Novel guanylyl cyclase inhibitor, ODQ reveals role of nitric oxide, but not of cyclic GMP in endothelin-1 secretion

Friedrich Brunner*, Heike Stessel, Walter R. Kukovetz

Institut für Pharmakologie und Toxikologie, Universität Graz, Universitätsplatz 2, 8010 Graz, Austria

Abstract The role of nitric oxide (NO) and guanosine 3′,5′-cyclic monophosphate (cyclic GMP) in cellular regulation of endothelin-1 (ET-1) secretion was investigated in cultured porcine aortic endothelial cells. NO synthase was inhibited with N^G-nitro-L-arginine (L-NNA) and guanylyl cyclase with the novel selective inhibitor, ODQ (1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one) (3 μM). Basal and phorbol ester (PMA)-stimulated ET-1 secretion were unaffected by ODQ, but stimulated secretion was increased by L-NNA. In the presence of the NO donors, spermine/NO, S-nitroso-glutathione (GSNO), and nitroprusside (NP) ET-1 secretion was reduced, but ODQ had no effect on this inhibition, although it effectively inhibited cyclic GMP production. NO release from donors, measured with a sensitive NO electrode, was greatest for spermine/NO, intermediate for GSNO, minimal for NP and paralleled inhibition of ET-1 secretion. The data suggest that in cultured endothelial cells, curtailment of ET-1 secretion is mediated by NO and independent of cyclic GMP.

Key words: Guanylyl cyclase inhibitor; Endothelin-1 secretion; Nitric oxide electrode; Cultured endothelial cell

1. Introduction

Endothelin-1 (ET-1) is a recently discovered endothelium-derived polypeptide with potent vasoconstrictor activity [1]. Secretion of ET-1 by cultured endothelial cells is stimulated by several components of normal plasma [2,3], and inhibited by heparin [4], atrial natriuretic peptides [5], and prostacyclin [6,7]. Agents that mimic the effect of endothelium-derived NO and raise intracellular levels of cyclic GMP have been shown to reduce agonist-stimulated ET-1 secretion [8].

The second messengers involved in inhibition of endothelial ET-1 secretion are not well understood. In cultured endothelial cells, NO [9] and/or cyclic GMP [10] may control ET-1 production, whereas in intact vessels inhibition was surmised to be due to cyclic GMP derived from smooth muscle cells. Shear stress-induced inhibition of ET-1 secretion was also attributed to NO-dependent elevations in cyclic GMP [11]. Exposure of cultured endothelial cells to hypoxia resulted in a considerable increase in ET-1 gene expression and peptide secretion, which were abolished in the presence of NP and potentiated by

L-NNA, an inhibitor of NO synthase [12]. These authors interpreted their data as evidence for an active inhibitory role of NO, rather than cyclic GMP in ET-1 secretion. Recently, 8-bromo-cyclic GMP was found to be ineffective in inhibiting basal or stimulated ET-1 production in cultured human endothelial cells, and cAMP had a complex modulatory effect with both stimulatory and inhibitory pathways mediated by distinct protein kinases [13].

Due to its potent biological effects of ET-1, the inhibitory mechanisms of ET-1 secretion are of considerable physiologic importance. To determine if cyclic GMP or NO, or both mediate(s) the suppression of ET-1 production, cyclic GMP production must be inhibited selectively, while leaving NO unaffected. This has been attempted with methylene blue [7,8,12], but cannot be achieved with this dye since it inhibits both guanylyl cyclase and NO synthase, as we have shown recently [14]. The other tool used in the above studies, inhibition of NO synthase by L-arginine derivatives, will reduce both endogenous NO and cyclic GMP levels, thus making it impossible to differentiate between the two putative mediators. Recently, a specific guanylyl cyclase inhibitor, ODQ without effects on NO synthase has become available [15]. Such a compound appears to be singularly useful in probing the role of cyclic GMP vs. NO in inhibition of ET-1 secretion. Therefore, the present study was designed: (i) to examine the effect of several NO-based vasodilators on ET-1 secretion; (ii) to determine the effect of ODQ on ET-1 secretion and cyclic GMP levels; and (iii) to measure NO donor-derived NO release into the culture medium using a sensitive NO electrode and to compare NO levels with rates of ET-1 release.

