Up-regulation of endothelial nitric oxide synthase expression by cyclic guanosine 3′,5′-monophosphate

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Abstract In this study, the role of cyclic GMP, the end product of the NO-cyclic GMP signalling pathway, in the regulation of ecNOS was investigated. Bovine pulmonary endothelial cells were exposed to 8-bromo-cyclic GMP and its effect on NO production, ecNOS protein, and mRNA levels was analyzed. Endothelial cells on exposure to 8-bromo-cyclic GMP produced significantly increased amounts of NO, detected as increased cyclic GMP in cocultures with vascular smooth muscle cells both under basal conditions and with agonist stimulation. 8-Bromo-cyclic GMP significantly increased the ecNOS protein and mRNA levels as detected on Western and Northern blots respectively. This 8-bromo-cyclic GMP mediated increase of NO production, ecNOS protein and mRNA levels suggests that cyclic GMP up-regulates the expression of ecNOS. Thus, there may be an intercellular feedback mechanism involved at the molecular level in the expression of the NO-cyclic GMP signalling pathway in blood vessels.

Key words: Cyclic GMP; NO synthase; Molecular expression; Vascular smooth muscle cell

1. Introduction

In the vasculature, nitric oxide (NO) produced in the endothelial cells by the constitutive isoform of NO synthase (ecNOS) acts as an important signal transduction system for the activation of soluble guanylate cyclase and subsequent cyclic GMP production in the vascular smooth muscle (VSM) [1,2]. This cyclic GMP, in a cascade of reactions, produces vascular relaxation [1,3]. NO thereby acts as a potent vasodilator and regulator of vascular tone and controls basal blood pressure [1–3]. In the vasculature, in addition to its potent vasodilator effects, NO has also been implicated in a wide variety of physiologic events such as modulation of platelet aggregation and adhesion, inhibition of smooth muscle proliferation and endothelin generation [1–5]. In addition, endothelial NO production is impaired in many types of vascular diseases including atherosclerosis and hypertension [3,5].

The ecNOS is activated and NO release is stimulated by receptor mediated agonists such as acetylcholine, bradykinin, substance P, histamine and receptor independent calcium inophores such as A23187 and also by fluid shear stress [6,7]. In addition, it has been shown that certain factors influence the expression of the ecNOS mRNA. Tumor necrosis factor-α [8], shear stress [7,9], chronic exercise training [10], protein kinase C [11] and lysophosphatidylcholine [12] were reported to regulate the molecular expression of ecNOS. Thus, there is now sufficient evidence to suggest that the expression of ecNOS, though it is a constitutive isoform, is subject to regulation by a variety of stimuli.

Recently the ability of cyclic GMP to alter specific gene expression including the regulation of early gene expression such as c-*fos* and junB has been reported [13,14]. On the basis of these considerations, we examined the possibility that cyclic GMP, the end product of NO-cyclic GMP signalling pathway, may modulate the expression and/or the activity of ecNOS as a potential mechanism of feedback regulation of this intercellular signalling pathway.

2. Materials and methods

2.1. Chemicals

8-Bromo guanosine 3′,5′-cyclic monophosphate (8-bromo-cyclic GMP), L-arginine, NADPH, calmodulin, pepstatin A, EDTA, EGTA, leupeptin, ATP and bradykinin were obtained from Sigma Chemical Co. Bio-Rad protein assay reagent was obtained from Bio-Rad Laboratories. Medium 199, streptomycin, penicillin, thymidine and Dulbecco's phosphate-buffered saline (PBS) were obtained from Gibco BRL. Fetal bovine serum was from Hyclone Laboratories. Anti ecNOS mouse IgG_1 monoclonal antibody was obtained from Transduction Laboratories. 4091 bp *Eco*RI ecNOS cDNA fragment is a kind gift from William Sessa [15]. 780 pb *Xba*I-*Pst*I glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA fragment is from American Type Culture Collection. All other chemicals used were of analytical grade.

