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Human vascular smooth muscle cells express a constitutive nitric oxide synthase that insulin rapidly activates, thus increasing guanosine 3′:5′-cyclic monophosphate and adenosine 3′:5′-cyclic monophosphate concentrations

M. Trovati[1], P. Massucco[1], L. Mattiello[1], C. Costamagna[2], E. Aldieri[2], F. Cavalot[1], G. Anfossi[1], A. Bosia[2], D. Ghigo[2]

[1] Diabetes Unit, Department of Clinical and Biological Sciences, University of Turin, San Luigi Gonzaga Hospital, Orbassano (Turin), Italy
[2] Department of Genetics, Biology, and Medical Chemistry, University of Turin, Turin, Italy

Abstract

Aims/hypothesis. Insulin incubation of human vascular smooth muscle cells (hVSMC) for 120 min increases both guanosine 3′:5′-cyclic monophosphate (cGMP) and adenosine 3′:5′-cyclic monophosphate (cAMP) and these effects are blocked by inhibiting nitric oxide synthase (NOS). These data suggest that insulin activates a constitutive Ca^{2+}-dependent NOS (*c*NOS), not described at yet in hVSMC. To test this hypothesis, we evaluated in hVSMC: i) the kinetics of the insulin-induced enhancement of the two cyclic nucleotides; ii) the ability of nitric oxide (NO) to increase both cyclic nucleotides; iii) NO involvement in the short-term influence of insulin on both cyclic nucleotides; iv) the ability of insulin to increase NO production in a few minutes; v) the presence of a *c*NOS activity; vi) the expression of mRNA for *c*NOS.
Methods. In hVSMC incubated with insulin, NO donors and the Ca^{2+} ionophore ionomycin, we measured cAMP and cGMP (RIA); in hVSMC incubated with insulin and ionomycin we measured NO, evaluated as L-(^{3}H)-citrulline production from L-(^{3}H)-arginine; by northern blot hybridization, we measured the expression of *c*NOS mRNA.
Results. i) By incubating hVSMC with 2 nmol/l insulin for 0–240 min, we observed an increase of both cGMP and cAMP (ANOVA: $p = 0.0001$). Cyclic GMP rose from 0.74 ± 0.01 to 2.62 ± 0.10 pmol/10^6 cells at 30 min ($p = 0.0001$); cAMP rose from 0.9 ± 0.09 to 11.65 ± 0.74 pmol/10^6 cells at 15 min ($p = 0.0001$). ii) Sodium nitroprusside (100 μmol/l) and glyceryltrinitrate (100 μmol/l) increased both cGMP and cAMP ($p = 0.0001$). iii) The effects of insulin on cyclic nucleotides were blocked by NOS inhibition. iv) An increase of NO was observed by incubating hVSMC for 5 min with 2 nmol/l insulin ($p = 0.0001$). v) Ionomycin (1 μmol/l) enhanced NO production ($p = 0.0001$) and increased both cyclic nucleotides ($p = 0.0001$). vi) hVSMC expressed mRNA of *c*NOS.
Conclusion/interpretation. Human VSMC express *c*NOS, which is rapidly activated by insulin with a consequent increase of both cGMP and cAMP, suggesting that insulin-induced vasodilation in vivo is not entirely endothelium-mediated. [Diabetologia (1999) 42: 831–839]

Keywords Insulin, nitric oxide, constitutive nitric oxide synthase, vascular smooth muscle, adenosine 3′:5′-cyclic monophosphate, guanosine 3′:5′-cyclic monophosphate.

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Corresponding author: M. Trovati, MD, Diabetes Unit, Department of Clinical and Biological Sciences, University of Turin, San Luigi Gonzaga Hospital, I-10043 Orbassano (Turin), Italy

Abbreviations: NO, Nitric oxide; NOS, nitric oxide synthase; *c*NOS, constitutive NOS; *ec*NOS, endothelial isoform of *c*NOS; cAMP, adenosine 3′:5′-cyclic monophosphate; cGMP, guanosine 3′:5′-cyclic monophosphate; L-NMMA, N^G-monomethyl-L-arginine; MEM, minimal essential medium; HUVEC, human endothelial cells from umbilical vein.

The vasodilating effect of insulin in humans in vivo is blunted by the nitric oxide synthase (NOS) inhibitor N^G-monomethyl-L-arginine (L-NMMA) [1, 2]. Furthermore, insulin stimulates nitric oxide (NO) production by endothelial cells in vitro [3]. Insulin, therefore, could increase the endothelium-dependent vasorelaxation, whereby the endothelial cell-derived NO relaxes vascular smooth muscle cells (VSMC) through activation of VSMC-soluble guanylate cyclase and the consequent production of guanosine 3′:5′-cyclic monophosphate (cGMP) [4]. Cyclic GMP is known to be involved in vasodilation by reducing intracellular Ca^{2+} in VSMC [5].

The relaxant effect exerted by insulin in VSMC is not necessarily, however, only mediated by endothelial cells. Actually, a direct effect of insulin on cultured VSMC has been observed in vitro, showing that these cells are targets of insulin action. Insulin, in particular, attenuates in VSMC the cytosolic Ca^{2+} fluxes elicited by some vasoconstricting substances, such as angiotensin II, arginine vasopressin, norepinephrine, serotonin and endothelin [6]. Since the ability of insulin to inhibit agonist-induced Ca^{2+} fluxes is exerted against different vasoconstrictors [6] and insulin does not affect their receptor-mediated binding [7], the inhibition can occur at a common point in the signalling cascade for all these agonists. A good target for the insulin action could be the agonist-elicited Ca^{2+} release from the sarcoplasmic reticulum, which is mediated by inositol-trisphosphate (IP_3) [7]. Indeed, some authors have shown that the insulin-induced attenuation of the agonist-mediated increase of Ca^{2+} fluxes is caused by a reduction of IP_3-releasable Ca^{2+} [7], mimicked by the cGMP analogue 8-bromo-cGMP and blocked by the NOS inhibitor L-NMMA [7]. These observations suggested the presence of an insulin-induced, NO-mediated increase of cGMP in VSMC, which could inhibit the IP_3-releasable Ca^{2+} fluxes [7].

