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Effects of endogenous and exogenous nitric oxide on endothelin-1 production in cultured vascular endothelial cells

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Abstract

The effects of various spontaneous nitric oxide (NO) donors and NO synthase inhibitors on endothelin-1 production were examined using porcine cultured aortic endothelial cells. NO donors such as (±)-(E)-4-methyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexanamide (NOR 2), (±)-(E)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexanamide (NOR 3) and (±)-*N*-[(*E*)-4-ethyl-2-[(*Z*)-hydroxyimino]-5-nitro-3-hexen-1-yl]-3-pyridine carboxamide (NOR 4) suppressed effectively the release of endothelin-1 from the cells. Endothelin-1 mRNA expression was also attenuated by these compounds. Other NO donors such as 3-[2-hydroxy-1-(1-methylethyl)-2-nitrosohydrazino]-1-propanamine (NOC 5), 2,2′-(hydroxynitrosohydrazino)bis-ethanamine (NOC 18), *s*-nitroso-*n*-acetyl-DL-penicillamine, *N*-morpholino sydnonimine (SIN-1) had no effects on endothelin-1 production. Endothelial intracellular cyclic guanosine monophosphate (cGMP) levels were significantly increased by all NO donors. 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a selective soluble guanylyl cyclase inhibitor, had no effect on the NOR 3-induced decrease in endothelin-1 secretion, although cGMP production was abolished by ODQ. NOR 3 also inhibited endothelin-1 secretion even in the presence of 2-(4-carboxyphenyl)-4,4,5,5-tetrametylimidazole-1-oxyl 3-oxide (carboxy-PTIO), a NO scavenger. NOR 3-induced inhibitory effects on endothelin-1 secretion were abolished by preincubation of the compound in phosphate-buffered saline (37°C, 4 h), a procedure by which about 98% of the parent compound's ability to release NO was lost. NO synthase inhibitors such as N^G-nitro-L-arginine, N^G-monomethyl-L-arginine and N^G-nitro-L-arginine methyl ester (L-NAME) enhanced prepro endothelin-1 mRNA expression and significantly increased endothelin-1 release from endothelial cells. Endothelin-1 secretion was also increased effectively by carboxy-PTIO or ODQ. When the cells were exposed to L-NAME with carboxy-PTIO or ODQ, no significant further increase in endothelin-1 release was observed. These results suggest that endogenous NO inhibits endothelin-1 production through guanylyl cyclase/cGMP-dependent mechanisms. In contrast, it seems unlikely that exogenous NO has an inhibitory effect on endothelin-1 production in endothelial cells. NOR compounds inhibit endothelin-1 production perhaps through NO/cGMP-independent mechanisms, i.e., through an unknown effect of the parent compound itself.

Keywords: Endothelin-1; Endothelial cell; Nitric oxide (NO); Nitric oxide (NO) donor; cGMP

1. Introduction

Endothelin-1 is a potent vasoconstrictive peptide purified from the supernatant of cultured porcine aortic endothelial cells (Yanagisawa et al., 1988). In endothelial cells, endothelin-1 is produced from an inactive intermediate form big endothelin-1 by endothelin converting enzyme, which is a membrane-bound and phosphoramidon-sensitive metalloproteinase (Matsumura et al., 1990b; Opgenorth et al., 1992). Endothelin-1 release from endothelial cells is constitutive, because little or no mature and big endothelin-1 is found intracellularly (Hexum et al., 1990). Thus, endothelin-1 biosynthesis and release appear to be regulated at the transcriptional level as well as at the level of endothelin-converting enzyme action. Several studies have indicated that various substances such as thrombin, angiotensin II, arginine-vasopressin and transforming growth factor-β1 stimulate the expression of prepro endothelin-1 mRNA in endothelial cells (Yanagisawa et al., 1988; Hilkert et al., 1992; Imai et al., 1992; Murata et al., 1995).

Nitric oxide (NO), known as an endothelium-derived relaxing factor, is formed from the terminal guanidino nitrogen atom of L-arginine by NO synthase (Moncada et al., 1991). Two major forms of NO synthase have been

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identified to date. The constitutive Ca^{2+}-dependent NO synthase isoform is present mainly in endothelial cells and brain. The inducible Ca^{2+}-independent NO synthase isoform activated by cytokines is found predominantly in macrophages and smooth muscle cells (Förstermann et al., 1991).

Previous studies have demonstrated that one of the effects of endothelin-1 is to increase in NO release (Tsukahara et al., 1994); endothelial NO synthase is activated upon stimulation of the endothelin ET_B receptor. On the other hand, there are several reports on the inhibitory regulation of endothelin-1 production by NO in the vascular endothelium (Boulanger and Lüscher, 1991; Kuchan and Frangos, 1993; Kourembanas et al., 1993; Brunner et al., 1995) and the adrenal glands (Hinson et al., 1996). However, in these reports, the authors' suggestion that NO had an inhibitory role on endothelin-1 production was mainly based on the finding that NO synthase inhibitors enhanced the basal or stimulated release of endothelin-1. The mechanisms underlying this inhibition are controversial (cyclic guanosine monophosphate (cGMP)-dependent or cGMP-independent).

