

Endothelin (ET)-3 stimulates cyclic guanosine 3',5'-monophosphate production via ETB receptor by producing nitric oxide in isolated rat glomerulus, and in cultured rat mesangial cells.

A Owada, ... , H Nonoguchi, F Marumo

J Clin Invest. 1994;**93**(2):556-563. <https://doi.org/10.1172/JCI117007>.

Research Article

We investigated the effects of endothelins on receptor-mediated cyclic nucleotide metabolism in rat glomerulus, inner medullary collecting duct (IMCD), and also in cultured rat glomerular mesangial cells. Endothelin (ET)-3 dose-dependently stimulated cGMP accumulation in glomerulus, which was higher than that of ET-1 or ET-2. ETB receptor agonist IRL 1620 produced cGMP in a dose-dependent manner, mimicking the effect of ET-3. ETA receptor antagonist BQ123-Na did not inhibit ET-3- or IRL 1620-stimulated cGMP generation. NG-monomethyl-L-arginine (L-NMMA) significantly inhibited ET-3- or IRL 1620-induced cGMP production, suggesting that ET-3- or IRL 1620-stimulated cGMP generation was mediated through nitric oxide (NO). Intracellular Ca chelator BAPTA/AM and calmodulin antagonist W-7, but not Ca channel blocker nifedipine, significantly inhibited ET-3- or IRL 1620-induced cGMP generation. In cultured rat mesangial cells, ET-3 stimulated cGMP generation through NO in the presence of fetal calf serum, which was not inhibited by addition of BQ123-Na. In IMCD, ET-3 had no stimulative effect on cGMP generation. We conclude that ET-3 stimulates NO-induced cGMP generation through ETB receptor in glomerulus. This effect seems to be mediated through intracellular Ca/calmodulin, but not through Ca influx via L-type Ca channel. Mesangial cells can be a source of NO coupled to ETB receptor activation in glomerulus. From these results, mesangial ETB receptor may work to counteract the vasoconstrictive effect of endothelin caused via ETA receptor [...]

Find the latest version:

<https://jci.me/117007/pdf>



Endothelin (ET)-3 Stimulates Cyclic Guanosine 3',5'-Monophosphate Production via ET_B Receptor by Producing Nitric Oxide in Isolated Rat Glomerulus, and in Cultured Rat Mesangial Cells

Akira Owada, Kimio Tomita, Yoshio Terada, Hisato Sakamoto, Hiroshi Nonoguchi, and Fumiaki Marumo
Second Department of Internal Medicine, Tokyo Medical and Dental University, Tokyo 113, Japan

Abstract

We investigated the effects of endothelins on receptor-mediated cyclic nucleotide metabolism in rat glomerulus, inner medullary collecting duct (IMCD), and also in cultured rat glomerular mesangial cells. Endothelin (ET)-3 dose-dependently stimulated cGMP accumulation in glomerulus, which was higher than that of ET-1 or ET-2. ET_B receptor agonist IRL 1620 produced cGMP in a dose-dependent manner, mimicking the effect of ET-3. ET_A receptor antagonist BQ123-Na did not inhibit ET-3- or IRL 1620-stimulated cGMP generation. N^G-monomethyl-L-arginine (L-NMMA) significantly inhibited ET-3- or IRL 1620-induced cGMP production, suggesting that ET-3- or IRL 1620-stimulated cGMP generation was mediated through nitric oxide (NO). Intracellular Ca chelator BAPTA/AM and calmodulin antagonist W-7, but not Ca channel blocker nifedipine, significantly inhibited ET-3- or IRL 1620-induced cGMP generation. In cultured rat mesangial cells, ET-3 stimulated cGMP generation through NO in the presence of fetal calf serum, which was not inhibited by addition of BQ123-Na. In IMCD, ET-3 had no stimulative effect on cGMP generation. We conclude that ET-3 stimulates NO-induced cGMP generation through ET_B receptor in glomerulus. This effect seems to be mediated through intracellular Ca/calmodulin, but not through Ca influx via L-type Ca channel. Mesangial cells can be a source of NO coupled to ET_B receptor activation in glomerulus. From these results, mesangial ET_B receptor may work to counteract the vasoconstrictive effect of endothelin caused via ET_A receptor in glomerulus. (*J. Clin. Invest.* 1994; 93:556-563.) Key words: microdissection • inner medullary collecting duct • endothelin receptor • Ca/calmodulin • protein kinase C

Introduction

Endothelins are the most potent vasoconstrictive peptides known (1). There are three isopeptides: endothelin (ET)¹-1, ET-2, and ET-3 (2). Although endothelins have both vasoconstrictive and vasodilative effects (2), it remains to be elucidated

how these opposite effects are interacted to modulate vascular tone and renal hemodynamics.

There are at least two subtypes of ET receptor, namely, ET_A and ET_B (3, 4). Sakurai et al. (3) demonstrated a non-isopeptide-selective subtype of endothelin receptor (ET_B receptor) from rat lung, which had roughly equipotent affinity to ET-1, ET-2, and ET-3 in transfected cells. At the same time, Arai et al. (4) showed a subtype of endothelin receptor (ET_A receptor) from bovine lung, which had much higher affinity to ET-1 than to ET-2 (~ 10 times) and to ET-3 (> 100 times). Functional differences between these two receptor subtypes in the intact kidney and signal transduction mechanisms coupled to each receptor subtype have not been shown to date. Recently, we demonstrated the presence of ET_A receptor in glomerulus and vessels of rat kidney, and the presence of ET_B receptor in glomerulus and inner medullary collecting duct (IMCD) using reverse transcription (RT)-PCR (5). These results prompted us to investigate the effects of ET on both glomerulus and IMCD.

cGMP causes relaxation of glomerular mesangial cells. Atrial natriuretic factor is known to stimulate cGMP generation in mesangial cells to increase GFR. There have been no data as to whether endothelins generate cyclic nucleotide in glomerulus or tubules to modulate renal hemodynamics. Nitric oxide (NO), which is synthesized from L-arginine in vascular endothelial cells, generates cGMP in vascular smooth muscle cells (6), resulting in dilatation of vessels. Our recent result suggests the presence of constitutive NO synthase both in glomerulus and IMCD (7), and both glomerular endothelial and mesangial cells have been shown to produce NO (8-11). Thus, ET may affect the tone of mesangial cells in glomerulus or sodium handling in IMCD, by NO-induced cGMP generation to modulate renal function.

We determined cGMP production by endothelins in both glomerulus and IMCD of rat, and in cultured rat mesangial cells, to specify the intraglomerular site of cGMP production. We also investigated the cellular mechanisms that generate cGMP, including specifying of the responsible ET receptor subtype.

Methods

Materials. ET-1, ET-2, and ET-3 were purchased from Peptide Institute (Osaka, Japan). ET-1 was dissolved with distilled water, and ET-2

Portions of this study were presented at the 24th and 25th annual meetings of the American Society of Nephrology, 1991 and 1992, respectively, Baltimore, MD.

Address correspondence to Dr. Kimio Tomita, Second Department of Internal Medicine, Tokyo Medical and Dental University, 5-45, Yushima 1-chome, Bunkyo-ku, Tokyo 113, Japan.