We recently showed in both human VSMC (hVSMC) and in platelets that insulin increases intracellular concentrations of cGMP [8–13], by a mechanism which is blocked both by the guanylate cyclase inhibitor methylene blue and by the NOS inhibitor L-NMMA [8, 9, 12, 13]. We also showed that insulin can, in both hVSMC and platelets, increase the intracellular concentrations of adenosine 3':5'-cyclic monophosphate (cAMP), with a mechanism blocked by L-NMMA [9, 13], that could be attributed to cGMP [14–17] or directly to NO [18] as we will explain more extensively in the Discussion.

In any case, our previous studies showed that, in both platelets and hVSMC, insulin increases the concentrations of both cyclic nucleotides, which are deeply involved in vasodilation and anti-aggregation via attenuation of Ca^{2+} fluxes, and that this effect is completely blunted by the NOS inhibitor L-NMMA [8, 9, 12, 13].

We measured the effects of insulin on cGMP and cAMP in platelets at close intervals ranging from a few seconds to 120 min to show that insulin action on both nucleotides is very rapid, reaching the maximum effect after only 2 min [13]. Furthermore, we observed that in these cells insulin increases NO production in a few minutes [13]. This observation was not surprising, since it has been shown that platelets, as endothelial cells, contain a constitutive NOS (*c*NOS), a cytosolic enzyme requiring calmodulin, Ca^{2+} and NADPH [19–21], that can be activated in a very short time.

In VSMC, however, as far as we know, the presence of a Ca^{2+}-dependent NOS has not yet been described. On the other hand, it has been observed that these cells contain an inducible NOS, which is Ca^{2+}/calmodulin-independent and is stimulated after hours by endotoxin, interleukin-1 and tumour necrosis factor. This NOS could be important in the pathogenesis of septic shock by a huge production of NO causing profound hypotension and hyporesponsiveness to vasoconstrictors [22, 23]. Our previous studies on cGMP and cAMP production in hVSMC were done using different insulin concentrations in the range 240–1920 pmol/l, at a fixed incubation time of 120 min [8, 9]. Because insulin increases the concentrations of both nucleotides via NO after only 2 h, we assumed the presence of a constitutive, Ca^{2+}-dependent NOS also in these cells.

The present study has been carried out to clarify, in hVSMC, the following points:

i) the kinetics of the insulin-induced increase of cGMP and cAMP concentrations;
ii) the ability of NO to enhance both cGMP and cAMP, evaluated by studying the effects of NO donors;
iii) the NO involvement in the short-term influence exerted by insulin on cGMP and cAMP;
iv) the ability of insulin to increase NO production in a few minutes;
v) the presence of Ca^{2+}-dependent NOS activity for which we measured NO production and cyclic nucleotide concentrations after hVSMC incubation with the Ca^{2+} ionophore ionomycin, a powerful short-term stimulus for the NO synthesis mediated by Ca^{2+}-dependent NOS activity [24]; and,
vi) the expression of mRNA for the endothelial isoform of *c*NOS (*ec*NOS), using the northern blot hybridization technique.

In an earlier study we looked at the dose-dependence of the insulin effects on cyclic nucleotides and observed that a 120 min incubation with insulin 240 pmol/l or above enhanced both cGMP and cAMP [8, 9]. Therefore, in this study we used a fixed insulin dose of 2 nmol/l to measure the greatest cyclic nucleotide increase.

Materials and methods

Design

Time-dependence of the insulin effects on both cGMP and cAMP in hVSMC. We incubated hVSMC at 37 °C for 0, 2, 4, 8, 10, 15, 30, 60, 90, 120, 180 and 240 min with 2 nmol/l human recombinant insulin; then measured intracellular concentrations of cGMP and cAMP ($n = 6$).

The dose-dependence of the short-term insulin effects on cyclic nucleotides was evaluated by incubating hVSMC at 37 °C for 30 min with 0.25, 0.5, 1 and 2 nmol/l insulin.

Ability of NO to increase both cGMP and cAMP in hVSMC. We incubated hVSMC at 37 °C, for 0, 2, 5, 10, 30, 60, 90, 120, 180 and 240 min, with NO donors: 100 μmol/l sodium nitroprusside ($n = 6$) or 100 μmol/l glyceryltrinitrate ($n = 6$); then measured intracellular concentrations of cGMP and cAMP ($n = 6$).

NO involvement in the short-term influence exerted by insulin on cGMP and cAMP in hVSMC. We incubated hVSMC at 37 °C for 30 min with 2 nmol/l insulin, with and without a 20 min preincubation with 1 mmol/l L-NMMA: then measured intracellular concentrations of cGMP and cAMP ($n = 6$). To exclude non-specific effects of this L-NMMA concentration, we measured cyclic nucleotide values after a 30 min hVSMC incubation with 100 μmol/l sodium nitrofrusside, both with and without a 20 min preincubation with 1 mmol/l L-NMMA.

Ability of insulin to increase NO production in hVSMC. We incubated hVSMC for 5 min at 37 °C with 1, 2, 16 and 32 nmol/l insulin ($n = 4$): then checked cells for NO synthesis, evaluated as L-(^{3}H)-citrulline production from L-(^{3}H)-arginine ($n = 4$).

Presence of Ca^{2+}-dependent NOS activity in hVSMC. Since *c*NOS is Ca^{2+}-dependent, we tried to get evidence of its existence in hVSMC by evaluating the effects on NO production and cyclic nucleotide concentrations of ionomycin, a substance that can rapidly increase intracellular Ca^{2+} concentrations. To do this we:

a) incubated hVSMC for 5 min at 37 °C with 1 μmol/l ionomycin; then checked cells for NO synthesis, evaluated as L-(^{3}H)-citrulline production from L-(^{3}H)-arginine ($n = 4$);
b) incubated hVSMC at 37 °C for 15 min with 1 μmol/l ionomycin, then measured intracellular concentrations of cGMP and cAMP ($n = 6$). A control experiment was done using 20 min preincubation with 1 mmol/l L-NMMA.

Expression of mRNA for ecNOS in hVSMC. We used the northern blot hybridization technique.

