0013-7227/99/$03.00/0
Endocrinology

Vol. 140, No. 3
Printed in U.S.A.

Human Vascular Endothelial Cells Express Oxytocin Receptors*

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ABSTRACT

Pharmacological studies in humans and animals suggest the existence of vascular endothelial vasopressin (AVP)/oxytocin (OT) receptors that mediate a vasodilatory effect. However, the nature of the receptor subtype(s) involved in this vasodilatory response remains controversial, and its coupled intracellular pathways are unknown. Thus, we set out to determine the type and signaling pathways of the AVP/OT receptor(s) expressed in human vascular endothelial cells (ECs).

Saturation binding experiments with purified membranes of primary cultures of ECs from human umbilical vein (HUVEC), aorta (HAEC), and pulmonary artery (HPAEC) and [^{3}H]AVP or [^{3}H]OT revealed the existence of specific binding sites with a greater affinity for OT than AVP (K_d = 1.75 *vs.* 16.58 nM). Competition binding experiments in intact HUVECs (ECV304 cell line) with the AVP antagonist [^{125}I]4-hydroxyphenacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2 or the OT antagonist [^{125}I]D$(CH_2)_5$[*O*-Me-Tyr-Thr-Orn-Tyr-NH_2]vasotocin, and various AVP/OT analogs confirmed the existence of a single class of surface receptors of the classical OT subtype.

RT-PCR experiments with total RNA extracted from HUVEC, HAEC, and HPAEC and specific primers for the human V1 vascular, V2 renal, V3 pituitary, and OT receptors amplified the OT receptor sequence only. No new receptor subtype could be amplified when using degenerate primers. DNA sequencing of the coding region of the human EC OT receptor revealed a nucleotide sequence 100% homologous to that of the uterine OT receptor reported previously.

Stimulation of ECs by OT produced mobilization of intracellular calcium and the release of nitric oxide that was prevented by chelation of extra- and intracellular calcium. No stimulation of cAMP or PG production was noted. Finally, OT stimulation of ECs led to a calcium- and protein kinase C-dependent cellular proliferation response.

Thus, human vascular ECs express OT receptors that are structurally identical to the uterine and mammary OT receptors. These endothelial OT receptors produce a calcium-dependent vasodilatory response via stimulation of the nitric oxide pathway and have a trophic action. (*Endocrinology* **140:** 1301–1309, 1999)

SEVERAL studies in various species, including humans, have suggested the existence of vascular endothelial arginine vasopressin (AVP)/oxytocin (OT) receptors that mediate a vasodilatory effect. However, the nature of the receptor subtype(s) involved in the process is controversial and remains to be established. Depending on the species studied, the type of preparation used [intact *vs.* deendotheliazed vascular vessel, whole vessel *vs.* isolated and cultured endothelial cells (ECs)], and the vascular territory explored, AVP V_1 vascular, AVP V_2 renal, or OT receptors have been thought to mediate this vasodilatory effect (1–3). A major reason for this controversy is the fact that the AVP/OT agonists and antagonists used in the aforementioned studies lose their selectivity and specificity when used at high concentrations. For instance, our own radioligand binding assays using mammalian cells stably transfected with the human V_1 vascular, V_2 renal, V_3 pituitary, or OT receptors revealed that DDAVP (considered a typical V_2 renal agonist) has, in fact, a rather narrow selectivity for the human V_2 renal receptor (4). Similarly, the peptide V_2 renal antagonists available to date are not selective for the human V_2 renal receptor subtype. Moreover, various vascular beds display different sensitivities to AVP and the distribution of these endothelial receptors seems to be regionally selective. For instance, AVP causes a direct, dose-dependent vasodilatation in the human forearm, but produces digital vasoconstriction (5). AVP produces in middle cerebral arteries an endothelium-independent vasoconstriction, whereas an endothelium-dependent relaxation was noted in the basilar arteries (6). By the same token, AVP was found to induce endothelium-dependent relaxation in canine basilar and posterior communicating arteries and endothelium-independent contraction in canine middle cerebral arteries (7). In chickens, OT, mesotocin (MT), arginine vasotocin (AVT), and AVP have short lasting vasodepressor effects (>20% drop in mean arterial pressure) in the following order of potency: OT = MT > AVT > AVP (3). Only AVT and AVP subsequently produce a vasopressor effect. Pretreatment with the OT antagonist D$(CH_2)_5$-*O*-Me-Tyr^2-Thr^4-Tyr^9-Orn^8-vasotocin abolishes the vasodepressor effect of all peptides and potentiates the vasopressor potency of AVP and AVT, thus suggesting that in chickens the vasodepressor effect of neurohypophysial peptides is probably mediated by an OT/MT-like receptor.

