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Activation of endothelial nitric oxide synthase by the pro-apoptotic drug embelin: Striking discrepancy between nitric oxide-mediated cyclic GMP accumulation and L-citrulline formation

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Abstract

The benzoquinone derivative embelin is a multifunctional drug that not only induces apoptosis by inhibiting XIAP, the X chromosome-linked inhibitor of apoptosis protein, but also blocks nuclear factor- κ B signaling pathways, thereby leading to down-regulation of a variety of gene products involved in tumor cell survival, proliferation, invasion, angiogenesis, and inflammation. Here, we report that embelin activates and modulates L-arginine/nitric oxide/cyclic GMP signaling in cultured endothelial cells. Embelin elicited a rapid increase of intracellular free Ca^{2+} , leading to activation of endothelial nitric oxide synthase (eNOS) and NO-induced cGMP accumulation. While the cGMP response was comparable to that caused by other Ca^{2+} -mobilizing agents, the stimulatory effect of embelin on L-citrulline formation (~4-fold) was substantially lower than that observed upon activation of eNOS with the Ca^{2+} ionophore A23187 (~18-fold), the receptor agonist ATP (~16-fold) or the sarco-endoplasmic reticulum Ca^{2+} -ATPase inhibitor thapsigargin (~14-fold). The apparent discrepancy between NO/cGMP and L-citrulline formation in embelin-treated cells was not due to enhanced metabolism and/or efflux of L-citrulline, increased NO bioavailability, inhibition of cGMP hydrolysis, sensitization of soluble guanylate cyclase (sGC) to NO, or enhanced formation of a sGC/eNOS complex. Our puzzling observations suggest that embelin improves coupling of endothelial NO synthesis to sGC activation through mobilization of an as yet unrecognized signaling pathway.

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Keywords

Embelin; Endothelial nitric oxide synthase; L-Citrulline; Nitric oxide; Soluble guanylate cyclase; cGMP

Introduction

Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone) has been shown to exhibit a variety of biological activities including antibacterial [1,2], antihelminthic [3,4], antifertility [5–7], analgesic [8,9], antiinflammatory [8] and antitumor [8,10,11] effects. While the precise mechanisms underlying most of these effects are unclear, recent evidence indicates that the antitumor activity can be attributed to the ability of embelin to bind to and inhibit XIAP,1 thereby inducing activation of caspase-3, -7 and -9, and apoptosis [12]. Accordingly, embelin selectively inhibits cell growth in cancer cells expressing high levels of XIAP and is considered as a promising lead compound for the design of new anticancer drugs [12].

In addition to blocking cell death in cancers, XIAP and other members of the inhibitor of apoptosis protein family are important survival factors in non-cancer cells and participate in a variety of signal transduction cascades, including nuclear factor- κ B, transforming growth factor- β and c-Jun N-terminal kinase signaling pathways [13–16]. At least for XIAP it has been demonstrated that most of the signaling properties of this protein are unrelated to caspase inhibition [17], suggesting a multifunctional role of XIAP in regulating cellular events. One of these events may be activation of eNOS due to direct interaction of XIAP with caveolin-1, resulting in a disruption of the inhibitory eNOS/caveolin-1 complex [18]. Considering that both caveolin-1 and embelin bind to the BIR3 domain of XIAP [12,18], we expected that embelin will prevent eNOS activation through interference with caveolin-1 binding to XIAP. Surprisingly, however, we observed the opposite effect, i.e. substantial activation of eNOS-mediated cGMP accumulation. Our attempts to clarify the molecular mechanisms underlying this unexpected effect of embelin revealed a striking discrepancy between eNOS-mediated cGMP accumulation and L-citrulline formation.

Experimental procedures

Materials

Culture media, antibiotics and fetal calf serum were purchased from PAA Laboratories (Linz, Austria). L-[2,3- 3 H]Arginine hydrochloride (1.5–2.2 TBq/mmol) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA) and purified as described earlier [19]. [α - 32 P]GTP (30 TBq/mmol) was from Perkin Elmer Life and Analytical Sciences (Boston, MA, USA). DEA/NO was obtained from Alexis Corporation (Lausen, Switzerland) and dissolved and diluted in 10 mM NaOH. Fura-2 acetoxymethyl ester was from Molecular Probes Europe BV (Leiden, The Netherlands) and dissolved in DMSO. Protein A/G agarose

¹ *Abbreviations used:* BAECs, bovine aortic endothelial cells; BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; cGMP, 3',5'-cyclic monophosphate; DEA/NO, 2,2-diethyl-1-nitroso-oxyhydrazine; eNOS, endothelial nitric oxide synthase; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazine; HSP90, heat shock protein 90; IBMX, 3-isobutyl-1-methylxanthine, NO, nitric oxide; PAECs, porcine aortic endothelial cells; sGC, soluble guanylate cyclase; XIAP, X chromosome-linked inhibitor of apoptosis protein.

was from Santa Cruz Biotechnology, Santa Cruz, CA, USA, anti-eNOS antibody from BD Biosciences San Diego, CA, USA, anti-sGC β_1 antibody from Cayman Chemical, Ann Arbor, MI, USA, and Super Signal West Pico Chemiluminescent Substrate from Pierce Chemical, Rockford, IL, USA). Purified bovine lung sGC [20] was kindly provided by Drs. M. Russwurm and D. Koesling, Department of Pharmacology and Toxicology, University Bochum, Germany. All other chemicals were from Sigma-Aldrich (Vienna, Austria).

