EXPERIMENTAL STUDY

Insulin influences the nitric oxide cyclic nucleotide pathway in cultured human smooth muscle cells from corpus cavernosum by rapidly activating a constitutive nitric oxide synthase

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Abstract

Aims: We have evaluated, in cultured human cavernosal smooth muscle cells, the expression and activity of calcium-dependent constitutive nitric oxide synthase (cNOS) and the ability of insulin to induce nitric oxide (NO) production and to increase intracellular cyclic nucleotides guanosine 3',5'-cyclic monophosphate (cGMP) and adenosine 3',5'-cyclic monophosphate (cAMP).

Methods: cNOS mRNA was detected by RT-PCR amplification, cNOS protein by immunofluorescence, cNOS activity as L-[³H]-citrulline production from L-[³H]-arginine and cyclic nucleotides by radioimmunoassay.

Results: cNOS mRNA and cNOS protein were found in cultured cells; cNOS activity was increased by 5-min exposure to 1 µmol/l calcium ionophore ionomycin (from 0.1094±0.0229 to 0.2685±0.0560 pmol/min per mg cell protein, *P* = 0.011) and to 2 nmol/l insulin (from 0.1214±0.0149 to 0.2045±0.0290 pmol/min per mg cell protein, *P* = 0.041). Insulin increased both cGMP and cAMP in a dose- and time-dependent manner (i.e. with 2 nmol/l insulin, cGMP rose from 2.71±0.10 to 6.80±0.40 pmol/10⁶ cells at 30 min, *P* = 0.0001; cAMP from 1.26±0.06 to 3.02±0.30 pmol/10⁶ cells at 60 min, *P* = 0.0001). NOS inhibitor N^G-monomethyl-L-arginine and phosphatidylinositol 3-kinase (PI 3-kinase) inhibitors wortmannin and LY 294002 blunted these effects of insulin. The action of insulin on cyclic nucleotides persisted in the presence of phosphodiesterase inhibition, guanylate cyclase activation by NO donors and adenylate cyclase activation by Iloprost or forskolin.

Conclusion: Human cavernosal smooth muscle cells, by expressing cNOS activity, are a source of NO and not only its target; in these cells, insulin rapidly activates cNOS through a PI 3-kinase pathway, with a consequent increase of both cyclic nucleotides, thus directly influencing the mechanisms involved in penile vascular tone and interplaying with classical haemodynamic mediators.

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Introduction

Diabetes mellitus is a relevant cause of erectile dysfunction (1–3). The complex mechanism of erection implies: (i) increase of arterial inflow via cavernosal arteries, (ii) relaxation of corporal smooth muscle cells which opens corporal sinusoids and (iii) decrease of venous outflow with a consequent increase of intra-corporal pressure (4, 5). Three separate components are involved in penile erection, namely: (i) central nervous system regulation, (ii) peripheral neurotransmission and (iii) haemodynamic response of the corporal bodies of the penis (5–8). The last process is both endothelium mediated and under neurologic

control, which involves cholinergic and nonadrenergic–noncholinergic neuroeffector systems (5, 7). As far as the neurological control is concerned, an essential role is played by the nonadrenergic–noncholinergic system and in particular by nitric oxide (NO), identified as a specific neurotransmitter (7–13). Thus, both nerve fibres expressing NO synthase (NOS) and endothelial cells play a relevant role in the haemodynamic events of penile erection (9, 11, 14–19). Effectors of the haemodynamic changes in septi and the arterial wall of corpus cavernosum are smooth muscle cells (20, 21): they respond to vasorelaxant stimuli (such as NO, prostaglandins and vasoactive intestinal peptide), mainly through an increase of intracellular

levels of cyclic nucleotides: guanosine 3',5'-cyclic monophosphate (cGMP) and adenosine 3',5'-cyclic monophosphate (cAMP) (9, 22). Cyclic nucleotides are responsible for smooth muscle relaxation mainly through a decreased cytosolic calcium (Ca^{2+}) availability (22–30). As far as the NO/cGMP pathway is concerned, it can be briefly summarized as follows: (i) smooth muscle cells from corpus cavernosum are targets of the NO released both by endothelial cells and nerve endings, (ii) in smooth muscle cells, NO activates soluble guanylate cyclase with a consequent synthesis of cGMP and (iii) cGMP reduces cytosolic Ca^{2+} content by inhibiting the inositol 1,4,5-trisphosphate-mediated Ca^{2+} release from intracellular stores (25, 27–32). Thus, biochemical pathways well known in the modulation of systemic arterial tone are operating in the cultured smooth muscle cells of corpus cavernosum (26, 28, 29).