It is appropriate to use spontaneous NO donor compounds to investigate the action of exogenous NO. NOR compounds, which are based on (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexanamide (NOR 3), also known as FK409 (Kita et al., 1994), and NOC compounds (Hrabie et al., 1993) both spontaneously generate NO in phosphate-buffered saline (pH 7.4). *S*-nitroso-*N*-acetyl-DL-penicillamine (Elizabeth and Fung, 1990) and *N*-morpholino sydnonimine (SIN-1) (Feelisch and Noack, 1987) have been also reported to be NO releasers that liberate NO spontaneously. These four types of NO donors have different structures and profiles of NO formation. It was reported that NOR 3 and SIN-1 have different profiles of NO formation and liberate NO at different rates (Kita et al., 1995), this may result in different biological actions. Thus, it seems reasonable to use a variety of spontaneous NO donors when evaluating effects of exogenous NO on endothelin-1 production in endothelial cells. Conventional NO synthase inhibitors were used to determine if endogenous NO tonically regulates endothelin-1 production under basal conditions. The possible involvement of guanylyl cyclase/cGMP system in exogenous and endogenous NO-mediated effects was also investigated.

2. Materials and methods

2.1. Cell culture

Porcine aortic endothelial cells were cultured as described (Matsumura et al., 1990a). When endothelial cells cultured in 12 well gelatin-coated plates or 60-mm Petri dishes became confluent, the culture medium was changed to serum-free Dulbecco's modified Eagle's medium containing 0.01% heat-inactivated bovine serum albumin. Next, endothelial cells were incubated in a CO_2 incubator for 3–24 h, with or without various agents. After the incubation, medium was aspirated off, boiled for 5 min then centrifuged at $8000 \times g$ for 10 min. The resulting supernatant was stored at −30°C until assay for endothelin-1. For Northern blot analysis, endothelial cells grown in 60-mm gelatin-coated Petri dishes were incubated with

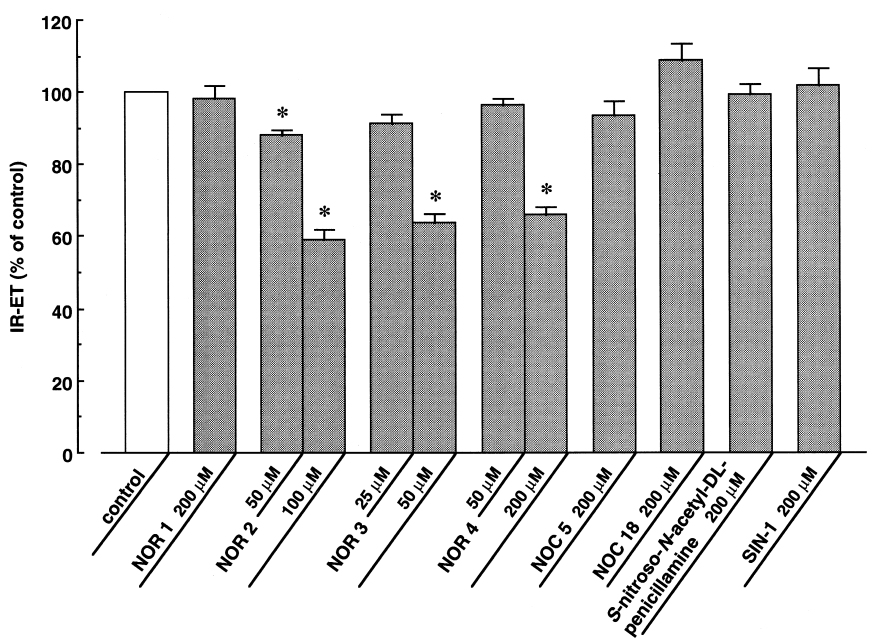

Fig. 1. Effects of NO donors on endothelin-1 release from porcine aortic endothelial cells. The cells were incubated with NO donors at indicated concentrations for 6 h. The basal endothelin-1 level (control) was 138.7 ± 7.5 fmol/10^5 cells/6 h. Each column and bar represents the mean ± S.E.M. ($n = 7$–10). $* P < 0.01$, compared with control. IR-ET, immunoreactive endothelin.

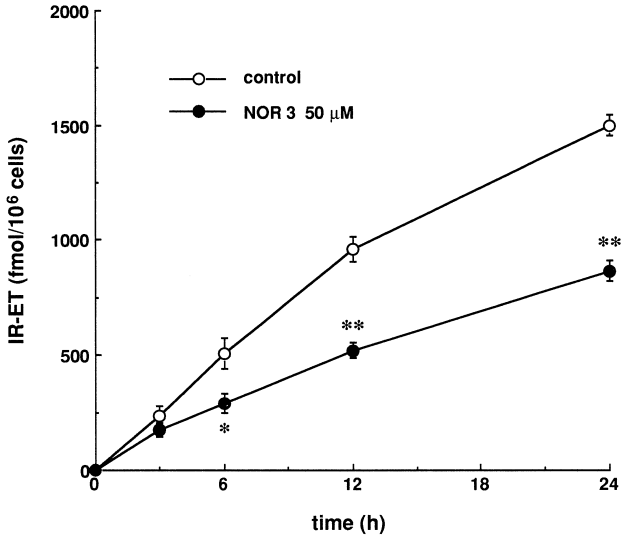

Fig. 2. Time course of the effect of NOR 3 on endothelin-1 release from porcine aortic endothelial cells. The cells were incubated for 3–24 h, with or without 50 mM NOR 3. Each point and bar represents the mean ± S.E.M. ($n = 6$). $* P < 0.05$, $** P < 0.01$, compared with control. IR-ET, immunoreactive endothelin.

various agents for 2 h, and these cells served as samples for total RNA extraction.