Materials

Chemicals. Human recombinant insulin was obtained from Calbiochem (La Jolla, Calif., USA); L-NMMA and sodium nitroprusside were obtained from Sigma (St. Louis, Mo., USA); glyceryltrinitrate was obtained from Astra Farmaceutici S.P.A. (Milan, Italy); L-arginine was purchased from Sigma-Aldrich (Milan, Italy); ionomycin was obtained from Boehringer Mannheim (Mannheim, Germany); L-(2,3,4,5-^{3}H)-arginine monohydrochloride 2.3×10^{12} Bq/mmol (62 Ci/mmol) was from Amersham International (Amersham, Bucks, UK); cDNA for *ec*NOS was a gift from Dr. T. Michel (Brigham and Women's Hospital, Boston, Mass., USA). Insulin was dissolved in modified Tyrode's buffer containing bovine serum albumin (BSA) (144 mmol/l NaCl, 2.7 mmol/l KCl, 11.9 mmol/l $NaHCO_3$, 0.4 mmol/l NaH_2PO_4 and 0.04 mmol/l BSA, pH 7.4); ionomycin was dissolved in dimethyl sulphoxide (DMSO) in a final concentration that did not exceed 0.2 %. In each experiment, controls and samples received the same amount of solvent.

Methods

Cell cultures. Experiments were done with hVSMC derived from microarterioles obtained from patients undergoing abdominal surgery. Isolation and characterization were made according to classical procedures [25], i.e. cells were characterized by phenotype and checked for the presence of smooth muscle cell α-actin and the absence of factor VIII (staining with a fluorescein isothiocyanate-conjugated antibody specific for factor VIII antigen). Cells were cultured using minimal essential medium (MEM) supplemented with 10 % fetal calf serum, 10 mmol/l glutamine, and antibiotics, and buffered with 10 mmol/l *N*-Tris (hydroxymethyl) methyl-2-aminoethane-sulphonic acid (TES) and 10 mmol/l *N*-(2-hydroxyethyl) piperazine-N^1-(2-ethanesulphonic acid) (HEPES). For the experiments, cells were used at the 6th-8th passage and cultured in six-well plates until 70 % confluence was achieved; then the medium with serum was removed and cells were made quiescent by serum starvation and culture in MEM containing 0.1 % BSA, glutamine, buffers and antibiotics (MEM/BSA). After 48-h quiescence, the medium was removed and, following three washes with Hank's solution, 2 ml fresh MEM/BSA was added to the cell layer.

Cyclic nucleotide determination. Cyclic nucleotide levels were measured after incubation of hVSMC with insulin or ionomycin or NO donors, both with and without preincubation with L-NMMA. At the end of the different incubation periods, the medium was removed from each well and 300 μl of absolute ethanol was added to make the cells permeable. Complete evaporation of ethanol was obtained by shaking. Cyclic nucleotides were then dissolved in 300 μl acetate buffer and kept at –70 °C until the assays. To measure cAMP and cGMP RIA kits were used (Diagnostic Products, Los Angeles, Calif., USA and Immuno Biological Laboratories, Hamburg, Germany, respectively). For the cAMP assay, the discrimination is 0.1 pmol/ml, the cross-reactivity with the nucleotides is: 100 % for cAMP, < 0.1 % for cGMP, < 0.001 % for AMP, GDP, ATP, GTP, intra-assay coefficient of variation 4.8 %; for the cGMP assay, the discrimination is 0.05 pmol/ml, the cross-reactivity is 100 % for cGMP, 0.0004 % for cAMP, 0.0001 % for GMP, GDP, ATP and GTP, intra-assay coefficient of variation 4.4 %.

Measurement of citrulline synthesis as a reliable marker of nitric oxide production. Since NO formation is catalysed by NOS, which induces the conversion of L-arginine to L-citrulline and NO with a 1: 1 stoichiometry [26], we measured L-^{3}H-citrulline synthesis from L-^{3}H-arginine to evaluate NO production in VSMC. The method used here to measure NO in VSMC is a modification of a technique described previously [27] and has been used by our research group in human umbilical vein endothelial cells [24]. Briefly, cultured hVSMC, put in 35-mm dishes, were washed once with HEPES buffer (145 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l $MgSO_4$, 10 mmol/l HEPES sodium salt, 10 mmol/l glucose and 1 mmol/l $CaCl_2$, pH 7.4) and incubated in 1 ml of the same buffer at 37 °C for 20 min. We added L-(^{3}H)-arginine, 185×10^3 Bq (5 μCi) corresponding to a final concentration of 0.8 nmol/l to each dish and after 1 min stimulated cells with insulin or ionomycin. After a 5 min incubation at 37 °C, the reaction was stopped by washing dishes with 2 ml cold PBS containing 5 mmol/l L-arginine and

4 mmol/l EDTA. Ethanol (0.5 ml, 96%) was then added and, after evaporation, 2 ml of 20 mmol/l HEPES-Na (pH 6) were added. The supernatant was collected, applied to 2-ml columns of Dowex AG50WX-8 (Na^+ form) and eluted with 4 ml water. The radioactivity corresponding to L-^{3}H-citrulline content in 6 ml eluate was measured with liquid scintillation counting. Blank was done by incubating cells in 1 mmol/l cold L-arginine to remove the small percentage of L-^{3}H-citrulline contaminating the L-^{3}H-arginine preparation. We calculated that L-^{3}H-citrulline present in the L-^{3}H-arginine preparation did not exceed 3% of radioactivity.

Expression of mRNA for ecNOS. Total RNA was obtained by the guanidine isothiocyanate/cesium chloride method [28]. We electrophoresed 10–15 μg of total RNA on a 1% agarose gel containing 6.3% formaldehyde in 4-morpholinepropanesulphonic acid (MOPS) buffer and blotted on a Nylon Duralon-UV membrane (Stratagene, La Jolla, Calif., USA) by the traditional capillary system in 10 × SSC [29]. Prehybridization and hybridization steps were carried out overnight in 50% deionized formamide, 10% dextran sulphate, 1 mol/l NaCl, with 100 μg/ml denaturated salmon sperm DNA at 42 °C. The cDNA for *ec*NOS [30] was labelled with a (^{32}P) 2′-Deoxycytidine 5′-triphosphate, 111×10^{12} Bq/mmol (3000 Ci/mmol), (Amersham International) at 2.2×10^8 cpm/μg specific activity, by the random primer labelling kit (Amersham International) according to the manufacturer's instructions. Post-hybridization washes were done at high stringency (once in 2 × SSC plus 0.1% SDS for 30 min at room temperature, once in 0.2 × SSC plus 0.1% SDS for 30 min at room temperature, and twice in 0.1% SDS for 30 min at 55 °C) and the membrane was exposed on autoradiography with Hyperfilm-MP (Amersham International) and intensifying screens at –80 °C.

This procedure was applied both to hVSMC and to human endothelial cells from umbilical vein (HUVEC) used as a positive control.