Thus, the aforementioned studies suggest the presence of specific AVP/OT receptors in vascular endothelial cells whose nature, regional distribution, and intracellular signal-

Received September 14, 1998.
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* This work was supported by NIH Grants HL-39757 and HL-41618, a grant from Sanofi Research (to M.T.), and Grant HL-03117 (to S.C.E.).

ing pathways remain to be determined. Therefore, we set out to address these issues in the present manuscript.

Materials and Methods

Materials

AVP, OT, and the peptide AVP and OT agonists and antagonists were obtained from Bachem (Torrance, CA) or provided by Dr. Maurice Manning from the Medical College of Ohio (Toledo, OH). Tris-HCl and other reagents, unless stated otherwise, were obtained from Sigma Chemical Co. (St. Louis, MO). Ionomycin was purchased from Calbiochem (La Jolla, CA). Human vascular ECs from umbilical vein (HUVEC), aorta (HAEC), and pulmonary artery (HPAEC) and MCDB 131 EC growth medium were purchased from Clonetics (San Diego, CA). ECV304 cells were obtained from the American Type Culture Collection (ATCC CRL-1998, Manassas, VA) and grown in medium 199 (Life Technologies, Grand Island, NY). FBS was purchased from HyClone Laboratories, Inc. (Logan, UT). Restriction and modification enzymes were obtained from Promega Corp. (Madison, WI) or New England Nuclear Biolabs (Beverly, MA). Iodogen (1,3,4,6-tetrachloro-3α-6α-diphenylglycoluril) was obtained from Pierce (Rockford, IL). Fura-2/AM was purchased from Molecular Probes, Inc. (Eugene, OR). [^{125}I]Na (SA, 131 mCi/ml), [^{3}H]AVP (SA, 59 Ci/mmol), [^{3}H]OT (SA, 32 Ci/mmol), and the [^{125}I]OT antagonist D(CH$_2$)$_5$-*O*-Tyr(Me)-Thr-Tyr-Orn-vasotocin ([^{125}I]OVTA; SA, 2200 Ci/mmol) were obtained from New England Nuclear Life Science (Boston, MA). The peptide linear V_1 antagonist 4-hydroxy-phenylacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH$_2$ (OHPhaa) was a gift from Dr. Maurice Manning, Medical College of Ohio, and was radioiodinated ([^{125}I]OHPhaa) with the Iodogen technique and purified by HPLC, as previously described (8). The nonpeptide V_1 antagonist SR 49059 (batch MY10−075) was provided by Dr. C. Serradeil-Le Gal, Sanofi Recherche (Toulouse, France). The nonpeptide rat V_1 antagonist OPC 21268 (batch 93F92M) and the V_2 antagonist OPC 31260 (batch 93D96M) were provided by Dr. J. F. Liard, Otsuka America Pharmaceutical, Inc. (Rockville, MD). Bis-aminophenoxyethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), *N*-nitro-L-arginine methyl ester (L-NAME), PD98059, bisindolylmaleimide I, and wortmaninn were obtained from Calbiochem. KN-93 was purchased from RBI (Natick, MA).