2.2. Endothelial and VSM cell culture

Bovine pulmonary endothelial and VSM cells were isolated and maintained as described before [16,17]. Briefly, a pure culture of endothelial cells was obtained by fluorescent-activated cell sorting using acetylated low density lipoprotein as the fluorescent marker. Endothelial cultures were then further identified by: (1) typical cobblestone morphology, (2) antibody staining for factor VIII antigen, and (3) demonstrating by Northern blot a single messenger RNA for endothelial cell β-actin and, on immunocytochemistry, the absence of immunostaining for α-actin. The VSM cells were identified by immunocytochemistry for smooth muscle α-actin using a monoclonal antibody. For Western and Northern blotting studies, pulmonary endothelial cells grown in roller bottles in medium 199 supplemented with fetal calf serum (10%), thymidine (2.4 μg/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml) were used. For co-culture studies, endothelial cells were placed in microcarrier culture by seeding 2×10^7 cells on 0.6 g of Cytodex 3 microcarrier beads (Pharmacia, Uppsala, Sweden) (4600 cm^2 surface area/g) in 200 ml medium 199 containing fetal calf serum (20%), thymidine (2.4 μg/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml) and maintained in 2 liter roller bottles. The VSM cells were grown in 24-well plates in medium 199 supplemented with fetal calf serum (10%), penicillin (100 U/ml) and streptomycin (100 μg/ml) and were used 3–5 days post-confluency.

2.3. Co-culture of endothelial and VSM cells

Co-culture studies were performed as published [16]. Briefly, confluent bovine pulmonary endothelial cells grown on the surface of the

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Abbreviations: NO, nitric oxide; ecNOS, endothelial constitutive nitric oxide synthase; cyclic GMP, cyclic guanosine 3′,5′-monophosphate; VSM cells, vascular smooth muscle cells.

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microcarrier beads were exposed to 8-bromo-cyclic GMP (100 μM), a lipophilic and a non-phosphodiesterase hydrolyzable cyclic GMP analog for 48 h in medium 199 supplemented with fetal calf serum (10%), penicillin (100 U/ml) and streptomycin (100 μg/ml). The coculture experiments were initiated by plating the microcarrier beads containing the equivalent of 5×10^7 endothelial cells on to the VSM cells grown after repeated washing with PBS to remove the residual 8-bromo-cyclic GMP. Bradykinin (1 μM) and ATP (100 μM) were used for stimulating ecNOS activity and endothelial NO release. The NO-dependent increase in cyclic GMP accumulation in the VSM cells was measured as an assay of ecNOS activity and NO production in the endothelium. Also, to rule out the cross over of 8-bromo-cyclic GMP with the cyclic GMP assay, endothelial cells on microcarriers that were exposed to 8-bromo-cyclic GMP were plated alone in 24-well plates at the same density as was done for cocultures and the residual cyclic GMP levels were measured and were used to rule out any cross over effect of 8-bromo-cyclic GMP. Further, we checked the soluble guanylate cyclase mediated production of cyclic GMP in the endothelial cells after stimulation with sodium nitroprusside (SNP; 10 μM). SNP failed to increase the cyclic GMP production in the pulmonary endothelial cells ($n = 4$, data not shown) that were used in the current experiments and these results are consistent with the early observation from our laboratory that pulmonary endothelial cells lacked the soluble guanylate cyclase [17].

2.4. Cyclic GMP assays

Cyclic GMP was extracted in 0.1 N HCl (0.5 ml) and measured by automated radioimmunoassay of acetylated HCl extracts according to the methods of Harper and Brooker [18], as modified by Patel and Linden [19].