Received for publication 24 September 1992 and in revised form 23 September 1993.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/94/02/0556/08 \$2.00

Volume 93, February 1994, 556-563

1. *Abbreviations used in this paper:* BAPTA/AM, bis-(*o*-aminopropoxy)-ethane-*N,N,N',N'*-tetraacetic acid, tetra(acetoxymethyl)-ester; BQ123-Na, C₃₁H₄₁O₇N₆Na; DiC8, 1,2-dioctanoyl-*sn*-glycerol; EDRF, endothelium-derived relaxing factor; ET, endothelin; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; IBMX, 3-isobutyl-1-methylxanthine; IMCD, inner medullary collecting duct; IRL 1620, Suc-[Glu⁹, Ala^{11,15}]-endothelin-1 (8-21); L-NMMA, N^G-monomethyl-L-arginine; PKC, protein kinase C; W-7, *N*-(6-aminoethyl)-5-chloro-naphthalenesulfonamide hydrochloride.

and ET-3 were dissolved with 0.1% aqueous acetic acid. Methylene blue, hemoglobin, protein kinase C (PKC) activator 1,2-dioctanoyl-*sn*-glycerol (DiC8), phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), TCA, Ca ionophore, and BSA were obtained from Sigma Chemical Co. (St. Louis, MO). PKC inhibitor 1-(5-isquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), and calmodulin antagonist *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7), were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). *N*^G-monomethyl-L-arginine (L-NMMA) and intracellular Ca chelator bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid, tetra(acetoxymethyl)-ester (BAPTA/AM) were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). ET_A receptor antagonist BQ123-Na, C₃₁H₄₁O₇N₆Na, was kindly donated by Banyu Pharmaceutical Co., Ltd. (Tokyo, Japan). ET_B receptor agonist IRL 1620, Suc-[Glu², Ala^{11,15}]-endothelin-1 (8-21), was donated by International Research Laboratories, Ciba-Geigy Ltd. (Hyogo, Japan). Nicardipine was kindly provided by Yamanouchi Pharmaceutical Ltd. (Tokyo, Japan). RPMI 1640 and FCS for mesangial cell culture were purchased from Gibco Laboratories (Grand Island, NY). All other chemicals were of first-grade purity.

Solutions. The microdissection solution (solution 1) was a Hepes-buffered solution of the following concentration (millimolar): 130 NaCl, 5 KCl, 1 NaH₂PO₄, 1 MgSO₄, 1 Ca lactate, 2 Na acetate, 5.5 glucose, 5 L-alanine, 2 L-leucine, and 10 Hepes, and 0.05% BSA was added after pH adjustment to 7.4 by NaOH. The collagenase solution (solution 2) was of the same composition as the microdissection solution (solution 1), except it contained 0.1% BSA and 0.1% collagenase.

Isolation of rat glomerulus and IMCD. Microdissection of glomerulus and IMCD was done as previously described (12). Male Sprague-Dawley rats (120–150 g) were anesthetized with pentobarbital sodium (50 mg/kg i.p.). The left kidney was removed after in situ perfusion with solution 2 after clamping the abdominal aorta and the right renal artery. 1-mm-thick slices of the kidney suspended in 10 ml of solution 2 were incubated for 40 min at 37°C with bubbling of 100% O₂. The slices were then washed with solution 1 and were transferred to a chilled petri dish containing solution 1 that was kept at 4°C by a cooling plate. Glomerulus and IMCD were dissected under a dissecting microscope (Olympus Corp., Tokyo, Japan). Microdissected nephrons were transferred with 2 μl of solution 1 using a siliconized glass pipette into 1.5-ml plastic centrifuge tubes containing 18 or 28 μl of solution 1 with 0.5 mM IBMX.

Microdissected glomeruli or IMCD in 20 or 30 μl of solution 1 containing 0.5 mM IBMX were preincubated for 20 min at 37°C with or without methylene blue, hemoglobin, L-NMMA, H-7, W-7, nicardipine, BAPTA/AM, or BQ123-Na, which was dissolved in 10 μl of solution 1 containing 0.5 mM IBMX. After preincubation, an appropriate concentration of ET-1, ET-2, ET-3, IRL 1620, DiC 8, or Ca ionophore in 10 μl of solution 1 with 0.5 mM of IBMX, was added. The incubation was terminated after 1–10 min by the addition of 50 μl of chilled 10% TCA with complete mixing (total amount, 90 μl). 70-μl samples from the supernatant were obtained after centrifuging the tubes for 10 min at 3,000 rpm, and they were stored at –40°C until the assay of cGMP.

Mesangial cell culture. Rat mesangial cells were cultured as previously described (13). Subcultured mesangial cells (5–11th passage) were prepared on 6-well dishes. After 48 h-serum depletion, confluent mesangial cells washed with phosphate buffered saline (PBS) were preincubated for 3 h with RPMI 1640 in the presence or absence of 20% FCS. Subsequently, the cells were prewashed with Hepes-buffered solution 1 (without BSA), and incubated for 3 min at 37°C with ET-3, L-NMMA, or BQ123-Na, which was dissolved in 1 ml of solution 1 with 0.5 mM IBMX (without BSA). The solution was aspirated, and the incubation was stopped by the addition of 1 ml of chilled 10% TCA. The samples were centrifuged and each 100-μl supernatant was stored at –40°C until cGMP assay.

cGMP assay. In each experiment, blank samples (containing medium and/or TCA only) were also processed for cGMP standards. After TCA extraction, each sample was tested for cGMP content. Ap-

propriate cGMP standards were added to blank samples. Samples of unknowns and cGMP standards were then assayed for cGMP content (radioimmunoassay kits; New England Nuclear, Boston, MA). The values of cGMP content were expressed per glomerulus per min, per millimeter per min (IMCD), or per μg protein per min (mesangial cells). The results of cGMP generation are values after subtracting basal values. Cellular protein was measured by a dye-binding method (Bio-Rad Laboratories, Richmond, CA) using BSA as a standard.

Statistical analysis was performed using Student's *t* test and ANOVA. *P* < 0.05 was considered significant.

Results

Effects of endothelins on cGMP generation in rat glomerulus and IMCD (Fig. 1). All of the endothelin isopeptides (ET-1, ET-2, and ET-3) stimulated cGMP generation in isolated rat glomerulus. The rank order of cGMP generation was ET-3 (*n* = 4, 0.21 ± 0.01 fmol/Gl per min, means ± SEM), ET-1 (*n* = 4, 0.15 ± 0.01 fmol/Gl per min), ET-2 (*n* = 6, 0.09 ± 0.01 fmol/Gl per min) and basal (*n* = 5, 0.02 ± 0.00 fmol/Gl per min). Therefore, ET-3 was used in further experiments. In IMCD, ET-3 did not stimulate cGMP production (*n* = 4, undetectable, < 0.01 fmol/mm per min). The subsequent results of cGMP generation are values after subtracting basal values.

Time course of the effect of ET-3 on cGMP generation in rat glomerulus (Fig. 2). ET-3 (10^{–7} M) stimulated cGMP generation in rat glomerulus in a time-dependent manner from 1–5 min (*n* = 3 or 4). cGMP accumulation by ET-3 linearly increased until 5 min. Therefore, glomeruli were incubated for 3 min in further experiments.

Dose-dependent effect of ET-3 on cGMP generation in rat glomerulus (Fig. 3). ET-3 stimulated cGMP generation in rat glomerulus in a dose-dependent manner, from 10^{–10} to 10^{–6} M (*n* = 3–5).

Effects of guanylate cyclase inhibitors methylene blue and hemoglobin on ET-3-induced cGMP production in rat glomerulus (Fig. 4). Two guanylate cyclase inhibitors were used to

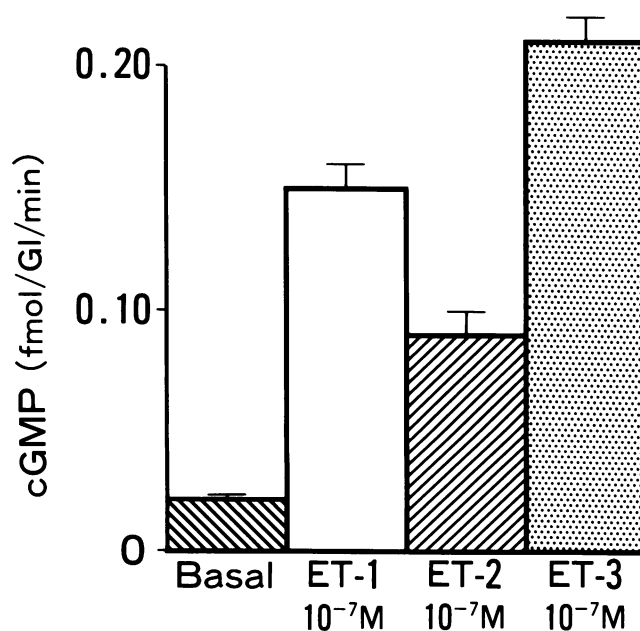


Figure 1. cGMP generation by endothelins in rat glomerulus. The rank order of cGMP generation was ET-3 > ET-1 > ET-2. Means ± SEM, *n* = 4–6.