Cell culture

Porcine aortic endothelial cells (PAECs) were isolated as described previously [21] and cultured at 37 °C, 5% CO₂, for up to three passages in Dulbeccó's modified Eagle's medium, containing 10% (v/v) heat-inactivated foetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 1.25 µg/ml amphotericin B. Rat lung fibroblasts (RFL-6 cells) were obtained from ATCC (CCL-192) and cultured in Ham's F-12 medium (37 °C, 5% CO₂), containing 20% (v/v) heat-inactivated fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 1.25 µg/ml amphotericin B. Bovine aortic endothelial cells (BAECs) were purchased from VEC Technologies, Inc., Rensselaer, NY, USA and cultured in medium 199 supplemented with 10% foetal calf serum, 5% iron-supplemented calf serum, 0.6 µg/ml thymidine, 2.2 mg/ml sodium bicarbonate, 500 U/ml penicillin, and 0.5 mg/ml streptomycin.

Determination of endothelial cGMP formation

Accumulation of intracellular cGMP was determined as previously described [22]. Briefly, PAECs 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 MgCl₂, 2.5 mM CaCl₂, 1 mM IBMX, and 1 µM indomethacin. Reactions were started by addition of the compounds to be tested and terminated at the time points indicated in figures and legends 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 endothelial L-[³H]citrulline formation

Intracellular conversion of L-[³H]arginine into L-[³H]citrulline was measured as previously described [22]. Briefly, PAECs grown in 6-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 MgCl₂ and 2.5 mM CaCl₂. Reactions were started by addition of L-[2,3-³H]arginine (~ 10⁶ dpm) and the compounds to be tested and terminated at the time points indicated in figures and legends by washing the cells with chilled incubation buffer. Subsequent to lysis of the cells with 0.01 N HCl, an aliquot was removed for determination of incorporated radioactivity. To the remaining sample, 200 mM sodium acetate buffer (pH 13.0) containing 10 mM L-citrulline was added (final pH ~5.0), and L-[³H]citrulline separated from L-[³H]arginine by cation exchange chromatography [22].

Determination endothelial L-citrulline levels

Based on a previously published rapid and precise HPLC/mass spectrometry method for determination of L-citrulline in blood and urine samples [23], we used a newly developed method for analysis of endothelial L-citrulline levels. Incubation conditions were identical to

those described above for the determination of intracellular L-[³H]citrulline formation with the exception that no radioactive L-arginine was added. Cells were washed with chilled incubation buffer, harvested, suspended in 0.5 ml of distilled water and lysed by sonication. Lysates were mixed with 1 ml of acetonitrile, precipitated proteins removed by centrifugation (15 min at 3000g), and supernatants stored at -80 °C until analysis. After thawing, 50 µl of the internal standard solution (0.9 µM homoarginine) was added to each sample and the solvent evaporated at 40 °C in vacuum. Residues were re-dissolved in 100 µl of water for HPLC analysis. Prior to injection, 50-µl aliquots of the samples were automatically derivatized with 25 µl of 200 mM boric acid buffer, pH 9.5, containing 37.3 mM α -phthalaldehyde, 68 mM 2-mercaptoethanol, and 10% (v/v) ethanol. The derivatization reaction was complete within seconds, producing the corresponding isoindoles of the amino acids L-citrulline and L-homoarginine [24]. Chromatographic separation of the analytes was accomplished by gradient elution. Solvent A consisted of 0.1% formic acid and 0.1% ammonium formate in water, resulting in a pH of 3.5. Solvent B was pure methanol. Elution was started with 30% solvent B that was linearly increased to 50% within 20.5 min. Strongly retained substances were then eluted with 85% solvent B. Recording of chromatograms was terminated after 27 min, followed by post-run of 5 min to re-equilibrate the column. The flow rate was 0.7 ml/min, but was lowered to 0.5 ml/min from 12.5 to 20.5 min. Column temperature was set to 40 °C. Under these conditions, the retention times for the derivatives of L-citrulline and L-homoarginine were 18.6 and 19.1 min, respectively. The analytes were detected by electrospray ionization-mass spectrometry. The electrospray ion source operated in the positive ion mode, producing molecular ions of a mass/charge ratio of 352 and 365 for the derivatives of L-citrulline and the internal standard L-homoarginine, respectively. From the recorded mass spectra, these ion traces were extracted for subsequent peak integration and quantification. The limit of detection was 5 µM and the intra-day coefficient of variation was found to be 5.2%.

In some samples, L-citrulline levels were also measured by a classical α -phthalaldehyde precolumn derivatization HPLC method with fluorescence detection [25], using a selective solid phase extraction procedure and a novel internal standard (N⁶-carbamoyl-DL-lysine). The method yielded virtually identical results as the HPLC-MS procedure and will be described in detail elsewhere. All L-citrulline data are expressed as intracellular concentrations, assuming a cell volume of 1.8 pl [26].