2.5. Preparation of ecNOS from cultured endothelial cells

The membrane bound ecNOS was solubilized as previously described [20,21] with slight modifications. Briefly, the bovine pulmonary endothelial cells that were exposed to 8-bromo-cyclic GMP or the vehicle for 48 h were scraped and washed in PBS and then suspended and homogenized in ice-cold 50 mM Tris.HCl (pH 7.4), containing 0.1 mM EDTA, 0.1 mM EGTA, 0.1% 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin and 1 μM pepstatin A. The homogenate was centrifuged at 100,000 × g for 60 min at 4°C. The particulate fraction was then solubilized with the detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; 20 mM) in homogenizing buffer containing 10% glycerol and protease inhibitors.

2.6. Western blotting

The particulate fractions that were collected, as described above were then analyzed by electrophoresis on a 7% SDS polyacrylamide gel, and then transferred electrophoretically to nitrocellulose paper to give a Western blot. The blot was blocked for non-specific binding with 1% BSA and 0.1% Tween at room temperature for 1 h. After blocking, the blot was washed with Tris-HCl buffered saline (pH 7.4), then incubated with the primary mouse anti-ecNOS antibody for 2 h and washed. The blot was then incubated with a secondary antibody, a peroxidase-conjugated goat anti-mouse IgG, which detects the primary antibody. After washing, the blot was incubated in ECL (Amersham), a chemiluminescent substrate for horseradish peroxidase. The luminescence was detected by exposure of the blot to X-ray film and quantitated using a densitometer.

2.7. Northern blotting

For mRNA analysis by Northern blotting of poly(A)$^+$ RNA, total RNA was isolated by the method of Chomczynski [22] after lysing the cells with Tri reagent (Molecular Research Center Inc.). Poly(A)$^+$ RNA was then purified from the total RNA using an oligo(dT)-cellulose affinity spin column and following the manufacturers instructions (5 Prime-3 Prime Inc.). Northern blot analysis of 2–4 μg poly(A)$^+$ RNA was performed by following the standard procedure as described by Sambrook et al. [23]. After random primed labeling, the [α-^{32}P]dCTP labeled cDNA probes for bovine ecNOS and GAPDH were then used for the hybridization of the blots overnight at 65°C. The blots were then washed twice in 2 × standard saline citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) for 15 min at room temperature and then twice in 0.4 × SSC/0.1% SDS for 15 min at 65°C, rinsed in 0.1 M phosphate buffer, pH 7.5, dried and the blots with the hybridized probes were detected by autoradiography and quantitated using a phosphorimager and IMAGEQUANT software (Molecular Dynamics).

3. Results

Bioassay experiments were performed using endothelial and VSM cell coculture where the production of cyclic GMP by the VSM cells was taken as a measure of NO production by the cocultured endothelial cells. When the VSM cells were cocultured with 8-bromo-cyclic GMP treated endothelial cells, significantly increased cyclic GMP levels (*$P < 0.05$ level, $n = 4$ and representative of three independent experiments) were observed, both under basal and agonist (bradykinin and ATP) stimulated conditions as compared to the respective controls (Fig. 1). This observed increase in cyclic GMP levels reflects the actual production of endothelial NO-dependent cyclic GMP since cross over of 8-bromo-cyclic GMP present in the endothelial cells with the cyclic GMP assay has been ruled out as described in section 2. Under basal conditions, the production of cyclic GMP by the VSM cells was about 2.8-fold higher (from 35.49 ± 9.25 to 137.65 ± 15.89 pmol cyclic GMP/well) with the endothelial cells that were exposed to 8-bromo-cyclic GMP, as compared to the controls. Similarly, bradykinin and ATP stimulated NO production, and hence cyclic GMP formation, were increased by ~40% (from 129.61 ± 6.56 to 182.33 ± 3.76 pmol cyclic GMP/well) and 70% (from 138.53 ± 7.33 to 240.02 ± 18.19 pmol/well) with 8-bromo-cyclic GMP treated endothelial cells as compared to the respective controls.

