Effects of β-adrenoceptor stimulation on endothelial nitric-oxide synthase phosphorylation of human umbilical vein endothelial cells[1]

YAO Kang, XU Biao[2], LIU Ya-Ping, Albert FERRO[3]

Department of Cardiology, Affiliated Zhong Da Hospital of Southeast University, Nanjing 210009, China;
[3]Department of Clinical Pharmacology, St Thomas' Hospital, King's College, London, England

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ABSTRACT

AIM: To investigate the effects of isoprenaline on the phosphorylation level of endothelial nitric oxide synthase (eNOS) of human umbilical vein endothelial cells (HUVEC) and to elucidate the regulation mechanisms of β-adrenoceptor (β-AR) on eNOS activity. **METHODS:** HUVEC were exposed to isoprenaline (1 μmol/L) for 30 min, in the absence or presence of selective β_1- or β_2-adrenoceptor antagonists. eNOS was then isolated by immunoprecipitation using a specific eNOS antibody. eNOS activity was determined by the conversion of *L*-[^{3}H] arginine to *L*- [^{3}H]citrulline. The levels of eNOS expression and eNOS phosphorylation were measured by Western blotting. **RESULTS:** Treatment of HUVEC with isoprenaline (1 μmol/L) for 30 min caused no measureable change in eNOS expression. However, eNOS activity and serine phosphorylation level were significantly increased (P<0.01). This effect could be abolished by a selective β_2-adrenoceptor antagonist ICI 118551. **CONCLUSION:** Isoprenaline increases eNOS activity of HUVEC, this is mediated by β_2-adrenoceptor and associated with an increase of eNOS serine phosphorylation level.

INTRODUCTION

Stimulation of β-adrenoceptors (β-AR) on vascular smooth muscle results in vasorelaxation through activation of andenylyl cyclase and consequent increase in tracellular cyclic adenosine-monophosphate (cAMP). Vascular endothelial cells also may express β-adrenoceptors, and there is increasing evidence that these may contribute to β-adrenergic vasorelaxation because vascular relaxation to β-AR stimulation is impaired by removal of the endothelium or by inhibition of endothelial nitric oxide synthase (eNOS)[1,2]. But the signal transduction mechanism involved is unclear. We have recently demonstrated in cultured human umbilical vein endothelial cells (HUVEC) and in rabbit femoral artery *in vivo* that stimulation of β-AR with isoprenaline increased NO production. cAMP generation (mediated either by β-AR stimulation or by other pathway) can give rise to activation of eNOS[3,4]. Agonists such as acetylcholine or histamine cause an influx of excellular Ca^{2+} which, by promoting the binding of calmodulin to

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[2] Correspondence to Prof XU Biao, MD.
Phn 86-25-327-2018. Fax 86-25-327-2010.
E-mail yaokangd@hotmail.com
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NOS, leads to activation[5]. However, we have found that isoprenaline increases adenylyl cyclase activity and intracellular cAMP in HUVEC, but that this is not associated with an increase in tracellular [Ca^{2+}] as assessed by Furo-2 fluorescence[3]. Indeed, recent evidence suggests that eNOS can also be activated in a calcium-independent manner by fluid shear stress[6] and estrogen[7], and that phosphorylation of eNOS is possiblely crucial for this novel calcium-independent activation. In the present study , we try to investigate the effect of isoprenaline on the activity of eNOS in HUVEC, and to examine whether phosphorylation of eNOS is involved in the process.

MATERIALS AND METHODS

Isolation and culture of HUVEC Fresh umbilical cords were obtained following delivery of healthy babies to healthy normotensive mothers. Approval was granted by the affiliated hospital of Southeast University. Human umbilical vein endothelial cells were obtained[9]. The cells were cultured in medium 199 (Gibco, USA), supplemented with 20 % fetal bovine serum and endothelial cell growth supplement 120 mg/L (Sigma,USA), into a 25-mL gelatin-coated culture flask, at 37 °C in 5 % CO_2+95 % air. At confluence, cells were detached from the substratum by brief exposure to trypsin-edetic acid (at 37 °C for 2 min), pelled (400×*g*, 5 min), and passed at a split ratio of 1:3. Confluent cells at passage 2 were used for all experiments.