Statistical analysis. Data in the text and in the figures are expressed as means ± SEM. Statistical analysis was carried out using ANOVA, to determine the significance of the time-dependent or dose-dependent effects, and using the unpaired Student's *t* test when only two values were compared.

Results

Time-dependence of the insulin effects on both cGMP and cAMP in hVSMC. Insulin induced an increase of cGMP and cAMP in hVSMC (ANOVA, $p = 0.0001$) (Fig. 1). Concentrations of cGMP were higher than basal values from 2 to 180 min ($p = 0.0001$) but not at 240 min. All cAMP concentrations were higher than basal values ($p = 0.0001$).

At 30 min, when cGMP concentrations reached their peak, the dose-dependent experiment showed a progressive increase of cGMP (ANOVA, $p = 0.0001$). All the insulin doses used induced increases of cGMP ($p = 0.0001$). Cyclic GMP values with 0, 0.25, 0.5, 1 and 2 nmol/l were 0.67 ± 0.01, 0.89 ± 0.02, 1.22 ± 0.01, 1.47 ± 0.01 and 2.49 ± 0.2 pmol/10^6 hVSMC, respectively. A similar dose-response curve was observed for cAMP (ANOVA, $p = 0.0001$).

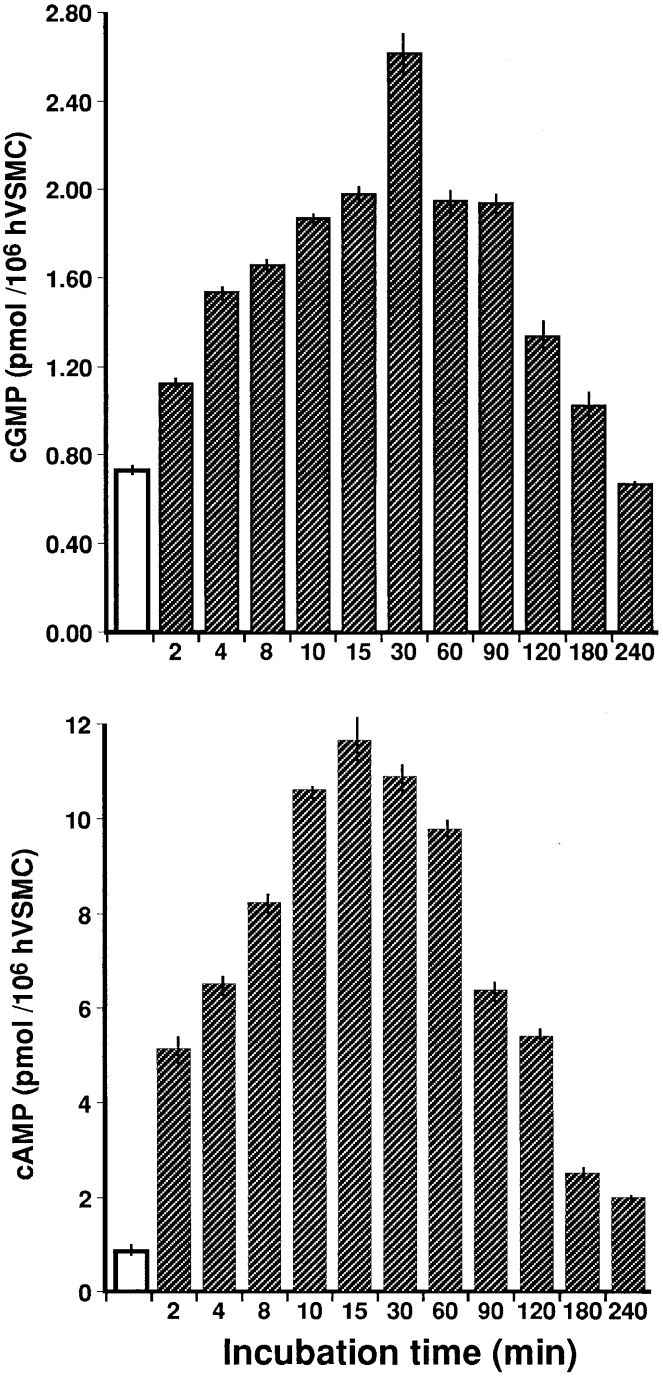

Fig. 1. Time-dependence of the effects exerted by 2 nmol/l insulin incubation at 37 °C on cGMP and cAMP concentrations in hVSMC. Insulin increased both cyclic nucleotides ($n = 6$, ANOVA for repeated measures $p = 0.0001$). Values for cGMP from 2 to 180 min were significantly higher than basal values ($p = 0.0001$). Values for cAMP from 2 to 240 min were significantly higher than basal values ($p = 0.0001$). □ Control; ▨ insulin 2 nmol/l

Ability of NO to increase both cGMP and cAMP in hVSMC. The NO donor sodium nitroprusside induced an increase of both cGMP and cAMP throughout the study (ANOVA, $p = 0.0001$) (Fig. 2). All concentrations measured were at least threefold higher