Radioligand binding assays

Purified plasma membranes of HUVECs, HAECs, and HPAECs grown in MCDB 131 medium were obtained by homogenization and centrifugation in Percoll gradient as previously described (9). Confluent ECs at passages 4–5 were washed twice with 10 ml ice-cold PBS and scrapped with 1 ml 5 mM Tris-HCl, 5 mM EDTA (pH 7.4), and enzyme inhibitors (20 μg/ml phenylmethylsulfonylfluoride, 20 μg/ml bacitracin, 10 μg/ml benzamidine, 10 μg/ml pepstatin, 10 μg/ml antipain, 10 μg/ml leupeptin, and 10 μg/ml soybean trypsin inhibitor). After centrifugation at 3,000 × *g* for 15 min at 4 C, the pellets were resuspended in buffer containing 5 mM Tris-HCl and 5 mM EDTA (pH 7.4) with enzyme inhibitors and 250 mM sucrose. After homogenization with 10 strokes of glass-Teflon pestle, 30% Percoll was added, and centrifugation was performed at 30,000 × *g* for 60 min at 4 C. The plasma membrane fraction was centrifuged at 100,000 × *g* for 1 h at 4 C and resuspended in 5 ml 50 mM HEPES, 10 mM $MgCl_2$, and 20% glycerol, pH 7.8. Saturation experiments were performed with purified membranes in 250 μl PBS, 10 mM $MgCl_2$, 0.2% BSA (pH 7.4), and increasing concentrations of [^{3}H]AVP with or without 1 μM unlabeled AVP or [^{3}H]OT with or without 1 μM unlabeled OT. After incubation at 30 C for 30 min, the membranes were filtered through Whatman GF/F filters (Clifton, NJ) soaked in 50 mM Tris-HCl (pH 7.8) and 1% polyethyleneimine and washed with 5 ml ice-cold 50 mM Tris-HCl (pH 7.4) buffer. The filters were mixed with scintillation liquid, and radioactivity was measured by liquid scintillation spectrometry (Beckman Coulter, Inc. Palo Alto, CA; counter LS 5801; yield, 64%). The affinity (K_d) and capacity of the receptors were calculated using a nonlinear least square analysis program (10). The protein concentration was measured with Pierce's bicinchoninic acid reagent, using ovalbumin as an internal standard.

ECV304 cells were grown to confluence in six-well dishes and washed twice with PBS, 10 mM $MgCl_2$, and 0.2% BSA, pH 7.4. Competition binding experiments were performed in triplicate by incubating the cells in the same medium with one fixed concentration of [^{125}I]OHPhaa or [^{125}I]OVTA (0.30 nM) and increasing concentrations of unlabeled OT, AVP, the V_2 AVP agonists DDAVP and dVDAVP, the nonpeptide V_1 AVP antagonists SR 49059 and OPC21268, the nonpeptide V_2 AVP antagonist OPC31260, or the OT antagonists D(CH$_2$)$_5$[*O*-Me-Tyr^2-Thr^4-Orn^8]vasotocin and D(CH$_2$)$_5$[*O*-Me-Tyr^2-Thr^4-Orn^{8}Tyr^9-NH$_2$]vasotocin for 30 min at 30 C. The cells were washed three times with ice-cold PBS and lysed with 0.5 ml 0.2 N NaOH-1% SDS. Cell-bound [^{125}I]OHPhaa or [^{125}I]OVTA radioactivity was counted in a γ-counter. IC_{50} values were derived from nonlinear least square analysis, and K_i values were calculated using the equation of Cheng and Prusoff: $K_i = IC_{50}/(1 + L_f/K_d)$.