Fluorescence measurements

Intracellular free Ca²⁺ was determined using the fluorescence probe fura-2 as previously described [27]. Briefly, PAECs from one petri dish (diameter, 90 mm) were harvested, suspended in 5 ml culture medium and incubated with 2 µM fura-2 acetoxymethyl ester at 37 °C. After 45 min, cells were washed, resuspended in 50 mM Tris buffer, pH 7.4, containing 100 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and either 2.5 mM CaCl₂ (incubation buffer) or 0.1 mM EGTA (nominally Ca²⁺-free incubation buffer), transferred into a thermostated cuvette, and fluorescence measured at excitation wavelengths of 340, 380, and 510 nm emission.

Intrinsic fluorescence of A23187 was measured in a thermostated cuvette containing 50 mM Tris buffer, pH 7.4, 100 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2.5 mM CaCl₂, 1 μ M A23187, and where indicated 0.1 mM embelin. The excitation wavelength was set to 308 nm and the emission wavelengths were scanned from 320 to 600 nm.

Determination of eNOS activity

Enzymatic activity of purified eNOS was determined by monitoring either the conversion of L-[³H]arginine into L-[³H]citrulline or NO formation as previously described [28,29]. For measuring L-[³H]citrulline formation, purified eNOS (100 ng) was incubated for 10 min at 37 °C in 0.1 ml of 50 mM triethanolamine/HCl buffer, pH 7.4, containing 0.1 mM L-[2,3-³H]arginine (~200,000 dpm), 0.5 mM CaCl₂, 0.2 mM NADPH, 10 μ M tetrahydrobiopterin, 5 μ M FAD, 5 μ M FMN, 10 μ g/ml calmodulin, 0.2 mM CHAPS, and the compound(s) to be tested. Subsequent to the addition of 1 ml of chilled 20 mM sodium acetate buffer containing 1 mM L-citrulline, L-[³H]citrulline was separated from L-[³H]arginine by cation exchange chromatography [28]. In parallel experiments, NO formation was monitored with a Clark-type electrode (Iso-NO, World Precision Instruments, Berlin, Germany) upon addition of calmodulin (10 μ g/ml) to 1 ml of 50 mM triethanolamine/HCl buffer, pH 7.4, containing 10 μ g purified eNOS, 1 mM L-arginine, 0.5 mM CaCl₂, 0.2 mM NADPH, 10 μ M tetrahydrobiopterin, 5 μ M FAD, 5 μ M FMN, 1000 U/ml superoxide dismutase, 0.2 mM CHAPS, and test compounds as indicated in text and figures.

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 mM triethanolamine/HCl buffer, pH 7.4, containing 0.5 mM [α -³²P]GTP (~300,000 cpm), 3 mM MgCl₂, 1 mM cGMP, 2 mM dithiothreitol, 10 μ M DEA/NO, and test compounds as indicated in text and figures. Reactions were terminated by ZnCO₃ precipitation, and ³²P-cGMP was isolated by column chromatography as described previously [30].

Determination of oxygen consumption

PAECs were harvested, resuspended in culture medium (~10⁶ cells/ml) and placed into the 2 ml glass-chambers of a two-channel high-resolution respirometer (Oroboros *Oxygraph*, Innsbruck, Austria). Air-saturated buffer was used for calibration of the polarographic oxygen sensors [31]. Mitochondrial oxygen consumption was measured at 37 °C upon addition of the compounds to be tested.

Immunoprecipitation and immunoblotting

BAECs were serum-starved overnight and then either not treated (control) or treated with embelin (0.1 mM) or bradykinin (1 μ M) for 2 min. Cells were lysed in ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.4, 100 mM NaF, 15 mM Na₄P₂O₇, 1 mM Na₃VO₄, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged at 10,000 *g* to remove insoluble material and were precleared by adding 50 μ l of protein A/G agarose followed by incubation for 2 h at 4 °C with rocking. The agarose beads were then pelleted by centrifugation at 1000 *g*. About 10 μ l of anti-eNOS antibody was added to the precleared

lysates and samples were incubated overnight at 4 °C with rocking. Protein A/G agarose was then added and samples were incubated for an additional 4 h at 4 °C. Beads were subsequently washed three times in ice-cold TBS wash buffer. Immunoprecipitated proteins were eluted from the beads by boiling for 5 min in SDS sample buffer. The supernatants were subjected to SDS polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and immunoblotted with anti-sGC β_1 antibody (1:1000 dilution). Bound antibody was visualized using Super Signal West Pico Chemiluminescent Substrate after exposure of the blot to peroxidase-conjugated secondary antibody.