In recent years, we have demonstrated the following. (i) Human arteriolar vascular smooth muscle cells express the constitutive, Ca^{2+} /calmodulin-dependent endothelial type constitutive NOS (cNOS), with a consequent basal production of NO (33). (ii) In these cells, insulin is able to stimulate cNOS, with a consequent increase of NO production and guanylate cyclase activation (33). (iii) Via this mechanism, insulin increases not only cGMP but also cAMP (33); this last phenomenon is not surprising, since NO donors, such as sodium nitroprusside (SNP) and glyceryl trinitrate (GTN), are able to increase both cGMP and cAMP in vascular smooth muscle cells (33) and in platelets (34, 35). It could be hypothesized that NO increases cGMP and that cGMP, by inhibiting a cAMP phosphodiesterase, increases cAMP concentrations (36); alternatively, it is possible that cAMP is produced by guanylate cyclase directly since this enzyme activity shows striking modifications when stimulated by NO, becoming able also to synthesize cAMP (37). (iv) Insulin increases – via NO – the effects on cAMP exerted by substances activating adenylate cyclase via receptor-dependent or receptor-independent mechanisms (33, 38, 39). Thus, we have demonstrated that vascular smooth muscle cells are not only targets of the NO released by endothelial cells, but produce NO by themselves through a Ca^{2+} -dependent NOS activity that insulin rapidly activates. The ability of insulin to activate cNOS has already been demonstrated by experiments *in vivo* (40, 41) and *in vitro* in other cell types: i.e. endothelial cells, where this effect of insulin is ascribed to the phosphatidylinositol 3-kinase (PI 3-kinase) pathway of insulin signalling (42), and in platelets (43).

Since smooth muscle cells from corpus cavernosum are a specialized type of vascular smooth muscle cells (20), we designed the present study in order to verify whether these cells are simple effectors of NO or play an active role in its production, as vascular smooth muscle cells. The presence of cNOS in these cells is a

matter of recent debate (44, 45) and has never been shown by molecular biology techniques. Furthermore, the ability of these cells to produce NO in a constitutive way has never been studied and, therefore, the biological meaning of a putative cNOS activity in these cells is completely unknown.

In this study, we describe a technique to obtain primary cultures of smooth muscle cells from human corpus cavernosum (CSMC) and to characterize them from a biochemical point of view. The possibility of investigating the behaviour of this cell type in controlled conditions, such as in *in vitro* cultures, represents a relevant tool with which to investigate the mechanism of the physiological erectile response and the putative role of different mediators.

In particular, the present study aims to clarify the following in human CSMC: (i) the cell proliferation rate and dependence on growth factors and insulin; (ii) the expression of cNOS; (iii) the ability of ionomycin, a calcium ionophore, to induce production of NO, as biological proof of the presence of an active Ca^{2+} /calmodulin-dependent cNOS enzyme; (iv) the ability of insulin to rapidly activate cNOS and, consequently, to increase the production of NO; (v) the ability of insulin to increase, via NO, both cGMP and cAMP; (vi) the dependence of the putative effect exerted by insulin on the NO-cyclic nucleotide cascade on the PI 3-kinase pathway of insulin signalling; (vii) the ability of insulin to interplay with NO donors (GTN and SNP) as far as cyclic nucleotide levels are concerned; (viii) the ability of insulin to interplay with forskolin and the prostacyclin analogue Iloprost as far as cell cAMP levels are concerned.

Materials and methods

Study design

The study was carried out on CSMC of a 42-year-old otherwise healthy subject (without obesity, diabetes mellitus, arterial hypertension, dyslipidaemia or other known diseases), who underwent corporoplasty according to Nesbit for congenital penile curvature abnormality. Since corpus cavernosum biopsy is not part of the intervention, which relies on the reconstruction of tunica albuginea, the patient gave his informed consent according to the Helsinki Declaration of 1975, as revised in 1983, after being reassured that erectile tissue excision would be so small as to avoid any risk of impotence. Cells isolated from small pieces of about 1 mm² gave six different proliferating foci, which were cultured and characterized according to the techniques described below. In these cells, we aimed to evaluate: (i) the proliferating features, according to standardized procedures; (ii) the presence of endothelial type cNOS mRNA by RT-PCR; (iii) the presence of cNOS protein by immunofluorescence; (iv) the functional activity of cNOS, which is a Ca^{2+} -dependent enzyme, by measuring