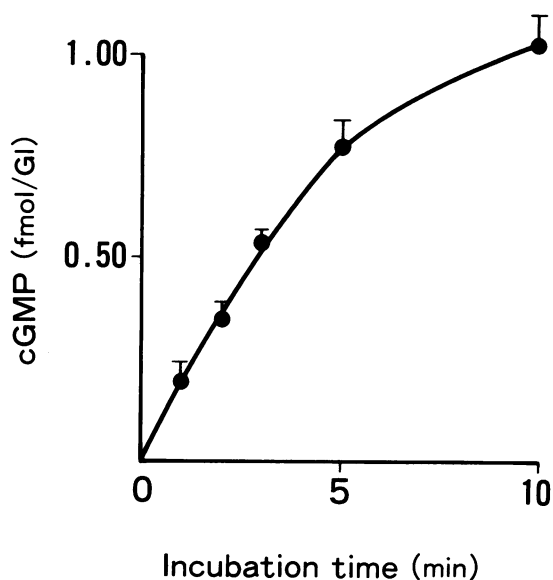


Figure 2. Time course of the effect of ET-3 on cGMP generation in rat glomerulus. ET-3 (10^{-7} M) stimulated cGMP generation in a time-dependent fashion from 1–5 min. The results of cGMP generation are values after subtracting basal values. Means \pm SEM, $n = 3$ or 4.

determine whether the guanylate cyclase was involved in the effect of ET-3 on cGMP production in rat glomerulus. Methylene blue (10^{-5} M) significantly inhibited ET-3-induced cGMP production, from 0.22 ± 0.02 to 0.11 ± 0.00 fmol/Gl per min ($n = 6$, $P < 0.01$), and hemoglobin (10^{-5} M) also significantly inhibited ET-3-induced cGMP production, from 0.22 ± 0.02 to 0.15 ± 0.01 fmol/Gl per min ($n = 5$, $P < 0.05$).

Effect of L-arginine analogue L-NMMA on ET-3-induced cGMP generation in rat glomerulus (Fig. 5). L-arginine analogue L-NMMA was used to determine whether NO synthesis

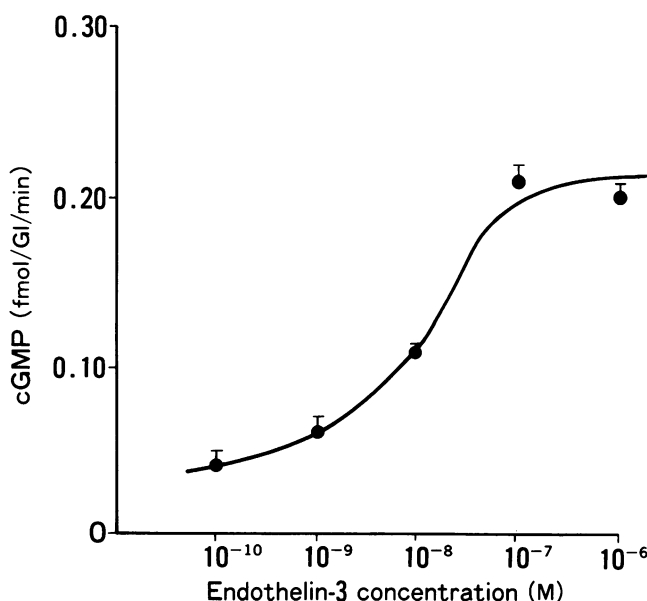


Figure 3. Dose-dependent effect of ET-3 on cGMP generation in rat glomerulus. ET-3 dose-dependently (10^{-10} M to 10^{-6} M) stimulated cGMP generation. The results of cGMP generation are values after subtracting basal values. Means \pm SEM, $n = 3$ –5.

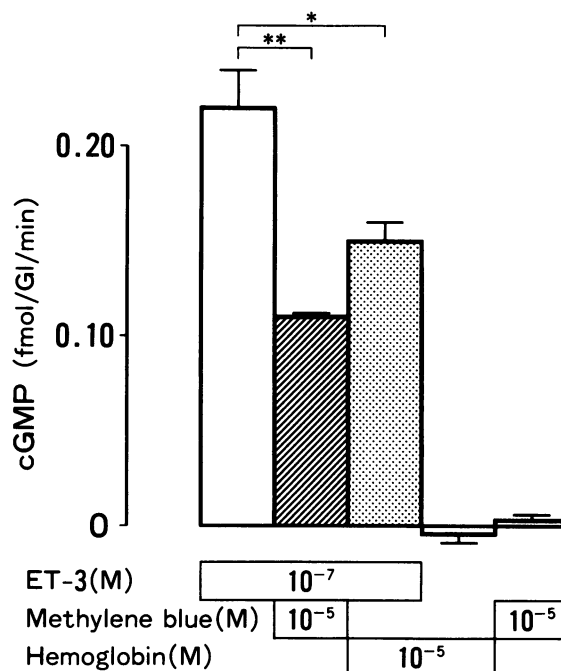


Figure 4. Effects of guanylate cyclase inhibitors methylene blue and hemoglobin on ET-3-induced cGMP production in rat glomerulus. cGMP production by ET-3 (10^{-7} M) is depicted (open bar); glomeruli were preincubated with methylene blue (10^{-5} M), and incubated with ET-3 (10^{-7} M), as graphed (hatched bar). Glomeruli were preincubated with hemoglobin (10^{-5} M) and incubated with ET-3 (10^{-7} M), as indicated (stippled bar). Methylene blue and hemoglobin significantly inhibited ET-3-induced cGMP production. The results of cGMP generation are values after subtracting basal values. Means \pm SEM, $n = 5$ or 6. * $P < 0.05$, ** $P < 0.01$.

was involved in ET-3-induced cGMP generation in rat glomerulus. L-NMMA (10^{-3} M) significantly inhibited ET-3-induced cGMP generation in rat glomerulus, from 0.20 ± 0.01 to 0.07 ± 0.00 fmol/Gl per min ($n = 5$, $P < 0.01$).

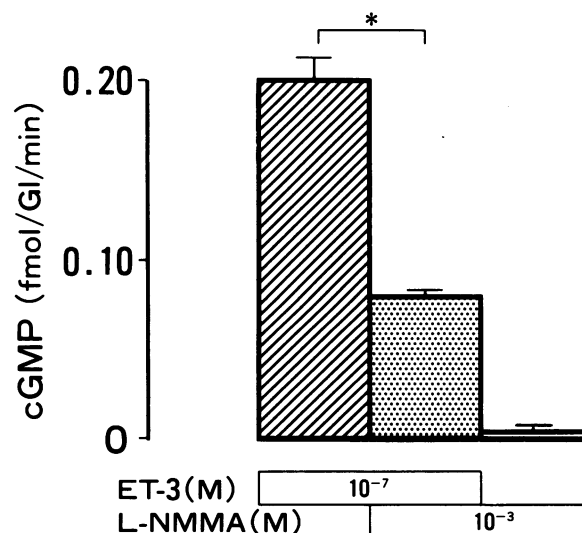


Figure 5. Effect of L-arginine analogue L-NMMA on ET-3-induced cGMP generation in rat glomerulus. L-NMMA (10^{-3} M) significantly inhibited ET-3 (10^{-7} M)-induced cGMP generation. The results of cGMP generation are values after subtracting basal values. Means \pm SEM, $n = 5$. * $P < 0.01$.