2.2. Radioimmunoassay for determination of endothelin-1

The radioimmunoassay for endothelin-1 was performed as described (Ikegawa et al., 1990). Endothelin-1 antiserum (a gift from Dr. M.R. Brown, University of California, San Diego, USA) did not cross-react with big endothelin-1 (Hexum et al., 1990).

2.3. Quantification of cGMP

The endothelial cells were incubated with NO donors for 10 min in 12-well plates, after a 5-min preincubation with 0.5 mM 3-iso-butyl-1-methylxantine at 37°C. The reaction was then stopped by placing the plates on ice for 5 min. After the cells were washed with PBS, intracellular cGMP was extracted with 0.1 N HCl. The amount of cGMP was determined by using a cGMP RIA kit (Yamasa Shoyu, Chiba, Japan).

2.4. Northern blot analysis

Total RNA was extracted from porcine aortic endothelial cells by the acid guanidium thiocyanate-phenol-chloroform extraction method. The isolated total RNA was separated in formaldehyde-1.1% agarose gel electrophoresis and transferred to a nylon membrane (Hybond-N^+, Amersham, Tokyo, Japan) in the presence of 20 × standard sodium citrate (3 M sodium chloride, 0.3 M sodium citrate, pH 7.2). The nylon membrane was hybridized in hybridization buffer (6 × standard sodium citrate, 0.01 M EDTA, 5 × Denhart's solution, 0.5% sodium dodecyl sulfate and 100 μg/ml of sheared, denatured salmon sperm DNA) at 67°C with porcine prepro endothelin-1 cDNA probe (a gift from Dr. K. Goto, University of Tsukuba, Tsukuba, Japan) and glyceraldehyde-3-phosphate dehydrogenase cDNA probe (Clontech, Palo Alto, CA) labeled with [α-^{32}P]dCTP by using a random Primer DNA Labeling Kit (Takara Shuzo, Kyoto, Japan). After hybridization, the membrane was washed twice in 2 ×, once in 1 × and twice in 0.1 × standard sodium citrate containing 0.1% sodium dodecyl sulfate, and autoradiography was then done with intensifying screens at −80°C by exposure to X-Omat AR film (Kodak) for 12 h.

The autoradiograms of ET-1 were quantified by densitometoric analyses, and the signals of ET-1 mRNA were normalized for each sample with respect to the density of the corresponding signal for glyceraldehyde-3-phosphate dehydrogenase mRNA.

2.5. Chemicals

(±)-(*E*)-4-methyl-2-[(*E*)-hydroxyimino]-5-nitro-6-methoxy-3-hexanamide (NOR 1), (±)-(*E*)-4-methyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexanamide (NOR 2), (±)-*N*-[(*E*)-4-ethyl-2-[(Z)-hydroxyimino]-5-nitro-3-hexen-1-yl]-3-pyridine carboxamide (NOR 4), 3-[2-hydroxy-1-(1-methylethyl)-2-nitrosohydrazino]-1-propanamine (NOC 5), 2,2′-(hydroxynitrosohydrazino)bis-ethanamine (NOC 18), SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine, SIN-1 and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide (carboxy-PTIO) were purchased from Dojindo Laboratories (Kumamoto, Japan). NOR 3 (FK409) was a gift from Fujisawa Pharmaceutical, (Osaka, Japan). 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was purchased from Tocris Cookson (Bristol, UK). N^G-nitro-L-arginine (L-NA) was purchased from Peptide Institute (Osaka, Japan). N^G-nitro-L-arginine methyl ester (L-

Table 1
Effects of NO donors on cyclic GMP production in porcine cultured aortic endothelial cells

Compound		Cyclic GMP (fmol/10^5 cells)
Control		44.5 ± 5.0
NOR 1	200 μM	219.4 ± 12.0[a]
NOR 2	50 μM	185.5 ± 9.5[a]
	100 μM	232.2 ± 16.9[a]
NOR 3	25 μM	179.1 ± 15.0[a]
	50 μM	213.0 ± 14.8[a]
NOR 4	50 μM	165.1 ± 10.2[a]
	200 μM	240.3 ± 18.7[a]
NOC 5	200 μM	179.0 ± 26.9[a]
NOC 18	200 μM	114.0 ± 14.1[a]
S-nitro-*N*-acetyl-DL-penicillamine	200 μM	171.2 ± 5.9[a]
SIN-1	200 μM	103.7 ± 4.1[a]

Each value represents the mean ± S.E.M. ($n = 6$–8).
[a] $P < 0.01$, compared with control.