Results

Embelin triggers NO-induced cGMP formation

Accumulation of cGMP in PAECs was monitored as measure for NO formation. As shown in Fig. 1A, embelin increased intracellular cGMP levels within 10 min from 6.8 ± 1.2 to 58.2 ± 6.1 pmol cGMP/ 10^6 cells. The maximal effect occurred at an embelin concentration of ~ 0.3 mM and was comparable to that elicited by stimulation of eNOS with a maximally active concentration of the Ca^{2+} ionophore A23187 ($0.3 \mu\text{M}$, 51.2 ± 8.9 pmol cGMP/ 10^6 cells) or direct activation of sGC with a maximally active concentration of the NO donor DEA/NO ($1 \mu\text{M}$, 73.5 ± 12.6 pmol cGMP/ 10^6 cells). The effect of embelin closely resembled that of A23187 in that it was completely blocked by the eNOS inhibitor L-NNA (0.3 mM) and the NO scavenger oxyhemoglobin (0.1 mM, Fig. 1B). Superoxide generated by hypoxanthine (1 mM)/xanthine oxidase (10 mU/ml) or FMN (0.1 mM), as well as chelating of extracellular Ca^{2+} by EGTA (0.1 mM) also markedly reduced the effect of both, embelin and A23187 (Fig. 1B). Activation of sGC by DEA/NO was not affected by L-NNA (71.0 ± 13.9 pmol cGMP/ 10^6 cells) or EGTA (72.7 ± 5.1 pmol cGMP/ 10^6 cells), but abolished by oxyhemoglobin, hypoxanthine/xanthine oxidase, and FMN (2.7 ± 1.1 , 5.0 ± 1.2 , and 3.5 ± 1.4 pmol cGMP/ 10^6 cells, respectively). Together with findings showing that cGMP formation in cells lacking NO synthase (such as RFL-6 rat lung fibroblast or HEK 293 human embryonic kidney cells) was stimulated by DEA/NO but not by embelin or A23187 (K. Schmidt and B. Mayer, unpublished work), these data clearly indicate that eNOS-derived NO accounts for embelin-triggered cGMP formation in endothelial cells.

Embelin promotes Ca^{2+} influx

Based on the EGTA data suggesting Ca^{2+} as trigger for eNOS activation by embelin, we investigated the effect the drug on $[\text{Ca}^{2+}]_i$ using the fluorescence dye fura-2. As shown in Fig. 2A, stimulation of PAECs with $10 \mu\text{M}$ embelin in the presence of extracellular Ca^{2+} (2.5 mM) evoked a continuous rise in $[\text{Ca}^{2+}]_i$. The sharp decrease in the signal observed immediately after addition of embelin was due to a quench of the fluorescence signal at 340 nm. At 0.1 mM embelin (Fig. 2B), both quench and Ca^{2+} -elicited increase in the fluorescence ratio ($\sim 1.2/\text{min}$ vs. $\sim 0.4/\text{min}$ with $10 \mu\text{M}$ embelin) were more pronounced. Due to the quench, reliable quantification of the intracellular Ca^{2+} concentration was not possible, but based on the maximum and minimum fluorescence ratio obtained upon addition of Triton X-100 and EGTA, respectively, we roughly estimated an elevation of $[\text{Ca}^{2+}]_i$ from 0.1 to 0.4 – $0.6 \mu\text{M}$ upon addition of 0.1 mM embelin. In nominally Ca^{2+} -free buffer, embelin ($10 \mu\text{M}$) did not elicit a detectable increase in the fluorescence ratio while a

pronounced Ca^{2+} signal was obtained when extracellular Ca^{2+} (2.5 mM) was subsequently added (Fig. 2C). In the absence of embelin, addition CaCl_2 was virtually ineffective (Fig. 2D). These data suggest that embelin increases $[\text{Ca}^{2+}]_i$ through influx of extracellular Ca^{2+} .

Embelin barely increases L-citrulline formation

To corroborate the cGMP data suggesting that embelin activates eNOS, we determined NOS activity as conversion of L-[^3H]arginine into L-[^3H]citrulline. As evident from Fig. 3A, embelin only slightly increased L-[^3H]citrulline levels (from 6.8 ± 1.9 to 12.3 ± 5.1 fmol/ 10^6 cells within 10 min), whereas a pronounced effect was obtained with A23187 (175 ± 74 fmol L-[^3H]citrulline/ 10^6 cells). A similar discrepancy was observed in time course studies shown in Fig. 3B. The amount of incorporated radioactivity measured after 1, 2, 3, 4, 6, and 10 min of incubation was not affected by embelin or A23187, indicating that these compounds do not interfere with the uptake/accumulation of L-[^3H]arginine (data not shown). In addition to measuring L-[^3H]arginine into L-[^3H]citrulline conversion, we also determined total intracellular L-citrulline levels to exclude unknown peculiarities of the L-[^3H]arginine assay (Fig. 3C). In agreement with the above data, we observed that A23187 caused a pronounced increase in L-citrulline formation (e.g. from 18.4 ± 2.6 to 54.6 ± 3.0 , and 92.1 ± 7.0 μM after 2 and 5 min, respectively), whereas the effect of embelin was marginal (25.6 ± 4.4 μM and 21.7 ± 4.6 μM L-citrulline after 2 and 5 min, respectively).

