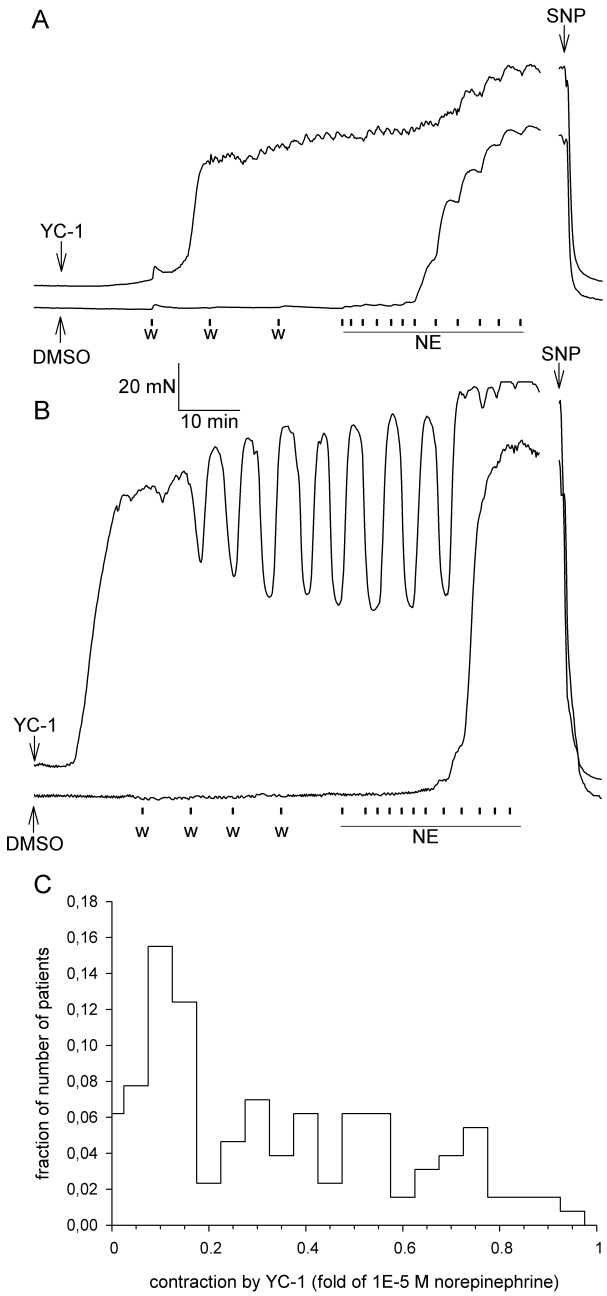

Figure 1: YC-1 induced contractions of HSV and HRA. (**A**) Representative organ bath tracing showing the effects of 3 µmol L^{-1} YC-1 (upper tracing) and the solvent control (lower tracing; DMSO, dimethyl sulfoxide) on HSV. Arrows indicate time of administration. w, washing; NE, norepinephrine concentration series from 1×10^{-11}

mol L^{-1} to 3×10^{-6} mol L^{-1} ; SNP, 50 µmol L^{-1} sodium nitroprusside added to both rings. (**B**) Same as (A) using HRA. Note the spontaneous oscillations in this example. (**C**) Distribution of maximum tension induced by 3 µmol L^{-1} YC-1 in the patient pool. Constrictions by both YC-1 and NE were determined in HSV from 134 patients.