RT-PCR experiments

The presence and type of endothelial AVP/OT receptors of several human vascular endothelial beds were established by RT-PCR. Total RNA was isolated from ECV304 cells as well as from HUVECs, HAECs, and HPAECs grown up to passage 4 in 100-mm petri dishes using the RNeasy kit from Qiagen following the manufacturer's protocol. The RNA aliquots (260/280 ratio, >1.8) were stored at −80 C until use. After treatment with deoxyribonuclease I to eliminate possible DNA contamination, the reverse transcriptase reaction was carried out by mixing 5–10 μg total RNA previously denatured at 70 C for 10 min in the presence of 0.5 μg/μl oligo(deoxythymidine) primer, 4 μl 5-fold concentrated RT buffer, 2 μl 100 mM dithiothreitol, 1 μl 10 mM deoxy-NTPs, 1 μl 40 U/μl RNasin, and 1 μl Superscript RT (200 U/μl; Life Technologies, Gaithersburg, MD). After incubation at 42 C for 50 min, then at 50 C for 10 min, the RT enzyme was inactivated by heating at 70 C for 15 min. Thereafter, 2-μl aliquots of the RT reaction were used for PCR reaction (initial denaturation at 95 C for 5 min followed by 30 cycles, 95 C for 1 min, 56 C for 1 min, 72 C for 2 min, and an additional cycle with extension at 72 C for 15 min) using specific primers for the human V_1 vascular, V_2 renal, and V_3 pituitary AVP and OT receptors, as presented in Table 1. To eliminate possible amplification of genomic DNA, these primers were chosen to overlap the intronic region present between the corresponding sixth and seventh transmembrane domains of all AVP/OT receptor sequences. Furthermore, to look for the possibility of a new AVP/OT receptor subtype being expressed in endothelial cells, we also conducted RT-PCR experiments with a set of degenerate primers derived from the second and sixth transmembrane domains, the region of highest nucle-

TABLE 1. Structure of the oligonucleotide primers used for amplification of human AVP/OT receptor cDNAs

Receptor subtype	Primer type	Oligonucleotide sequence	Size (bp)
V_1-vascular	Sense	5′-AACATCTGGTGCAACGTCC-3′	352
	Antisense	5′-CAGTCTTGAAGGAGATGGCC-3′	
V_2-renal	Sense	5′-ATTCATGCCAGTCTGGTGC-3′	422
	Antisense	5′-TCACGATGAAGTGTCCTTGG-3′	
V_3-pituitary	Sense	5′-CCTGGCTATCTTCGTTCTGC-3′	659
	Antisense	5′-AAGATGATGGTCTCAGCGG-3′	
Oxytocin	Sense	5′-ATCACATGGATCACGCTAGC-3′	740
	Antisense	5′-TCATCTTCCATCATGGAGGC-3′	
Degenerate	Sense	5′-GACCTGGYSGTGGCDBTSTTY	734
	Antisense	5′-RTAKATCCAGGGRTTRSWGCAGC-3′	

otide sequence homology between the AVP/OT receptor subtypes. We verified with complementary DNAs (cDNAs) coding for the various AVP/OT receptor subtypes that all of these sets of primers amplified bands of the appropriate sizes. Finally, as a positive control for each RNA preparation, a 753-bp fragment of the glyceraldehyde 3-phosphate dehydrogenase sequence was amplified (sense primer, 5′-GACCTCAACTACATGG TCTACATG-3′; antisense primer, 5′ TGTCGCTGTTGAAGTCAGAGGAGAC-3′). Double strand nucleotide sequencing of the DNA fragments obtained by RT-PCR was achieved by fluorescent labeling (*Taq* DyeDeoxy Terminator Cycle Sequencing kit, PE Applied Biosystems, Inc., Foster City, CA).

Measurement of intracellular calcium

OT mobilization of intracellular calcium was measured in subconfluent monolayers of HUVECs grown on glass coverslips. Cells were loaded with fura-2 at 37 C for 40 min, then placed in fura-2-free medium for 20 min at 37 C before measurement of the fluorescence signal in Krebs-Henseleit-HEPES buffer as described previously (11).

Nitric oxide (NO) production assay

As NO production in vascular ECs is much less abundant than that in macrophages, the formation of nitrites and nitrates was measured by chemiluminescence using a Sievers (Boulder, CO) NOA280 instrument (12). Subconfluent primary cultures of HUVECs isolated by the technique of Jaffe (13) were grown in 24-well dishes, washed twice in Molecular Cell and Developmental Biology medium and 2% FCS, and stimulated by 1 mM sodium nitroprusside, 100 nM bradykinin, or 1 μM OT alone or in the presence of 4 mM EGTA and 10 μM BAPTA. After 1-min incubation at 37 C, the medium was removed and spun, and the supernatant was kept at 4 C until chemiluminescence measurement.

cAMP production

cAMP production was measured in subconfluent monolayers of HUVECs grown in 24-well dishes using the cAMP[^{125}I]SPA kit from Amersham (Arlington Heights, IL). ECs were stimulated by 1 μM OT or 10 μM forskolin for 15 min at 37 C in the presence of 0.5 mM isobutyl-1-methylxanthine.

