Vol. 174, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1991 Pages 228-235

Endothelin-3 Stimulates Production of Endothelium-Derived Nitric Oxide via Phosphoinositide Breakdown

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Received November 26, 1990

Summary: Cultured bovine endothelial cells (EC) have specific receptors for endothelin (ET)-3 functionally coupled to phosphoinositide breakdown. We studied whether ET-3 stimulates synthesis of nitric oxide (NO), an endothelium-derived relaxing factor that activates soluble guanylate cyclase in EC, and whether the ET-3-induced NO formation involves G-proteins. ET-3 dose-dependently stimulated production of intracellular cGMP in EC, of which effects were abolished by pretreatment with N^G-monomethyl $_L$-arginine, an inhibitor of NO synthesis, and methylene blue, an inhibitor of soluble guanylate cyclase. The stimulatory effects of ET-3 on cGMP production, inositol trisphosphate formation and increase in cytosolic free Ca^{2+} concentration were similarly blocked by pretreatment with pertussis toxin (PTX). These data suggest that ET-3 induces synthesis of NO mediated by phosphoinositide breakdown via PTX-sensitive G-protein in EC.

There is a growing awareness that endothelium plays an important role in intrinsic modulation of vascular tone by elaborating several potent vasoactive substances (1), including

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Abbreviations: EC, endothelial cells; ET, endothelin; NO, nitric oxide; $_L$-NMMA, N^G-monomethyl $_L$-arginine; IP_3, inositol-1,4,5-trisphosphate; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; PTX, pertussis toxin, EDRF; endothelium-derived relaxing factor; SNP, sodium nitroprusside; VSMC, vascular smooth muscle cells.

0006-291X/91 $1.50

vasodilators, such as endothelium-derived relaxing factors (EDRF) and prostacyclin, and vasoconstrictors, such as thromboxane A_2 and endothelin (ET)-1. EDRF has recently been shown to be mainly ascribed to nitric oxide (NO) (2), which is synthesized from the terminal guanidido nitrogen atom of L-arginine, and stimulates soluble guanylate cyclase in vascular smooth muscle cells (VSMC) (3) as well as in EC (4). Recently, N^G-monomethyl L-arginine (L-NMMA), a stereospecific arginine analogue, has shown to antagonize the formation of NO from L-arginine (5).

ET-1 is a novel 21-residue vasoconstrictor peptide, originally characterized from porcine endothelium (6). Subsequent genomic DNA cloning of human ET genes revealed the presence of three ET isopeptides, designated ET-1, ET-2 and ET-3 (7). Among three ET isopeptides, ET-3 shows the most potent initial depressor response through the endothelium-dependent mechanism (8). We have recently demonstrated the presence of specific ET-3 receptors in cultured bovine EC, which are functionally coupled to phosphoinositide breakdown and increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) (9). Therefore, the present study was designed to investigate whether ET-3 stimulates synthesis of NO in cultured bovine EC, and whether the receptor-mediated NO formation involves GTP-binding proteins (G-proteins).

MATERIALS AND METHODS

Drugs: ET-3 was obtained from Peptide Institute Inc. (Osaka, Japan), L-NMMA from Calbiochem Co., Ltd. (La Jolla, CA, USA), L-arginine, sodium nitroprusside (SNP) and 3-isobutyl-1-methylxanthine from Sigma Chemical (St. Louis, MO, USA), methylene blue from Schmid GMBH & Co.(Stuttgart-Unterturkheim, FRG), pertussis toxin (PTX) from Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan) and fura-2 acetoxymethyl ester (AM) from Dojin Chemicals (Kumamoto, Japan).
Cell culture: Bovine ECs were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal calf serum as

described (9); the subcultured EC (9-14th passage) was used in the experiments.

Determination of intracellular cGMP: Confluent ECs were incubated at 37°C for 10 min in Hanks' medium containing 0.5 mM 3-isobutyl-1-methylxanthine as described (10). After extraction with 6 % trichloroacetic acid, concentrations of intracellular cGMP were determined by cGMP radioimmunoassay kit (New England Nuclear, Boston, MA).

Measurement of $[Ca^{2+}]_i$: Suspended ECs were incubated with 4 μ M fura-2 AM at 37°C for 20 min, and the fluorescence of Ca^{2+}-fura-2 of the suspended cells ($\sim 5x10^6$ cells/ml) was measured by a spectrofluorimeter (CAF-100; Jasco Co., Ltd., Tokyo, Japan) as decribed (9).

Measurement of inositol-1,4,5-trisphosphate (IP_3): Confluent ECs were incubated at 37°C for 30 sec in Hanks' medium containing 10 mM LiCl, and IP_3 levels were determined by a competitive protein binding assay kit (Amersham Japan, Tokyo), as described (9).