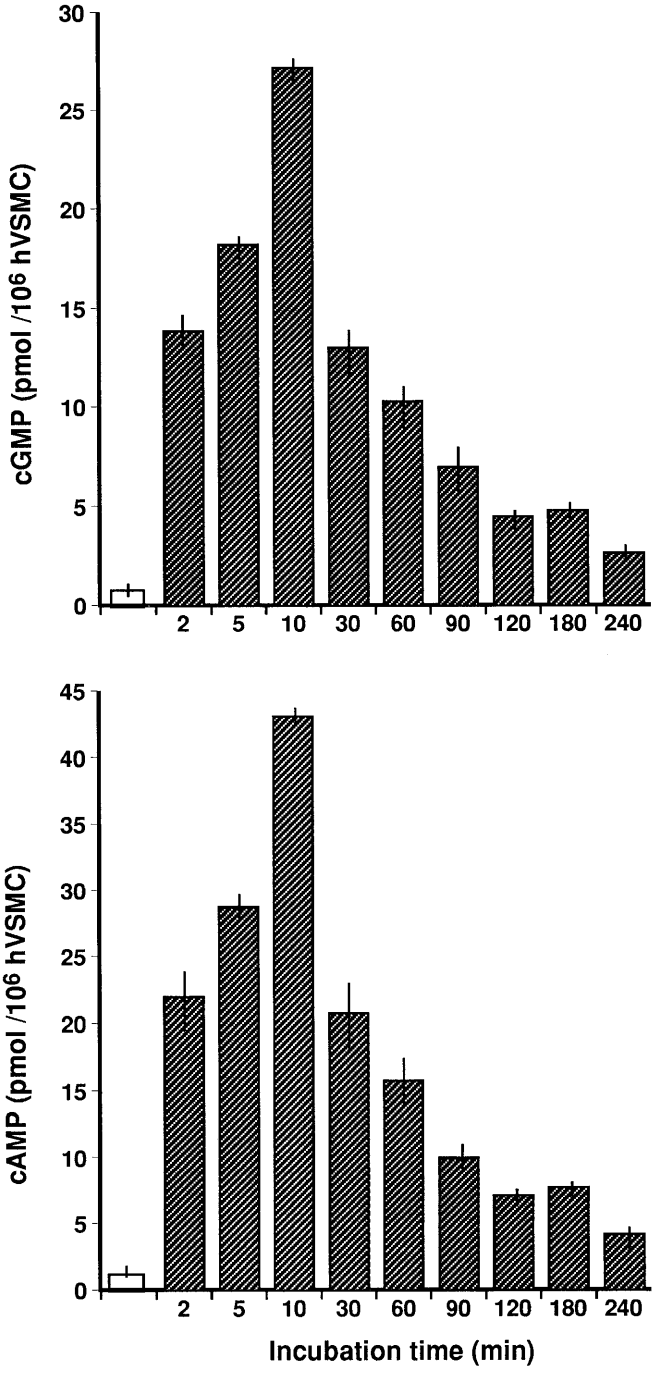

Fig. 2. Time-dependence of the effects exerted by 100 μmol/l sodium nitroprusside incubation at 37 °C on cGMP and cAMP concentrations in hVSMC. Sodium nitroprusside increased both cyclic nucleotides ($n = 6$, ANOVA for repeated measures, $p = 0.0001$). All cGMP and cAMP values measured were significantly higher than basal values ($p = 0.0001$). □ Control; ▨ sodium nitroprusside 100 μmol/l

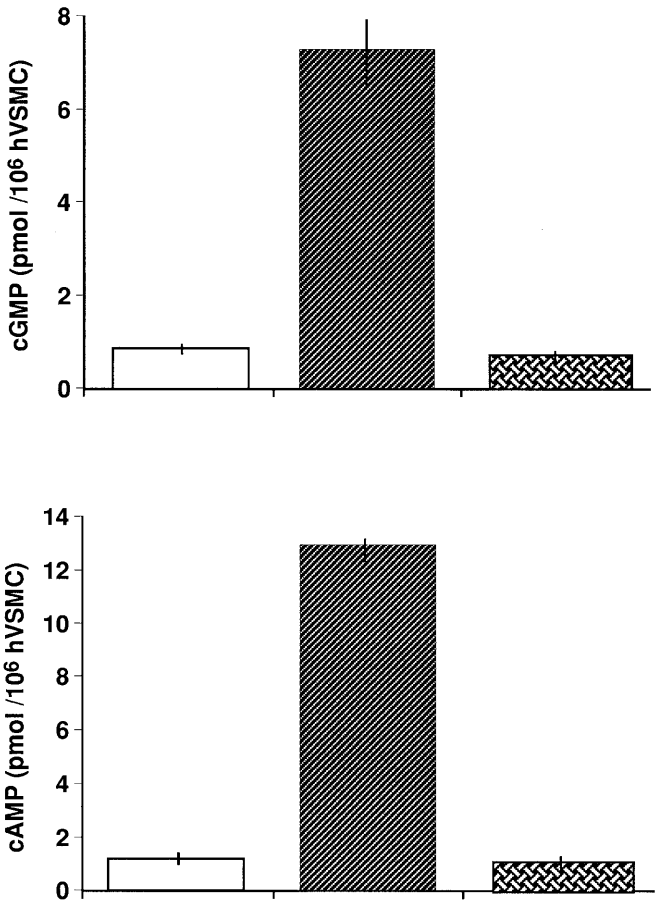

Fig. 3. Influence of a 20-min preincubation with 1 mmol/l L-NMMA on the effects exerted by a 30-min incubation with 2 nmol/l insulin on cGMP and cAMP in hVSMC. The significant insulin effects on both cyclic nucleotides ($p = 0.0001$) were completely blunted by L-NMMA. □ Control; ▨ insulin 2 nmol/l; ▩ insulin 2 nmol/l + L-NMMA 1 mmol/l

than baseline, both for cGMP and for cAMP ($p = 0.0001$). The highest value was reached at 10 min, followed by a decrease.

Also glyceryltrinitrate increased both cyclic nucleotides. In particular, cGMP concentrations increased from 0.84 ± 0.06 to 10.01 ± 0.33 pmol/10^6 hVSMC at 10 min and then decreased to 4.64 ± 0.08 pmol/10^6 hVSMC at 240 min, whereas cAMP concentrations increased from 1.33 ± 0.09 to 15.85 ± 0.53 pmol/10^6 hVSMC at 10 min and then decreased to 7.35 ± 0.12 pmol/10^6 hVSMC at 240 min. During incubation with glyceryltrinitrate, all cGMP and cAMP concentrations were higher than basal values ($p = 0.0001$).

To compare the effects exerted by NO donors and those exerted by insulin, we also measured the influence on cyclic nucleotides of 30 min incubation with 2 nmol/l insulin and found that cGMP increased from 0.84 ± 0.06 to 7.28 ± 0.70 pmol/10^6 hVSMC ($p = 0.0001$) and cAMP increased from 1.33 ± 0.09 to 12.93 ± 0.30 pmol/10^6 hVSMC ($p = 0.0001$).