Correlation between cGMP and L-citrulline levels

In view of these puzzling observations, we determined the correlation between L-[^3H]citrulline and cGMP formation in response to embelin and several established eNOS activators. Upon incubation for 2 min, all agonists caused a concentration-dependent increase in the formation of cGMP (Fig. 4A) and L-[^3H]citrulline (Fig. 4B), but a plot of cGMP vs. L-[^3H]citrulline levels (Fig. 4C) revealed striking differences. In response to ATP, bradykinin and thapsigargin, a virtually linear correlation ($r = 0.974$) between cGMP and L-[^3H]citrulline levels was obtained over the whole range of the plot (from ~ 5 to ~ 50 fmol L-[^3H]citrulline corresponding to ~ 10 and ~ 55 pmol cGMP per 10^6 cells). A similar correlation cGMP vs. L-[^3H]citrulline correlation was obtained with a maximally active concentration of A23187 (55.3 ± 4.2 fmol L-[^3H]citrulline corresponding to 54.3 ± 6.5 pmol cGMP per 10^6 cells, data not shown). Due to an all-or-none response of the cells to A23187, it was not possible to establish reliable concentration–response curves with the Ca^{2+} ionophore. The effect of embelin was strikingly different from that of all other agonists tested. Again, L-[^3H]citrulline and cGMP levels showed a linear correlation ($r = 0.988$) but with about 5-fold steeper slope; maximal cGMP levels of 55 pmol/ 10^6 cells occurred at L-[^3H]citrulline levels as low as ~ 13 fmol/ 10^6 cells.

Embelin does not affect L-citrulline homeostasis

Enhanced degradation and/or enhanced extrusion of eNOS-derived L-citrulline were considered as possible explanation of our observations. To test this hypothesis, we studied the effect of embelin on recovery/metabolism of exogenously applied and endogenously formed L-citrulline. In the first set of experiments, cells were incubated for 30 min with L-[^{14}C]citrulline ($\sim 500,000$ dpm) in the absence and presence of embelin, and radioactivity determined in cell lysates and in the L-citrulline fractions eluting from cation exchange

columns. Under control conditions, 1363 ± 220 dpm/ 10^6 cells was found in cell lysates and 1058 ± 184 dpm/ 10^6 cells in the L-citrulline fractions, corresponding to a recovery of 77.6%. In cells treated with 0.1 mM embelin neither uptake (1469 ± 143 dpm/ 10^6 cells) nor recovery of L-[^{14}C]citrulline (1073 ± 215 dpm/ 10^6 cells = 73.0%) were significantly altered. In a further approach, we studied the effect embelin on the fate of endogenously formed L-[^3H]citrulline. PAECs were stimulated with 0.3 μM A23187 for 10, 20, and 30 min in the presence of L-[^3H]arginine, which resulted in L-[^3H]citrulline levels of 268 ± 12 , 270 ± 15 , and 246 ± 13 fmol/ 10^6 cells, respectively (Fig. 5A, open squares). When eNOS was stimulated for 10 min with 0.3 μM A23187 and then inhibited by addition of L-NNA (final concentration 0.3 mM), L-[^3H]citrulline levels declined to 144 ± 7 and 75 ± 4 fmol/ 10^6 cells within 10 and 20 min, respectively (open circles). To test for the effect of embelin, the compound (final concentration 0.1 mM) was added after 20 min to cells that had been treated with A23187 (time point 0) and L-NNA (time point 10 min), followed by detection of L-[^3H]citrulline after further 10 min of incubation (filled circle). At that time point, L-[^3H]citrulline levels were 76 ± 2 fmol/ 10^6 cells and thus identical to those measured in the absence of embelin (75 ± 4 fmol/ 10^6 cells, see above).

Although all experimental approaches applied so far clearly indicated that embelin does not affect L-citrulline metabolism and/or extrusion, we performed a further set of experiments in which we treated cells with embelin and the presence of other eNOS activators (Fig. 5B). As expected, embelin did not affect L-[^3H]citrulline accumulation induced by ATP or bradykinin but, intriguingly, abolished the effect of A23187. Since this effect of embelin was not observed upon stimulation of the cells with the Ca^{2+} ionophore ionomycin, embelin appears to specifically block the pore-forming function of A23187. To test whether this intriguing effect is due to a direct chemical reaction between A23187 and embelin, we monitored the intrinsic fluorescence of A23187 (1 μM in buffer) in the absence and presence of 0.1 mM embelin. As evident from the emission scans shown in Fig. 5C, A23187 elicited a pronounced fluorescence signal that was almost completely quenched by embelin. These data clearly suggest that embelin directly reacts with A23187, thereby inhibiting of function of the ionophore.

Embelin does not potentiate DEA/NO-induced cGMP formation

Based on the lack of effect of embelin on endothelial L-citrulline degradation, we speculated that the drug may sensitize sGC to NO or potentiate NO-induced cGMP accumulation through inhibition of cGMP hydrolysis. To test these hypotheses, we investigated the effect embelin on cGMP production induced by DEA/NO. The experiments were performed with PAECs pretreated with L-NNA (to block endogenous NO formation, Fig. 6A) and cells lacking NO synthase (RFL-6 fibroblasts, Fig. 6B). In both experimental setups, embelin (0.1 mM) did not significantly affect DEA/NO-induced cGMP formation, demonstrating that the drug acts neither as sGC sensitizer nor as PDE inhibitor.