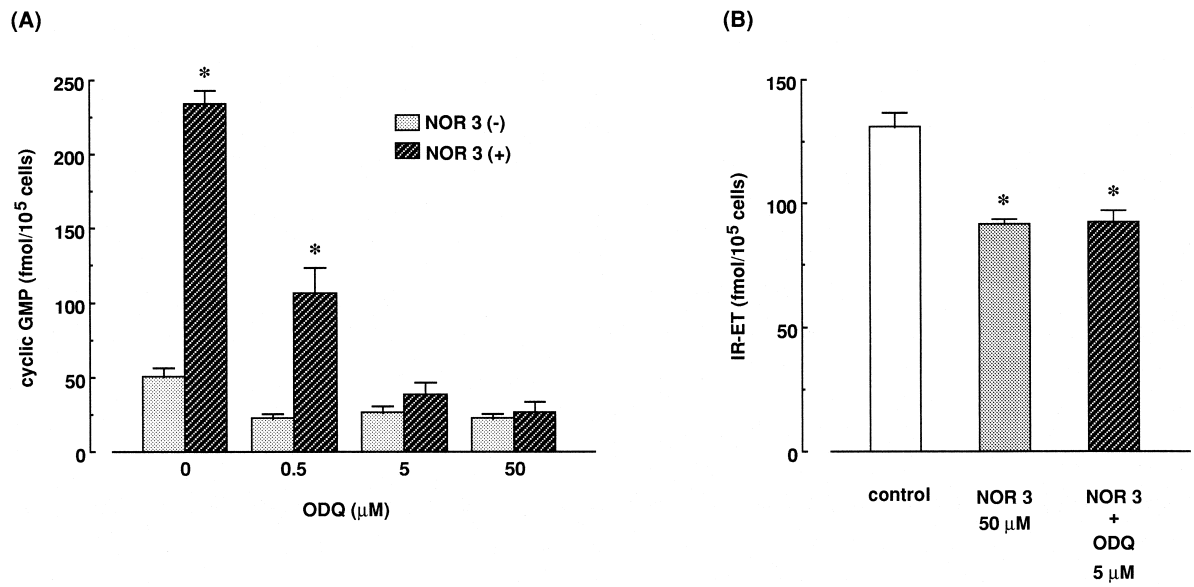

Fig. 3. Effects of ODQ on NOR 3-induced endothelial cGMP production (A) and decrease in endothelin-1 release (B). (A) Endothelial cells were incubated for 10 min in the absence or presence of 50 μM NOR 3 after 5-min preincubation with 0.5 mM 3-iso-butyl-1-methylxantine. The cells were treated with ODQ (0.5, 5, 50 μM) 5 min prior to the addition of NOR 3. Each column and bar represents the mean ± S.E.M. ($n = 7–9$). * $P < 0.001$, compared with the value in the absence of NOR 3. (B) Endothelial cells were exposed to 50 μM NOR 3, with or without 5 μM ODQ, for 6 h. Each column and bar represents the mean ± S.E.M. ($n = 9$) * $P < 0.01$, compared with control. IR-ET, immunoreactive endothelin.

NAME) and N^G-monomethyl-L-arginine (L-NMMA) were purchased from Wako (Osaka, Japan). [^{125}I]Endothelin-1 and [α-^{32}P]dCTP were obtained from Amersham Japan (Tokyo, Japan).

2.6. Statistical analysis

All values were expressed as mean ± S.E.M. For statistical analysis, we used unpaired Student's *t*-test for two-group comparisons and one-way analysis of variance combined with Dunnett's or Bonferroni's multiple range test for multiple comparisons. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effects of NO donors on endothelin-1 release from endothelial cells

Fig. 1 shows effects of various NO donors on endothelin-1 release from endothelial cells incubated for 6 h. NOR 2, NOR 3 and NOR 4 produced dose-related decreases in endothelin-1 release: the release was 59.2, 64.0, and 66.0% of basal release (138.7 ± 7.5 fmol/10^5 cells) with NOR 2 (100 μM), NOR 3 (50 μM) and NOR 4 (200 μM), respectively. NOR 3 also produced a time-dependent decrease in endothelin-1 release, and the level of endothelin-1 released was reduced in a linear fashion up to 24 h (Fig. 2). NOR 1, NOC 5, NOC 18, *S*-nitroso-*N*-acetyl-DL-penicillamine and SIN-1, at concentrations of ~200 μM, did not affect endothelin-1 release from the cells (Fig. 1, only results at 200 μM are shown).

3.2. NO donor-induced endothelial intracellular cGMP production

As shown in Table 1, NOR 2, NOR 3, NOR 4 produced dose-related increases in endothelial intracellular cGMP

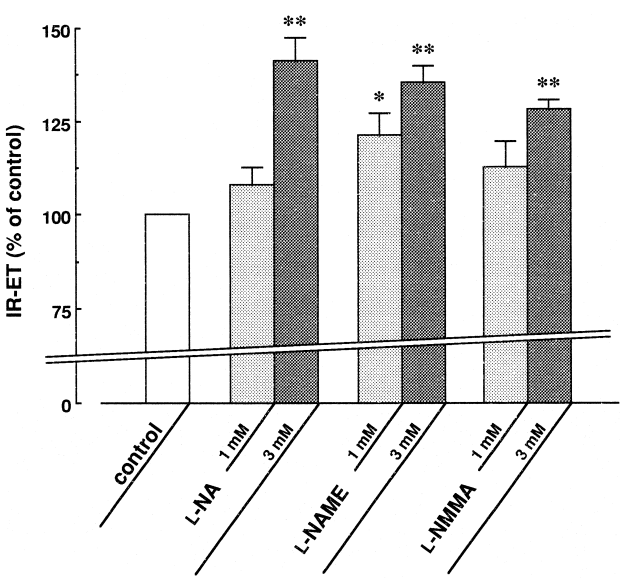

Fig. 4. Effects NO synthase inhibitors on endothelin-1 release from porcine aortic endothelial cells. The cells were incubated with NO synthase inhibitors (1, 3 mM) for 6 h. Basal endothelin-1 level (control) was 111.3 ± 4.5 fmol/10^5 cells/6 h. Each column and bar represents the mean ± S.E.M. ($n = 8–9$). * $P < 0.05$, * * $P < 0.01$, compared with control. IR-ET, immunoreactive endothelin.