2. Materials and methods

2.1. Materials and solutions

Spermine/NO was obtained from Midwest Research Institute, Kansas City, MO, USA. NP (sodium salt), nitrite (sodium salt), glutathione, 8-bromo-cyclic GMP, PMA, Ca^{2+} ionophore A23187, thapsigargin, L-NNA, 3′,5′-cyclic nucleotide phosphodiesterase (PDE, from bovine heart) and superoxide dismutase (SOD) were purchased from Sigma Chemie, Vienna, Austria. ODQ was synthesized by Dr. E.B. Nielsen (Novo Nordisk A/S, Novo Nordisk Park, Måløv, Denmark). The anti-ET-1 antibody and ET-1 standards were from Peninsula Laboratories, Belmont, CA, USA. Dulbecco's minimum essential medium (DMEM) was from Life Technologies, Vienna, Austria. The source of all other chemicals and tissue culture media was described previously [16]. The following stock solutions were prepared [solvent]: spermine/NO (10 mM, [10 mM NaOH]); PMA (1 mM, [DMSO]), thapsigargin (0.2 mM, [DMSO]), ODQ (100 mM, [DMSO]); A23187 (1 mM [ethanol]); L-NNA (10 mM [H_2O]), NP (1 mM [H_2O]). GSNO (10 mM) was prepared by mixing 0.1 ml glutathione (100 mM) + 0.8 ml HCl (10 mM) + 0.1 ml nitrite (100 mM). All stock solutions were stored at −20°C and diluted in distilled water or culture medium to the appropriate concentration just prior to the experiment (maximum water content: 10%).

*Corresponding author. Fax: (43) (316) 323-5414.

2.2. Cell culture and experimental protocol

Porcine aortic endothelial cells were isolated and cultured as previously described [17]. Only cells of first passage were used (~1 × 10⁶ cells per well). For experiments, cells were incubated in fresh DMEM together with all agents for 3 h at 37°C. ODQ (3 μM) was added to the medium 15 min prior to NO donors [15]. To prevent cyclic GMP degradation, IBMX (1 mM) was also included. Following incubation, the media were harvested and frozen at −70°C pending analysis.

2.3. Measurement of ET-1 and cyclic GMP

ET-1 was measured by radio immunoassay (RIA) as previously described [17]. Briefly, thawed media were diluted with RIA-buffer 1 to 5 and 0.1 ml was incubated with [3(¹²⁵I)Tyr]-ET-1 (0.1 ml, ~10,000 cpm/tube) and anti-ET-1 antibody (0.1 ml; RAS 6901) for 24 h at room temperature. To terminate incubation, 0.1 ml of γ-globulin (11 mg/ml) and 0.75 ml of polyethylene glycol 6000 (20%) was added, and centrifuged for 20 min to separate bound from free radioactivity. The pellet was counted in a gamma-counter (Packard Canberra, Vienna, Austria). Cyclic GMP was measured in culture media and cell lysates by RIA as described previously [18]. To determine whether residual cyclic GMP in the presence of ODQ was of biological importance, cells were incubated in the additional presence of PDE (0.02 U/ml incubation medium) to completely remove the nucleotide.

2.4. Electrochemical determination of NO

The electrochemical determination of NO was performed with a Clark-type NO-sensitive electrode (ISO-NO, World Precision Instruments, Mauer, Germany) as described previously [19]. Measurements were made at 37°C in a small glass vial containing 0.9 ml of DMEM and ~10⁶ endothelial cells (baseline). The contents of the vial was constantly stirred. When the baseline was stable, 0.1 ml of the NO donor was added and the NO concentration recorded by an Apple Macintosh computer via an analogue-to-digital converter (MacLab, WissTech GmbH, Spechbach, Germany).

2.5. Determination of cell viability

Cell viability was determined morphometrically by trypan blue exclusion and cell counting. In addition, lactate dehydrogenase (LDH) activity was measured as described previously [20].