On the Western blot, using a mouse monoclonal antibody

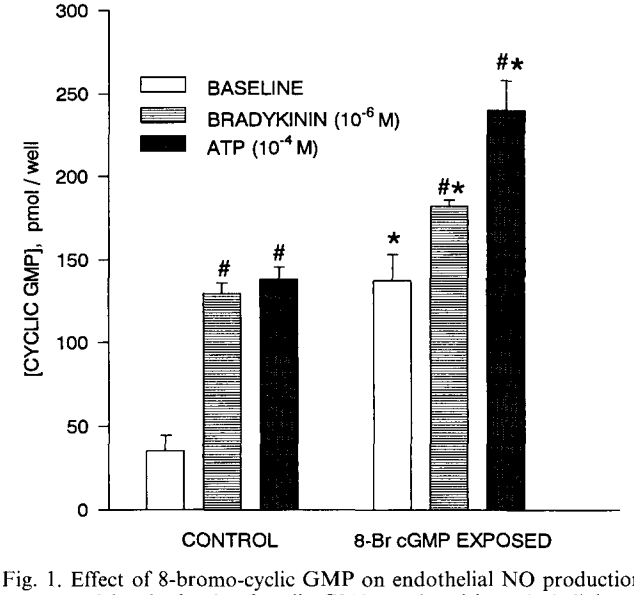

Fig. 1. Effect of 8-bromo-cyclic GMP on endothelial NO production as assessed by the levels of cyclic GMP produced in endothelial and VSM cell cocultures. After exposure to 8-bromo-cyclic GMP (100 μM) for 48 h, the endothelial cells grown on the surface of the microcarrier beads were repeatedly washed with PBS and were plated on to the VSM cells. The NO mediated cyclic GMP production was measured under basal and agonist (bradykinin and ATP) stimulated conditions. Values are mean ± S.E.M. of four determinations and representative of three independent experiments. The values represent the actual endothelial NO-dependent increase in cyclic GMP after ruling out the cross over of 8-bromo-cyclic GMP with the cyclic GMP assay as described in section 2. #Significantly different from baseline ($P < 0.05$). *Significantly different from corresponding control ($P < 0.05$).

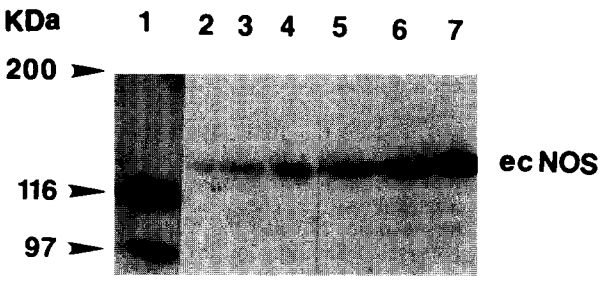

Fig. 2. Western blot demonstrating the effect of 8-bromo-cyclic GMP on ecNOS protein levels in endothelial cells. After exposure to 8-bromo cyclic GMP (100 μM) for 48 h, the particulate fractions of the endothelial cells were separated and Western blotting was performed with 40 μg protein as described in section 2 and probed for ecNOS using a monoclonal antibody specific to ecNOS. Lane 1 = molecular weight standards; lanes 2–4 = protein from control endothelial cells; and lanes 5–7 = protein from 8-bromo-cyclic GMP exposed endothelial cells.

specific to ecNOS, significantly increased ecNOS protein levels ($^*P < 0.05$ level) were detected in the particulate fractions of 8-bromo-cyclic GMP treated endothelial cells as compared to untreated controls (Fig. 2). The ecNOS protein levels were increased by 368 ± 52% ($n = 8$ from three independent experiments) on exposure to 8-bromo-cyclic GMP as compared to the control protein levels.

The Northern blot results showed that 8-bromo-cyclic GMP treatment increased the level of ecNOS mRNA (Fig. 3). After normalizing for loading with the housekeeping gene GAPDH mRNA , the ecNOS mRNA levels of the 8-bromo-cyclic GMP exposed endothelial cells were found to be significantly enhanced (54 ± 16%) as compared to the control ecNOS mRNA ($^*P < 0.05$ level, $n = 5$ from three independent experiments).

