Intracellular Endothelin Type B Receptor-driven Ca\(^{2+}\) Signal Elicits Nitric Oxide Production in Endothelial Cells*

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Elena Deliu\(^{1,2},\) G. Cristina Braiiloiu\(^{1,2},\) Karthik Mallilankaraman\(^{3},\) Hong Wang\(^{4},\) Muniswamy Madesh\(^{4},\) Ashiwell S. Undieh\(^{5,6},\) Walter J. Koch\(^{4,6},\) and Eugen Braiiloiu\(^{4,2}\

From the \(^{1}\)Department of Pharmacology and \(^{2}\)Center for Translational Medicine, Temple University School of Medicine, Philadelphia, Pennsylvania 19140 and \(^{3}\)Department of Pharmaceutical Sciences, Jefferson School of Pharmacy, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Background: The intracrine nature of endothelin-1 is largely correlated with nuclear signaling events.

Results: In endothelial cells, endothelin-1 acting on endolysosomal ET\(_B\) receptors increases cytosolic Ca\(^{2+}\) and nitric oxide.

Conclusion: Endolysosomal ET\(_B\) receptors are functional.

Significance: We identify a new pathway for ET-1-induced intracrine signaling and provide the first evidence that intracellular G protein-coupled receptors are involved in redox signaling.

Endothelin-1 exerts its actions via activation of ET\(_A\) and ET\(_B\) G\(_{q/11}\) protein-coupled receptors, located in the plasmalemma, cytoplasm, and nucleus. Although the autocrine/paracrine nature of endothelin-1 signaling has been extensively studied, its intracrine role has been largely attributed to interaction with receptors located on nuclear membranes and the nucleoplasm.

Because ET\(_B\) receptors have been shown to be targeted to endolysosomes, we used intracellular microinjection and concurrent imaging methods to test their involvement in Ca\(^{2+}\) signaling and subsequent NO production. We provide evidence that microinjected endothelin-1 produces a dose-dependent elevation in cytosolic calcium concentration in ET\(_B\)-transfected cells and endothelial cells; this response is sensitive to ET\(_B\) but not ET\(_A\) receptor blockade. In endothelial cells, the endothelin-1-induced Ca\(^{2+}\) response is abolished upon endolysosomal but not Golgi disruption. Moreover, the effect is prevented by inhibition of microautophagy and is sensitive to inhibitors of the phospholipase C and inositol 1,4,5-trisphosphate receptor. Furthermore, intracellular endothelin-1 increases nitric oxide via an ET\(_B\)-dependent mechanism. Our results indicate for the first time that intracellular endothelin-1 activates endolysosomal ET\(_B\) receptors and increase cytosolic Ca\(^{2+}\) and nitric oxide production. Endothelin-1 acts in an intracrine fashion on endolysosomal ET\(_B\) to induce nitric oxide formation, thus modulating endothelial function.

Compelling evidence indicates that in addition to the classical localization and signaling at the plasma membrane, a wide variety of G protein-coupled receptors (GPCRs)\(^{3}\) are expressed and fully functional intracellularly (1–3). Endothelin-1 (ET-1) acts on ET\(_A\) and ET\(_B\) receptors, G\(_{q/11}\)-coupled GPCRs, exerting opposing effects on vascular functions. In the cardiovascular human and rodent cells, ET\(_A\) and ET\(_B\) are localized to the plasma membrane, cytoplasm, nuclear membranes, and nucleoplasm (4–6). ET-1 elevates both cytosolic and nuclear Ca\(^{2+}\) concentrations; the rise in cytosolic Ca\(^{2+}\) concentration, [Ca\(^{2+}\)]\(_{i}\), in response to ET-1 is seen in both intact and membrane-perforated cells (5). Moreover, ET-1 is involved in nitric oxide (NO) and reactive oxygen species generation both in the cytosol and in the nucleus (5).

Although the stimulation of nuclear membrane receptors elicits events largely restricted to the nucleus (3, 5), receptors located on the membrane of other organelles trigger signaling cascades within the cytoplasm, resulting in rapid cell responses. Because ET\(_B\) receptors were identified in the endolysosomal system (7–9), in this study, we examined whether or not endolysosomal ET\(_B\) receptors are involved in Ca\(^{2+}\) signaling and NO production in rat pulmonary microvascular endothelial cells (RPMVEC), which express ET\(_B\) receptors (10–12).