NO synthesis as L-[³H]-citrulline synthesis from L-[³H]-arginine in cells exposed for 5 min at 37°C to 1 µmol/l ionomycin, a substance that rapidly activates cNOS via an increase of cytosolic Ca²⁺ (46); (v) the ability of insulin to increase NO synthesis, by measuring NO production as L-[³H]-citrulline synthesis from L-[³H]-arginine in cells incubated for 5 min at 37°C with 2 nmol/l insulin; (vi) the ability of insulin to increase both cGMP and cAMP via NO through the PI 3-kinase pathway of insulin signalling by measuring these cyclic nucleotides in cells incubated for 30 min at 37°C with human recombinant insulin at a concentration of 2 nmol/l both with and without a 20-min preincubation with the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) at final concentration of 1 mmol/l and with the PI 3-kinase inhibitors wortmannin at final concentrations of 1 and 2 µmol/l and LY 294002 at a final concentration of 100 µmol/l; (vii) the dose-dependence of the effect of insulin on cGMP and cAMP by cell incubation for 30 min at 37°C with human recombinant insulin at concentrations of 0, 0.125, 0.25, 0.5, 1 and 2 nmol/l; (viii) the time-dependence of the effect of insulin on cyclic nucleotide levels in the interval between 30 s and 360 min; (ix) the influence of phosphodiesterase inhibition on the effects of insulin on cyclic nucleotides by carrying out experiments with a 20-min preincubation with theophylline (500 µmol/l) or 3-isobutyl-1-methylxanthine (IBMX) (500 µmol/l); (x) the ability of insulin to interplay with the effects of guanylate cyclase activators by incubating the cells for 30 min with both insulin and SNP at 40 and 100 µmol/l and with both insulin and GTN at 40 and 100 µmol/l; (xi) the ability of insulin to interplay with the effects of adenylate cyclase activators by incubating the cells for 30 min with both insulin and the stable prostacyclin analogue Iloprost (500 pg/ml) or forskolin (2 µmol/l).

Chemicals

Human recombinant insulin was obtained from Lilly Humulin R (Lilly France S.A., St Cloud, Paris, France); L-NMMA, type IA collagenase, L-arginine, forskolin, SNP, theophylline, IBMX, wortmannin and LY 294002 hydrochloride were obtained from Sigma (St Louis, MO, USA); GTN was obtained from Astra Farmaceutici S.p.A. (Milan, Italy); Iloprost, a stable prostacyclin analogue, was a gift from Schering S.p.A. (Milan, Italy); ionomycin was obtained from Boehringer Mannheim (Mannheim, Germany); L-[^{2,3,4,5}-³H]-arginine monohydrochloride (2.3 × 10¹² Bq/nmol; 62 Ci/mmol) was from Amersham International (Amersham Pharmacia, Amersham, Bucks, UK); forskolin and wortmannin were dissolved in dimethylsulfoxide in a final concentration that did not exceed 0.25% (35 µmol/l). In each experimental condition, each sample received the same amount of solvent.

Cell isolation and culture

Tissue samples of corpus cavernosum were obtained during the surgical procedure of corporoplasty. Penile specimens were surgically debrided and washed in physiological saline to remove blood. Samples were cut into fragments of about 1 mm² by a sterile lancet and incubated for 2 h in a Petri dish in the presence of Hank's balanced salt solution (HSS) containing type IA collagenase (2000 IU/ml). After repeated washes with HSS, fragments were cultured under a coverslip in the presence of minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mmol/l glutamine and antibiotics, and buffered with 10 mmol/l N-Tris (hydroxymethyl) methyl-2-aminoethane-sulfonic acid and 10 mmol/l Hepes. Incubation was carried out at 37°C in a humidified incubator with an atmosphere of 5% O₂:95% CO₂ for 2 weeks and medium was replaced with fresh medium every 4 days. After 14 days, microscopic inspection allowed the observation of cell outgrowth and the coverslip was then removed with sterile forceps and incubated in another Petri dish. After a further 2 weeks, cells were detached by trypsinization (2.5 g porcine trypsin, 1 g EDTA, 100 ml physiological saline), centrifugally washed and plated in other dishes. Cultures were morphologically homogeneous; neither a cobblestone morphology nor flattened, spread-out shapes were observed. The transfer procedure was repeated for subsequent passages and cells were cultured following standardized procedures in medium containing 10% FCS. The large majority of experiments were carried out for four to five passages. Human umbilical vein endothelial cells (HUVEC) were a generous gift from Professor Federico Bussolino, Institute for Cancer Research and Treatment, Candiolo-Turin, Italy.

Immunofluorescence staining, fluorescence-activated cell sorting (FACS) separation and Western immunoblotting for cell characterization

CSMC were cultured on glass coverslips until 70% confluence, washed twice with phosphate-buffered saline (PBS) and fixed in freshly prepared 3.7% formaldehyde solution for 10 min at room temperature. Aspecific binding was blocked by treating cells with 1% serum bovine albumin (BSA) in PBS for 15 min. Cells were then incubated in PBS with 1% BSA containing primary antibody at 37°C for 60 min, washed three times for 10 min each in PBS and then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibody diluted 1:40 in PBS for 60 min at room temperature. The primary antibodies employed were monoclonal anti-smooth muscle actin and anti-desmin (Sigma-Aldrich, St Louis, MO, USA; dilution 1:30) and monoclonal anti-vimentin

(BioMakoz, Rehovot, Israel; dilution 1:30). After washing in PBS three times for 10 min each, the coverslips were mounted in mounting medium on a microscope slide and viewed with a fluorescence LSM Zeiss confocal microscope. As a negative control, fixed cells treated only with FITC-conjugated anti-mouse secondary antibody were used. To exclude the presence of endothelial cells in the preparation, fixed cells were immunostained with an antiserum against the endothelial marker, von Willebrand Factor (clone F8/86; Dako, Carpinteria, CA, USA).