4. Discussion

The present study demonstrates that cyclic GMP up-regulates the expression of ecNOS based on the following evidence. (1) Cyclic GMP production increased in endothelial and VSM cell co-cultures when endothelial cells were pretreated with 8-bromo cyclic GMP. (2) EcNOS protein levels increased after endothelial cells were exposed to 8-bromo-cyclic GMP as observed on Western blot using an antibody specific to ecNOS. (3) Steady state levels of ecNOS mRNA increased in response to 8-bromo-cyclic GMP.

8-Bromo cyclic GMP, in the current study, consistently caused an increase in the steady state levels of ecNOS mRNA. Under the current hybridization and washing conditions, we and others [15] have shown that the ecNOS cDNA probes do not cross-react with inducible NOS or brain NOS. Also, since the size of the brain NOS (about 10.4 kb) is more than twice the size of ecNOS (about 4.8 kb) and there exists a large difference between the ecNOS and cytokine-inducible forms of NOS (only 50% amino acid homology), the increased steady state levels of mRNA observed in the present study, in response to 8-bromo-cyclic GMP, reflects an up-regulation of the ecNOS gene. Thus the increased ecNOS protein levels in response to 8-bromo-cyclic GMP and the increased cyclic GMP production in co-cultures of VSM cells and 8-bromo-cyclic GMP treated endothelial cells are consistent with the increase of ecNOS mRNA and support the hypothesis that 8-bromo-cyclic GMP up-regulates ecNOS gene expression.

The expression of ecNOS, although it is a constitutive enzyme, is subject to regulation by a variety of factors. In human umbilical vein endothelial cells (HUVEC), tumor necrosis factor-α was shown to decrease the ecNOS mRNA levels in a dose-dependent manner by acting at the posttranscriptional level, thereby decreasing its half-life [8]. There are in vitro data showing that prolonged mechanical deformation of the endothelium by defined shear or cyclic stretching increases ecNOS gene expression, protein and activity [9,24]. In dogs, chronic exercise training was reported to increase ecNOS mRNA and coronary vascular NO production [10]. Inhibition of protein kinase C by staurosporine or chelerythrine was shown to enhance the levels of ecNOS mRNA, NO synthase protein and activity [11]. In HUVECs it was reported that lysophosphatidylcholine increased the ecNOS mRNA levels by increasing its rate of synthesis and this was followed by increased ecNOS protein levels and activity [12].

Recently cyclic GMP has been implicated in regulating the expression of several proteins. Cyclic GMP was shown to down-regulate the atrial natriuretic peptide clearance receptors on cultured vascular endothelial cells [25]. In the HepG2 cell line, the human asialoglycoprotein receptor was shown to be posttranscriptionally regulated by cyclic GMP [26]. Rp-8-bromo cyclic GMPs (a cyclic GMP dependent protein kinase inhibitor) was reported to produce a dose-dependent decrease in erythropoietin mRNA levels in Hep3B cells in response to hypoxia [27]. The expression of cellular immediate early genes c-*fos* and junB, was shown to increase in response to cyclic GMP in pheochromocytoma PC12 cells [13]. Similarly, in transient and stable transfection experiments involving mammalian cells, it was shown that cyclic GMP analogs activated phorbol ester response element (TRE)-regulated promoters, increased DNA binding of AP-1 and increased c-*fos* and junB expression [14]. In hematopoietic cells, NO releasing agents and cyclic GMP analogs were reported to change the expression of differ-