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Endothelin-1, bafilomycin A1, brefeldin A, BQ-123 sodium salt, BQ-788 sodium salt, ryanodine, and xestospongin C were purchased from EMD Millipore (Billerica, MA), U-73122, L-NAME, and heparin were from Sigma-Aldrich, and hirapin were from Sigma-Aldrich, and Ned-19 was from Tocris Bioscience (R&D Systems, Minneapolis, MN).

**Cell Culture, DNA Constructs, and Transfection**—RPMVEC were cultured in M199 medium (Thermo Fisher Scientific) containing 15% fetal bovine serum (Atlanta Biologicals, Inc., Lawrenceville, GA), 1% GlutaMax, 1% penicillin-streptomycin-amphterin B (both from Invitrogen), 50 μg/ml endothelial cell growth supplement (BD Biosciences), 1% non-essential amino acids (ATCC, Manassas, VA) on glass coverslips coated with endothelin receptor type A; ET\(_A\), endothelin receptor type B; IP\(_{A}\), inositol 1,4,5-trisphosphate; IP\(_{B}\), inositol 1,4,5-trisphosphate receptor; L-NAME, L-NG-nitroarginine methyl ester; RPMVEC, rat pulmonary microvascular endothelial cells.
with gelatin. U2OS cells (ATCC) were cultured in DMEM (Mediatech, Herndon, VA) containing 10% fetal bovine serum (Atlanta Biologicals). U2OS cells were transiently transfected with GFP-tagged ET<sub>β</sub> (rat) cDNA or pCMV6-AC-mGFP vector (OriGene Technologies Inc, Rockville, MD) using Turbofectin 8 transfection reagent according with the manufacturer’s instructions (OriGene Technologies Inc). Cells were used 24–48 h after transfection.

**Intracellular Injection**—RPMVEC were transiently transfected with GFP-tagged ET<sub>β</sub> (rat) using Turbofectin 8. The cells were incubated with LysoTracker Red (1 μM) (Invitrogen) following pretreatment with either the vehicle (dimethyl sulfoxide 0.1% (v/v)), baflomycin A1 (1 μM) or glycy1-1-phenylalanine 2-naphthylamide (100 μM) for 1 h, as reported (13). Cells were fixed in 4% paraformaldehyde, mounted with DAPI Fluoromount G (Southern Biotech, Birmingham, AL), and examined under a confocal scanning microscope (Leica TCS SP5) with excitation wavelengths set to 405 nm for DAPI, 488 nm for GFP, and 585 nm for LysoTracker Red, in the sequential mode.

**Calcium Imaging**—Measurements of [Ca<sup>2+</sup>] were performed as described previously (13, 14). Cells were incubated with 5 μM Fura-2 AM (Invitrogen) in Hanks’ balanced salt solution at room temperature for 45 min, in the dark, washed three times with dye-free Hanks’ balanced salt solution, and then incubated for another 45 min to allow for complete de-esterification of the dye. Coverslips (25-mm diameter) were subsequently mounted in an open bath chamber (RP-40LP, Warner Instruments, Hamden, CT) on the stage of an inverted microscope Nikon Eclipse TiE (Nikon, Inc., Melville, NY). The microscope is equipped with a Perfect Focus System and a Photometrics CoolSnap HQ2 CCD camera (Photometrics, Tucson, AZ). During the experiments, the Perfect Focus System was activated. Fura-2 AM fluorescence (emission, 510 nm), following alternate excitation at 340 and 380 nm, was acquired at a frequency of 0.25 Hz. Images were acquired and analyzed using NIS-Elements AR software (version 3.1, Nikon, Inc.). The ratio of the fluorescence signals (340/380 nm) was converted to Ca<sup>2+</sup> concentrations (15).