To detect the content of vascular smooth muscle-specific α -actin (α -SMA), 20 μ g total protein extract was separated by SDS-PAGE and electrotransferred to nitrocellulose membrane. The blot was first incubated with a monoclonal antibody against α -SMA from Sigma Chemical Co. (1:1000 dilution), then with an anti-mouse horseradish peroxidase-linked second antibody. The blot was then detected with an EXL-plus kit (Amersham Life Sciences).

The purity of CSMC culture was evaluated by flow cytometry. The cells were detached with trypsin, washed in PBS supplemented with 0.2% BSA and 0.1 NaN_3 and permeabilized with 0.05% saponin for 5 min at 4 °C. After two further washings, the cell samples were incubated (30 min, 4 °C) with the following monoclonal antibodies: anti-human α -smooth muscle actin (clone 1 A4; Sigma), anti-human CD31 (Serotec, Oxford, Oxon, UK) and anti-human von Willebrand Factor (clone F8/86; Dako, Carpinteria, CA, USA). After two further washings, the cells were incubated (30 min, 4 °C) with FITC-conjugated anti-goat anti-mouse immunoglobulins (Dako). Antigen expressions were analyzed with a FACS scan flow cytometer (Beckton Dickinson, Milan, Italy). Each analysis represents the results from 10 000 events. As negative controls, we used cells exposed only to the second antibody. HUVEC were used as positive controls for CD31 and von Willebrand Factor.

Cell proliferation assay

CSMC at initial passages were made quiescent by serum starvation and cultured in MEM/BSA. After 48 h quiescence, CSMC were detached by trypsinization and plated in 35 mm culture dishes at 1×10^4 cells/ml in 2 ml fresh MEM containing different concentrations of FCS (0, 2, 5, 10 and 20%). CSMC were trypsinized at intervals and counted in a haemocytometric chamber. Viability was evaluated by the trypan blue exclusion technique.

Doubling time, expressed in hours, was calculated following the formula: $(t_2 - t_1) \times \log 2 / \log N_{t_2} / N_{t_1}$ where $t_2 - t_1$ was the time-interval expressed in hours between the cell count, N_{t_1} the cell number at time t_1 and N_{t_2} the cell number at time t_2 .

Some experiments were carried out using the same procedure by exposing cultured cells to 2% FCS in the

presence of different concentrations of insulin (0.6–60 nmol/l).

Evaluation of mRNA for cNOS

Total RNA was isolated from cultured CSMC and from HUVECs by the acid guanidinium–isothiocyanate–phenol method (47). Total RNA (5 μ g) was reverse transcribed by first strand cDNA synthesis kit (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) with an HPLC-purified cNOS specific primer 5'-TGTTG-GTGTCTGAGCCGGG-3' (Amersham Pharmacia Biotech) designed on exon 26 of the published human endothelial NOS sequence (48). An aliquot of this cDNA solution was amplified in a total volume of 50 μ l with 30 mmol/l Tris–HCl buffer (pH 8.3), 2 mmol/l MgCl_2 , 2.5 U AmpliTaq Gold DNA polymerase (Perkin Elmer Biosystems, Foster City, CA, USA) and two specific primers (0.15 μ mol/l each) in a Perkin Elmer Gene Amp PCR System 2400. The sense (5'-GGGCCAGAGCTAC-GCACAGC-3') and the antisense (5'-GTGCCCAGGGCC-CCTGC-3') oligonucleotides, designed respectively on exon 12 and 16 of the published human endothelial NOS sequence (48), amplified a 345 bp fragment of the cDNA.

The single PCR product obtained was electrophorized through a 1% agarose gel, transferred onto a Nylon Membrane (Schleicher and Schuell, Keene, NH, USA) and fixed by UV radiation. The blot was then hybridized with a cDNA probe specific for human endothelial NOS (Alexis Corp., Lausen, Switzerland) labelled by [α - ^{32}P]dCTP Random Primer Labelling Kit (Amersham Pharmacia Biotech). The membrane was exposed for 2 days to Kodak Omat AR films (Kodak Co., Rochester, NY, USA) at -80°C with an intensifying screen (49).