2.6. Presentation of results

Group data are presented as arithmetic mean values ± S.E.M. and were compared using the unpaired Student's t-test. All ET-1 secretion data were calculated as secretion rates (pg/3 h and ~10⁶ cells) and are presented as a percentage of control secretion which is indicated in the figures. The time course of NO measurements (inset of Figs. 4 and 5) was directly transcribed from the MacLab disc file. A $P < 0.05$ was considered as significant. $P < 0.01$ or lower were not indicated separately. The number of culture dishes is indicated as n.
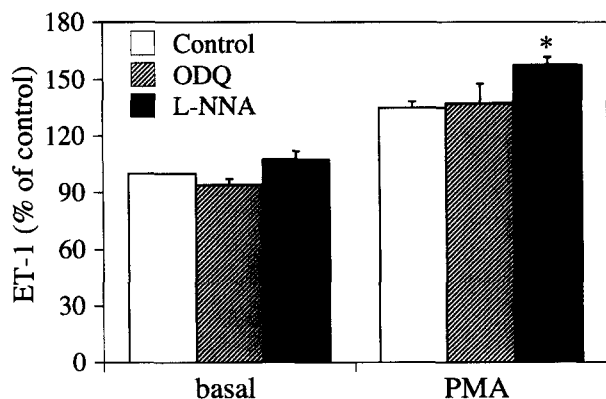

Fig. 1. ET-1 secretion into culture medium under basal and stimulated conditions (PMA; 0.4 μM). Stimulated secretion was increased by L-NNA (0.1 mM); $P < 0.05$). Data are means ± S.E.M., $n = 6$–9. Reference (100%): 327 ± 12 pg/3 h. *$P < 0.05$ vs. Control (PMA).
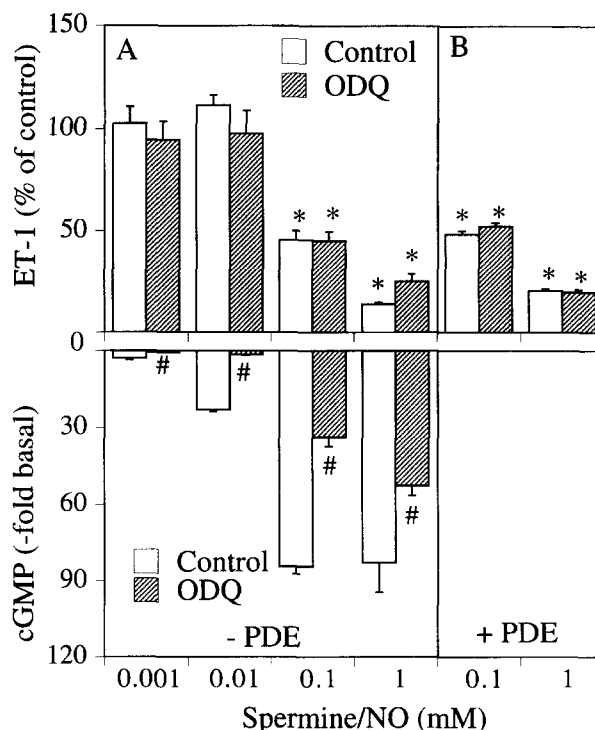

Fig. 2. Effect of ODQ (3 μM) on spermine/NO-induced inhibition of ET-1 secretion (upper panel) and cyclic GMP accumulation (lower panel). (A) In the absence of PDE and presence of IBMX (1 mM). (B) In the presence of PDE (0.02 U/ml) and absence of IBMX. Note the complete removal of cyclic GMP and unchanged ET-1 secretion in (B). *$P < 0.05$ vs. baseline; #$P < 0.05$, ODQ vs. Control. Data are means ± S.E.M., $n = 6$ (A) and $n = 3$ (B). Reference (100%): 1.4 ± 0.4 pmol/3 h cyclic GMP; for ET-1, see Fig. 1.

3. Results

Fig. 1 illustrates the effect of NO synthase and guanylyl cyclase inhibition on basal and stimulated ET-1 secretion. Basal ET-1 secretion into culture medium measured over 3 h was unaffected by either enzyme inhibitor ($P > 0.05$). The phorbol ester PMA (0.4 μM) stimulated ET-1 secretion by 35% as we have shown previously [17]. Inhibition of guanylyl cyclase with ODQ had no effect on stimulated ET-1 secretion ($P > 0.05$), but blocking NO synthase with L-NNA (0.1 mM) slightly increased ET-1 secretion rate (17%; $P < 0.05$ vs. Control). The cell-permeant cyclic GMP derivative, 8-bromo-cyclic GMP (0.1 mM), had no effect on ET-1 secretion ($P > 0.05$, $n = 6$; data not shown).