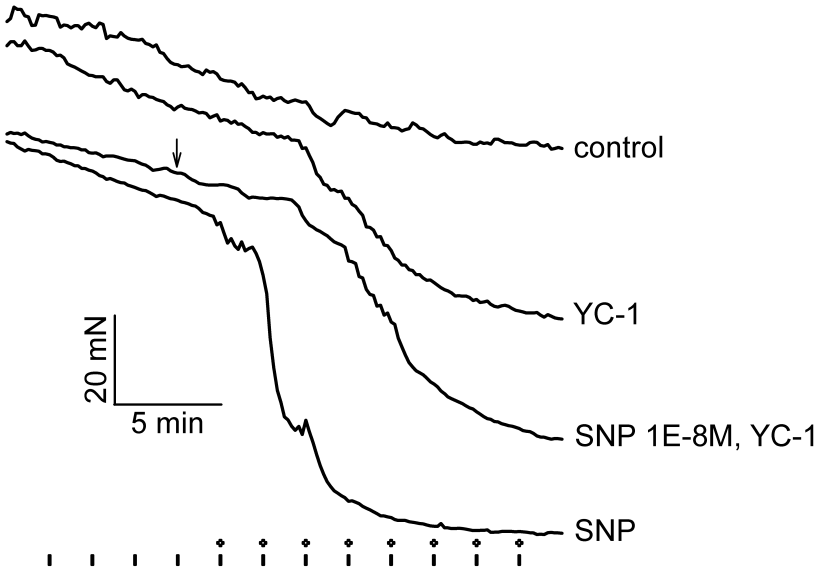

Figure 2: Organ bath tracings of YC-1 induced relaxations of HSV preconstricted with NE. Arrow indicates addition of 1×10^{-8} mol L^{-1} SNP. Vertical ticks indicate SNP dose-response curve from 1×10^{-10} mol L^{-1} to 3×10^{-5} mol L^{-1}. Crosses indicate YC-1 dose-response curve from 1×10^{-8} mol L^{-1} to 3×10^{-5} mol L^{-1}. Tracings are representative for 5 independent experiments.