PG formation

PG production was measured in subconfluent monolayers of HUVECs grown in six-well dishes and stimulated by 1 μM OT for 15 min at 37 C. PGE_2 and 6-keto-$PGF_{1\alpha}$ were measured using enzyme immunoassay kits from Cayman Chemicals (Ann Arbor, MI; catalog no. 514010 and 515211).

Cell proliferation assay

Cell proliferation was measured using the CellTiter 96 Aqueous One Solution cell proliferation assay from Promega Corp. (Madison, WI) based on the cellular conversion of the colorimetric reagent 3,4-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt into soluble formazan by dehydrogenase enzymes found only in metabolically active, proliferating cells. Primary cultures of HUVECs were grown to subconfluence in 96-well plates (30,000 cells/well), washed, and grown for 24 h in 200 μl MCDB 131 and 0.5% FBS. Cells were treated with 2% FBS or OT for 24 h, followed by incubation with 20 μl dye solution for 3–5 h according to the manufacturer's instructions. Subsequently, absorbance was recorded at 490-nm wavelength using an enyzme-linked immunosorbent assay plate reader (650-nm reference wavelength).

Data analysis

Nucleotide sequences were analyzed and compared using MacVector software package (Oxford Molecular Group, Oxford, UK). Data were expressed as the mean ± SEM. Statistical analysis was based on Student's *t* test and ANOVA. $P < 0.05$ was considered significant. Regression lines were calculated by the least squares method.

Results

Radioligand binding characteristics of the AVP/OT receptors expressed in HVEC

To demonstrate the presence of specific AVP/OT receptors in human ECs, radioligand binding studies were initially performed with the tritiated endogenous ligands [^{3}H]AVP and [^{3}H]OT and purified plasma membranes from HUVECs (Fig. 1). [^{3}H]AVP and [^{3}H]OT bound to a single class of specific binding sites (binding capacity, 132 ± 29 and 198 ± 36 fmol/mg protein, respectively). As the affinity for OT is greater than that for AVP (K_d = 1.75 ± 0.29 *vs.* 16.58 ± 3.15 nM, respectively), these results suggested that the receptor expressed in human ECs belonged to an OT subtype rather than to an AVP subtype. Similar findings were noted with purified membrane preparations from HAECs and HPAECs (data not shown).

These experiments performed with purified membranes of primary cultures of ECs were found to be impractical for several reasons, including the elevated cost of purchasing and growing primary cultures of ECs, the limited amount of material available for binding assays, the instability with time of stored purified membranes, and the low specific activity of the tritiated radioligands used. Thus, we replaced