**Intracellular Microinjection**—Injections were performed using Femtotips II, InjectMan NI2, and FemtoJet systems (Eppendorf) as reported previously (16–19). Pipettes were back filled with an intracellular solution composed of 110 mM KCl, 10 mM NaCl, and 20 mM HEPES (pH 7.2) (20) or the specific chemicals. The injection time was 0.4 s at 60 hectoPascal with a compensation pressure of 20 hPa to maintain the microinjected volume to <1% of cell volume, as measured by microinjection of a fluorescent compound (Fura-2-free acid) (20). The intracellular concentration of chemicals was determined based on the concentration in the pipette and the volume of injection. The cellular volume was estimated to 1000 μm<sup>3</sup> (21).

**Measurement of NO Levels**—Intracellular NO was monitored with DAF-FM (4-amino-5-methylamino-2',7'-difuorofluorescein, Invitrogen), a pH-insensitive fluorescent dye, as described previously (22). Cells were incubated at room temperature for 45 min in Hanks’ balanced salt solution containing a low concentration (0.5 μM) of DAF-FM. This condition significantly reduced the background autofluorescence and improved the signal-to-noise ratio of NO detection in single cells. After loading, cells were rinsed three times with saline. NO fluorescence was measured at a rate of 0.1 Hz using excitation/emission wavelengths of 488 nm/540 nm.

**Data Analysis**—Data were expressed as mean and S.E. One-way analysis of variance, followed by post hoc Bonferroni and Tukey tests, was used to assess significant differences between groups; p < 0.05 was considered statistically significant.

**RESULTS**

To avoid any possible plasmalemmal effect of ET-1, in all series of experiments, the cells were pretreated for 5 min with a mixture of ET<sub>α</sub> and ET<sub>β</sub> plasmalemmal non-permeant antagonists (BQ-123 and BQ-788, both 10<sup>−7</sup> M).

Microinjection of ET-1 (10<sup>−10</sup> M) produced small and non-significant increases in [Ca<sup>2+</sup>]<sup>i</sup>, in untransfected or GFP-transfected U2OS cells (∆[Ca<sup>2+</sup>]<sup>i</sup>), was 22 ± 4 nm and 19 ± 3 nm, respectively, Fig. 1, A and B). Similar non-significant responses were observed upon intracellular administration of ET<sub>α</sub> antagonist BQ-123 or ET<sub>β</sub> antagonist BQ-788 (both 10<sup>−7</sup> M) to ET<sub>β</sub>-GFP-transfected U2OS cells (∆[Ca<sup>2+</sup>]<sup>i</sup>), was 34 ± 4 nm and 32 ± 2 nm, respectively, Fig. 1, C and D) or to endothelial cells (∆[Ca<sup>2+</sup>]<sup>i</sup>), was 29 ± 4 nm and 27 ± 4 nm, respectively, Fig. 1, E and F). Six cells were injected in each of the above mentioned control experiments.

**Intracellular Injection of ET-1 Elevates [Ca<sup>2+</sup>]<sup>i</sup> in ET<sub>β</sub> Expressing Cells**—In U2OS cells transiently transfected with ET<sub>β</sub>-GFP, microinjection of ET-1 (10<sup>−11</sup> M, 10<sup>−10</sup> M, and 10<sup>−9</sup> M final concentrations inside the cell) produced a concentration-dependent elevation of [Ca<sup>2+</sup>]<sup>i</sup> by 361 ± 7 nm, 706 ± 16 nm, and 1394 ± 28 nm, respectively (n = 6 for each concentration tested) (Fig. 2, A, B, and D). Intracellular microinjection of control buffer in ET<sub>β</sub>-expressing cells resulted in a small and non-significant increase in [Ca<sup>2+</sup>]<sup>i</sup> by 34 ± 4 nm (n = 6 cells) (Fig. 2, A–C). The small increase in [Ca<sup>2+</sup>]<sup>i</sup> followed by a rapid return and steady Ca<sup>2+</sup> base line after control microinjections indicate the optimization of injection parameters to avoid any potential artifacts produced by cell damage at the level of organelles or plasma membrane. Untransfected and GFP-transfected U2OS cells did not respond to ET-1 microinjection (Fig. 1, A and B). When the cells were co-injected with the ET<sub>α</sub> antagonist BQ-123 (10<sup>−7</sup> M), the rise in [Ca<sup>2+</sup>]<sup>i</sup> in response to ET-1 (10<sup>−10</sup> M) was 698 ± 12 nm (n = 6), largely similar to that produced by ET-1 (10<sup>−10</sup> M) alone (Fig. 2, A, B, D, and E). However, co-administration of the ET<sub>β</sub> antagonist BQ-788 (10<sup>−7</sup> M) and ET-1 (10<sup>−10</sup> M) prevented ET-1-induced response, elevating [Ca<sup>2+</sup>]<sup>i</sup> by 22 ± 3 nm (n = 6), a response resembling that of control buffer (Fig. 2, A, C, and F). ET<sub>α</sub> and ET<sub>β</sub> antagonists alone did not affect [Ca<sup>2+</sup>]<sup>i</sup>, when injected into U2OS-ET<sub>β</sub>-GFP (Fig. 1, C and D).