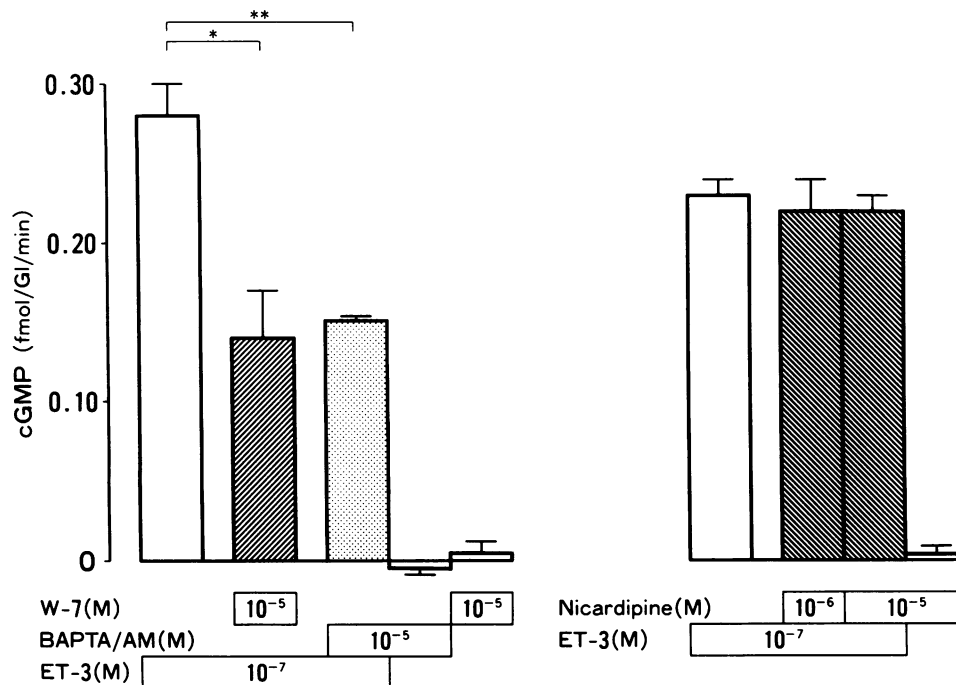


Figure 6. Effects of intracellular Ca chelator BAPTA/AM, calmodulin antagonist W-7, and Ca channel blocker nicardipine on ET-3-stimulated cGMP generation in rat glomerulus. Both BAPTA/AM (10^{-5} M) and W-7 (10^{-5} M) significantly inhibited ET-3 (10^{-7} M)-stimulated cGMP generation (left). Nicardipine (10^{-6} M or 10^{-5} M) did not inhibit ET-3 (10^{-7} M)-stimulated cGMP generation (right). The results of cGMP generation are values after subtracting basal values. Means \pm SEM, $n = 4$ or 5 . * $P < 0.01$, ** $P < 0.001$.

Effects of intracellular Ca chelator BAPTA/AM, calmodulin antagonist W-7, Ca ionophore, and Ca channel blocker nicardipine on ET-3-stimulated cGMP generation in rat glomerulus (Fig. 6). Effects of BAPTA/AM, W-7, Ca ionophore, and nicardipine were studied to investigate whether Ca/calmodulin complex was involved in ET-3-stimulated cGMP generation in glomerulus. BAPTA/AM (10^{-5} M) significantly inhibited ET-3-stimulated cGMP generation, from 0.28 ± 0.02 to 0.16 ± 0.00 fmol/Gl per min ($n = 4$, $P < 0.001$). W-7 (10^{-5} M) also significantly inhibited ET-3-stimulated cGMP generation, from 0.28 ± 0.02 to 0.14 ± 0.03 fmol/Gl per min ($n = 5$, $P < 0.01$). Ca ionophore (10^{-6} M) stimulated cGMP production ($n = 4$, 0.05 ± 0.01 fmol/Gl per min). Nicardipine (10^{-6} M or 10^{-5} M) did not inhibit ET-3-stimulated cGMP generation, from 0.23 ± 0.01 fmol/Gl per min ($n = 4$) to 0.22 ± 0.03 fmol/Gl per min ($n = 5$) at 10^{-6} and 10^{-5} M, respectively.

Effect of PKC inhibitor H-7 on ET-3-stimulated cGMP generation, and effect of PKC activator DiC 8 on cGMP generation in rat glomerulus (Fig. 7). Effects of modulation of PKC activity were examined to investigate whether PKC activation was involved in ET-3-stimulated cGMP generation in glomerulus.

H-7 did not inhibit ET-3-stimulated cGMP generation, from 0.28 ± 0.02 fmol/Gl per min ($n = 5$) to 0.28 ± 0.01 fmol/Gl per min ($n = 5$), and to 0.23 ± 0.01 fmol/Gl per min ($n = 5$) at 10^{-6} and 10^{-5} M, respectively. DiC 8 did not mimic the stimulating effect of ET-3 on cGMP generation (0.02 ± 0.00 fmol/Gl per min, $n = 3$, and 0.01 ± 0.00 fmol/Gl per min, $n = 4$, at 2.5 and 25 $\mu\text{g/ml}$, respectively).

Effects of ET_A receptor antagonist BQ123-Na on ET-3-stimulated cGMP generation in rat glomerulus (Fig. 8). BQ123-Na did not inhibit ET-3-stimulated cGMP generation in rat glomerulus from 0.15 ± 0.01 fmol/Gl per min ($n = 7$) to 0.14 ± 0.01 ($n = 3$), 0.13 ± 0.00 ($n = 5$), and 0.15 ± 0.01 fmol/Gl per min ($n = 6$), at concentrations of 10^{-10} , 10^{-8} , and 10^{-6} M, respectively. However, a higher concentration (10^{-3} M) of BQ123-Na significantly inhibited ET-3-stimulated cGMP gen-

eration in glomerulus, from 0.15 ± 0.01 to 0.08 ± 0.00 fmol/Gl per min, probably due to occupation of both ET_A and ET_B receptors ($n = 7$, $P < 0.01$).

Dose-dependent effect of ET_B receptor agonist IRL 1620 on cGMP generation, and effect of L-arginine analogue L-NMMA on IRL 1620-induced cGMP generation in rat glomerulus (Fig. 9). IRL 1620 (10^{-10} – 10^{-6} M) produced cGMP in a dose-dependent manner, mimicking the effect of ET-3. L-NMMA (10^{-3} M) significantly inhibited IRL 1620 (10^{-6} M)-stimu-

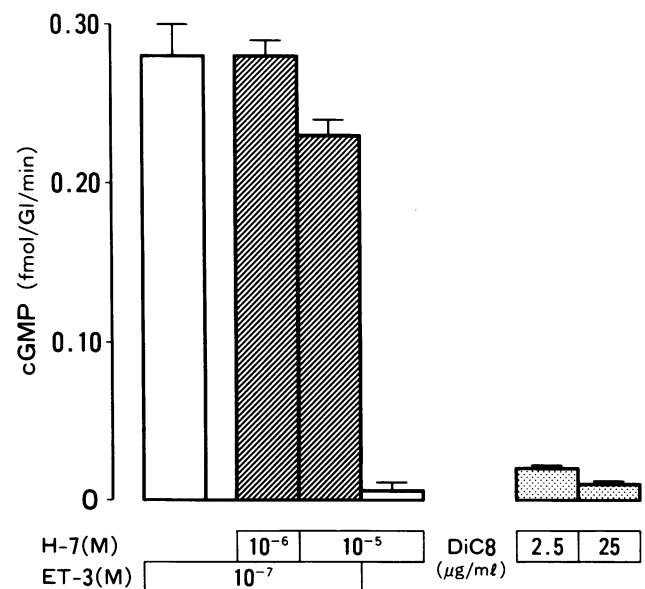


Figure 7. Effect of protein kinase C inhibitor H-7 on ET-3-stimulated cGMP generation, and effect of protein kinase C activator DiC 8 on cGMP generation in rat glomerulus. H-7 (10^{-6} M or 10^{-5} M) did not inhibit ET-3 (10^{-7} M)-induced cGMP generation (left). DiC 8 (2.5 or 25 $\mu\text{g/ml}$) did not mimic the stimulating effect of ET-3 on cGMP generation. The results of cGMP generation are values after subtracting basal values. Means \pm SEM, $n = 3$ – 5 .