RESULTS

ET-3 dose-dependently (10^{-9}-10^{-7} M) stimulated synthesis of intracellular cGMP in bovine EC (Fig. 1); the approximate half-maximal dose to induce cGMP formation was $5x10^{-9}$ M. As shown in Table 1, the stimulatory effect of ET-3 (10^{-8} M) on cGMP production was completely abolished by the simultaneous addition of L-NMMA ($2x10^{-4}$ M), of which effect was reversed when

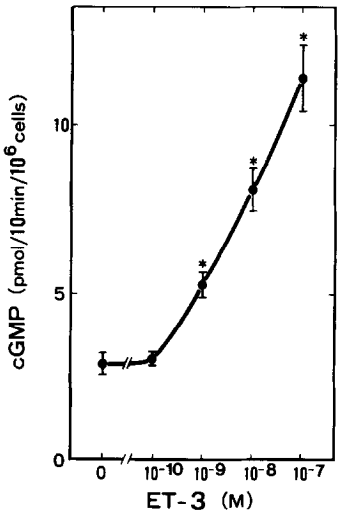

Fig. 1. Dose-responsive effect of ET-3 on cGMP production in cultured bovine EC.
Confluent cells were incubated at 37°C for 10 min with various doses (10^{-10}-10^{-7} M) of ET-3 in the presence of isobutyl-methylxanthine. Each point is the mean of 6 dishes; bars shows SE. *P<0.05 vs. control.

Table 1. ET-3- and SNP-induced cGMP production in cultured bovine EC

Drugs (Concentration)	cGMP (pmol/10 min/10^6 cells) mean ± SE (n=6)
Experiment 1	
Control	3.47 ± 0.13
ET-3 (10^{-8} M)	9.37 ± 0.50*
$_L$-NMMA ($2x10^{-4}$ M)	2.04 ± 0.16*
ET-3 (10^{-8} M) + $_L$-NMMA ($2x10^{-4}$ M)	3.56 ± 0.29
ET-3 (10^{-8} M) + $_L$-NMMA ($2x10^{-4}$ M) + $_L$-arginine (10^{-3} M)	10.5 ± 0.67*
ET-3 (10^{-8} M) + methylene blue (10^{-5} M)	2.99 ± 0.30
SNP (10^{-3} M)	12.5 ± 0.92*
SNP (10^{-3} M) + $_L$-NMMA ($2x10^{-4}$ M)	12.5 ± 0.76*
SNP (10^{-3} M) + methylene blue (10^{-5} M)	4.05 ± 0.38
Experiment 2	
Control	3.44 ± 0.37
SNP (10^{-3} M)	16.5 ± 0.71*
PTX (100 ng/ml)	3.87 ± 0.39
SNP (10^{-3} M) + PTX (100 ng/ml)	16.1 ± 0.97*

Confluent cells were incubated at 37°C for 10 min with ET-3 (10^{-8} M) or SNP (10^{-3} M) in the presence of 0.5 mM isobutylmethylxanthine. (Experiment 1) $_L$-NMMA with or without $_L$-arginine, and methylene blue were coincubated with ET-3 and SNP. (Experiment 2) After preincubation with or without PTX (100 ng/ml) for 6 hrs, cells were incubated with or without SNP (10^{-3} M) in the same manner as in Experiment 1.
*$P<0.05$ *vs.* control.

$_L$-arginine (10^{-3} M) was coincubated with $_L$-NMMA. The ET-3-induced cGMP production was also abolished by methylene blue (10^{-5} M). SNP also stimulated synthesis of intracellular cGMP in bovine EC, whose effect was inhibited by methylene blue, but not by $_L$-NMMA.

To study whether G-protein is involved in the ET-3-induced phosphoinositide breakdown, $[Ca^{2+}]_i$ increase and cGMP formation, ECs were pretreated without or with PTX (100 ng/ml) for 6 hrs. As shown in Fig. 2, pretreatment with PTX completely abolished increase in $[Ca^{2+}]_i$ induced by ET-3 (10^{-8} M). Pretreatment with PTX similarly inhibited the ET-3-induced cGMP production (Fig. 3A) as well as IP_3 formation (Fig. 3B). In contrast, SNP significantly stimulated cGMP formation in PTX-treated cells to the same extent as in non-treated cells (Table 1), suggesting

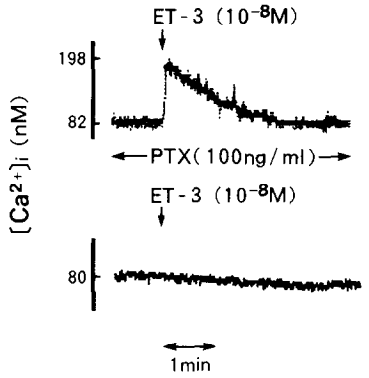

Fig. 2. Effect of pertussis toxin on ET-3-induced $[Ca^{2+}]_i$ increase in bovine EC.
After pretreatment without (upper) and with (lower) pertussis toxin (PTX), cell suspensions loaded with fura-2 were challenged with ET-3 (10^{-8} M).

that PTX does not directly affect soluble guanylate cyclase activity.