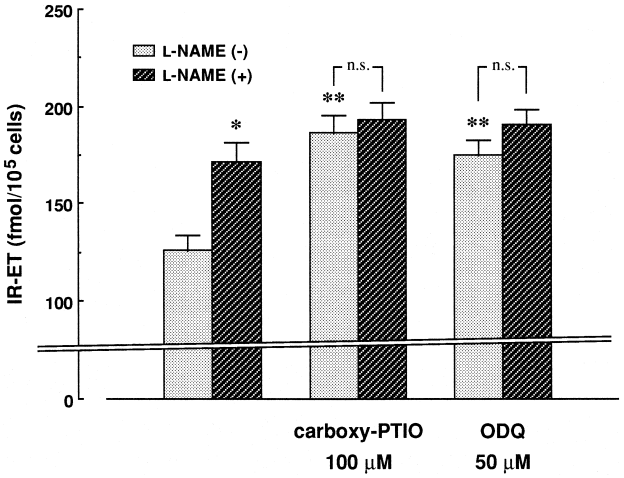

Fig. 5. Effects of L-NAME with carboxy-PTIO or ODQ on endothelin-1 release from porcine aortic endothelial cells. The cells were incubated for 6 h with carboxy-PTIO (100 μM) or ODQ (50 μM) in the absence or presence of 3 mM L-NAME. Each column and bar represent the mean ± S.E.M. ($n = 9$). $*P < 0.01$, $**P < 0.001$, compared with control. IR-ET, immunoreactive endothelin.

production from the basal value of 44.5 ± 5.0 fmol/10^5 cells. Despite the lack of effects on endothelin-1 release, NOR 1 markedly increased cGMP production. NOC 5, NOC 18, *S*-nitroso-*N*-acetyl-DL-penicillamine and SIN-1 also increased cGMP production although these compounds produced relatively small increases compared with those elicited by the NOR compounds.

3.3. NOR 3-induced inhibition of endothelin-1 release and increase in cGMP production

Only NOR compounds (except NOR 1) showed inhibitory effects on endothelin-1 release from endothelial cells, although all the NO donors used markedly increased cGMP production. Next, we investigated whether the NO/guanylyl cyclase/cGMP system is involved in the NOR compounds-induced inhibition of endothelin-1 release, using NOR 3. As shown in Fig. 3A, NOR 3 (50 μM) markedly increased endothelial cGMP production to 4.6-fold of the control, an effect which was abolished by ODQ, a novel selective inhibitor of NO-stimulated guanylyl cyclase (Garthwaite et al., 1995) (≥ 5 μM). In contrast, ODQ (5 μM) had no effect on the decrease of endothelin-1 secretion induced by NOR 3 (Fig. 3B). This concentration of ODQ had no significant effect on the basal release of endothelin-1. When the cells were exposed to NOR 3 (50 μM) in the presence of 50 and 100 μM carboxy-PTIO, a NO scavenger (Akaike et al., 1993), significant decreases in endothelin-1 secretion of 45% and 44% were observed,