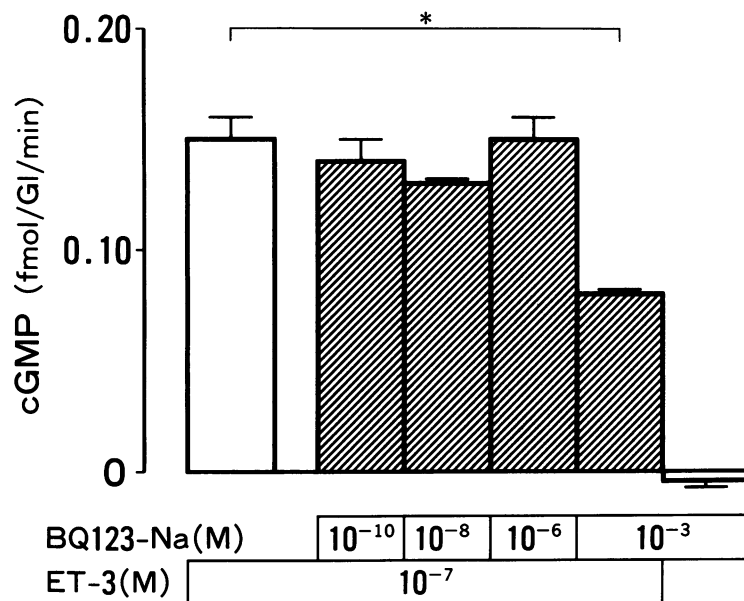


Figure 8. Effects of ET_A receptor antagonist BQ123-Na on ET-3-stimulated cGMP generation in rat glomerulus. BQ123-Na did not inhibit ET-3 (10⁻⁷ M)-stimulated cGMP generation at concentrations of 10⁻¹⁰, 10⁻⁸, and 10⁻⁶ M. However, a quite high concentration (10⁻³ M) of BQ123-Na significantly inhibited ET-3-stimulated cGMP generation. The results of cGMP generation are values after subtracting basal values. Means±SEM, *n* = 3–7. **P* < 0.01.

lated cGMP generation, from 0.62±0.07 to 0.04±0.01 fmol/Gl per min (*n* = 3, *P* < 0.01).

Effect of ET_A receptor antagonist BQ123-Na on IRL 1620-stimulated cGMP production in rat glomerulus (Fig. 10). We examined the possibility that ET_B receptor agonist IRL 1620 might also occupy the ET_A receptor, resulting in cGMP production, because higher concentrations of IRL 1620 may bind to both ET_B and ET_A receptor. We added a sufficient concentration (10⁻⁶ M) of BQ123-Na to occupy ET_A receptor together with IRL 1620 (10⁻⁸ or 10⁻⁷ M). BQ123-Na (10⁻⁶ M) did not inhibit IRL 1620 (10⁻⁸ or 10⁻⁷ M)-stimulated cGMP generation, from 0.23±0.03 to 0.25±0.04 fmol/Gl per min (*n* = 3), and from 0.49±0.04 to 0.52±0.03 fmol/Gl per min (*n* = 3), respectively. Therefore, most of cGMP production was considered to be due to ET_B receptor.

Effects of intracellular Ca chelator BAPTA/AM, calmodulin antagonist W-7, and Ca channel blocker nifedipine on IRL 1620-stimulated cGMP production in rat glomerulus (Fig. 11). BAPTA/AM (10⁻⁵ M) and W-7 (10⁻⁵ M) significantly inhibited IRL 1620-stimulated cGMP generation, from 0.23±0.03 to 0.06±0.01 fmol/Gl per min (*n* = 3), and to 0.11±0.01 fmol/Gl per min (*n* = 3), respectively. In contrast, nifedipine (10⁻⁵ M) did not inhibit IRL 1620-stimulated cGMP generation, from 0.23±0.03 to 0.20±0.03 fmol/Gl per min (*n* = 3).

Effect of ET-3 on cGMP production, and effect of L-arginine analogue L-NMMA, and effect of ET_A receptor antagonist BQ123-Na on ET-3-induced cGMP production in cultured rat mesangial cells (Fig. 12). Basal cGMP production by cultured mesangial cells was 0.07±0.01 fmol/μg protein per min when mesangial cells were preincubated in the absence of 20% FCS, and 0.15±0.04 fmol/μg protein per min when mesangial cells were preincubated for 3 h in the presence of 20% FCS. The subsequent results of cGMP generation are values after subtracting basal values. ET-3 (10⁻⁷ M) did not stimulate cGMP production when mesangial cells were preincubated in the absence of 20% FCS. In contrast, ET-3 (10⁻⁷ M) stimulated cGMP production in the presence of 20% FCS (*n* = 3, 0.15±0.03 fmol/μg protein per min). L-NMMA (10⁻³ M) significantly inhibited ET-3-induced cGMP production in the

presence of 20% FCS (*n* = 3, 0.07±0.02 fmol/μg protein per min, *P* < 0.001 vs. ET-3 alone). BQ123-Na (10⁻⁸ M) did not inhibit ET-3-stimulated cGMP production in the presence of 20% FCS (*n* = 3, 0.14±0.01 fmol/μg protein per min).

Discussion

Our study showed that ET-3 as well as ET-1 and ET-2 stimulated cGMP production in rat glomerulus, and that ET-3 stimu-

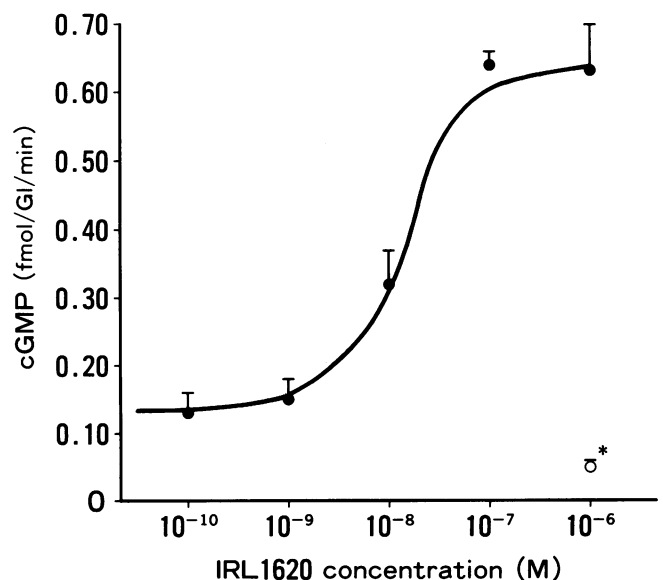


Figure 9. Dose-dependent effect of ET_B receptor agonist IRL 1620 on cGMP generation, and effect of L-arginine analogue L-NMMA on IRL 1620-induced cGMP generation in rat glomerulus. IRL 1620 dose-dependently (10⁻¹⁰ to 10⁻⁶ M; closed circles) stimulated cGMP production. Glomeruli (open circle) were preincubated with L-NMMA (10⁻³ M) and incubated with IRL 1620 (10⁻⁶ M). L-NMMA significantly inhibited IRL 1620-induced cGMP generation. The results of cGMP generation are values after subtracting basal values. Means±SEM, *n* = 3. **P* < 0.01.