**Intracellular Injection of ET-1 Elevates [Ca<sup>2+</sup>]<sup>i</sup> in RPMVEC**—ET<sub>β</sub> microinjection to RPMVEC, which endogenously express ET<sub>β</sub> (23), produced fast and transient increases in [Ca<sup>2+</sup>]<sup>i</sup>. RPMVEC responded to intracellular administration of ET-1 (10<sup>−11</sup> M, 10<sup>−10</sup> M, and 10<sup>−9</sup> M final concentrations inside the cell) with [Ca<sup>2+</sup>]<sup>i</sup>, increases of 170 ± 6 nm, 447 ± 9 nm, and 751 ± 14 nm, respectively (n = 6), whereas control buffer microinjection produced a minor and non-significant [Ca<sup>2+</sup>]<sup>i</sup> elevation, of 22 ± 4 nm (Fig. 3, A–D). In RPMVEC, co-admin
istration of BQ-123 (10^-7 M) with ET-1 (10^-10 M) did not significantly affect ET-1-induced response (Δ[Ca^{2+}]) for BQ-123 + ET-1 was 428 ± 7 nM versus 447 ± 9 nM for ET-1 alone, Fig. 3, A, B, D, and E), whereas concomitant microinjection of BQ-788 (10^-7 M) and ET-1 (10^-10 M) completely abolished the ET-1 response (Δ[Ca^{2+}]) for BQ-788 + ET-1 was 24 ± 4 nM, similar to control buffer microinjection, Fig. 3, A–C, and F). Neither the ETA nor ETB antagonist had an effect on its own when injected into RPMVEC (Fig. 1, E and F).

**Endolysosomal Localization of Functional ET_B Receptors**—In endothelial cells, the increase in [Ca^{2+}] by microinjected ET-1 (10^-10 M) was insensitive to brefeldin A (10 μM, 1-h incubation), a Golgi apparatus disruptor (24) (Δ[Ca^{2+}]_i was 423 ± 7 nM in the presence versus 447 ± 9 nM in the absence of brefeldin A, n = 6; Fig. 4, A–D). Conversely, exposure to bafilomycin A1 (1 μM, 1 h), a V-type ATPase inhibitor (25), abolished the effect of microinjected ET-1 on endothelial [Ca^{2+}]_i (Δ[Ca^{2+}]_i = 28 ± 6 nM, n = 6, Fig. 4, A, B, and E). Furthermore, inhibition of microautophagy with rapamycin (30 μM, 1 h) (26) prevented ET-1 from eliciting a significant rise in [Ca^{2+}]_i (Δ[Ca^{2+}]_i = 14 ± 5 nM, n = 6, Fig. 4, A, B, and F).

In ET_B-GFP-expressing RPMVEC, endolysosomes were identified with LysoTracker Red (Fig. 5A). Treatment with bafilomycin A1 (1 μM, 1 h), or glycyl-L-phenylalanine 2-naphthylamide (100 μM, 1 h), a basic amine inducing lysosomal permeabilization (27), markedly diminished the LysoTracker Red and GFP-ETB fluorescence (Fig. 5, B and C).