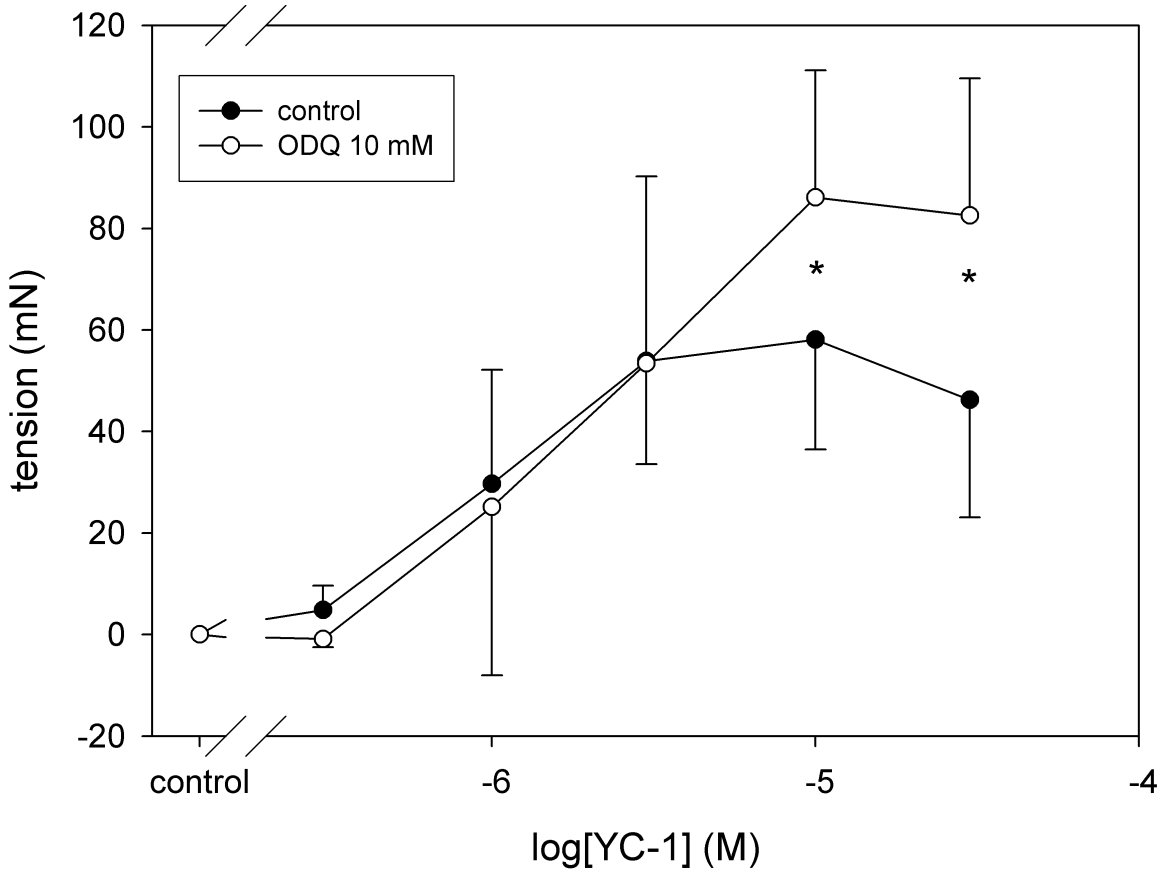

Figure 3: Differentiation of relaxing and constricting effects of YC-1. HSV were constricted with increasing doses of YC-1 in the absence (filled circles) or presence (open circles) of the sGC inhibitor ODQ (10 mmol L^{-1}). *, significantly different (p=0.012, ANOVA, n=6)