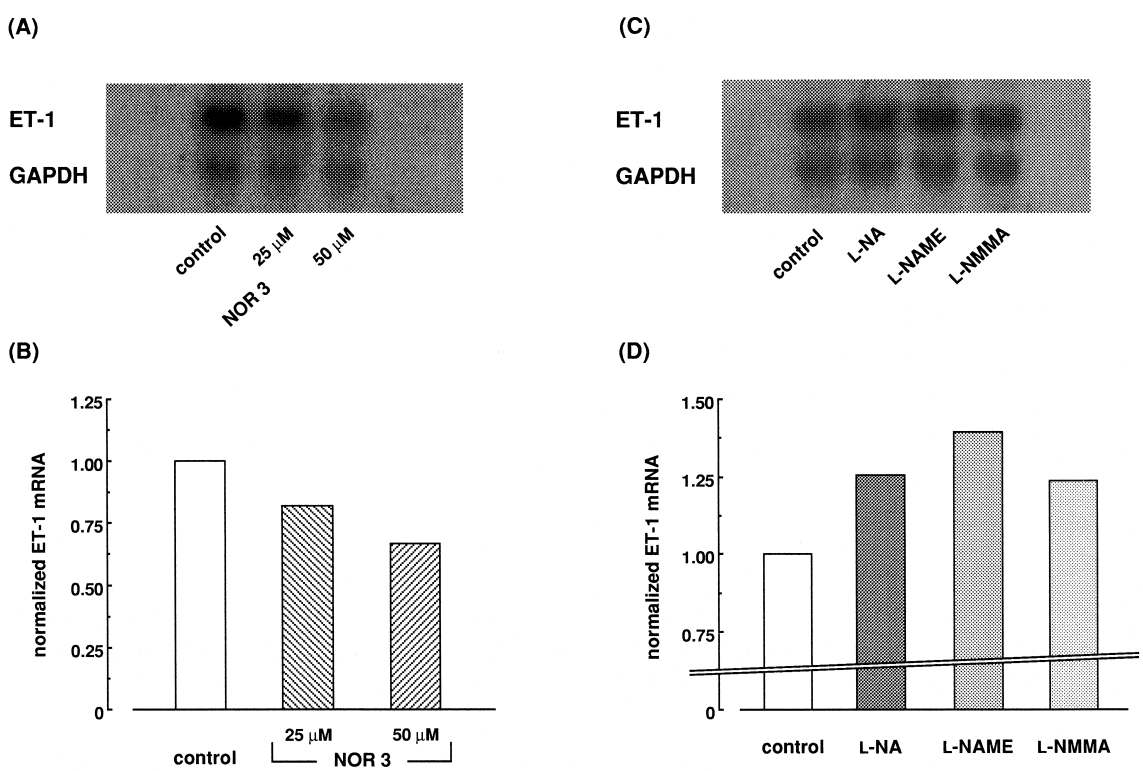

Fig. 6. Effects of NOR 3 (A, B) and NO synthase inhibitors (C, D) on prepro endothelin-1 mRNA expression in porcine aortic endothelial cells. Total RNA (16 μg/lane) was hybridized with porcine prepro endothelin-1 (ET-1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA as probes. The cells were incubated with NOR 3 (A, B) and 3 mM NO synthase inhibitors (C, D) for 2 h. The signals for GAPDH mRNA for each lane are shown as internal control (A and C). The signals for ET-1 mRNA were normalized to the corresponding GAPDH signals, quantified by densitometric analyses (B and D). Each column represents the mean of two experiments.

respectively. These results suggest that the NO/guanylyl cyclase/cGMP system does not contribute to the NOR 3-induced decrease in endothelin-1 production. We next examined whether the decomposed product of NOR 3, after releasing NO spontaneously, could decrease the release of endothelin-1. This 'inactive' NOR 3 was prepared by preincubating the compound in PBS at 37°C for 4 h before addition to the cells. It has been reported that about 98% of the activity of NOR 3 as NO releaser is lost after a 4-h incubation in PBS (pH 7.4) at 37°C (Kita et al., 1994). In contrast to the effect of active NOR 3 (50 μM), inactive NOR 3 at the same concentration had no significant effect on the endothelin release (data not shown).

3.4. Effects of NO synthase inhibitors on endothelin-1 release from endothelial cells

As shown in Fig. 4, when endothelial cells were incubated with L-NA, L-NAME, and L-NMMA (1, 3 mM) for 6 h, endothelin-1 secretion was increased in a dose-dependent manner. The basal release (111.3 ± 4.5 fmol/10^5 cells) was increased significantly by 30–40% by each NO synthase inhibitor at 3 mM.

3.5. Effects of L-NAME with carboxy-PTIO or ODQ on endothelin-1 release from endothelial cells

As shown in Fig. 5, the NO scavenger, carboxy-PTIO (50, 100 μM) significantly enhanced the release of endothelin-1 from the cells. In addition, guanylyl cyclase inhibitor ODQ at 50 μM, the concentration which suppresses markedly endothelial cGMP production, also increased the endothelin-1 release. These findings suggest that the suppression of the endogenous NO/guanylyl cyclase/cGMP system leads to enhancement of endothelin-1 production. When endothelial cells were exposed to L-NAME (3 mM) in the presence of carboxy-PTIO or ODQ, no significant further increase in endothelin-1 release was observed, thereby suggesting that the L-NAME-induced increase in endothelin-1 release is due to inhibition of endogenous NO formation in endothelial cells.