Immunofluorescence evidence of cNOS protein

The presence of cNOS protein was evaluated as previously described by immunostaining fixed cells with a monoclonal anti-cNOS antibody (Transduction Laboratories, Lexington, KY, USA; dilution 1:50).

To show the simultaneous presence of cNOS protein and α -SMA, we carried out double immunostaining experiments using the following antibodies: for α -SMA, a rabbit antibody from Zymed Laboratories (San Francisco, CA, USA), and a tetramethyl-rhodamine isothiocyanate (TRITC)-goat anti-rabbit IgG-conjugated (Zymed Laboratories) secondary antibody; for cNOS the previously described monoclonal antibody and a FITC-conjugated antibody, goat anti-mouse immunoglobulins (Dako). The procedure was the same as that previously described and the cells were examined with a confocal microscope using FITC and TRITC filters simultaneously with a programme for co-localization assay.

Evaluation of NOS activity

Since NO synthesis is catalyzed by NOS, which induces the conversion of L-arginine to L-citrulline and NO with a 1:1 stoichiometry (46, 50), the presence of NOS activity in cultured CSMC was checked as L-[³H]-citrulline production from L-[³H]-arginine.

Briefly, cultured CSMC in 35 mm dishes were washed once with Hepes buffer (145 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l MgSO₄, 10 mmol/l Hepes sodium salt, 10 mmol/l glucose and 1 mmol/l CaCl₂, pH 7.4) and incubated in 1 ml of the same buffer at 37°C for 20 min. We added L-[³H]-arginine (185 × 10³ Bq; 5 µCi) corresponding to a final concentration of 0.8 nmol/l to each dish and after 1 min cells were stimulated with 1 µmol/l ionomycin (to assess the calcium dependence of NOS activation) or 2 nmol/l insulin (to assess its ability to activate cNOS). After a 5-min incubation at 37°C, the reaction was stopped by washing the 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 20 mmol/l Hepes-Na (pH 6) was added. The supernatant was collected, applied to 2 ml columns of Dowex AG50WX-8 (Na⁺ form) and eluted with 4 ml distilled water. The radioactivity corresponding to L-[³H]-citrulline content in 6 ml eluate was measured with liquid scintillation counting. The blank was prepared by incubating cells in 1 mmol/l cold L-arginine to remove the small percentage of L-[³H]-citrulline contaminating the L-[³H]-arginine preparation. We calculated that L-[³H]-citrulline present in the L-[³H]-arginine preparation did not exceed 3% of radioactivity.

Cyclic nucleotide determination

For the experiments investigating cyclic nucleotide determination, cells were cultured into 6-well plates with medium containing 10% FCS until 70% confluence was achieved; the medium was then removed and replaced overnight with medium containing 1% FCS before exposure of the cells to the different substances as described in the section on study design. At the end of the different incubation periods, medium was removed from each well and 300 µl absolute ethanol was added to permeabilize the cells. A complete evaporation of ethanol was obtained under shaking. Cyclic nucleotides were then dissolved in 300 µl acetate buffer and kept at -70°C until the assays. cAMP and cGMP were measured by radioimmunoassay kits (Diagnostic Products Corporation Inc., Cambridge, MA, USA and Immuno Biological Laboratories, Hamburg, Germany respectively).

For the cGMP assay, the sensitivity was 0.05 pmol/ml, the specificity was 100% for cGMP, 0.0004% for cAMP, 0.0001% for GMP, GDP, ATP and GTP and the intra-assay coefficient of variation was 4.4%. For the cAMP assay, the sensitivity was less

than 0.1 pmol/ml, cross-reactivity with the other nucleotides was 100% for cAMP, <0.1% for cGMP, <0.001% for AMP, GDP, ATP, GTP and the intra-assay coefficient of variation was 4.8%.

Statistical analysis

Data are expressed as means ± S.E.M. Six to twelve experiments were carried out for each experimental series. Statistical analysis was performed by means of analysis of variance (ANOVA) to determine the statistical significance of dose-response effects and by the unpaired Student's *t*-test when only two values were compared.

Results

CSMC characterization and proliferation

Human CSMC grew in culture as a homogeneous population of spindle-shaped cells with centrally located nuclei. The cells were positive for α-SMA (granular pattern) (Fig. 1B) and desmin (data not shown). Figure 1C shows the immunofluorescence for vimentin, which was only used to indicate cytoskeleton morphology since it is not specific for vascular smooth muscle cells. Cells were negative for the endothelial marker von Willebrand Factor.

Immunoblotting showed the presence of vascular smooth muscle-specific α-actin as a single band of 43 kDa.

The purity of cultured CSMC has been demonstrated by FACS positivity for α-SMA (97%) and negativity for the endothelial markers von Willebrand Factor and CD31. HUVEC presented the expected positivity for the endothelial markers.