**Lyosomal ET_B Activation Releases Ca^{2+} from IP3-dependent Stores**—In Ca^{2+}-free saline, microinjection of ET-1 (10^-10 M) to RPMVEC increased [Ca^{2+}]_i by 431 ± 11 (n = 6), largely similar to the Δ[Ca^{2+}]_i produced in Ca^{2+}-containing Hanks’ balanced salt solution (447 ± 9 nM, n = 6). This response was

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**Figure 1. Control experiments.** A, averaged Ca^{2+} responses to intracellular administration of endothelin-1 (10^-10 M) in untransfected (black) or GFP-transfected (gray) U2OS cells. B, comparison of the amplitudes of ET-1 effects on [Ca^{2+}]_i in untransfected and GFP-transfected U2OS control cells. C, averaged Ca^{2+} responses to BQ-123 (ET_A antagonist, 10^-7 M, black) or BQ-788 (ET_B antagonist, 10^-7 M, gray) microinjection in ET_B-GFP-transfected U2OS cells. D, comparison of the Ca^{2+} responses of ET_B-GFP-U2OS cells to intracellular administration of BQ-123 or BQ-788. E, averaged Ca^{2+} responses to BQ-123 (10^-7 M, black) or BQ-788 (10^-7 M, gray) microinjection in rat endothelial cells, endogenously expressing ET_B. F, comparison of the Ca^{2+} responses of endothelial cells to intracellular administration of BQ-123 or BQ-788.
basically absent in cells pretreated with xestospongion C and heparin (36 ± 2 nM, n = 6, Fig. 6, A and B), which block inositol 1,4,5-trisphosphate (IP3) receptors (IP3R), or with the phospholipase C inhibitor U-73122 (21 /H11006 3n M, n = 6, Fig. 6, A and B).

Conversely, blocking NAADP-dependent Ca2+/H11001 release from endolysosomes with Ned-19 (5 /H9262 M, 15 min) (28) or inhibition of ryanodine receptors with ryanodine (1 /H9262 M, 15 min) was ineffective in counteracting ET-1 effect (424 /H11006 8n M, n = 6, respectively; Fig. 6, A and B).

Lysosomal ETB-dependent NO Production in RPMVEC—Cytosolic NO levels were measured in RPMVEC using DAF-FM fluorescence. Intracellular administration of ET-1 (10 /H11002 10 M) significantly increased DAF-FM fluorescence by 14.45 ± 0.73% (p < 0.05; n = 6), whereas control buffer microinjection had a small and non-significant effect (0.53 ± 0.08%, n = 6, Fig. 7, A and B). The NO increase produced by ET-1 was not affected by BQ-123 (10 /H9004 7 M, DAF reduced to 14.33 ± 0.7%, n = 6), which blocks ETα but was lost upon BQ-788 (10 /H11002 7 M) treatment (ETB antagonist, ΔDAF reduced to 0.25 ± 0.12%, n = 6, Fig. 7, A and B). In the presence of the NO synthase inhibitor L-NAME (100 /H9262 M) or of the microautophagy blocker rapamycin (30 /H9262 M), intracellularly injected ET-1 no longer produced significant increases in NO levels (ΔDAF was 0.25 ± 0.09%, and 0.19 ± 0.09%, respectively; n = 6, Fig. 7, A and B).

DISCUSSION

ET-1, first identified as an endothelium-derived vasoconstrictor peptide, is an autocrine and paracrine signaling factor with extensive modulatory effects on vascular function (29). ET-1 acts also as an intracrine, activating intracellular cognate receptors (2). Stimulation of ET-1 receptors at the plasma or nuclear membrane induces Ca2+/H11001 release, NO, and reactive oxygen species formation in the cytosol or nucleus (5, 30). Cardiovascular disease pathogenesis involves oxidative stress and further alteration of ET-1 and NO signaling pathways (30, 31).
ET-1 immunoreactivity is expressed within the cytoplasm on the membranes of the endoplasmic reticulum (ER), mitochondria, and cytosolic vesicles of endothelial cells from human (4) and various rodent species, including rats (6). Moreover, the components responsible for ET-1 generation are present intracellularly in endothelial cells, as well as in other cardiovascular cells (6, 32, 33). These findings suggest that ET-1 is available within the cytoplasm to activate its intracellular receptors.