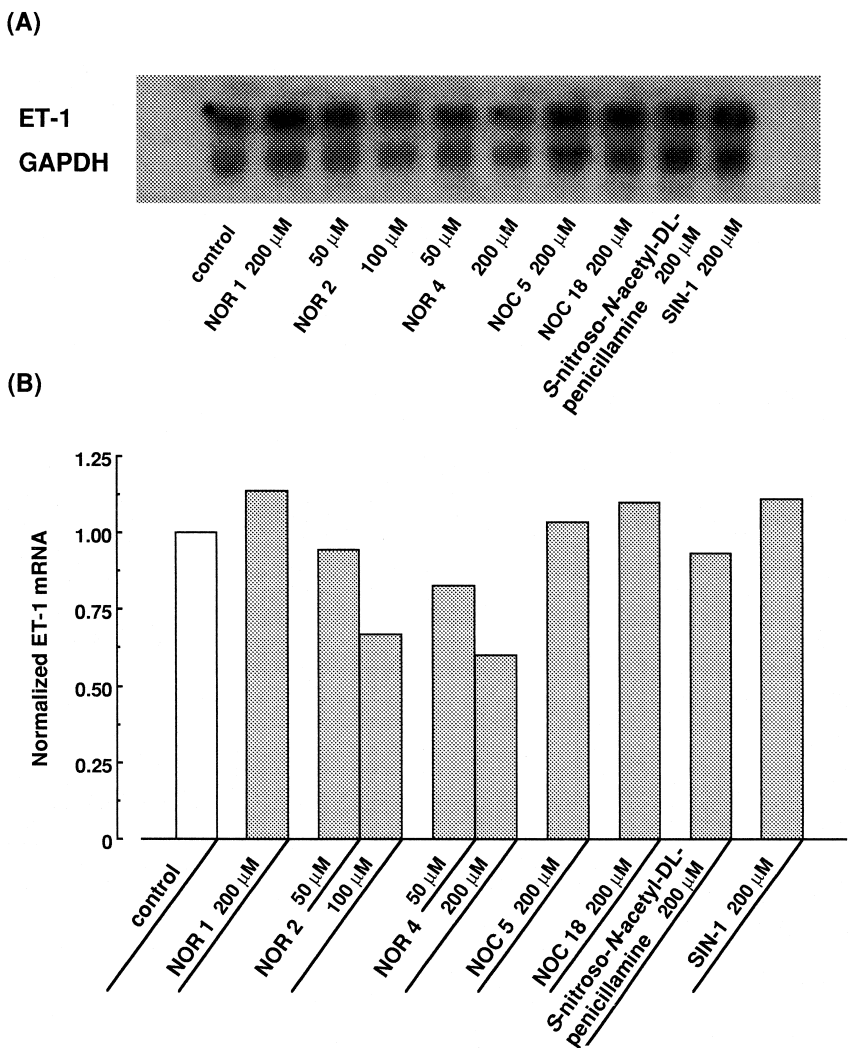

Fig. 7. Effects of NO donors on prepto endothelin-1 mRNA expression in porcine aortic endothelial cells. The cells were incubated with NO donors for 2 h. Details are similar as in Fig. 6.

3.6. Effects of NO donors and NO synthase inhibitors on prepro endothelin-1 mRNA expression in endothelial cells

To determine whether changes in endothelin-1 release result from a regulation at the transcription level, effects of NO donors and NOS inhibitors on prepro endothelin-1 mRNA expression were examined using Northern blot analysis. Exposure of NOR 3 (25, 50 μM) for 2 h dose dependently inhibited the prepro endothelin-1 mRNA expression (Fig. 6A,B). A similar inhibition of prepro endothelin-1 mRNA expression was observed with NOR 2 (50, 100 μM) and NOR 4 (50, 200 μM), whereas other NO donors had no apparent effect on endothelin-1 mRNA expression (Fig. 7). In contrast, the NO synthase inhibitors (3 mM) enhanced the expression of endothelin-1 mRNA (Fig. 6C,D). Thus, there was a good correlation between changes of endothelin-1 mRNA expression and changes in endothelin-1 release.

4. Discussion

Our results indicated that, of the various NO donors used, only NOR compounds (NOR 2, NOR 3 and NOR 4) suppressed both endothelin-1 secretion and prepro endothelin-1 mRNA expression. Thus, we investigated the possible mechanisms of NOR compounds-induced inhibition of endothelin-1 production, using NOR 3 which showed the most potent inhibitory effect on endothelin-1 production. First, involvement of guanylyl cyclase/cGMP system in the NOR 3-induced inhibition was evaluated using ODQ, a selective inhibitor of NO-stimulated guanylyl cyclase (Garthwaite et al., 1995). ODQ did not affect NOR 3-induced decreases in endothelin-1 secretion, although the cGMP production stimulated by this compound was abolished by ODQ. These findings suggest that the guanylyl cyclase/cGMP system activated by NO liberated from NOR compounds is not responsible for the inhibition of endothelin-1 production by these compounds. Second, to determine if the inhibition of endothelin-1 release occurs through a NO-dependent mechanism, we examined the effect of NOR 3 in the presence or absence of carboxy-PTIO, a NO scavenger (Akaike et al., 1993). NOR 3 significantly decreased endothelin-1 release in the presence of carboxy-PTIO, to the same extent as seen with NOR 3 alone. Third, we examined whether the decomposed product of NOR 3, after NO had been spontaneously released, could decrease the release of endothelin-1. Our results clearly indicated the ineffectiveness of this 'inactive' NOR 3 on endothelin-1 release. Based on these findings, we suggest that NOR compounds inhibit endothelin-1 production through probably NO/cGMP-independent mechanisms. The chemical characteristics of the parent compound itself may be related to inhibitory effects on endothelin-1 production, but mechanisms involved remain to be clarified. Based on results obtained with other NO donors, it is unlikely that exogenous NO and exogenous NO-stimulated cGMP have an inhibitory effect on the basal endothelin-1 production in endothelial cells.

We noted that the inhibition of endogenous NO synthesis by NO synthase inhibitors such as L-NA, L-NAME and L-NMMA increased endothelin-1 production at the transcriptional level. Carboxy-PTIO also increased endothelin-1 secretion in a dose-dependent manner, probably by scavenging of endogenous NO. When the cells were exposed to carboxy-PTIO at 100 μM plus L-NAME at 3 mM, the concentration which had a maximal effect on endothelin-1 release, the enhancement of endothelin-1 release was not additive. These findings suggest that endogenous NO tonically modulates endothelin-1 production as an inhibitory factor in endothelial cells. Moreover, the release of endothelin-1 was significantly enhanced by ODQ (50 μM). When the cells were exposed to ODQ (50 μM) plus L-NAME (3 mM), endothelin-1 secretion was enhanced to similar extent as observed in ODQ alone. Thus, endogenous NO seems to inhibit endothelin-1 production, via guanylyl cyclase/cGMP-dependent mechanisms.