Effects of embelin on purified sGC and eNOS

Interestingly and in striking contrast to its lack of effect in intact cells, embelin completely blocked NO-induced activation of purified sGC in enzyme assays (Fig. 7A). The inhibitory effect of embelin was less pronounced in the presence of 0.1 mg/ml BSA (IC_{50} of ~ 20 μM

vs. $\sim 2 \mu\text{M}$ in the absence of BSA) and virtual abolished at 1 mg of albumin per ml. In accordance with these data, which suggest non-specific interaction of embelin with proteins, treatment of purified eNOS with embelin also resulted in loss of enzymatic activity measured as formation of NO (Fig. 7B) or L-citrulline (Fig. 7C). Similar to its interaction with sGC, embelin inhibited L-citrulline formation with an IC_{50} in the low μM olar range, and no effect was observed in the presence of 1 mg/ml BSA. Together with our findings showing that embelin affects neither L-citrulline nor cGMP formation in endothelial cell lysates (data not shown), the protective effect of BSA clearly suggests that embelin does not directly affect eNOS and sGC activity inside cells.

Effect of embelin on sGC/eNOS complex formation

Several eNOS activating agonists, including bradykinin, have been shown to facilitate the interaction between eNOS, HSP90, and sGC, thereby targeting intracellular NO produced by eNOS directly to sGC [32–35]. We, therefore, tested whether such complex formation also occurs in response to embelin, and if so, whether it is more pronounced than that induced by bradykinin. As shown in Fig. 8, treatment of BAECs with 0.1 mM embelin for 2 min did indeed evoke an increased association of sGC with eNOS, as measured by immunoprecipitation experiments. However, the effect of embelin was similar to that of bradykinin, suggesting that an enhanced sGC/eNOS complex formation does not account for the highly efficient activation of sGC in embelin-treated cells.

No role for mitochondria in embelin-induced cGMP formation

Based on a report suggesting that embelin induces partial uncoupling of oxidative phosphorylation in mitochondria [36], we used the classical mitochondrial uncoupler FCCP to test whether this phenomenon contributes to the observed effects of embelin. In accordance with literature data [36], we found that treatment of PAECs with 0.1 mM embelin slightly increased oxygen consumption (from 20.7 ± 1.0 to $27.7 \pm 2.5 \text{ pmol s}^{-1} \times 10^{-6}$ cells), but the effect was less pronounced than that elicited by $0.3 \mu\text{M}$ FCCP ($70.4 \pm 5.2 \text{ pmol s}^{-1} \times 10^{-6}$). Importantly, FCCP ($0.3 \mu\text{M}$) failed to increase endothelial cGMP accumulation (6.8 ± 1.2 and $7.1 \pm 1.5 \text{ pmol}/10^6$ cells in absence and presence of FCCP, respectively), demonstrating that the effect of embelin on NO/cGMP signaling cannot be attributed to mitochondrial uncoupling. Finally, we observed that inhibition of mitochondrial Ca^{2+} signaling by blocking mitochondrial $\text{Na}^{+}/\text{Ca}^{2+}$ exchange with CGP-37157, inhibition of mitochondrial ATPase with oligomycin, inhibition of complex I with rotenone, or inhibition of complex III with myxothiazole neither affected cGMP nor L-[^3H]citrulline formation in response to embelin (data not shown), excluding that mitochondria play a role in embelin-induced cGMP accumulation.

Discussion

In the present study, we investigated the effect of embelin on eNOS activity in endothelial cells. As a first approach, we determined intracellular cGMP accumulation as measure for NO formation and observed a pronounced stimulatory effect of embelin that was comparable to that elicited by the Ca^{2+} ionophore A23187. In accordance with eNOS-derived NO as trigger for cGMP formation, the effect of both A23187 and embelin was abolished upon

inhibition of eNOS with L-NNA or scavenging of NO with oxyhemoglobin or superoxide. Based on findings showing that embelin was virtually inactive in the absence of extracellular Ca^{2+} , we monitored the cytosolic Ca^{2+} signaling with fura-2 and found that embelin increased $[\text{Ca}^{2+}]_i$ up to $\sim 0.5 \mu\text{M}$, demonstrating that eNOS activation is triggered by Ca^{2+} . Since it was not aim of this study to clarify the mechanism underlying the modulation of Ca^{2+} signaling by embelin, we only performed some basic experiments suggesting that Ca^{2+} influx rather than release of Ca^{2+} from intracellular store was responsible for embelin-triggered elevation of the endothelial Ca^{2+} concentration.

Although the finding that embelin promotes Ca^{2+} influx resulting in eNOS activation is novel and interesting, the most surprising observation of the present study is certainly the pronounced accumulation of cGMP that was triggered by embelin at very low apparent rates of eNOS-catalyzed L-citrulline formation. This phenomenon did not occur in response to agents that induce Ca^{2+} release from intracellular stores and thus store-operated Ca^{2+} entry (ATP, bradykinin and thapsigargin), suggesting that it may reflect activation of a signaling cascade that is coupled to influx of Ca^{2+} through embelin-sensitive ion channels or an as yet unknown pore-forming activity of embelin.