Immunoprecipitation of eNOS Cells were washed twice with warm PBS and treated with isoprenaline or vehicle for 30 min, in the absence or presence of β_1-AR or β_2-AR antagonist. At the end of the incubation, the cells were washed twice with ice-cold PBS, and lysed on ice for 30 min in 0.5 mL of lysis buffer (Tris-HCl 25 mmol/L, edetic acid 1 mmol/L, NaCl 150 mmol/L, NaF 50 mmol/L, 1 % Triton-100, PMSF 1 mmol/L, leupeptin 1 mg/L, aprotinin 1 μmol/L, pH 7.6). Lysates were transferred to an eppendorf tube and rotated at 15000×*g*, 4 °C for 15 min. The supernatant containing equal amounts of protein were added to Protein A-Sepharose CL4B beads (Amersham, UK) precoated with anti-eNOS antibody (Santa Cruz, Germany), at 4 °C for 2 h. Having been extensively washed with PBS, the beads were divided into two equal aliquots: one was used for NOS activity measurements, and the other for Western blotting studies.

Western blotting of immunoprecipitated eNOS Immunoprecipitates were boiled in SDS-PAGE sample buffer (glycerol 8 %, SDS 1.6 %, 2-mercapatoethanol 64 mmol/L, Tris-HCl 0.5 mol/L, pH 6.8) for 5 min, and separated by SDS-PAGE on an 8 % gel, followed by electroblotting for 1 h onto a nitrocelluose membrane (Amersham, UK). Membranes were blocked by a 2-h incubation at 4 °C in TBST (Tris-HCl 20 mmol/L pH 7.6, NaCl 137 mmol/L, 0.1 % Tween-20) containing 5 % blocking agents (Amersham USA), followed by a 1-h incubation in rabbit anti-eNOS antiserum (Santa Cruz , Germany) at dilution 1:1000. Having been extensively washed in TBST, membranes were incubated for 1 h with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody at room temperature. They were then extensively washed in TBST, and developed using enhanced chemiluminescene substrate (ECL, Amersham UK). Bands thus revealed were analyzed by scanning densitometry.

Analysis of serine phosphorylation To assay for serine phosphorylation of eNOS, nitrocellucose membranes were stripped by incubation in stripping buffer (Tris-HCl 62.5 mmol/L, pH 6.9, 2 % SDS, 2-mercapatoethanol 100 mmol/L) at 50 °C for 30 min. Stripped membranes were reprobed with anti-phosphoserine antibody (Santa Cruz,Germany) as before.

Activity of immunoprecipatated eNOS eNOS immunoprecipitated and immobilized on protein A-Sepharose CL4B beads was resuspended in 100 μL of NOS assay buffer of the following composition: Tris-HCl 50 mmol/L, edetic acid 0.1 mmol/L, egtazic acid 0.1 mmol/L, NaF 10 μmol/L, leupeptin 2 μmol/L, aprotinin 1 μmol/L, PMSF 1 mmol/L, NADPH 1 mmol/L, tetrahydrobiopterin 3 μmol/L, calmodulin 100 nmol/L, $CaCl_2$ 2.5 mmol/L, *L*-arginine 10 μmol/L, pH 7.5. *L*-[^{3}H]arginine (Sigma,USA) 7400 Bq was added and, following incubation at 37 °C for 30 min, the reaction was quenched by the addition of 1 mL of ice-cold stop buffer (HEPES 20 mmol/L, edetic acid 2 mmol/L, egtazic acid 2 mmol/L, pH 5.5). An excess of Dowex resin (Sigma, USA) was added, this was mixed thoroughly and allowed to settle for 10 min. The resultant supernatant, containing the *L*-citrulline fraction, was counted by liquid scintillation.