Boulanger and Lüscher (1991), using intact porcine aorta and L-NMMA (200 μM), and Brunner et al. (1995), using cultured porcine endothelial cells and L-NA (100 μM), found that these NO synthase inhibitors augmented thrombin or phorbol ester-stimulated, but not the basal, release of endothelin-1. Brunner et al. (1995) also found that NO donors such as spermine/NO (0.1–1 mM) and *S*-nitroso-glutathione (1 mM) inhibited basal endothelin-1 secretion through a cGMP-independent mechanism. Kuchan and Frangos (1993) noted that exposure of cultured endothelial cells to shear (6–25 dyn/cm^2 for $\geq$ 6 h) markedly inhibited endothelin-1 release, and that the inhibitory effect of shear was potentiated and diminished by 3-iso-butyl-1-methylxantine and by methylene blue, respectively, suggesting that elevation of the endogenous NO/cGMP system is responsible for the shear-induced inhibition of endothelin-1 release. In addition, they found that L-NA (400 μM) reversed the above shear-induced inhibition, although this concentration of L-NA did not increase the basal endothelin-1 release in stationary cultures. Such evidence is qualitatively consistent with our results. The increasing effects of NO synthase inhibitors on the basal endothelin-1 release observed in our study may be related to the higher concentration of the agents used. Our results are consistent with those of Hinson et al. (1996), who found that L-NAME (5 mM) enhanced and that sodium nitroprusside (0.1 mM) did not suppress endothelin-1 release from the intact perfused rat adrenal gland. Further, Kourembanas et al. (1993) reported that L-NA (2.5 mM) enhanced both basal and hypoxia-induced prepro endothelin-1 mRNA expression and endothelin-1 release from cultured endothelial cells. Taken together, it is likely that an endogenous NO is a physiologically relevant and local inhibitory signal for endothelin-1 production in various tissues.

NOR compounds and NOC compounds have different half-lives, 2.6 min–21 h in PBS (NOR 1, 2.6 min; NOR 2, 28 min; NOR 3, 46 min; NOR 4, 107 min; NOC 5, 25 min; NOC 18, 21 h) (Kato et al., 1996; Keefer, in press). The rate of NO liberation from NOR 2, NOR 3 and NOC 5 is similar. In the present study, NOC 5 had no inhibitory effect on endothelin-1 production, in contrast to NOR 2 and NOR 3. Thus, the different rates of NO release from NO donors probably do not contribute to effects on endothelin-1 production. Only NOR 1 among the NOR compounds tested had no effects on endothelin-1 gene expression and endothelin-1 release. This may be due to extremely short half-life (2.6 min) of this compound. This view is compatible with our proposal that chemical characteristics of the parent compound itself are related to the NOR compound-induced inhibition of endothelin-1 production. It has been reported that NOR 3 decomposes via several intermediates and that NO is generated from an intermediate formed by the degradation of NOR 3 (Fukuyama et al., 1995). Thus, endothelin-1 production might be also inhibited by intermediates formed during degradation of NOR compounds.

One would have to question whether inhibitory actions of NOR compounds on endothelin-1 production were due to cytotoxicity, since NO and related substances such as peroxynitrite (reaction product of NO with superoxide anion) are reported to have cytotoxic effects on endothelial and other cells under certain conditions (Estrada et al., 1992; Xia et al., 1996; Wink et al., 1997). In the present study, when the release of lactate dehydrogenase from the cells during incubation with NOR compounds was measured, no significant changes were observed compared with the basal condition. Furthermore, we noted that superoxide dismutase did not affect the NOR 3-induced decrease in endothelin-1 release (data not shown). Décout et al. (1995) demonstrated that reduced oxygen species such as superoxide anion and hydroxyl radical were not generated during decomposition of NOR 3, and they suggested that this novel NO donor is a less cytotoxic compound. However, the possibility that NOR compounds suppress endothelin-1 production in endothelial cells partly through cytotoxic effects will need to be ruled out.

In summary, endogenous NO appears to suppress endothelin-1 production at the transcriptional level, probably via guanylyl cyclase/cGMP-dependent mechanisms. This effect may partly contribute to regulation of vascular tone by NO. Several investigators (Thompson et al., 1995; Richard et al., 1995; Banting et al., 1996) have noted that the presser effect induced by NO synthase inhibitors is markedly attenuated by treatment with endothelin ET_A or ET_A/ET_B receptor antagonists, thereby suggesting that the enhancement of endothelin-1 production is involved in the NO synthase inhibitors-induced vasoconstriction. However, it seems unlikely that exogenous NO or the exogenous NO-activated guanylyl cyclase/cGMP system is an inhibitory signal for endothelin-1 production in endothelial cells. One possible explanation is that the high NO concentration in endothelial cells is achieved by stimulating its endogenous production. If such is the case, additional stimulation by exogenous NO may not be an inhibitory signal for endothelin-1 production in cells. The NOR compounds-induced inhibition of endothelin-1 production is probably related to chemical characteristics of the parent compound itself. Further investigations are required for clarification of the mechanisms of the NOR compound-induced inhibition of endothelin-1 production and of the ineffectiveness of exogenous NO.

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