NO involvement in the short-term influence exerted by insulin on cGMP and cAMP in hVSMC. A complete inhibition of the insulin-induced cGMP and cAMP increases was observed with 1 mmol/l L-NMMA

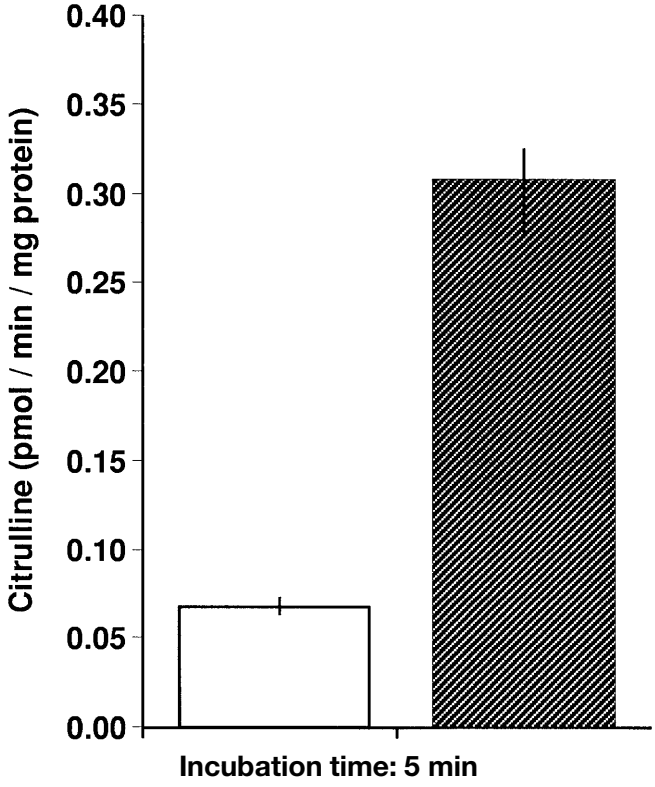

Fig. 4. Influence of a 5-min hVSMC incubation with 2 nmol/l insulin on NO generation, evaluated by the measurement of L-(^{3}H)-citrulline after incubation with L-(^{3}H)-arginine ($n = 4$). The insulin effects were significant ($p = 0.0001$). □ Control; ▨ insulin 2 nmol/l

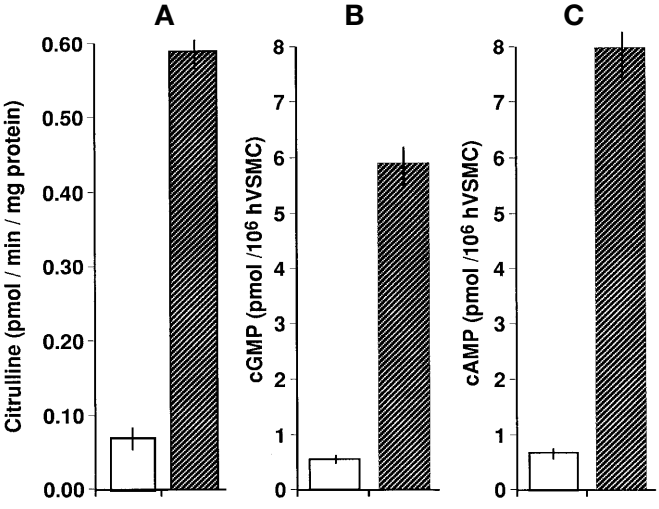

Fig. 5 A–C. (**A**) Influence of a 5-min incubation of hVSMC with 1 μmol/l ionomycin on NO generation, evaluated by the measurement of L-(^{3}H)-citrulline after incubation with L-(^{3}H)-arginine ($n = 4$). The ionomycin effect was significant ($p = 0.0001$). (**B**) Influence of a 15-min incubation of hVSMC with 1 μmol/l ionomycin on cGMP concentrations. The ionomycin effect was significant ($p = 0.0001$). (**C**) Influence of a 15-min incubation of hVSMC with 1 μmol/l ionomycin on cAMP concentrations. The ionomycin effect was significant ($p = 0.0001$). □ Control; ▨ ionomycin 1 μmol/l

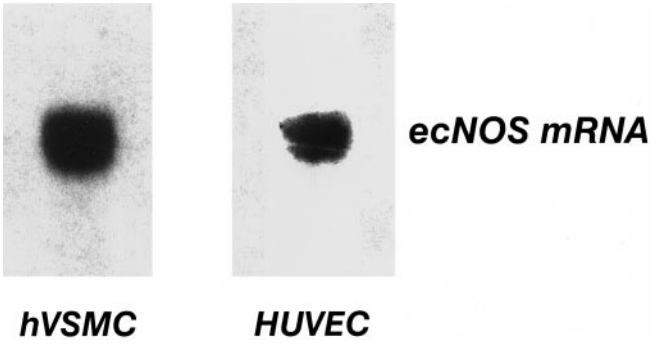

Fig. 6. Expression of the *ec*NOS mRNA in hVSMC. This northern blot experiment is representative of two similar experiments. As a control for *ec*NOS transcript HUVEC (human umbilical vein endothelial cells) mRNA was checked

(Fig. 3). With the same L-NMMA concentration, sodium nitroprusside-induced increases of cGMP ($p = 0.0001$) and cAMP ($p = 0.0001$) remained unmodified: in particular cGMP and cAMP values with sodium nitroprusside + L-NMMA were 103 % and 104 % of values with sodium nitroprusside alone, respectively (NS). The quantitative difference of the cGMP and cAMP responses to insulin shown in Fig. 1 and in Fig. 3 are due to the different cell passages used for the experiments.

Ability of insulin to increase NO production in hVSMC. Incubation with 2 nmol/l insulin increased L-(^{3}H)-citrulline production from L-(^{3}H)-arginine (Fig. 4), a reliable marker of NO synthesis ($p = 0.0001$). Insulin at 1, 2, 16 and 32 nmol/l induced an increase of citrulline from 0.07 ± 0.003 to 0.16 ± 0.06, 0.30 ± 0.03, 0.34 ± 0.07 and 0.38 ± 0.08 pmol · min^{-1} · mg protein^{-1}, respectively (ANOVA, $p = 0.007$).

Presence of Ca^{2+}-dependent NOS activity in hVSMC. Ionomycin rapidly increased L-(^{3}H)-citrulline production from L-(^{3}H)-arginine, a reliable marker of NO synthesis ($p = 0.0001$) and enhanced both cGMP and cAMP ($p = 0.0001$) (Fig. 5). The increase of both cyclic nucleotides was completely blunted by L-NMMA. These results suggest the presence of a Ca^{2+}-dependent NOS activity in hVSMC, responsible for the increase of both cGMP and cAMP.

Expression of mRNA for ecNOS in hVSMC. Figure 6 shows that hVSMC express a specific *ec*NOS transcript.

Discussion

This study shows that hVSMC express the endothelial isoform of the constitutive NOS. This result can account for many of the different actions exerted by insulin in VSMC in vitro [6, 31].

The demonstration of *ec*NOS expression in hVSMC is the final step of a research work carried out by our team in the last ten years, involving also platelets, since they share with VSMC many biological features [6]. The last steps of this work are presented in the first part of the present study.