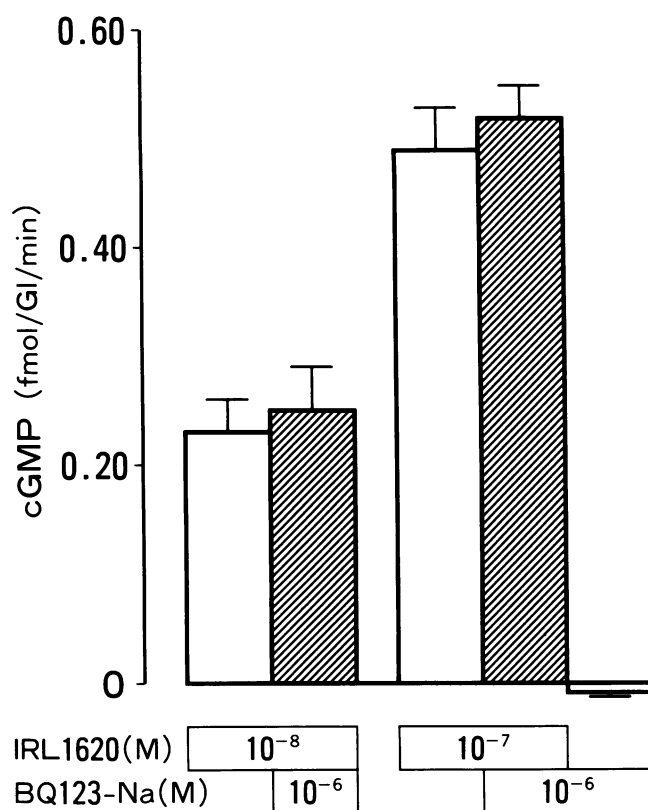


Figure 10. Effect of ET_A receptor antagonist BQ123-Na on IRL 1620-stimulated cGMP production in rat glomerulus. BQ123-Na (10^{-6} M) did not inhibit IRL 1620 (10^{-8} M or 10^{-7} M)-stimulated cGMP production. The results of cGMP generation are values after subtracting basal values. Means \pm SEM, $n = 3$.

lated cGMP production in cultured rat glomerular mesangial cells in the presence of FCS. These effects were considered to be mediated through NO, because L-arginine antagonist L-NMMA abolished ET-3-induced cGMP production in glomerulus and in mesangial cells. These results are compatible with previous reports of in vitro studies using rat mesenteric arteries (14–16).

As for the specification of receptor subtypes, ET_B receptor, but not ET_A receptor, was considered to be responsible for ET-3-mediated cGMP generation through NO in glomerulus or in mesangial cells, because our study demonstrated that an ET_B receptor agonist (IRL 1620) mimicked the effect of ET-3 on NO-dependent cGMP generation in glomerulus (Fig. 9), and that an ET_A receptor antagonist (BQ123-Na) did not inhibit the stimulating effects of ET-3 or IRL 1620 on cGMP generation in glomerulus or in mesangial cells (Figs. 8, 10, 12). The binding affinity of BQ123-Na to ET_A receptor was over one-thousandfold more than to ET_B receptor; the IC_{50} for ^{125}I -labeled ET-1 binding values to vascular smooth muscle cells (ET_A receptor) and to cerebellum membranes (ET_B receptor) were 7.3×10^{-9} and 1.8×10^{-5} M, respectively (17). IRL 1620 was a specific ligand for ET_B receptor, as judged by the K_i values for ET_A (1.9×10^{-6} M) and ET_B (1.6×10^{-11} M) receptors (18). Our study demonstrated that IRL 1620 dose-dependently (10^{-10} to 10^{-6} M) stimulated cGMP generation in glomerulus (Fig. 9). The possibility is less likely that higher concentrations of IRL 1620 bound to ET_A receptor as well as to ET_B receptor, thereby inducing cGMP generation through ET_A

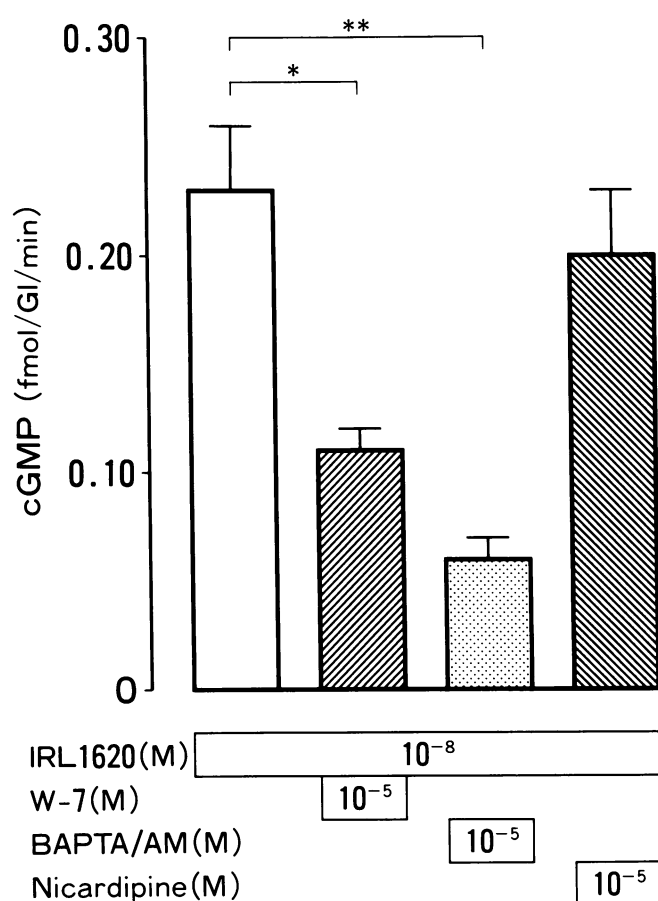


Figure 11. Effects of intracellular Ca chelator BAPTA/AM, calmodulin antagonist W-7, and Ca channel blocker nicardipine on IRL 1620-stimulated cGMP production in rat glomerulus. Both BAPTA/AM (10^{-5} M) and W-7 (10^{-5} M) significantly inhibited IRL 1620 (10^{-8} M)-stimulated cGMP production. In contrast, nicardipine (10^{-5} M) did not inhibit IRL 1620-stimulated cGMP production. The results of cGMP generation are values after subtracting basal values. Means \pm SEM, $n = 3$. * $P < 0.05$, ** $P < 0.01$.

receptor, because our data showed that 10^{-6} M of BQ123-Na (which almost completely occupied ET_A receptor) did not affect IRL 1620 (10^{-8} or 10^{-7} M)-stimulated cGMP production in glomerulus (Fig. 10). Our data suggest that IRL 1620-stimulated cGMP production was mediated only through ET_B receptor.

Our results showed that IRL 1620 stimulates glomerular cGMP generation better than any of the native ET isoforms. Takai et al. (18) demonstrated that the K_i value of IRL 1620 for ET_B receptor (1.6×10^{-11} M) was twofold higher than that of ET-1 or ET-3 (both were 8×10^{-12} M) in porcine lung membranes, suggesting that the binding affinity of IRL 1620 for ET_B receptor was half as much as that of ET-1 or ET-3. Thus, ET-1 or ET-3 would stimulate cGMP generation better than IRL 1620. However, the K_i value of IRL 1620 for ET_A receptor (1.9×10^{-6} M) was much higher than that of ET-1 (4×10^{-11} M) or ET-3 (1.5×10^{-8} M) in porcine lung membranes, suggesting that the binding affinity of IRL 1620 for ET_A receptor was much less than that of ET-1 or ET-3. Therefore, ET_B receptor selectivity (K_{iET_A}/K_{iET_B}) of IRL 1620 (a value of 120,000) was extremely higher than that of ET-1 (a value of 5) or ET-3 (a value of 1,900) in porcine lung membranes (18).

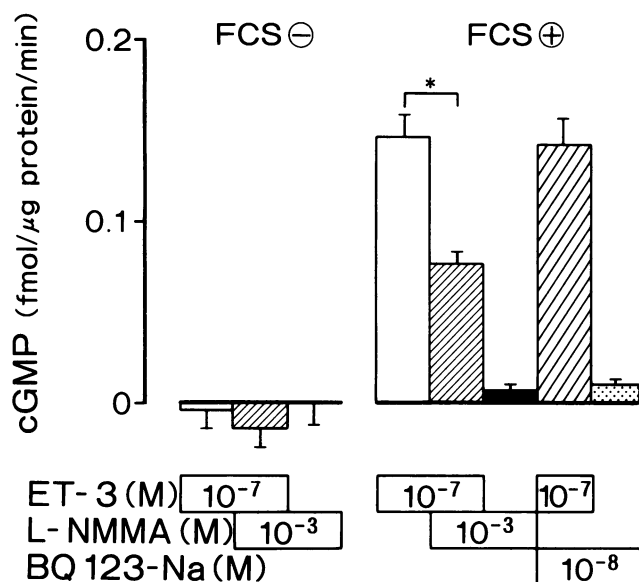


Figure 12. Effect of ET-3 on cGMP production, effect of L-arginine analogue L-NMMA, and effect of ET_A receptor antagonist BQ123-Na on ET-3-induced cGMP production in cultured rat mesangial cells. ET-3 (10⁻⁷ M) stimulated cGMP production when mesangial cells were preincubated for 3 h in the presence of 20% FCS (right), but did not stimulate in the absence of 20% FCS (left). L-NMMA (10⁻³ M) significantly inhibited ET-3-induced cGMP production in the presence of 20% FCS. BQ123-Na (10⁻⁸ M) did not inhibit ET-3-stimulated cGMP production in the presence of 20% FCS. The results of cGMP generation are values after subtracting basal values. Means±SEM, *n* = 3. **P* < 0.001.