Fig. 2 illustrates the effect of spermine/NO, which releases NO in a controlled fashion [21], on ET-1 secretion and cyclic GMP accumulation in the culture medium. ET-1 secretion and cyclic GMP production were inhibited in a concentration-dependent manner, but not at matched concentrations. In the presence of ODQ, cyclic GMP accumulation was greatly reduced at all concentrations of spermine/NO, but ET-1 secretion was unchanged compared to Control ($P > 0.05$, Fig. 2A). A possible role for cyclic GMP in suppression of ET-1 synthesis observed at 0.1 and 1 mM spermine/NO was examined by co-incubating cells with PDE to immediately degrade any cyclic nucleotide produced (Fig. 2B). The same inhibitory pattern of ET-1 accumulation was observed despite complete removal of
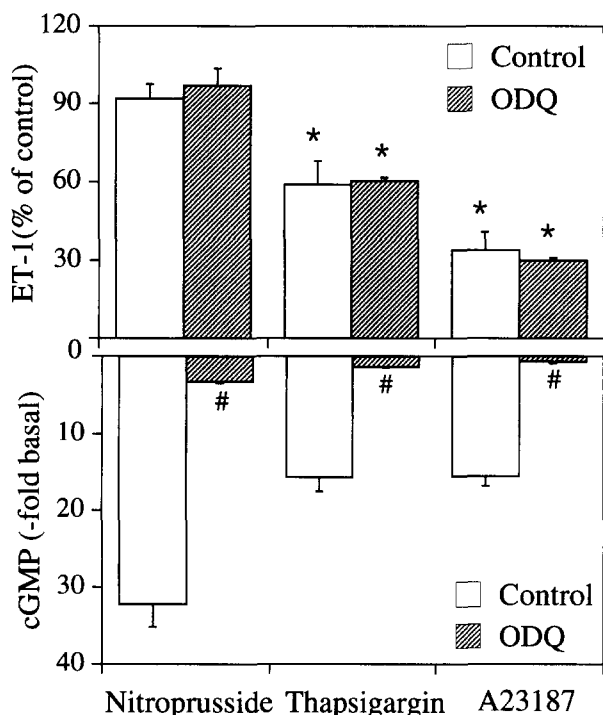
Fig. 3. Effect of ODQ (3 μM) on inhibition of ET-1 secretion (A) and cyclic GMP accumulation (B) induced by nitroprusside (1 mM), thapsigargin (100 nM), and A23187 (1 μM). * P < 0.05 vs. baseline; #P < 0.05, ODQ vs. Control. Data are means ± S.E.M., n = 6. For reference values see Fig. 2.

cyclic GMP, and the degree of inhibition was identical to that in the absence of PDE. A similar mismatch between inhibition of ET-1 secretion and cyclic GMP levels was also observed with the organic nitrate, NP [22] (1 mM) and two experimental agents known to stimulate cyclic GMP production via increased intracellular free Ca^{2+} [17], thapsigargin (0.1 μM) and the Ca^{2+} ionophore A23187 (1 μM) (Fig. 3). However, whereas the latter two compounds reduced ET-1 secretion to 60% and 38% of control, respectively NP had no effect (P > 0.05). All three compounds increased cyclic GMP levels, and ODQ prevented these increases, but it was again without effect on inhibition of ET-1 secretion. Similarly, in the presence of 1 mM GSNO [14,23], ET-1 secretion was inhibited by 45% and cyclic GMP production was stimulated 23-fold. ODQ was without effect on ET-1 levels in the culture medium (P > 0.05), but potently reduced cyclic GMP generation (n = 6; data not shown). Control incubations with glutathione or spermine were without effect on ET-1 secretion and cyclic GMP accumulation (n = 3).