We and others (17, 34–36) have previously reported that stimulation of intracellular GPCRs such as angiotensin II AT1 receptors, CB1, and CB2 cannabinoid receptors (16, 37, 38), or estrogen receptor GPER/GPR30 (18, 19, 39) leads to \([\text{Ca}^{2+}]_i\) elevation. Using a similar approach in this study, we tested the hypothesis that ET-1 may act in an intracrine fashion on intracellular ETB receptors to modulate endothelial functions. Using imaging methods and concurrent intracellular injection of ET-1, we provide the first evidence of functionality of intracellular ETB receptors in cells transiently transfected with the receptor. Because ET-1 is an endothelium-derived peptide, and ETB is the predominant type of endothelin receptor in endothelial cells, thenext series of experiments were designed to evaluate whether intracellular ETB receptors are functional in RPMVEC. Similar to ETB-transfected cells, RPMVEC responded to ET-1 microinjection with a dose-dependent increase in \([\text{Ca}^{2+}]_i\). Blocking intracellular ETB receptors abolished the effect of ET-1, whereas ETA antagonism did not affect it.

Interestingly, a previous study demonstrating cytoplasmic distribution of both ETA and ETB in aortic human vascular cells (5).
endothelial cells also showed that the responses initiated by ET-1 at the plasma membrane are not dependent on plasmalemmal ETB but on ETA (4). According to the present study, the reverse is true in RPMVEC where the effects of microinjected ET-1 are mediated through intracellular ETB receptors. Our findings are particularly relevant considering the ability of endothelial cells to synthesize ET-1 (40–42) and make it available within the cytoplasm (32, 33, 40) to activate its intracellular targets. Importantly, a majority of the responses elicited by ET-1 in the endothelium, including the release of vasorelaxant factors such as NO, prostacyclin, and endothelium-derived hyperpolarizing factor are ETB receptor-dependent (11).

We further examined the intracellular location of functional ETB receptors in endothelial cells. Previous studies have identified that ETB receptors, similar to other GPCRs, may be targeted to the endolysosomes (7–9) or Golgi apparatus (43). Disruption of the Golgi apparatus did not affect the response of endothelial cells to intracellular ET-1, whereas inhibition of lysosomal acidification completely abolished it. Our results indicate that degradation is not the only fate of endolysosomal ETB; they are also functional and involved in ET-1 signaling. This may correlate with the remarkable stability of ETB receptors in these organelles (44).

In addition to playing a role in cellular degradation, increasing evidence supports lysosomes as key regulators of cell home-
ostasis (45) and as platforms for continued receptor-mediated signaling (1). Similar to the plasma membrane, the membrane of the endocytic vesicles is organized into specialized domains, working as a platform for the assembly of specific signaling complexes; these features allow the endolysosomal targeted receptors to initiate signaling from this intracellular compartment (46). Accordingly, various types of receptors, including GPCRs, have been reported to trigger signal transduction pathways upon their endolysosomal activation (1, 47). However, the ET-1 binding pocket on the ETB receptor is located on the N-terminal side, thus within the lysosomal lumen (11). We have previously demonstrated that angiotensin II is transferred inside the endolysosomal vesicles via microautophagy (17), a process in which soluble cytosolic molecules are engulfed (48). Thus, we further tested whether a similar mechanism was applicable to endothelin. Indeed, rapamycin, an inhibitor of the final uptake reaction in the microautophagic process (26), prevented the cellular responses to microinjected endothelin-1. Two major events may be delimited in the microautophagic process: lysosomal membrane invagination/formation of autophagic tubes and vesicle scission (48). Although the former may occur with a 30-min lag, the latter is very rapid, occurring in seconds (26). Given that microautophagy is an ongoing process, important in housekeeping and in the maintenance of cytosolic mass (45), membrane invaginations are formed continuously and cytosolic components may be rapidly uptaken into the endolysosomal lumen. Indeed, activation of lysosomal ETB receptors occurred readily in response to microinjected ET-1.