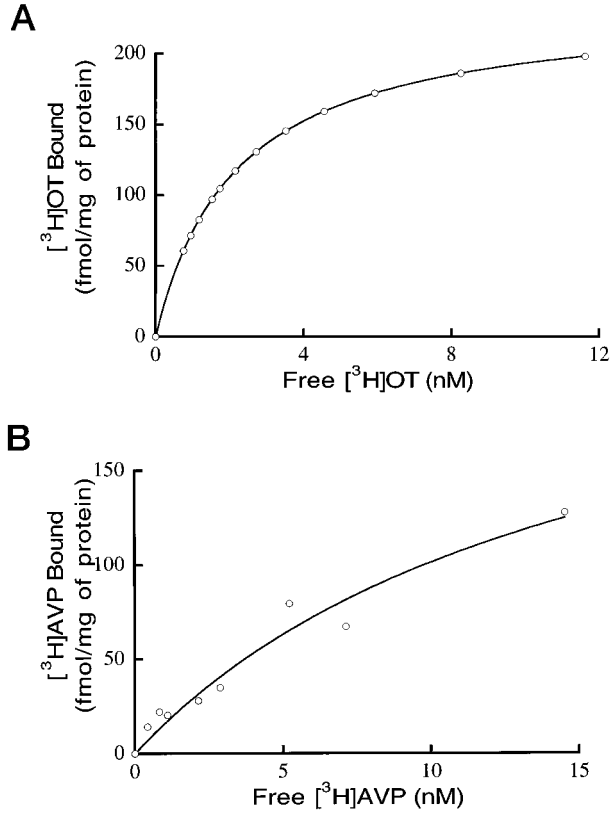

FIG. 1. Saturation equilibrium specific binding of [^{3}H]AVP and [^{3}H]OT to purified membranes of human endothelial cells. Purified membranes of primary cultures of HUVECs were prepared as described in *Materials and Methods* and incubated at 30 C for 30 min with increasing concentrations of tritiated AVP or OT. These graphs represent specific binding of typical experiments with a protein concentration of 70 μg/tube. A total of four different experiments were performed in duplicate for both [^{3}H]AVP and [^{3}H]OT.

the tritiated (low specific activity) ligands AVP and OT with the radioiodinated (high specific activity) ligands [^{125}I]OHPhaa and [^{125}I]OVTA, respectively, AVP and OT antagonists. To reduce the cost of acquiring and growing primary cultures of human ECs, we used instead the ECV304 human EC line. This spontaneously transformed immortal EC line was established from the vein of a normal human umbilical cord. These ECs are characterized by a typical cobblestone monolayer growth pattern and no specific growth factor requirement. These cells display several endothelial markers and have been used by various investigators as a sturdy model of human vascular ECs.

Competition binding experiments with [^{125}I]OH-Phaa and increasing concentrations of cold AVP, the V_1 vascular antagonist SR 49050, and the V_2 renal agonist DDAVP revealed the existence of a single class of specific receptors in ECV304 cells (Fig. 2). The affinity constants for AVP, the V_1 vascular antagonist and the V_2 renal agonist were 14, 19, and 154 nM, respectively. These data suggested that the receptor present in ECV304 cells did not belong to the classical V_1 vascular or V_2 renal subtype. Subsequently, we performed competition binding experiments with the OT antagonist [^{125}I]OVTA and increasing concentrations of various AVP/OT analogs (Table 2). The affinities of OT and the two OT antagonists for the receptor on ECV304 cells were in the nanomolar range, whereas the affinities of AVP and its classical V_2 agonists and antagonists as well as V_1 antagonists were much weaker. From these experiments, we concluded that, as in primary cultures of vascular ECs, a single class of receptors is present on ECV304 ECs and that this receptor belongs to the OT subtype.

Cloning and sequencing of the human vascular endothelial AVP/OT receptor

The structure and type of vascular endothelial AVP/OT receptors expressed in several human vascular endothelial beds were established by RT-PCR. Initial RT-PCR experiments with total RNA extracted from ECV304 cells generated bands of appropriate sizes with the glyceraldehyde 3-phosphate dehydrogenase and OT primers only, whereas no band was observed with the primers specific for the other known AVP/OT receptor subtypes (Fig. 3). Nucleotide sequencing revealed that the cDNA fragment amplified with these OT receptor primers (bp +659 to +1340) was identical to the sequence of the human uterine OT receptor originally cloned by Kimura *et al.* (14). The RT-PCR reaction with the degenerate primers produced a band of 734 bp. The nucleotide sequence of this fragment amplified with the degenerate primers matched only the human OT receptor sequence, thus ruling out the presence of a new AVP/OT receptor subtype in human ECs. These results suggest that the ECV304 ECs express an OT receptor subtype structurally similar to that present in uterine and mammary glands.