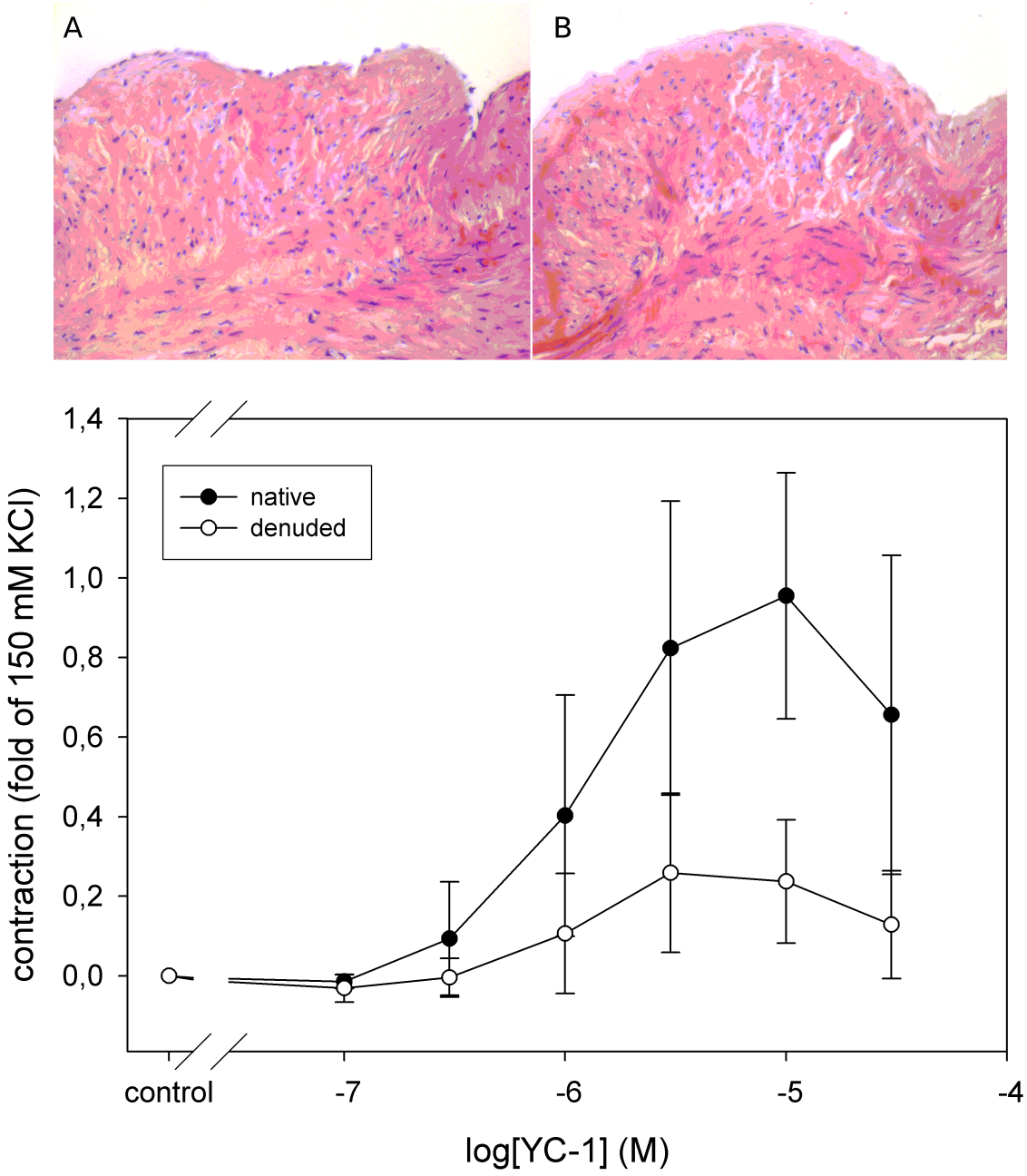

Figure 4: Dependence of YC-1 induced contractions on endothelium. YC-1 dose-response curves of native (filled circles) and endothelium-denuded HSV (open circles). Curves are significantly different ($p<0.001$, ANOVA, n=8). Inserts A and B show representative H&E-stained cross sections of native and denuded HSV, respectively.