Based on established NOS enzymology [37] it is beyond any doubt that the enzyme generates L-citrulline and NO in a 1:1 stoichiometry. Thus, our data suggest that embelin either increases L-citrulline degradation/extrusion or promotes more efficient sGC activation at given rates of endothelial NO synthesis. Since the drug did not affect the metabolism/recovery of endogenous or exogenously added L-citrulline, it appears safe to exclude potential effects of embelin on endothelial L-citrulline homeostasis. Several processes could be responsible for apparently increased cGMP accumulation at given rates of NO synthesis: (i) increased NO bioavailability, e.g. through inhibition of basal endothelial superoxide formation, (ii) inhibition of cGMP hydrolysis catalyzed by an IBMX-insensitive phosphodiesterase, (iii) sensitization of sGC to NO, as described for several benzyl indazole derivatives [38–40], and finally (iv) translocation of eNOS and/or sGC, resulting in improved functional and/or physical association of the two proteins. Since embelin did not potentiate cGMP accumulation induced by DEA/ NO, effects on NO bioavailability, cGMP hydrolysis and NO sensitivity of sGC are most likely not involved. Thus, it appears that embelin promotes more efficient coupling of NO synthesis to sGC activation than known agonists of NO/cGMP signaling acting through mobilization of intracellular Ca^{2+} .

So far, there is evidence indicating that upon elevation in the intracellular free Ca^{2+} concentration, sGC dynamically translocates to the plasma membrane, thereby increasing its sensitivity to NO [41]. The mechanisms underlying these phenomena are unclear but could be related to the formation of an eNOS/HSP90/sGC complex, which targets intracellularly formed NO directly to sGC [32–35]. Accordingly it is conceivable that embelin facilitates translocation, membrane association, and/or complex formation of sGC more efficiently than other agonists. However, immunoprecipitation experiments revealed that the effect of embelin on the interaction between eNOS and sGC is not more pronounced than that elicited by bradykinin. Although it cannot be excluded that embelin triggers the formation of a complex that is rather labile and thus not detectable under the conditions employed, our data suggest that enhanced translocation of sGC and eNOS/sGC complex

formation does not account for the highly efficient activation of sGC in embelin-treated cells.

Next we hypothesized that treatment of the cells with embelin could lead to a preferential activation of already membrane-associated (and thus sensitized) sGC, e.g. by inducing selective elevation in subplasmalemmal Ca^{2+} . Mitochondria are known to buffer subplasmalemmal Ca^{2+} [42], and embelin was reported to interfere with mitochondrial function [36]. However, we did not obtain any evidence for a role of mitochondria in embelin-induced cGMP accumulation, suggesting that the striking difference between embelin and agents acting through mobilization of intracellular Ca^{2+} may not result from an elevation of subplasmalemmal Ca^{2+} through embelin-triggered Ca^{2+} influx. Thus, we cannot exclude that the improved coupling of NO synthesis to sGC activation caused by embelin is unrelated to Ca^{2+} influx. Further work is needed to identify the signaling cascade that mediates the unique effect of embelin on NO/cGMP signaling.

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Fig. 1.

Effect of embelin on cGMP accumulation. PAECs were preincubated for 15 min in isotonic Tris buffer, containing 5 mM KCl, 1 mM MgCl_2 , 2.5 mM CaCl_2 , 1 mM IBMX and 1 μM indomethacin, and then stimulated for 4 min with embelin, A23187 or DEA/NO at concentrations as indicated (A, B). When inhibitors/scavengers were tested (B), the respective drugs were either already present during the preincubation (L-NNA, 0.3 mM; EGTA, 0.1 mM (instead of 2.5 mM CaCl_2)) or added immediately prior to stimulation of the cells (OxyHb (oxyhemoglobin, 0.1 mM); FMN, 0.1 mM; HX/XO (hypoxanthine; 1 mM / xanthine oxidase, 10 mU/ml)). Subsequent to lysis of the cells, cGMP was quantified by radioimmunoassay as described in the methods section. Data are mean values $\pm$ SEM of three to five independent experiments.

Fig. 2.

Effect of embelin on intracellular free Ca^{2+} concentration. PAECs loaded with fura-2 were suspended in isotonic Tris buffer, containing 5 mM KCl, 1 mM MgCl_2 , and 2.5 mM CaCl_2 , (A and B) or 0.1 mM EGTA (C and D), and changes in the intracellular free Ca^{2+} concentration monitored by measuring the fluorescence intensity at 340/380 nm. Embelin (10 or $100 \mu\text{M}$) or CaCl_2 (1 mM) was added at time points indicated by arrows. At the end of each experiment, maximal and minimal fluorescence ratios were determined by adding 0.1% Triton X-100 and 100 mM EGTA, respectively. The traces shown are representative for three independent experiments yielding similar results.

Fig. 3.