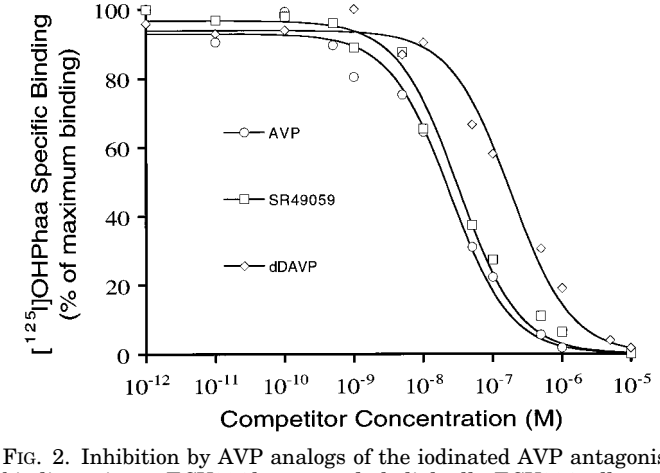

FIG. 2. Inhibition by AVP analogs of the iodinated AVP antagonist binding to intact ECV304 human endothelial cells. ECV304 cells were grown as described in *Materials and Methods* and were incubated at 30 C for 30 min with one concentration of the iodinated AVP antagonist [^{125}I]OHPhaa and increasing concentrations of unlabeled AVP analogs.

The same RT-PCR protocol was applied to RNA samples extracted from HUVECs, HAECs, and HPAECS grown up to passage 4. We observed identical results, *i.e.* DNA amplification with the OT receptor-specific primers, but no DNA amplification with the primers specific for the V_1 vascular, V_2 renal, and V_3 pituitary AVP receptors. Furthermore, no new AVP/OT receptor subtype was identified in these ECs.

Subsequently, the 5′-coding region of the EC receptor was generated by additional RT-PCR reactions with upstream primers (sense, 5′-TCAACTTTAGGTTCGCCTGC-3′; antisense, 5′-TCTTGAAGCTGATAAGGCCG-3′) derived from the human OT receptor sequence (bp −271 to +678; Fig. 4). Amplification of this GC-rich region required the addition of 4% dimethylsulfoxide during PCR. Finally, the whole open reading frame of the endothelial receptor was amplified by RT-PCR using a single set of primers (sense, 5′-TCAACTTTAGGTTCGCCTGC-3′; antisense, 5′-TCATCTTCCATCATGGAGGC-3′; bp −271 to +1340). Complete nucleotide sequencing of both strands of these DNA fragments obtained by RT-PCR revealed a perfect match with the sequence of the human uterine OT receptor.

To test the possibility of alternative splicing, parallel amplification of the human uterine OT receptor and the endothelial OT receptor was performed with the primers specific for the 5′-region, the 3′-region, and the whole open reading frame. As shown in Fig. 4, bands of equal size were amplified, thus suggesting that the uterine and endothelial OT receptors share the same coding region.

To rule out the possibility that the amplified OT receptor message was induced during cell culture, total RNA was obtained from primary cultures of HUVECs (first passage) and submitted to RT-PCR. Again, only the primers specific for the OT receptor led to amplification of DNA fragments of the right size (data not shown).

Signal transduction pathways of the OT receptor of human vascular ECs

Mobilization of intracellular calcium. An increase in $[Ca^{2+}]_i$ is the hallmark of activation of the vascular smooth muscle cell AVP V_1 vascular receptor, the AVP V_3 pituitary receptor, and the uterine OT receptor. Thus, we examined whether the OT receptor of human vascular ECs was also coupled to mobilization of intracellular Ca^{2+}. OT stimulation of HUVECs

TABLE 2. Affinity (K_i in nanomolar concentrations) of AVP, OT, and their structural analogs for the receptors expressed in ECV304 cells

Compound	Type	K_i (nM)
AVP		24
OT		1.35
dDAVP	V2 agonist	277
dVDAVP	V2 agonist	381
OPC31260	V2 antagonist	1,346
SR49059	V1 antagonist	36
OPC21268	V1 antagonist	228
$d(CH_2)_5$[O-Me-Tyr^2-Thr^4-Orn^8]Vasotocin	OT antagonist	1.37
$d(CH_2)_5$[O-Me-Tyr^2-Thr^4-Orn^8-Tyr^9-NH_2]Vasotocin	OT antagonist	0.76