Next, we defined the Ca2+ release in response to activation of endolysosomal ETB receptors. We examined the effect of microinjected endothelin on NO production. In endothelial cells, an elevation of \([Ca^{2+}]_i\) results in activation of endothelial NO synthase via Ca2+-calmodulin binding (52, 53) and endothelial NO synthase phosphorylation (54), which leads to the release of the vasorelaxant mediator NO. Indeed, intracellular administration of ET-1 resulted in NO release, an effect that was completely contingent on endothelial NO synthase. Our results also indicate that microautophagy and ETB activation are critical steps for NO production in response to intracellular ET-1.

Thus, we propose a new pathway for ET-1-induced intracellular ET-1 is transferred to the endolysosomal lumen via microautophagy to trigger ETB-phospholipase C-dependent IP3 generation. IP3 further activates specific Ca2+ release channels (IP3R) from the endoplasmic reticulum or endolysosomes; \([Ca^{2+}]_i\) elevation stimulates endothelial NO synthase, resulting in endothelial NO accumulation (Fig. 8). This mode of action of ET-1 is canonical, in that it is associated with its receptor, ETB, is second messenger-dependent, and occurs at a membranous compartment (55, 56).

ET-1 released in the circulation is a very potent vasoconstrictor that has been implicated in the pathophysiology of systemic and pulmonary hypertension and in atherosclerosis (57, 58). Although ET-1 acts on the vascular smooth muscle to produce vasoconstriction, activation of endothelial ETB receptors promotes vasorelaxation via NO release (59). ET-1 involvement in atherosclerosis is also dual. ET-1 promotes vasoprotection through ETB receptor-dependent generation of NO and reac-
Endolysosomal ET<sub>B</sub> Receptors Signal through NO

FIGURE 8. Proposed signaling of endothelin-1 via endolysosomal ET<sub>B</sub> receptor activation. Cytosolic endothelin-1 (ET-1), transferred to the endolysosomal lumen (Endo-Lys) via microautophagy, stimulates endolysosomal ET<sub>B</sub> receptors, which in turn activate phospholipase C (PLC) located in the membrane of endolysosomal Ca<sup>2+</sup> stores. Thus, IP<sub>3</sub> is released from membrane phosphoinositides and activates IP<sub>3</sub>R receptors from the endoplasmic reticulum (ER) or endolysosomes. The subsequent IP<sub>3</sub>-induced increase in cytosolic Ca<sup>2+</sup> activates endothelial NO synthase (eNOS) to produce NO. The NO released from the endothelial cells leads to relaxation of the vascular smooth muscle.

Innovative approaches previously employed to demonstrate endocrine release of NO and increased plasma endothelin-1 (60). The shift its overall effects toward vasorelaxation/vasoprotection and may also activate NO released from the endothelial cells leads to relaxation of the vascular smooth muscle. ET-1 to vascular endothelial cells may produce selective activation of lysosomal ET<sub>B</sub>, Ca<sup>2+</sup> mobilization and NO generation; this may shift its overall effects toward vasorelaxation/vasoprotection and prevent its potentially detrimental, vasoconstrictor/proliferative activity on adjacent vascular smooth muscle cells. Endothelial cell-specific ET<sub>B</sub> knock-out mice exhibit decreased endogenous release of NO and increased plasma endothelin-1 (60). Innovative approaches previously employed to demonstrate that another intracrine, angiotsensin II, increases blood pressure upon intracellular trapping at the kidney level (55, 61), may also prove useful in the case of ET-1. Should our findings be supported by in vivo data, selective targeting of ET<sub>B</sub> agonists to the endothelial cytosol may prove therapeutically beneficial in cardiovascular disorders associated with dysfunction of ET-1/NO pathway.

To summarize, this study provides the first evidence that activation of endolysosomal ET<sub>B</sub> receptors increases cytosolic Ca<sup>2+</sup> concentration and nitric oxide production. Furthermore, we show here for the first time that intracellular receptors (namely, ET<sub>B</sub>) may be involved in redox signaling. Our study suggests a novel mechanism for ET<sub>B</sub>-mediated endothelium-dependent vasorelaxation and extends the current knowledge on intracrine signaling.

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