Proliferation curves in the presence of different concentrations of FCS showed that the rate of cell proliferation increased in the presence of increasing FCS concentrations (0, 2, 5, 10%) up to 10%, no further effect being elicited by FCS at 20%.

As shown in Table 1, doubling times decreased in the presence of increasing FCS concentrations. Insulin concentrations ranging between 6 and 60 nmol/l increased the proliferation rate induced by low FCS levels (2%) as shown in Fig. 2.

Table 1 Doubling time in hours (see text for formula) of CSMC in the presence of different concentrations of FCS.

FCS (%)	Day 4	Day 10
2	42.5	70.1
5	34.1	58.7
10	29.1	54.7
20	29.4	54.8

Figure 1 Immunofluorescence pattern for CSMC. (A) Control, (B) immunostaining with monoclonal antibody anti- α SMA, (C) immunostaining with monoclonal antibody anti-vimentin and (D) immunostaining with monoclonal antibody anti-cNOS (immunofluorescence pictures were obtained using confocal microscopy).

Expression of mRNA for cNOS

The expression of cNOS mRNA in CSMC was shown by RT-PCR. To confirm that the 345 bp PCR product amplified from the cDNA reverse transcribed from total RNA using primers specific for cNOS belongs to cNOS mRNA, it was blotted and hybridized with a probe specific for the constitutive isoform of NOS (Fig. 3). mRNA expression for cNOS in HUVECs is also shown as a positive control.

Figure 2 Effects of insulin on the proliferation of CSMC elicited by 2% FCS in the absence or the presence of different insulin concentrations (0.6–60 nmol/l).

Evidence of cNOS protein

Figure 1D shows that CSMC exhibited a clearly positive immunostaining with monoclonal anti-human cNOS antibody.

Figure 4 shows the simultaneous positivity for both α -SMA and cNOS in a representative cell.

Presence of a Ca^{2+} -dependent NOS activity

Ionomycin rapidly increased L-[³H]-citrulline production from L-[³H]-arginine in CSMC ($P = 0.011$) as shown in Fig. 5. Citrulline synthesis is a reliable marker of NO formation. These results indicate the presence of a Ca^{2+} -dependent NOS activity in these cells.

Evidence that insulin increases NO synthesis

In CSMC, insulin rapidly increased NO synthesis, measured as L-[³H]-citrulline production from L-[³H]-arginine ($P = 0.041$), as shown in Fig. 6.

Figure 3 The 345 bp fragment obtained by RT-PCR and hybridized with specific human cNOS probe in both (A) HUVEC and (B) CSMC.

Figure 4 Double immunostaining of CSMC for α -SMA (primary antibody: rabbit anti- α -SMA; secondary antibody: TRITC-conjugated goat anti-rabbit IgG) and cNOS (primary antibody: monoclonal anti-cNOS; secondary antibody: FITC-conjugated goat anti-mouse IgG). The positivity for cNOS is green, for α -SMA it is red and for both it is orange–yellow.

Evidence that insulin increases cell concentrations of both cGMP and cAMP via NO production involving the PI 3-kinase pathway of insulin signalling

Figure 7 shows the effects of insulin on intracellular levels of both cyclic nucleotides. Insulin (2 nmol/l) induced an increase of cGMP after 30 min of incubation (Student's *t*-test: $P = 0.0001$ for both cGMP and cAMP). Preincubation with L-NMMA and wortmannin (2 μ mol/l) completely blunted the effects of insulin on

Figure 5 Influence of the calcium ionophore ionomycin (1 μ mol/l) on NO synthesis in CSMC, shown as L-[3 H]-citrulline production from L-[3 H]-arginine ($P = 0.011$) ($n = 12$).

both cGMP and cAMP (Fig. 7). A significant reduction in the action of insulin was observed also with 1 μ mol/l wortmannin ($P = 0.0001$ vs insulin alone). The involvement of PI 3-kinase in the action of insulin on both cyclic nucleotides has been further confirmed by the complete inhibition exerted by LY 294002, another PI 3-kinase inhibitor, when the cGMP values (pmol/ 10^6 cells) in the presence of LY 294002 (100 μ mol/l) were 3.6 ± 0.21 without and 2.88 ± 0.32 with 2 nmol/l insulin (not significant).

Dose-dependent effect of insulin on cell concentrations of both cGMP and cAMP

The dose-dependent effects of insulin (30-min exposure) on cGMP and cAMP are shown in Fig. 8. Dose-dependent effects were found for both cyclic nucleotides (ANOVA for repeated measures: $P = 0.0001$ for both cGMP and cAMP). For cGMP, the effects of insulin were significant at the following concentrations: 0.25 nmol/l, $P = 0.005$; 0.5, 1 and 2 nmol/l, $P = 0.0001$. For cAMP, the effects of insulin were significant at the following concentrations: 0.25, 0.5, 1 and 2 nmol/l, $P = 0.0001$.