Statistical analysis The data were expressed as mean±SD and statistically analyzed using one-way ANOVA. Values considered to be statistically significant were $P<0.05$.

RESULTS

Effect of isoprenaline on eNOS activity in HUVEC eNOS activity was evaluated from the conversion of *L*-[^{3}H]arginine to *L*-[^{3}H]citrulline production. Isoprenaline increased *L*-[^{3}H]citrulline production by 34.4 % as compared with basal (P<0.01). To determine whether the β-AR mediated increase in *L*-[^{3}H] citrulline production was mediated through β_1 or β_2-AR, we performed a large series of experiments with CGP 20712A 300 nmol/L (a β_1-AR selective antagonist) or ICI 118551 100 nmol/L (a β_2-AR selective antagonist). Coincubation with CGP 20712A did not affect *L*-[^{3}H]citrulline production in response to isoprenaline. By contrast, in the presence of ICI 118551, isoprenaline did not increase *L*-[^{3}H]citrulline formation suggesting that the activation of eNOS by isoprenaline was mediated exclusively through β_2-AR (Fig 1).

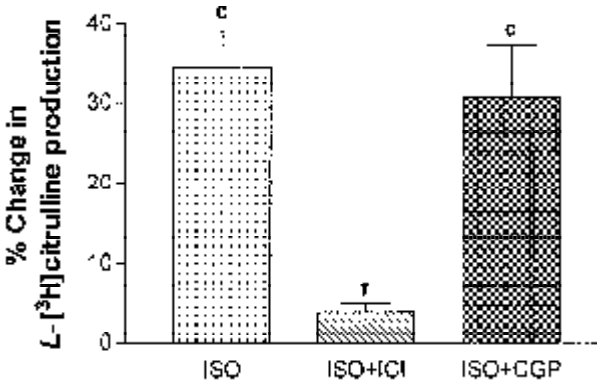

Fig 1. Effects of isoprenaline (ISO) on eNOS acvitity in HUVEC. Cells were incubated for 30 min with ISO (1 μmol/L), in the absence or presence of selective β_1- or β_2-adrenoceptor antagonists. eNOS was then immunoprecipitated from lysed cells. *L*-[^{3}H]arginine to *L*-[^{3}H]citrulline conversion was determined in the immunprecipitate. Results are expressed by percent change in *L*-[^{3}H]citrulline formation in response to different treatments compared with control cells (100 %). n=4. ^cP<0.01 *vs* control. ^fP<0.01 *vs* ISO.

Effect of isoprenaline on serine phosphorylation of eNOS in HUVEC To investigate whether the observed effects of isoprenaline on eNOS activity in HUVEC were caused by phosphorylative modification of the eNOS enzyme itself, rather than changing expression of eNOS, HUVEC were exposed to isoprenaline or vehile for 30 min. eNOS was then isolated from lysed HUVEC by immunoprecipitate using a specific antiserum to eNOS, and the resulting immunoprecipitate was divided into two equal aliquots: one was used for NOS activity measurements, and the other for Western blotting studies. Western blotting, using the same eNOS antiserum, revealed the presence of a band at 135 kDa, corresponding to the known molecular mass of human eNOS (Fig 2A, up panel). Scanning densito-