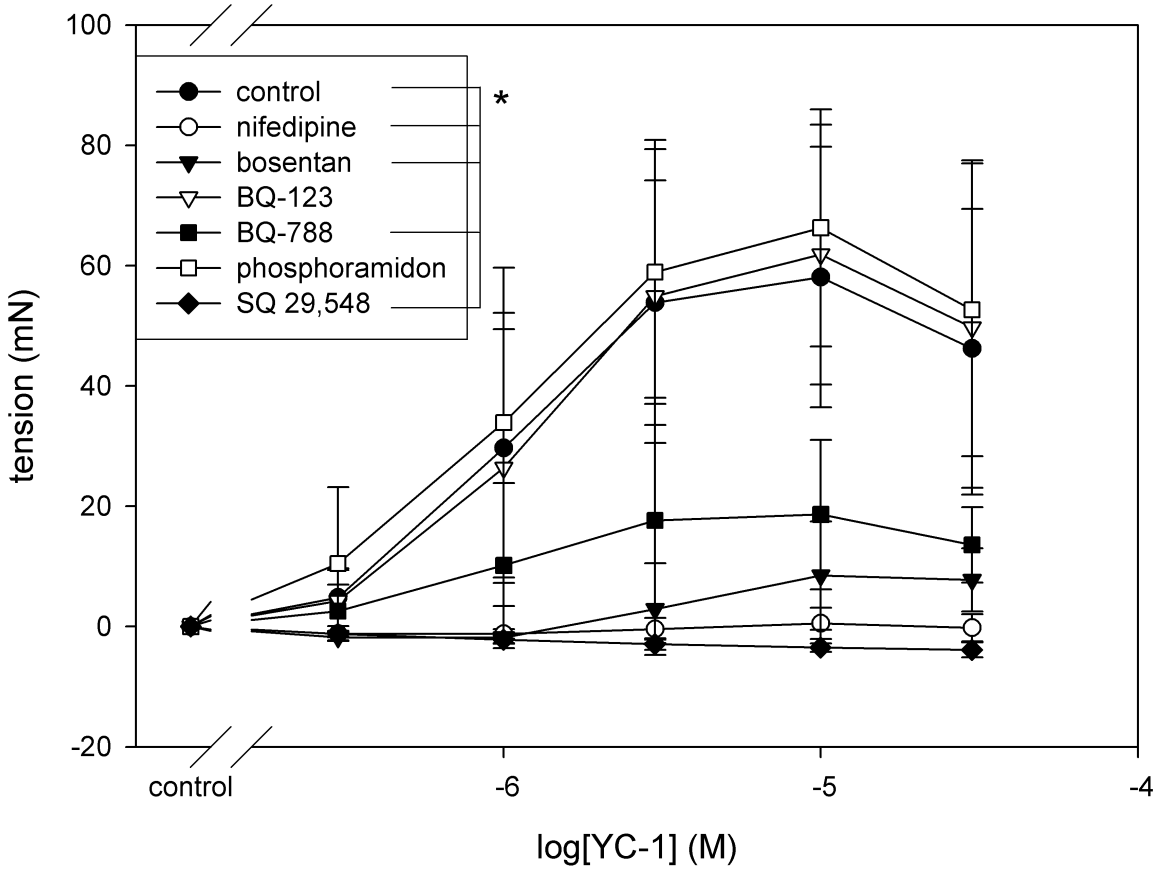

Figure 5: Effects of calcium channel, endothelin receptor, ECE converting enzyme, and TP receptor antagonists on YC-1 induced contractions in HSV. Cumulative YC-1 dose-response curves (control: filled circles) were constructed after administering nifedipine (1 µmol L^{-1}, open circles), bosentan (10 µmol L^{-1}, filled triangles), BQ-123 (3 µmol L^{-1}, open triangles), BQ-788 (0.5 µmol L^{-1}, filled squares), phosphoramidon (10µmol L^{-1}, open squares), and SQ 29,548 (0.3µmol L^{-1}, filled diamonds). * significantly different from control (ANOVA, $p<0.001$, n=6-7).

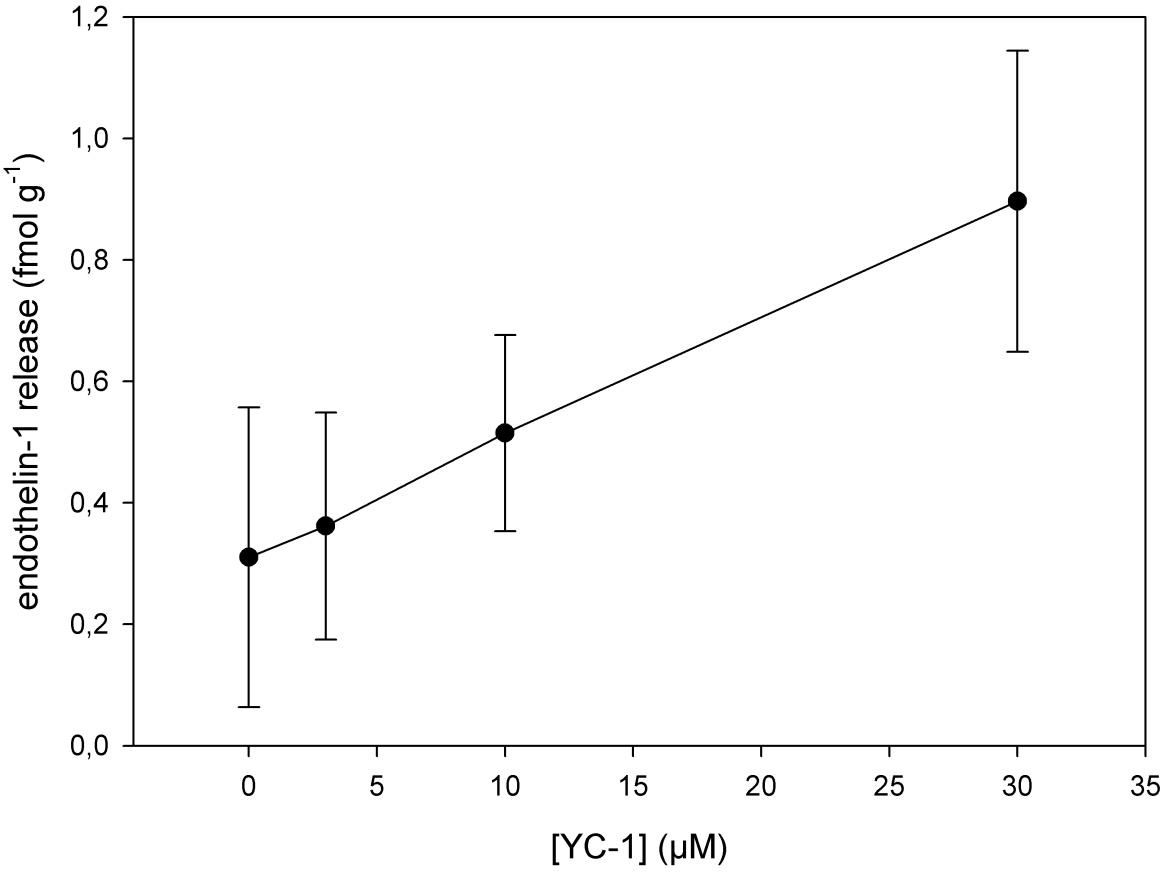

Figure 6: Endothelin-1 release from HSV rings in organ baths after administering YC-1 for 60 min. Release changed significantly with YC-1 concentration (ANOVA, p=0.032, n=7).