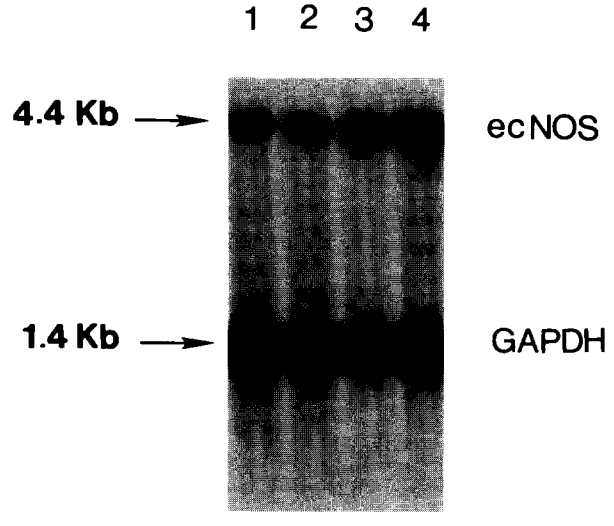

Fig. 3. Northern blot analysis of the effect of 8-bromo-cyclic GMP on ecNOS mRNA levels in endothelial cells. After exposure of endothelial cells to 8-bromo-cyclic GMP (100 μM) for 48 h, the poly(A)$^+$ RNA were isolated and Northern blotting was performed with 2–4 μg mRNA as described in section 2 and probed for ecNOS and GAPDH using cDNA's specific for ecNOS and GAPDH. Lanes 1 and 2 = control mRNA; lanes 3 and 4 = mRNA from 8-bromo-cyclic GMP exposed endothelial cells.

entiation-associated genes and induce cellular differentiation [28]. Most recently, it was reported that cyclic GMP up-regulated inducible NO synthase expression in VSM cells [29].

The current observation of up-regulation of ecNOS by 8-bromo-cyclic GMP indicate that cyclic GMP is involved in the regulation of the signalling pathway in which it can be produced suggesting an intercellular feedback mechanism. To our knowledge, this is the first report about the regulation of ecNOS expression by cyclic GMP. This cyclic GMP mediated increase in the expression of ecNOS may play an important role in various pathophysiological conditions where elevated cyclic GMP levels were observed.

Increased levels of cyclic GMP in the circulation and other body fluids has been reported in various clinical conditions. Plasma cyclic GMP levels are reported relatively sensitive to changes in clinical status with treatment of heart failure [30]. Elevated plasma cyclic GMP levels were reported in end-stage renal failure patients on chronic hemodialysis [31] and in cirrhotic patients [32]. Plasma and urinary cyclic GMP has been reported to be elevated in patients with schizophrenia [33] and in certain malignancies such as ovarian cancer [34]. However, the physiological significance of these findings remains to be elucidated. Under such pathophysiologic conditions the increased cyclic GMP levels may influence the expression of ecNOS and the NO-cyclic GMP signalling pathway.

A great deal of interest has focused on the role of endothelium derived factors in modulating smooth muscle cell function. It is now well established that the endothelium produces a variety of autacoids, including NO and endothelin, which influence vascular smooth muscle reactivity and growth. The results of the present study showing that cyclic GMP can modify the expression of ecNOS, suggest that the cells of the vascular media can in-turn alter the functions of the endothelium.

Our current observations suggest that cyclic GMP modulates, in part, ecNOS gene expression. It is possible that changes in the biological activity of soluble or particulate guanylate cyclases and cyclic GMP production under certain pathophysiological conditions are likely to regulate ecNOS and NO responses. Currently, the mechanisms or the significance behind these events are not clear. Recently, the presence of AP-1 sites have been reported in both the human [35] and bovine ecNOS promoter [36]. Also, cyclic GMP has been implicated in the induction of the immediate early genes c-*fos* and junB and the formation of AP-1 complex [13,14]. Hence, one likely possibility is that cyclic GMP might modulate the transcription of ecNOS gene through the increased formation and binding of AP-1 complex to the specific DNA sequence in ecNOS promoter. Currently, further studies are underway to define the mechanisms involved in this intercellular molecular regulation of ecNOS.

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