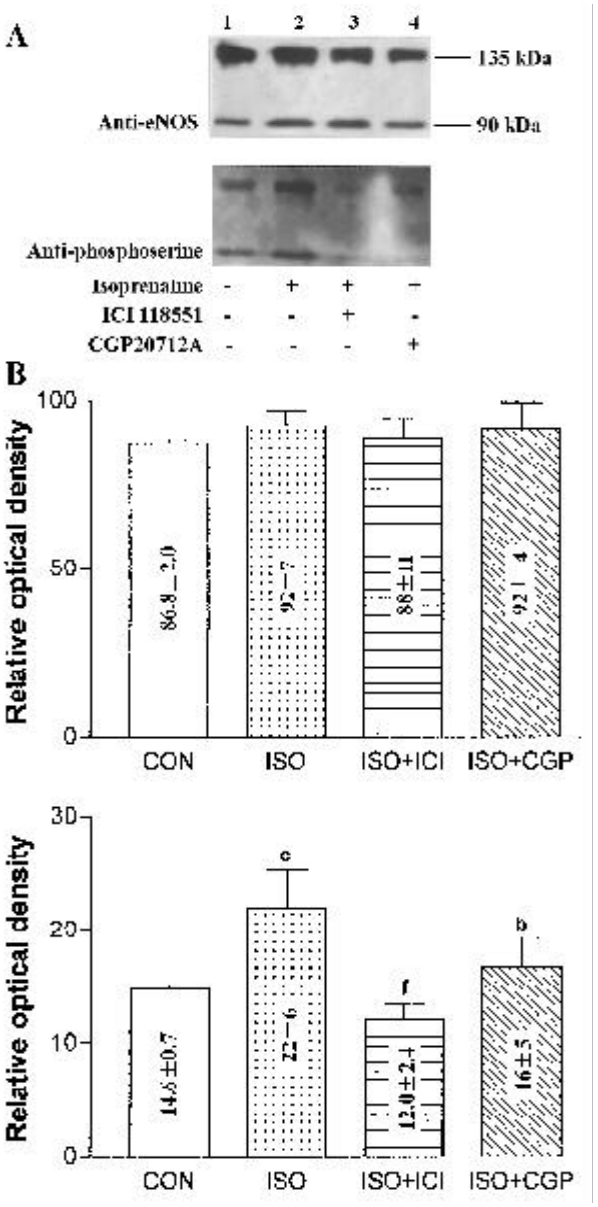

Fig 2. Effect of isoprenaline (ISO) on serine phosphorylation of eNOS in HUVEC. Cells were incubated for 30 min with isoprenaline 1 μmol/L, in the absence or presence of selective β_1- or β_2-adrenoceptor antagonists. eNOS was isolated by immunoprecipitation. A, Western blot of its phosphoserine content following different treaments. B, Serine phosphorylation of eNOS, quantified by scanning densitometry of bands following Western blotting and probing using anti-eNOS (top panel) and anti-phosphoserine (bottom panel) antibodies. n=4. ^bP<0.05, ^cP<0.01 *vs* control (CON). ^fP<0.01 *vs* ISO.

metric analysis revealed no change in the amount of eNOS present, following treatment with isoprenaline (1 μmol/L), in the absence or presence of β_1 or β_2-AR selective antagonist (Fig 2B, up panel). Following stripping of the membrane and reprobing with a specific rabbit antiserum to phosphoserine groups, a band was found once again at 135 kDa (Fig 2A, botom panel). Scanning densitometry demonstrated increased band density in response to isoprenaline, which was prevented by ICI 118551 100 nmol/L (Fig 2B, botom panel).

DISCUSSION

Previous *in vitro* and *in vivo* studies have suggested that β-andrenoceptor-mediated vasorelaxation may have an endothelium-dependant compoment in several animal species. Dawes *et al*[10] showed that infusion of isoprenaline into the brachiartery of human subjects induced an increase in forearm blood flow, and that the response was inhibited by coinfusion of the NOS inhibitor *L*-NMMA. We previously demonstrated in cultured HUVEC *in vitro* and in rabbit femoral artery *in vivo*, that β-adrenoceptor activation or cyclic AMP elevation by other means stimulated NO production. The present study showed that treatment of HUVEC with isoprenaline significantly increased serine phosphorylation level and eNOS activity, this effect could be abolished by a selective β_2-adrenoceptor antagonist ICI 118551.