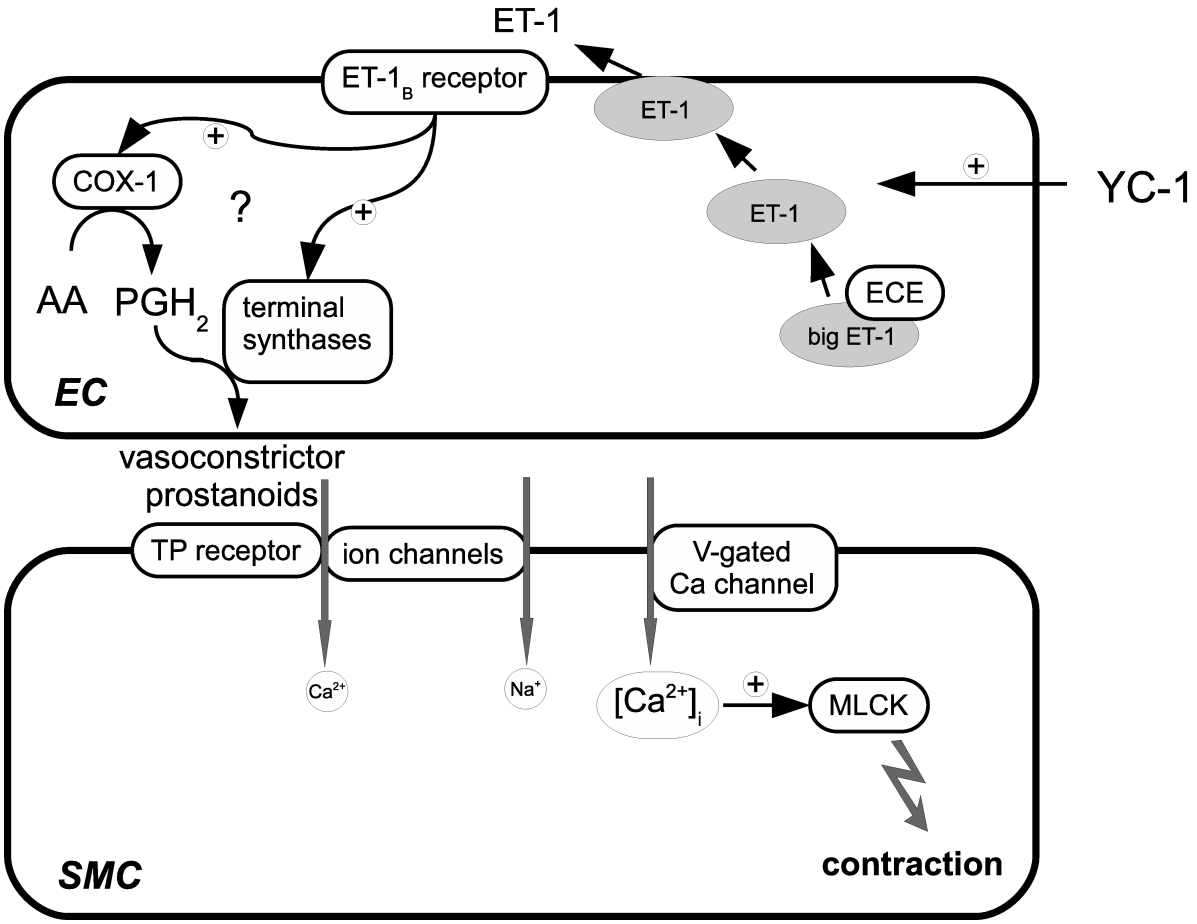

Figure 7: Putative mechanism of YC-1 induced contractions. AA, arachidonic acid; COX, cyclooxygenase; EC, endothelial cell; ECE, endothelin-converting enzyme; ET-1, endothelin-1; MLCK, myosin light chain kinase; PG, prostaglandin; SMC, smooth muscle cell.