Time-dependence of the effect of insulin on cell concentrations of both cGMP and cAMP

Figure 9 shows the behaviour of cyclic nucleotide concentrations at different times (0–360 min) after exposure to insulin (2 nmol/l). For both cyclic nucleotides,

Figure 6 Influence of 2 nmol/l insulin on NO synthesis in CSMC, shown as L-[3 H]-citrulline production from L-[3 H]-arginine ($P = 0.041$) ($n = 12$).

Figure 7 Influence of the NOS inhibitor L-NMMA (1 mmol/l) and the PI 3-kinase inhibitor wortmannin (2 μmol/l) on the effects of insulin on (A) cGMP and (B) cAMP in CSMC ($n = 6$).

Figure 8 Dose-dependence of the effect of insulin on intracellular levels of (A) cGMP and (B) cAMP in CSMC. Levels of significance are described in the text ($n = 6$).

the insulin-induced increase was highly significant (ANOVA; $P = 0.0001$) and a time-dependence of the response was clearly evident. In particular, (i) cGMP values were significantly higher than basal concentrations at 0.5, 1, 2, 4, 8, 10, 15, 30, 60, 90 and 120 min ($P = 0.0001$ at each control), with a peak at 30 min; the difference versus basal was no more significant starting from 180 min, with a return to basal values at 240 min and (ii) cAMP values were higher than basal concentrations at 0.5 ($P = 0.028$), 1, 2, 4, 8, 10, 15, 30, 60, 90 and 120 min ($P = 0.0001$) and 180 min ($P = 0.004$), with a peak at 60 min; values returned to basal at 240 min.

Evidence that the action of insulin on cyclic nucleotides persists with phosphodiesterase inhibition

The preincubation with the phosphodiesterase inhibitor theophylline (500 μmol/l) increased basal values of both cGMP and cAMP ($P = 0.0001$ for both cyclic

Figure 9 Time-dependence of the effect of insulin on cell concentrations of (A) cGMP and (B) cAMP in CSMC. Levels of significance are described in the text ($n = 6$).

nucleotides); also in these experimental conditions, insulin further increased the concentrations of cGMP, from 14.19 ± 1.39 to 16.72 ± 2.14 pmol/ 10^6 cells ($P = 0.0001$) and of cAMP, from 3.52 ± 0.17 to 8.72 ± 0.40 pmol/ 10^6 cells ($P = 0.0001$). The effects of insulin were also preserved in the presence of IBMX, another phosphodiesterase inhibitor: in particular, in the presence of 500 μ mol/l IBMX, cGMP values (pmol/ 10^6 cells) were 11.51 ± 0.32 without and 14.68 ± 0.66 with 2 nmol/l insulin ($P = 0.002$).

Evidence that the action of insulin on cyclic nucleotides persists in the presence of guanylate cyclase activation by NO donors

Stimulation of guanylate cyclase by SNP caused a significant increase in both cGMP ($P = 0.0001$ with both 40 and 100 μ mol/l) and cAMP ($P = 0.0001$ with both 40 and 100 μ mol/l). When insulin was co-incubated with SNP, cGMP and cAMP values were further increased (for cGMP: $P = 0.002$ and $P = 0.0001$ with 40 and 100 μ mol/l respectively; for cAMP $P = 0.04$ with both 40 and 100 μ mol/l).

Figure 10 Influence of 30-min incubation with insulin, SNP and insulin + SNP on (A) cGMP and (B) cAMP in CSMC. Levels of significance are described in the text ($n = 6$).

(Fig. 10). Similar results have been obtained with GTN (data not shown).

Evidence that insulin action on cAMP persists in the presence of adenylate cyclase activation by receptor-mediated (Iloprost) or non-receptor-mediated (forskolin) mechanisms

The stimulation of adenylate cyclase activity by Iloprost resulted in a significant increase in cAMP values ($P = 0.0001$). When insulin was co-incubated with Iloprost, cAMP values were further increased ($P = 0.0001$) (Fig. 11). The stimulation of adenylate cyclase activity by forskolin resulted in a significant increase in cAMP values ($P = 0.0001$). When insulin was co-incubated with forskolin, cAMP values were further increased ($P = 0.008$) (Fig. 11).

Figure 11 Influence of 30-min incubation with insulin, Iloprost, insulin + Iloprost, forskolin, and insulin+forskolin on cAMP in CSMC. Levels of significance are described in the text ($n = 6$).