The release of NO from spermine/NO is shown in Fig. 4. At 100 μM parent compound, the NO concentration measured in culture medium was ~4 μM. Between 100 μM and 600 μM, NO release increased linearly; at 1 mM (outside linear portion of calibration), the NO concentration reached was ~20–25 μM. Following a bolus concentration of 100 μM spermine/NO, NO release was rapid in onset and maintained above 1 μM for the entire incubation period of 3 h (inset in Fig. 4). By contrast, release of NO from NP (Fig. 5) was very small: upon addition of 1–10 mM NP, concentrations used in pharmacological ex-

periments, less than 0.5 μM NO was measured; a maximum of ~2.5 μM was achieved with 100 mM NP. No NO was detected at NP concentrations below 1 mM. Although NO release from this donor was also rapid in onset, it was short-lasted (~10 min; inset in Fig. 5). The low levels of NO were not due to metabolism of NO to superoxide anion because SOD (2,500 U/ml) was without effect (n = 3; data not shown). For GSNO (0.1–1 mM) peak NO levels achieved were comparable to those measured for spermine/NO (data not shown).

Because of the potentially toxic effects of micromolar concentrations of NO observed in a vaiety of different cells, the viability of endothelial cells at the end of the incubation period was evaluated. No changes were noticeable in the general appearance of cells, nor in their capacity to accumulate trypan blue or release LDH (data not shown).

4. Discussion

The present study demonstrates that inhibition of ET-1 release by NO-containing compounds cannot be reversed by selective inhibition of guanylyl cyclase, indicating that ET-1 secretion is not regulated by cyclic GMP. Of the three NO donors tested, only those reduced ET-1 secretion which also released measurable amounts of NO (spermine/NO, GSNO), whereas NP showed no inhibitory effect on ET-1 secretion and released no NO. This suggests that the active inhibitory principle is NO or NO-derived.

Nitrovasodilators such as NP and GSNO are well known to activate NO-dependent soluble guanylyl cyclase and increase tissue cyclic GMP levels [24]. These compounds have been suggested to attenuate ET-1 secretion via a mechanism involving the NO/cyclic GMP system (see section 1). In the present experiments, they also potently (≥ 1 μM) stimulated cyclic GMP production of cultured endothelial cells, but were only weak inhibitors of ET-1 secretion. The involvement of cyclic
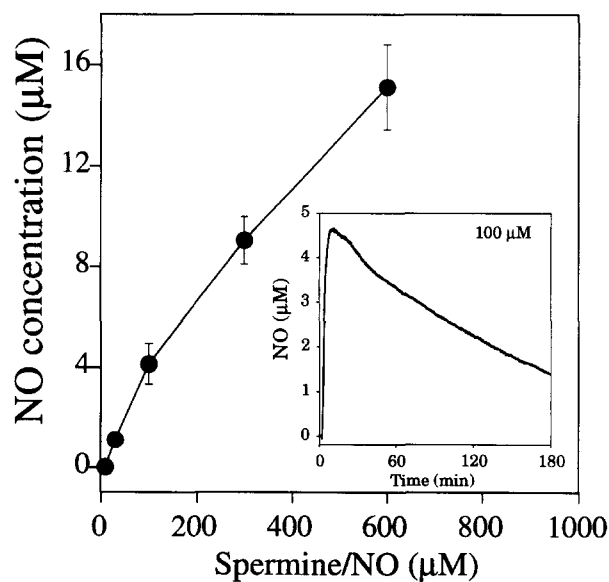

Fig. 4. Release of NO from spermine/NO into culture medium (pH 7.4, 37°C). The values represent the maximum concentration attained after a given addition of spermine/NO. Data are means ± S.E.M., n = 3. (Inset) Kinetics of NO release following a single addition of 100 μM spermine/NO to the reaction vial (single experiment).
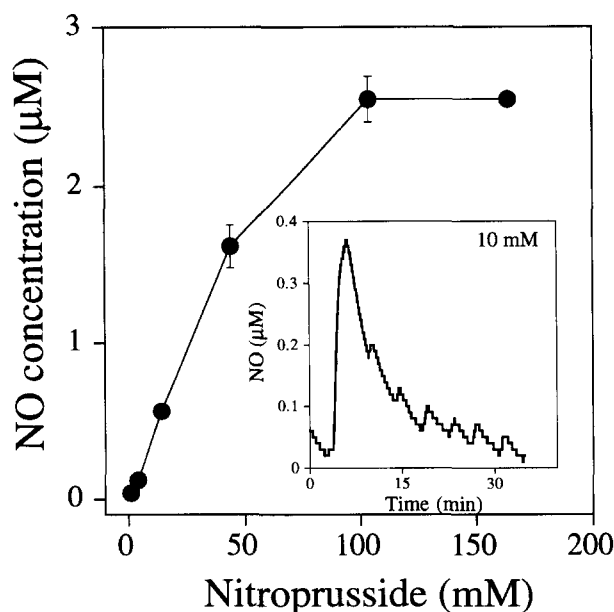
Fig. 5. Release of NO from nitroprusside into culture medium ($n = 3$). The values represent the maximum concentration attained after a given addition of nitroprusside. Data are means ± S.E.M., $n = 3$. (Inset) Kinetics of NO release following a single addition of 10 mM nitroprusside to the reaction vial (single experiment).