Effect of embelin on conversion of L-[³H]arginine into L-[³H]citrulline (A and B) and intracellular L-citrulline levels (C). (A) PAECs were incubated for 10 min with embelin (0.01–0.3 mM) or A23187 (A23, 0.3 μM) in the presence of isotonic Tris buffer, containing 5 mM KCl, 1 mM MgCl₂, 2.5 mM CaCl₂, and L-[³H]arginine. Where indicated, experiments were performed with cells pretreated for 15 min with 0.3 mM L-NNA. In time course studies (B), cells were stimulated with 0.1 mM embelin or 0.3 μM A23187. Subsequent to lysis of the cells, L-[³H]citrulline was isolated and quantified as described in the methods section. (C) Incubation conditions were identical to that described for (B), with the exception that experiments were performed in the absence of L-[³H]arginine. Intracellular L-citrulline levels were quantified by HPLC analysis as described the methods section. Data are mean values ± SEM of three to five independent experiments.

Fig. 4.

Correlation between cGMP and L-[³H]citrulline accumulation. PAECs were incubated for 2 min in isotonic Tris buffer (see legends to Figs. 1 and 3) with increasing concentrations of ATP (open circles), bradykinin (filled circles), thapsigargin (open squares) or embelin (filled squares), and accumulation of cGMP (A) and L-[³H]citrulline (B) was determined as described the methods section. (C) L-[³H]citrulline vs. cGMP plot of the data shown in A and B. Data are mean values $\pm$ SEM of three independent experiments.

Fig. 5.

Effect of embelin on L-citrulline metabolism (A and B) and the intrinsic fluorescence of A23187 (C). (A) PAECs were incubated in Tris buffer (see legend to Fig. 3) in the presence of L-[³H]arginine and 0.3 μ M A23187, and L-[³H]citruilline was quantified after 10, 20, and 30 min (open squares). In some experiments, L-NNA (0.3 mM, open circles) and embelin (0.1 mM, filled circles) were added to A23187-treated cells at time points indicated by arrows. (B) PAECs were stimulated for 2 min with ATP (1 mM), bradykinin (BK, 1 μ M), A23187 (A23, 0.3 μ M), or ionomycin (Iono, 1 μ M) in the absence and presence of embelin (0.1 mM). L-[³H]Citruilline was isolated and quantified as described the methods section. (C) Intrinsic fluorescence of A23187 was measured in a thermostated cuvette containing 50 mM Tris buffer, pH 7.4, 100 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2.5 mM CaCl₂, 1 μ M A23187, and where indicated 0.1 mM embelin. The excitation wavelength was set to 308 nm and the emission wavelengths were scanned from 320 to 600 nm. Data shown in (A) and (B) are mean values $\pm$ SEM of three independent experiments. In (C), original traces representative for three independent experiments are shown.

Fig. 6.

Effect of embelin on NO-induced cGMP accumulation in PAECs (A) and RFL-6 fibroblasts (B). Cells were preincubated for 15 min in the absence (fibroblasts) and presence of 0.3 mM L-NNA (endothelial cells) in Tris isotonic buffer (see legend to Fig. 1) and then stimulated for 4 min with DEA/NO at concentrations as indicated in the absence and presence of 0.1 mM embelin. Subsequent to lysis of the cells, cGMP was quantified by radioimmunoassay as described in the methods section. Data are mean values $\pm$ SEM of three independent experiments.

Fig. 7.

Effects of embelin on purified sGC (A) and eNOS (B and C). (A) Purified sGC (50 ng) was incubated for 10 min with increasing concentrations of embelin in buffer containing 0.5 mM [α - ^{32}P]GTP, 3 mM MgCl_2 , 1 mM cGMP, 2 mM dithiothreitol, and BSA as indicated. ^{32}P -cGMP was isolated and quantified as described in the methods section. (B) Purified eNOS (10 μg) was incubated with embelin at concentrations as indicated in buffer containing 1 mM arginine, 0.5 mM CaCl_2 , 0.2 mM NADPH, 10 μM tetrahydrobiopterin, 5 μM FAD, 5 μM FMN, 1000 U/ml superoxide dismutase and 0.2 mM CHAPS. At time point zero, calmodulin (10 $\mu\text{g}/\text{ml}$) was added, and NO formation monitored with a Clark-type electrode as described in the methods section. (C) Purified eNOS (0.1 μg) was incubated for 10 min with increasing concentrations of embelin in buffer containing 0.1 mM L- ^3H arginine, 0.5 mM CaCl_2 , 0.2 mM NADPH, 10 μM tetrahydrobiopterin, 5 μM FAD, 5 μM FMN, 10 $\mu\text{g}/\text{ml}$ calmodulin, 0.2 mM CHAPS, and BSA as indicated. L- ^3H citrulline was isolated and quantified as described in the methods section. Data shown in (A) and (C) are mean values $\pm$ SEM of three independent experiments. In (B), original traces representative for three independent experiments are shown.

Fig. 8.

Effect of embelin on sGC/eNOS complex formation. BAECs were incubated for 2 min in the absence and presence of 0.1 mM embelin or 1 μ M bradykinin. Then, cells were lysed, and equal quantities of lysate were immunoprecipitated with an anti-eNOS antibody. Immunoprecipitated proteins were then immunoblotted with either an anti-sGC β_1 antibody (A) or an anti-eNOS antibody (B). Similar results were obtained in four different experiments.