Discussion

In this study, we have described experiments carried out on cultured CSMC. As far as we know, it is one of the few investigations concerning isolation, primary culture and characterization of this cell type and the first one showing the effect of insulin on their proliferation. Under our conditions, cells grew in culture as a homogeneous spindle-shaped population with centrally located nuclei. They exhibited the specific markers of vascular smooth muscle cells α -actin (both in immunofluorescence, in Western immunoblotting and in the FACS scan) and desmin. Furthermore, they proliferated in a dose-dependent way according to the different FCS concentrations up to 10%. In the presence of low FCS concentrations, insulin at very high, absolutely non-physiological concentrations (6–60 nmol/l) slightly increased the proliferation rate.

The study clearly shows for the first time, via a molecular biology technique, that CSMC express an endothelial-type constitutive Ca^{2+} -dependent NOS, adding a further element to the present debate on this relevant topic. It also demonstrated for the first time that cNOS in CSMC is a biologically active calcium-dependent enzyme, able to produce detectable basal values of NO and to be further stimulated by a calcium ionophore. Our data, therefore, have shown that CSMC are not only targets of NO derived from endothelial cells or from nerve endings, but are also a source of NO potentially involved in the mechanism of erection, even if further studies are needed to evaluate the physiological role of NO constitutively produced by CSMC in human penile erection.

Furthermore, this study has shown that, in CSMC: (i) insulin activates cNOS; (ii) via NO, insulin increases cell concentrations of the vasodilating cyclic nucleotides cGMP and cAMP; (iii) the effects of insulin on the

NO–cGMP/cAMP cascade are mediated by the signalling pathway of PI 3-kinase, the same as the one by which insulin activates cNOS in endothelial cells (42), as the experiments with the PI 3-kinase inhibitor wortmannin clearly demonstrate; (iv) the effects of insulin on both cGMP and cAMP persist when the synthetic enzymes guanylate cyclase and adenylate cyclase are stimulated, and when the catabolic enzyme phosphodiesterases are inhibited by theophylline and IBMX, which inhibit all the phosphodiesterases, and therefore also phosphodiesterase V – which seems to be the main agent catalysing the breakdown of cGMP in corporal smooth muscle – without being specific for it.

Our study has, therefore, provided evidence that in the complex mechanism of penile erection insulin could play a role by increasing NO production in CSMC, with a consequent enhancement of both cGMP and cAMP and interplay with other physiologic mediators.

The involvement of NO in the insulin-induced increase, not only of cGMP but also of cAMP, in CSMC agrees with previous studies carried out in our laboratory both in platelets (43) and in vascular smooth muscle cells (38, 39); this study, on the other hand, has demonstrated that, also in CSMC, nitrates enhance not only cGMP but also cAMP, as previously described in human vascular smooth muscle cells (33) and in platelets (34, 35). The NO-mediated, insulin-induced increase in cAMP is preserved in the presence of phosphodiesterase inhibition; thus, the inhibition of a cAMP phosphodiesterase by cGMP (36) is less likely to account for the cAMP increase than the ability of NO by itself to modify the guanylate cyclase properties inducing an adenylate-cyclase activity (37).

As far as the well-known vasodilating agents are concerned, we have shown in CSMC the expected responses to adenylate cyclase activation by the prostacyclin analogue Iloprost and forskolin. As far as forskolin is concerned, our data provide further evidence of its direct effects on CSMC that provide the rationale for its use in erectile dysfunction (51, 52). Furthermore, we have demonstrated that insulin enhances the effect of both Iloprost and forskolin on cAMP content. The interplay with forskolin demonstrates that the effect of insulin on cAMP is not specific for receptor-activating agents, but is extended to other substances acting independently of receptor-mediated mechanisms. We believe that insulin increases cAMP *per se* via NO, and therefore enhances the effects of all the substances able to increase cAMP synthesis with different mechanisms.

Furthermore, insulin increases both cGMP and cAMP in CSMC also in the presence of NO donors activating guanylate cyclase. This phenomenon is interesting, since both insulin and NO donors act via NO in guanylate cyclase stimulation. Our study, therefore, supports the conclusion that, when NO donors are employed, the insulin-induced increase of endogenous NO continues to exert biological effects on guanylate cyclase.

In summary, this study has indicated a method to isolate, from human penile tissue, smooth muscle cells sensitive to different mediators and exhibiting a Ca^{2+} -dependent NOS activity able to be stimulated by insulin in a few minutes. The present results are potentially relevant to better understanding the mechanisms involved in penile erection in man. Even though it is known that insulin induces an NO-mediated systemic vasodilation *in vivo* (40, 41), no investigation has, as yet, considered the effects of insulin on penile circulation. Our results, therefore, provide the first evidence that insulin, via an increase of both the vasodilating cyclic nucleotides in smooth muscle cells from human corpus cavernosum, directly influences mechanisms deeply involved in the vascular tone of the penis and interplays with classical haemodynamic mediators.

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