Vasodilative substances such as acetylcholine, bradykinin have been shown previously to exert their action via an endothelium-dependent manner and NO production. Sympathoadrenal system and vascular endothelium *L*-arginine/NO system are both very important to cardiovascular homeostasis. But interactions between the two systems are known little. Here, we found that stimulation of β-adrenoceptor in HUVEC with isoprenaline elicited an increase in conversion of *L*-arginine to *L*-citrulline, indicating an increase in NOS activity. This effect was attributable to β_2-adrenoceptor subtype, because ICI 118551 completely blocked the increase of eNOS activity caused by isoprenaline. The physiological consequences of vascular endothelial NO production in response to β-adrenergic activation are present unknown, but it is possible that this process is important in the normal control of vessel tone by the sympatheadrenal system. Furthermore, certain disease states, including atheresclerosis, heart failure and essential hypertension are associated with both endothelial dysfunction and impaired β-adrenergic vasodilation[11,12]. The present findings suggest that these abnormalities may be causally related.

The eNOS is classified as a constitutive and strictly Ca^{2+}/calmodulin-dependent enzyme[13]. Agonists which have been shown previously to promote NO release from endothelium, including histamine and acetylcholine, cause an influx of extracellular Ca^{2+} by promoting the binding of calmodulin to the endothelial constitutive isoform of eNOS, leading to activation. Indeed, we have confirmed that histamine caused a transient rise in Ca^{2+} in HUVEC. However, isoprenaline was without demonstrable effect on Ca^{2+} as assessed by Furo-2 fluorescence, suggesting that activation of eNOS by β-adrenoceptor stimulation probably occurs by a novel mechanism not involving Ca^{2+} influx and calmodulin activation. It is unlikely to be due to activation of the inducible (Ca^{2+}-independent) isoform of NOS, since cultured HUVEC have previously been found to express the endothelial isoform only, even following stimulation with proinflammatory cytokines[14]. Protein phosphorylation plays a central role in signal transduction pathways regulating a variety of biological processes, including the activities, biosynthesis and degradation of many enzymes. Possible mechanisms of the stimulative effect of isoprenaline on endothelial NOS activation include either increase in eNOS expression or a change in post-translational modification perhaps through phosphorylation. To investigate the possibility that isoprenaline exerts an effect on the enzyme itself, we isolated eNOS from HUVEC by immunoprecipitation, following 30-min exposure of HUVEC to isoprenaline or vehile. we quantified the eNOS thus isolated by Western blotting and scanning densitometry. Treatment of HUVEC with isoprenaline (1 μmol/L) for 30 min caused no measureable change in eNOS expression. However, eNOS activity and serine phosphorylation level were significantly increased ($P<0.01$). This effect could be abolished by a selective β_2-adrenoceptor antagonist ICI 118551. It seems likely that the stimulation in eNOS activity in response to isoprenaline was caused by increasing phosphylation of serine residues in the eNOS enzyme. Indeed, recent evidence suggests that eNOS can also be activated in a calcium-independent manner by fluid shear stress and VEGF, presumable through phosphorylation on serine1179 mediated by Akt (protein kinase B)[15,16], thus enhancing the sensitity of the enzyme to calcium-activated calmodulin[17]. Further studies are required to elucidate the definite kinase and

mechanisms of β-adrenoceptor-cyclic AMP-mediated activation of eNOS in these cells.

From our result of Western blotting, you can see another band at 90 kDa position. We supposed that it was heat shock protein 90 (Hsp90). Indeed, Hsp90 has recently been identified as an eNOS-associated protein, and its binding to the enzyme increases catalytic activity[18,19]. A certain amount of Hsp90 appears to complex with eNOS in unstimulated endothelial cells as immunoprecipitation of Hsp90 results in the recovery of eNOS, and vice versa. In all cases the association of Hsp90 with eNOS increased NO production and was prevented by pretreatment with geldanamycin (a specific inhibitor of Hsp90). Further work is needed to elucidate the possible interaction between these two proteins.

In conclusion, isoprenaline significantly increased eNOS activity in cultured HUVEC associated with an increase in serine phosphorylation. Furthermore, all the measured responses to isoprena-lined were mediated exclusively throuth β_2-AR subtype activation. These finds clarify the mechanism of β_2-adrenoceptor action on human blood vessel cells and may be of physiological and pathophysiological importance if they are representative of other vascular beds.

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