GMP in this inhibition was probed using the novel guanylyl cyclase inhibitor, ODQ which is a specific inhibitor of NO-sensitive GC without effect on particulate guanylyl cyclase or adenylyl cyclase, as was shown previously [15]. Hence, this compound is singularly useful in determining the contribution of endogenous cyclic GMP in regulating ET-1 secretion. ODQ potently antagonized basal and agonist-induced cyclic GMP production without affecting ET-1 secretion rates (Figs. 2 and 3). Furthermore, the complete removal of cyclic GMP by PDE resulted in no change in the spermine/NO-induced inhibition of ET-1 secretion. This is strong evidence that the inhibitory effect on ET-1 secretion, at least of the agonists tested here, is not mediated by cyclic GMP. The same conclusion was reached using 8-bromo-cyclic GMP, confirming a recent report dealing with human endothelial cells [13]. Previous reports ascribing an inhibitory role to cyclic GMP using methylene blue do not contradict our conclusion, because this dye, besides blocking guanylyl cyclase, inhibits nitric oxide synthase at even higher potency than guanylyl cyclase [14]. Moreover, the dye stimulates the production of superoxide anion [25]. Both these actions will reduce the tissue NO concentration, so that the observed effect of methylene blue could also be due to changes in NO rather than cyclic GMP levels.

Our suggestion of an NO-mediated inhibition of ET-1 secretion is supported by the increase in ET-1 secretion following blockade of NOS by L-NNA and the measurements of donor-derived NO levels in culture medium. This conclusion is strengthened by the fact that control compounds (spermine and glutathione) did not affect ET-1 secretion and produced no measurable NO. Interestingly, a similar conclusion was reached recently using L-NNA [12], although this inhibitor cannot differentiate between a regulatory effect mediated by decreasing endogenous NO as opposed to cyclic GMP levels. Given the present evidence, in their study L-NNA probably reduced ET-1 levels by lowering endogenous NO production and not via reduced cyclic GMP levels. The reasons for the inconsistent effect of nitric oxide synthase inhibition on basal ET-1 secretion rates are not clear. The inhibition of basal ET-1 secretion reported by Kourembanas et al. [12] following 24 h of incubation with L-NNA (2.5 mM) was not consistently observed in the present and a previous study [17] using cultured cells, nor in several other ones using native endothelial cells [9] and whole blood vessels [8]. The reasons may include differences in incubation time (24 h in [12] vs. 3–6 h in the other studies), quality of cells (6th to tenth passage [12] vs one passage or native cells), and different levels of $[Ca^{2+}]_i$ which we have recently shown to affect basal ET-1 secretion rates [20].

Our measurements of NO released from several NO donors explain the differing potency of NO donors in inhibiting ET-1 release. Thus, NP was entirely ineffective at 1 mM, and no NO release was detectable under our experimental conditions. On the other hand, the activation of guanylyl cyclase is probably mediated by NP itself (parent compound) and not by NO released from it as suggested previously [26]. Both spermine/NO and GSNO reduced ET-1 secretion, but spermine/NO was more potent, possibly reflecting the higher NO concentration attained in the culture medium with this compound. The inhibitory action of NO on ET-1 secretion was in all probability a specific effect and not due to NO-induced cell-toxicity as judged by several standard criteria of cell viability [20,27]. The reason for the low absolute potency of all NO donors tested is unknown; too short an incubation time cannot account for their weak inhibitory effects, since peak NO levels were reached early and similar results were obtained following 5 h of incubation (data not shown).