Thus, in isolated glomerulus, which has both subtypes of ET receptor (5), it is possible that IRL 1620 stimulates cGMP production better than native ET isopeptides because it should bind to ET_B receptor more than the ET isopeptides.

Our data showed that the rank order of cGMP accumulation was ET-3 > ET-1 > ET-2. The three endothelin isopeptides have roughly equipotent affinity to ET_B receptor in transfected cells (3). However, it is not easy to explain the greater stimulation by ET-3 than ET-1 and ET-2 in our study. Small differences in cGMP generation would happen due to intracellular signal pathway to generate cGMP in intact glomerulus. Therefore, it is possible that cGMP accumulation is not equipotent among the three isopeptides in intact glomerulus. A similar result was also observed in a previous study (19), which showed that ET-3 had a more potent vasodilating action than ET-1, which seems to be mediated via ET_B receptor.

NO can be produced in glomerulus because constitutive NO synthase mRNA was observed in glomerulus in our previous report (7). That report could not differentiate intraglomerular localization of the enzyme between endothelial and mesangial cells. In this study, to examine the possible localization of ET_B receptor-mediated NO synthesis, we used cultured rat mesangial cells. Our study has clearly demonstrated that ET-3 stimulates cGMP generation in mesangial cells in the presence of FCS. Our previous report demonstrated cell cycle-dependent expression of ET receptor subtypes in cultured rat mesangial cells (20). The expression of ET_A receptor mRNA but not that of ET_B receptor mRNA was evident in quiescent cultured mesangial cells, whereas the expression of ET_B receptor mRNA was strongly enhanced in cycling cultured mesan-

gial cells by exposure to FCS (20). These results suggest that mesangial ET_B receptor seems to be activated by stimuli such as FCS. We do not know whether intact isolated glomerulus was quiescent or cycling in our study. It is speculated that glomerular endothelial cells may supply NO via endothelial ET_B receptor to mesangial cells in quiescent glomerulus, whereas mesangial ET_B receptor as well as endothelial ET_B receptor may cooperatively or independently mediate NO generation in cycling glomerulus. Coexistence of endothelial cells as a NO source with mesangial cells has been demonstrated to be crucial in NO-induced cGMP generation by agonists such as bradykinin or thrombin (9, 11). Mesangial ET_B receptor may work to preserve GFR with NO-induced cGMP generation by buffering the contractile effect of ET via ET_A receptor.

Our study showed that intracellular Ca chelator BAPTA/AM (21) and calmodulin antagonist W-7 (22) inhibited NO-dependent cGMP generation by ET-3 or ET_B receptor agonist IRL 1620 in glomerulus. It is not possible that both BAPTA/AM and W-7 might have inhibited soluble guanylate cyclase, instead of inhibiting NO synthase in our study, because our data showed that neither BAPTA/AM nor W-7 inhibited sodium nitroprusside-stimulated cGMP generation in glomerulus (data not shown), suggesting that these agents did not inhibit soluble guanylate cyclase. Ca ionophore mimicked stimulating effect of ET-3 on cGMP production, although the degree was smaller. The reason for the incomplete stimulation may be because Ca ionophore stimulates NO-dependent cGMP production, while it can also activate Ca-dependent cyclic nucleotide phosphodiesterase (23, 24), resulting in acceleration of cGMP hydrolysis. Ca channel blocker nifedipine had no inhibitory effect on NO-dependent cGMP generation by ET-3 or ET_B receptor agonist in glomerulus. This is in accordance with the findings that dihydropyridine calcium channel blockers nifedipine and diltiazem had no inhibitory effect on synthesis/release of endothelium-derived relaxing factor (EDRF) in rabbit aorta (25) or in cultured bovine aortic endothelial cells (26). In addition, Bay K 8644, a dihydropyridine that activates voltage-dependent calcium channels, did not release EDRF in rat aorta (27). However, it cannot be excluded that ET_B receptor-mediated NO synthesis in glomerulus is stimulated by Ca influx through dihydropyridine-insensitive Ca channels. These results suggest that ET_B receptor-mediated NO synthesis in glomerulus is dependent on intracellular Ca and calmodulin, but it is independent of Ca influx through L-type Ca channel. We do not know why increments of intracellular Ca in response to ET_A receptor activation do not increase NO and ultimately cGMP. At least six Ca-calmodulin-dependent protein kinases are reported. The lack of Ca²⁺ to induce cGMP may be because Ca mobilization by ET_A receptor is not related to Ca-calmodulin-dependent protein kinase, which activates NO synthase.

NO synthase in glomerulus or in mesangial cells responsible for NO synthesis in our study seems to be constitutive, because constitutive NO synthase, but not inducible NO synthase, has been shown to be dependent on Ca/calmodulin (28, 29). Our study showed that ET-3 stimulated NO-dependent cGMP generation even at 1 min of incubation time in glomerulus. This rapid reaction can be mediated by constitutive NO synthase, but not by inducible NO synthase. Previous studies demonstrated the presence of cytokine (IL-1 and TNFα)-inducible NO synthase in rat mesangial cells (10, 30). However, no study has reported the presence of constitutive NO synthase

in mesangial cells. We believe that the present study provides the first evidence that constitutive NO synthase is present in mesangial cells.

Our study showed that PKC inhibitor H-7 did not inhibit ET-3-induced cGMP generation, and that PKC activator DiC 8 did not mimic the stimulating effect of ET-3 on cGMP generation, in glomerulus. These results suggest that PKC activation is not involved in ET-3-induced cGMP generation in glomerulus.

ET-3 did not stimulate cGMP production in IMCD in our study. A recent study demonstrated that bradykinin stimulated cGMP generation in cultured cortical collecting duct cells in an EDRF-dependent manner only in combination with endothelial cells (31). Thus, it is possible that ET-3 could not stimulate cGMP production in isolated IMCD in the absence of EDRF-producing endothelial cells in our study. It is apparently controversial that ET-3 did not stimulate cGMP generation in IMCD, which possesses ET_B receptor, NO synthase, and soluble guanylate cyclase as demonstrated by our RT-PCR studies (5, 7), although ET-3 stimulated cGMP generation in glomerulus, which also possesses the three. One of the reasons why we could not see ET-3-stimulated cGMP generation in IMCD may be because the cGMP generation in IMCD was too small to detect in this study.

In conclusion, ET-3, via ET_B receptor, stimulates cGMP generation through intracellular Ca/calmodulin-dependent and NO-dependent mechanisms in glomerulus. Mesangial cells can be a source of NO coupled to ET_B receptor activation in glomerulus. Mesangial ET_B receptor may act to preserve GFR with NO-stimulated cGMP generation by counteracting the ET_A receptor-mediated vasoconstrictive effect of endothelin.

Acknowledgments

We thank Drs. M. Ihara, and M. Yano, Banyu Pharmaceutical Co., Ltd., for their kind gift of BQ 123-Na, and also thank Drs. M. Takai, and T. Okada, International Research Laboratories, Ciba Geigy Ltd., for their kind gift of IRL 1620. We acknowledge Dr. M. Shinohara, Tokyo Medical and Dental University, for his technical support.

This work was partly supported by a grant-in-aid for General Scientific Research in Japan (04454234, 04670383), and by the Mochida Memorial Foundation for Medical and Pharmaceutical Research in 1993.