DISCUSSION

The present study clearly demonstrates that ET-3 stimulates cGMP production in bovine EC, of which effects are completely

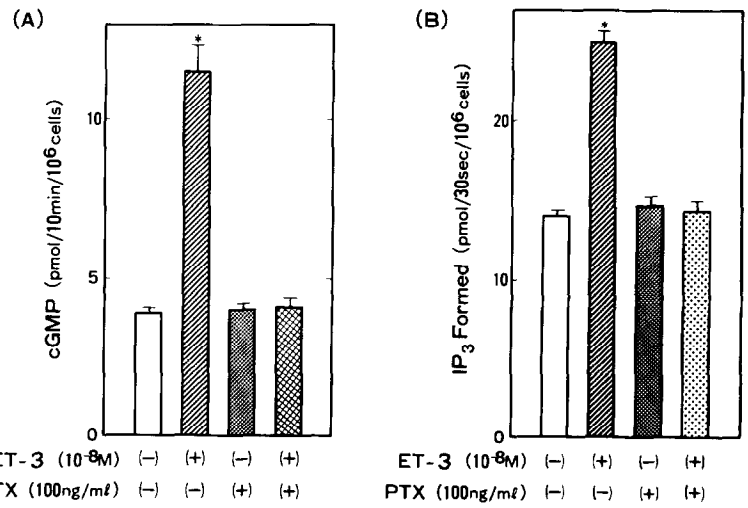

Fig. 3. Effects of pertussis toxin on formation of cGMP (A) and IP_3 (B) by ET-3 in cultured bovine EC.
After preincubation with or without PTX (100 ng /ml) for 6 hrs, ECs were incubated at 37°C with or without ET-3 (10^{-8} M) for 10 min in the presence of isobutylmethylxanthine (A) and for 30 sec in the presence of 10 mM LiCl (B). Each column is the mean of 6 dishes; bars shows SE. *$P<0.05$ vs. control.

abolished by methylene blue, a soluble guanylate cyclase inhibitor, as well as L-NMMA, a stereospecific inhibitor of NO synthesis (5), whereas the effect of SNP that directly activates soluble guanylate cyclase in EC is blocked by methylene blue, but not by L-NMMA. These data strongly suggest that ET-3 stimulates NO synthesis from L-arginine in EC, which then activates soluble guanylate cyclase to increase intracellular cGMP.

Recently, it has been reported that ET-3 in low doses induced endothelium-dependent vasodilatation in perfused rat mesentric arteries, whose effect was inhibited by L-NMMA and reverserd by L-arginine (11). These data are compatible with ours. Taken together, it is suggested that ET-3 may function as a vasodilator by stimulating production of NO from L-arginine in EC to activate soluble guanylate cyclase in VSMC.

The present study further shows that PTX completely abolished the ET-3-induced IP_3 formation and subsequent $[Ca^{2+}]_i$ increase in bovine EC. It is well recognized that interaction of Ca^{2+}-mobilizing ligands with their specific membrane receptors leads to phospholipase C (PLC) activation accompanied by hydrolysis of phosphoinositide-4,5-bisphosphate and the consequent generation of IP_3 and diacylglycerol (12,13), and G-proteins play an important regulatory role in coupling of receptors to PLC (14).

The present data suggest that endothelial ET-3 receptor is functionally coupled to PLC via PTX-sensitive G-protein(s). This is in contrast to ET-1 receptor in VSMC functionally coupled to PLC via PTX-insensitive G-protein(s) (15). In certain cells, PTX blocks hormone-dependent activation of phospho-

inositol turnover by ADP ribosylating and uncoupling G-proteins from receptors, while hormone-dependent phosphoinositide hydrolysis is insensitive to PTX in other cells. Even in the same human umbilical EC, histamine stimulates phosphoinositol turnover *via* PTX-sensitive G-protein, but bradykinin involves PTX-insensitive phosphoinositol response (16). The inhibitory effect of PTX on the ET-3-induced cGMP formation and the lack of its effect on the SNP-induced cGMP formation also lend strong support to the contention that the ET-3-induced NO synthesis is mediated by phosphoinositide breakdown *via* PTX-sensitive G-protein(s).

It has been shown that NO-forming enzyme(s) is critically dependent on Ca^{2+}, NADPH and tetrahydrobiopterin (17). Therefore, it is possible to speculate that the ET-3-induced $[Ca^{2+}]_i$ increase resulting from phosphoinositide breakdown may play an important role in synthesis and/or release of NO in EC. In fact, it has been reported that the formation of NO by other agonists, such as ATP and bradykinin, is triggered by Ca^{2+} mobilization in EC (4). Thus, characterization of the putative NO-forming enzyme(s) as well as its regulatory mechanism in EC should elucidate the control of vascular tonus by the endothelium-dependent NO.

ACKNOWLEDGMENTS

This study was supported in part by Grants-in-Aid from the Ministry of Education, Science & Culture (01480217, 01480286, 02304055) and the Ministry of Health & Welfare (63C-1), Japan.

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