We show in this study the time-dependence of the insulin effects on cyclic nucleotides in hVSMC: i) as in platelets, insulin elicits an increase after only 2 min of incubation [13]; ii) as in platelets, the insulin effects on cGMP and on cAMP are simultaneous [13]; iii) the cyclic nucleotide maximum increase (15–30 min) is delayed in comparison with that observed in platelets, where it is reached after only 2 min [13].

The differences in the cyclic nucleotide kinetics in the two cell types could reflect differences in the kinetics of post-receptor insulin signalling involving NOS activation or NO-induced guanylate cyclase activation, or both. Further studies are needed to clarify these points.

We have shown previously that the insulin effects on both cGMP and cAMP observed at 120 min are blunted both in hVSMC and in platelets by incubation with the inhibitor of guanylate cyclase methylene blue and by the inhibitor of NOS L-NMMA [8, 9, 12, 13]; this suggests insulin influence on both cGMP and cAMP was mediated by an insulin action on NO. Our present study shows that the insulin effect on both cyclic nucleotides is also very rapid in hVSMC and that this rapid increase is NO-dependent.

The involvement of NO in the insulin-induced increase not only of cGMP but also of cAMP agrees with the ability of NO donors sodium nitroprusside and glyceryltrinitrate to enhance cAMP. This allows us to better understand the molecular basis of the vasodilating effects exerted by these agents that is usually attributed only to cGMP. The ability of NO donors to increase cAMP, however, has already been observed in human platelets and has been attributed to a cGMP-stimulated inhibition of the so-called cGMP-inhibited cAMP phosphodiesterase [14–17]. In other words, it is possible that NO, derived from NO donors or endogenously synthesized, enhances cGMP production and, via cGMP, decreases cAMP catabolism, therefore increasing its cellular concentrations. Alternatively, when guanylate cyclase is stimulated by NO, it shows striking modifications in its activity, being able also to synthesize cAMP [18]. It is possible, therefore, that cAMP is produced by guanylate cyclase both in the presence of the exogenous NO provided by nitro-vasodilators and in the presence of the endogenous NO stimulated by insulin. The simultaneity of cGMP and cAMP increases in hVSMC during insulin incubation fits particularly well with this hypothesis.

The NO-mediated effect exerted by insulin on both cyclic nucleotides in hVSMC shows a consistent trend to reduce with time. It is known that pretreatment of tissues with NO donors results in a molecular desensitization of soluble guanylate cyclase [32, 33] and that activation of guanylate cyclase by NO is reversible over time [34, 35].

Interestingly, the experiments in this study in which hVSMC were incubated with fixed doses of sodium nitroprusside and glyceryltrinitrate show a reduction over time in the increase of both cyclic nucleotides.

Also note that insulin increases NO production in only 5 min. Results concerning the insulin effects on cyclic nucleotides and NO in hVSMC, therefore, clearly suggest the presence of a NOS able to be rapidly activated, as *c*NOS is.

Since *c*NOS is also Ca^{2+}-dependent, the ability of a Ca^{2+} ionophore to enhance NO production in hVSMC and to increase cyclic nucleotide concentrations with a NO-dependent mechanism is another important step in supporting the hypothesis of a *c*NOS expression in hVSMC. The evidence that a Ca^{2+} ionophore increases, via NO, not only cGMP but also cAMP is not surprising if we consider that cGMP increases induced through NO are accompanied by increases of cAMP, as shown in this study.

Thus, from the biological point of view, the results of this study strongly support the suggestion that hVSMC exhibit a Ca^{2+}-dependent NOS activity that insulin is able to activate in a few minutes. A similar suggestion, based on other evidence, has been done by other authors [31]. The transcript for *ec*NOS in hVSMC that we present in this study accounts for the results concerning cyclic nucleotides obtained in our laboratory [8, 9] and for other direct effects exerted by insulin in hVSMC [6, 31].

In summary, this study indicates that hVSMC express not only an inducible, Ca^{2+}-independent NOS activity, able to produce huge amounts of NO when stimulated by endotoxins and cytokines and therefore to induce the dramatic clinical picture of septic shock [22, 23] but also a constitutive, Ca^{2+}-dependent NOS activity, able to be stimulated by insulin in a few minutes. The mechanisms by which insulin activates *c*NOS in hVSMC need further investigation: a possible hypothesis is a covalent modification of the enzyme that would result in its activation [36], as it has been already shown for the insulin effects on the cGMP-inhibited cAMP phosphodiesterase [37].

Obviously, evidence that insulin can directly activate *c*NOS in hVSMC does not exclude the possibility that, in vivo, insulin-induced vasodilation is at least in part – and potentially in the greatest part – an endothelium-mediated phenomenon, since insulin is also able to increase NOS activity in endothelial cells [3]. In vivo, the direct insulin-mediated effects on hVSMC could be modest. Evidence of the ability of insulin to activate VSMC *c*NOS provides, however, an explanation for some of its direct effects on cyclic

nucleotides and Ca^{2+} fluxes previously observed in these cells [6, 31].