References

- Yanagisawa, M., H. Kurihara, S. Kimura, Y. Tomobe, M. Kobayashi, Y. Mitsui, Y. Yazaki, K. Goto, and T. Masaki. 1988. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature (Lond.)* 332:411-415.
- Inoue, A., M. Yanagisawa, S. Kimura, Y. Kasuya, T. Miyauchi, K. Goto, and T. Masaki. 1989. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc. Natl. Acad. Sci. USA* 86:2863-2867.
- Sakurai, T., M. Yanagisawa, Y. Takura, H. Miyazaki, S. Kimura, K. Goto, and T. Masaki. 1990. Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature (Lond.)* 348:732-735.
- Arai, H., S. Hori, I. Aramori, H. Ohkubo, and S. Nakanishi. 1990. Cloning and expression of a cDNA encoding an endothelin receptor. *Nature (Lond.)* 348:730-732.
- Terada, Y., K. Tomita, H. Nonoguchi, and F. Marumo. 1992. Different localization of 2 types of endothelin receptor messenger RNA in microdissected rat nephron segments using reverse transcription and polymerase chain reaction assay. *J. Clin. Invest.* 90:107-112.
- Busse, R. 1987. Stimulation of soluble guanylate cyclase activity by endothelium-derived relaxant factor: a general principle of its vasodilator and anti-aggregatory properties. *Thromb. Res.* VIII(Suppl.):3.
- Terada, Y., K. Tomita, H. Nonoguchi, and F. Marumo. 1992. Polymerase chain reaction localization of constitutive nitric oxide synthase and soluble guanylate cyclase messenger RNAs in microdissected rat nephron segments. *J. Clin. Invest.* 90:659-665.
- Zatz, R., and G. De Nucci. 1991. Effects of acute nitric oxide inhibition on rat glomerular microcirculation. *Am. J. Physiol.* 261:F360-F363.
- Marsden, P. A., T. A. Brock, and B. J. Ballermann. 1990. Glomerular endothelial cells respond to calcium-mobilizing agonists with release of EDRF. *Am. J. Physiol.* 258:F1295-F1303.
- Marsden, P. A., and B. J. Ballermann. 1990. Tumor necrosis factor activates soluble guanylate cyclase in bovine glomerular mesangial cells via an L-arginine-dependent mechanism. *J. Exp. Med.* 172:1843-1852.
- Shultz, P. J., A. E. Schorer, and L. Raij. 1990. Effects of endothelium-derived relaxing factor and nitric oxide on rat mesangial cells. *Am. J. Physiol.* 258:F162-F167.
- Tomita, K., H. Nonoguchi, and F. Marumo. 1990. Effects of endothelin on peptide-dependent cyclic adenosine monophosphate accumulation along the nephron segments of the rat. *J. Clin. Invest.* 85:2014-2018.
- Sakamoto, H., S. Sasaki, Y. Hirata, T. Imai, K. Ando, T. Ida, T. Sakurai, M. Yanagisawa, T. Masaki, and F. Marumo. 1990. Production of endothelin-1 by rat cultured mesangial cells. *Biochem. Biophys. Res. Commun.* 169:462-468.
- Botting, R. M., and J. R. Vane. 1990. Endothelins: potent releasers of prostacyclin and EDRF. *Pol. J. Pharmacol. Pharm.* 42:203-218.
- Fukuda, N., Y. Izumi, M. Soma, Y. Watanabe, M. Watanabe, M. Hatano, I. Sakuma, and H. Yasuda. 1990. L-N^G-monomethyl arginine inhibits the vasodilating effects of low dose of endothelin-3 on rat mesenteric arteries. *Biochem. Biophys. Res. Commun.* 167:739-745.
- Warner, T. D., J. A. Mitchell, G. Nucci, and R. Vane. 1989. Endothelin-1 and endothelin-3 release EDRF from isolated perfused arterial vessels of the rat and rabbit. *J. Cardiovasc. Pharmacol.* 13(Suppl. 5):S85-S88.
- Ihara, M., K. Noguchi, T. Saeki, T. Fukuroda, S. Tsuchida, S. Kimura, T. Fukami, K. Ishikawa, M. Nishikibe, and M. Yano. 1991. Biological profiles of highly potent novel endothelin antagonists selective for the ET_A receptor. *Life Sci.* 50:247-255.
- Takai, M., I. Umemura, K. Yamasaki, T. Watanabe, Y. Fujitani, K. Oda, Y. Urade, T. Inui, T. Yamamura, and T. Okada. 1992. A potent and specific agonist, Suc-[Glu⁹, Ala^{11,15}]-endothelin-1(8-21), IRL 1620, for the ET_B receptor. *Biochem. Biophys. Res. Commun.* 184:953-959.
- Namiki, A., Y. Hirata, M. Ishikawa, M. Moroi, J. Aikawa, and K. Machii. 1992. Endothelin-1 and endothelin-3-induced vasorelaxation via common generation of endothelium-derived nitric oxide. *Life Sci.* 50:677-682.
- Nakamura, Y., H. Sakamoto, Y. Terada, S. Sasaki, K. Tomita, and F. Marumo. 1991. Cells cycle-dependent expression of endothelin receptors mRNA in cultured mesangial cells. *J. Am. Soc. Nephrol.* 2:410. (Abstr.)
- Torre, V., H. R. Matthews, and T. D. Lamb. 1986. Role of calcium in regulating the cyclic GMP cascade of phototransduction in retinal rods. *Proc. Natl. Acad. Sci. USA* 83:7109-7113.
- Hidaka, H., Y. Sasaki, T. Tanaka, T. Endo, S. Ohno, Y. Fujii, and T. Nagata. 1981. N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, a calmodulin antagonist, inhibits cell proliferation. *Proc. Natl. Acad. Sci. USA* 78:4354-4357.
- Smith, J. B., and T. M. Lincoln. 1987. Angiotensin decreases cyclic GMP accumulation produced by atrial natriuretic factor. *Am. J. Physiol.* 253:C147-C150.
- Haneda, M., R. Kikkawa, S. Maeda, M. Togawa, D. Koya, N. Horide, N. Kajiwar, and Y. Shigeta. 1991. Dual mechanism of angiotensin II inhibits ANP-induced mesangial cGMP accumulation. *Kidney Int.* 40:188-194.
- Jayakody, R. L., C. T. Kappagoda, M. P. J. Senaratne, and N. Sreeharan. 1987. Absence of effect of calcium antagonists on endothelium-dependent relaxation in rabbit aorta. *Br. J. Pharmacol.* 91:155-164.
- Mugge, A., T. Peterson, and D. G. Harrison. 1991. Release of nitrogen oxides from cultured bovine aortic endothelial cells is not impaired by calcium channel antagonists. *Circulation* 83:1404-1409.
- Spedding, M., V. Schini, P. Schoeffter, and C. Miller. 1986. Calcium channel activation does not increase release of endothelium-derived relaxant factors (EDRF) in rat aorta although tonic release of EDRF may modulate calcium channel activity in smooth muscle. *J. Cardiovasc. Pharmacol.* 8:1130-1137.
- Moncada, S., R. M. J. Palmer, and E. A. Higgs. 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* 43:109-142.
- Connor, K. J., and S. Moncada. 1991. Glucocorticoids inhibit the induction of nitric oxide synthase and the related cell damage in adenocarcinoma cells. *Biochim. Biophys. Acta* 1097:227-231.
- Pfeilschifter, J., P. Rob, A. Mulsch, J. Fandrey, K. Vosbeck, and R. Busse. 1992. Interleukin 1 and tumor necrosis factor α induces a macrophage-type of nitric oxide synthase in rat renal mesangial cells. *Eur. J. Biochem.* 203:251-255.
- Stoos, B. A., O. A. Carretero, R. D. Farhy, G. Scicli, and J. L. Garvin. 1992. Endothelium-derived relaxing factor inhibits transport and increases cGMP content in cultured mouse cortical collecting duct cells. *J. Clin. Invest.* 89:761-765.