The inhibitory effect of increased levels of $[Ca^{2+}]_i$ on ET-1 secretion has been addressed previously [17]. As expected, cyclic GMP levels were also increased under these conditions, but inhibition of ET-1 secretion was similarly unaffected by ODQ as with NO donors (Figs. 2 and 3). Hence, cyclic GMP seems to be involved neither in NO donor nor Ca^{2+}-mediated inhibition of NO release.

References

[1] Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988) Nature 332, 411–415.
[2] Schini, V.B., Hendrickson, H., Heublein. D.M., Burnett Jr., J.C. and Vanhoutte, P.M. (1989) Eur. J. Pharmacol. 165, 333–334.
[3] Maemura, K., Kurihara, H., Morita, T., Ohhashi, Y. and Yazaki, Y. (1992) Gerontology 38, 29–35.
[4] Imai, T., Hirata, Y., Emori, T. and Marumo, F. (1993) Hypertension 21, 353–358.
[5] Hu, R.M., Levin, E.R., Pedram, A. and Frank, H.J.L. (1992) J. Biol. Chem. 267, 17384–17389.
[6] Yokokawa, K., Kohno, M., Yasunari, K., Murakawa, K.-I. and Takeda, T. (1991) Hypertension 18, 304–315.
[7] Prins, B.A., Hu, R.M., Nazario, B., Pedram, A., Frank, H.J.L., Weber, M.A. and Levin, E.R. (1994) J. Biol. Chem. 269, 11938–11944.
[8] Boulanger, C. and Lüscher, T.F. (1990) J. Clin. Invest. 85, 587–590.

[9] Warner, T.D., Schmidt, H.H.H.W. and Murad, F. (1992) Am. J. Physiol. 262, H1600–H1605.

[10] Boulanger, C.M. and Lüscher, T.F. (1991) Circ. Res. 68, 1768–1772.

[11] Kuchan, M.J. and Frangos, J.A. (1993) Am. J. Physiol. 264, H150–H156.

[12] Kourembanas, S., Mcquillan, L.P., Leung, G.K. and Faller, D.V. (1993) J. Clin. Invest. 92, 99–104.

[13] Stewart, D.J., Cernacek, P., Mohamed, F., Blais, D., Cianflone, K. and Monge, J.C. (1994) Am. J. Physiol. 266, H944–H951.

[14] Mayer, B., Brunner, F. and Schmidt, K. (1993) Biochem. Pharmacol. 45, 367–374.

[15] Garthwaite, J., Southam, E., Boulton, C.L., Nielsen, E.B., Schmidt, K. and Mayer, B. (1995) Mol. Pharmacol. (in press, August issue).

[16] Graier, W.F., Groschner, K., Schmidt, K. and Kukovetz, W.R. (1992) Biochem. J. 288, 345–349.

[17] Brunner, F., Stessel, H., Simecek, S., Graier, W. and Kukovetz, W.R. (1994) FEBS Lett. 350, 33–36.

[18] Schmidt, K., Mayer, B. and Kukovetz, W.R. (1989) Eur. J. Pharmacol. 170, 157–166.

[19] Schmidt, K., Klatt, P. and Mayer, B. (1994) Biochem. J. 301, 645–647.

[20] Brunner, F. (1995) Biochem. Pharmacol. 49, 1785–1791.

[21] Maragos, C.M., Morley, D., Wink, D.A., Dunams, T.M., Saavedra, J.E., Hofman, A., Bove, A.A., Isaac, L., Hrabie, J.A. and Keefer, L.K. (1991) J. Med. Chem. 34, 3242–3247.

[22] Feelisch, M. and Kelm, M. (1991) Biochem. Biophys. Res. Commun. 180, 286–293.

[23] Mathews, W.R. and Kerr, S.W. (1993) J. Pharmacol. Exp. Ther. 267, 1529–1537.

[24] Waldman, S.A. and Murad, F. (1987) Pharmacol. Rev. 39, 163–196.

[25] Furchgott, R.F. and Vanhoutte, P.M. (1989) FASEB J. 3, 2007–2018.

[26] Smith, M.P., Humphrey, S.J., Kerr, S.W. and Mathews, W.R. (1994) Methods Findings Exp. Clin. Pharmacol. 16, 323–335.

[27] van der Laarse, A., Hollaar, L. and van der Valk, L.J.M. (1979) Cardiovasc. Res. 13, 345–353.