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References

1. Steinberg HO, Brechtel G, Johnson A, Fineberg N, Baron A (1994) Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release. J Clin Invest 94: 1172–1179
2. Scherrer U, Randin D, Vollenweider P, Vollenweider L, Nicod P (1994) Nitric oxide release accounts for insulin's vascular effects in humans. J Clin Invest 94: 2511–2515
3. Zeng G, Quon MJ (1996) Insulin-stimulated production of nitric oxide is inhibited by wortmannin. Direct measurement in vascular endothelial cells. J Clin Invest 98: 894–898
4. Palmer RMJ, Ferridge AG, Moncada S (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 327: 524–526
5. McDaniel NL, Chen X-L, Singer HA, Murphy RA, Rembold CM (1992) Nitrovasodilators relax arterial smooth muscle by decreasing $(Ca^{2+})_i$ and uncoupling stress from myosin phosphorylation. Am J Physiol 263: C461–C467
6. Trovati M, Anfossi G (1998) Insulin, insulin resistance and platelet function: similarities with insulin effects on cultured vascular smooth muscle cells. Diabetologia 41: 609–622
7. Saito F, Hori MT, Fittingoff M, Hino T, Tuck ML (1993) Insulin attenuates agonist-mediated calcium mobilization in cultured rat vascular smooth muscle cells. J Clin Invest 92: 1161–1167
8. Trovati M, Massucco P, Mattiello L et al. (1995) Insulin increases cyclic nucleotide content in human vascular smooth muscle cells: a mechanism potentially involved in insulin-induced modulation of vascular tone. Diabetologia 38: 936–941
9. Trovati M, Massucco P, Mattiello L et al. (1996) Studies on the insulin influence on cyclic adenosine monophosphate in human vascular smooth muscle cells: dependence on cyclic guanosine monophosphate and modulation of catecholamine effects. Diabetologia 39: 1156–1164
10. Trovati M, Massucco P, Mattiello L, Mularoni E, Cavalot F, Anfossi G (1994) Insulin increases guanosine-3′,5′-cyclic monophosphate in human platelets: a mechanism involved in the insulin anti-aggregating effect. Diabetes 43: 1015–1019
11. Trovati M, Mularoni E, Burzacca S et al. (1995) Impaired insulin-induced platelet anti-aggregating effect in obesity and in obese non-insulin-dependent diabetes mellitus. Diabetes 44: 1318–1322
12. Trovati M, Massucco P, Mattiello E et al. (1996) The insulin-induced increase of guanosine-3′,5′-cyclic monophosphate in human platelets is mediated by nitric oxide. Diabetes 45: 768–770
13. Trovati M, Anfossi G, Massucco P et al. (1997) Insulin stimulates nitric oxide synthesis in human platelets, and, through nitric oxide, increases platelet concentrations of both guanosine-3′,5′-cyclic monophosphate and adenosine-3′,5′-cyclic monophosphate. Diabetes 46: 742–749
14. Gryglewski RJ, Korbut R, Kalecinska A, Zembowicz A (1989) Interaction between stimulators of adenylate and guanylate cyclases in human leukocytes, platelets and arteries. Int J Tissue React 11: 269–274
15. Maurice DH, Haslam RJ (1990) Molecular basis of the synergistic inhibition of platelet function by nitrovasodilators and activators of adenylate cyclase: inhibition of cyclic AMP breakdown by cyclic GMP. Mol Pharmacol 37: 671–681
16. Anfossi G, Massucco P, Mularoni E, Cavalot F, Mattiello L, Trovati M (1993) Organic nitrates and compounds that increase intraplatelet cyclic guanosine monophosphate (cGMP) levels enhance the antiaggregating effects of the stable prostacyclin analogue iloprost. Prostaglandins Leuko Essent Fatty Acids 49: 839–845
17. Anfossi G, Massucco P, Mularoni E, Cavalot F, Mattiello L, Trovati M (1994) Effects of forskolin and organic nitrate on aggregation and intracellular cyclic nucleotide content in human platelets. Gen Pharmacol 25: 1093–1100
18. Mittal KC, Murad F (1977) Formation of adenosine 3′,5′-monophosphate by preparations of guanylate cyclase from rat liver and other tissues. J Biol Chem 252: 3136–3140
19. Bredt DS, Snyder SH (1990) Isolation of nitric oxide synthase, a calmodulin-requiring enzyme. Proc Natl Acad Sci USA 87: 682–685
20. Radomski MW, Palmer RMJ, Moncada S (1990) An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. Proc Natl Acad Sci USA 87: 5193–5197
21. Muragandam A, Mutus B (1994) Isolation of nitric oxide synthase from human platelets. Biochim Biophys Acta 1200: 1–6
22. Rees DD, Cellek S, Palmer RMJ, Moncada S (1990) Dexamethasone prevents the induction by endotoxin of a nitric oxide synthase and the associated effects on vascular tone: an insight into endotoxin shock. Biochem Biophys Res Commun 173: 541–547
23. Schini VB, Junquero DC, Scott-Burden T, Vanhoutte PM (1991) Interleukin-1 beta induces the production of an L-arginine-derived relaxing factor from cultured smooth muscle cells from rat aorta. Biochem Biophys Res Commun 176: 114–121
24. Ghigo D, Alessio P, Foco A et al. (1993) Nitric oxide synthesis is impaired in glutathione-depleted human umbilical vein endothelial cells. Am J Physiol 265: C728–C732
25. Scott-Burden T, Resink TJ, Hahn AWA, Baur U, Box RJ, Buhler FR (1989) Induction of growth-related metabolism in human vascular muscle cells by low density lipoprotein. J Biol Chem 264: 12582–12589
26. Palmer RMJ, Ferrige AG, Moncada S (1989) A novel citrulline-forming enzyme implicated in the formation of nitric oxide by vascular endothelial cells. Biochem Biophys Res Commun 158: 348–352
27. Bredt DS, Snyder SH (1989) Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. Proc Natl Acad Sci USA 86: 9030–9033
28. Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18: 5294–5299
29. Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor, New York
30. Lamas S, Marsden PA, Li GK, Tempst P, Michel T (1992) Endothelial nitric oxide synthase: molecular cloning and characterization of a novel constitutive enzyme isoform. Proc Natl Acad Sci USA 89: 6348–6532

31. Sowers JR (1997) Insulin and insulin-like growth factor in normal and pathological cardiovascular physiology. Hypertension 29: 691–699
32. Axelsson KL, Andersson RGG (1983) Tolerance towards nitroglycerin, induced in vivo, is correlated to a cGMP response and an alteration in cGMP turnover. Eur J Pharmacol 88: 71–79
33. Waldman SA, Rapoport RM, Murad F (1987) Desensitization to nitroglycerin in vascular smooth muscle from rat and human. Biochem Pharmacol 35: 3525–3531
34. Lewicki JA, Brandwein HJ, Mittal CK, Arnold WP, Murad F (1982) Properties of purified soluble guanylate cyclase activated by nitric oxide and sodium nitroprusside. J Cyclic Nucleotide Res 8: 17–25
35. Arnold WP, Mittal CK, Katsuki S, Murad F (1977) Nitric oxide activates guanylate cyclase and increases guanosine 3′,5′-cyclic monophosphate levels in various tissue preparations. Proc Natl Acad Sci USA 74: 3203–3207
36. Michel T, Li GK, Busconi L (1993) Phosphorylation and subcellular translocation of endothelial nitric oxide synthase. Proc Natl Acad Sci USA 90: 6252–6256
37. Lopez-Aparicio P, Belfrage P, Manganiello VC, Kono T, Dagerman E (1993) Stimulation by insulin of a serine kinase in human platelets that phosphorylates and activates the cGMP-inhibited cAMP phosphodiesterase. Biochem Biophys Res Commun 193: 1137–1144