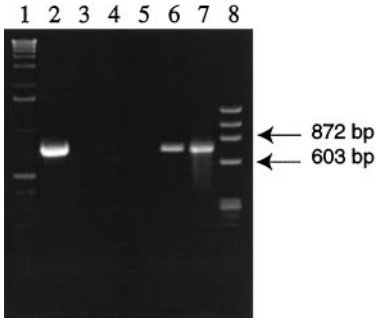

FIG. 3. AVP and OT receptor mRNA expression in human vascular endothelial cells. Total RNA isolated from ECV304 cells was used for RT-PCR reactions as described in *Materials and Methods*. Lane 1 from the *left* is the 1-kb ladder control; lane 2 is the GAPDH-positive control; lanes 3, 4, 5, and 6 correspond to the primers specific for the human V_1 vascular, V_2 renal, V_3 pituitary, and OT receptors, respectively. Lane 7 corresponds to the degenerate primers. Lane 8 is the ϕX174 DNA *Hae*III digest.

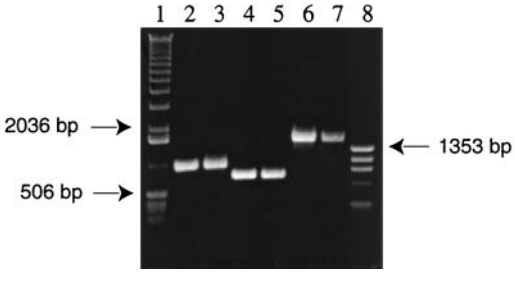

FIG. 4. Amplification of the OT receptor mRNA of human vascular endothelial cells. Total RNA isolated from ECV304 cells was used for amplification of the whole coding region of the endothelial OT receptor. The human uterine OT receptor sequence was simultaneously amplified with the same sets of primers. Lane 1 from the *left* is the 1-kb ladder control, lanes 2 and 3 are amplifications of the 5′-coding region of the uterine and endothelial OT receptors, respectively. Lanes 4 and 5 are amplifications of the 3′-coding region of the uterine and endothelial OT receptors, respectively. Lanes 6 and 7 are amplifications of the whole coding region of the uterine and endothelial OT receptors, respectively. Lane 8 is the ϕX174 DNA *Hae*III digest.

loaded with fura-2 produced within seconds a rapid spike followed by a more sustained mobilization of intracellular Ca^{2+}, with a return to the baseline within 3–5 min after the addition of OT (Fig. 5). This is similar to our previous observations after stimulating vascular smooth muscle cells with AVP (11). OT mobilization of intracellular calcium in HUVECs was specifically blocked by the specific OT antagonist D$(CH_2)_5$-*O*-Tyr(Me)-Thr-Tyr-Orn-vasotocin and was reduced when extracellular calcium was chelated by EDTA (data not shown).

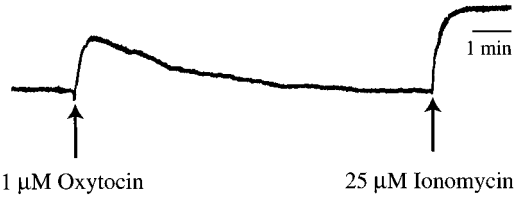

FIG. 5. OT stimulation of calcium mobilization in human vascular endothelial cells. ECV304 cells were grown on glass coverslips and loaded with fura-2 as described in *Materials and Methods*. Basal intracellular Ca^{2+} was measured fluorometrically after reaching thermal equilibrium at 37 C. Cells were stimulated with 1 μM OT in the presence of extracellular calcium. Maximum mobilization of intracellular calcium was induced by 25 μM ionomycin.

Stimulation of the NO pathway. Evidence in the literature suggests that activation of the AVP/OT receptor of ECs leads to a vasodilatory response, but the underlying mechanisms are not known. We assessed directly the production of NO induced by OT in ECs. Consequently, OT stimulation of NO production in primary cultures of HUVECs was measured by quantitating nitrites and nitrates by chemiluminescence using a Sievers NOA280 instrument (12). The following results (n = 4 for each condition) were obtained after 1 min of stimulation (Table 3). Sodium nitroprusside led to a dramatic increase in NO levels (48-fold increase; $P < 0.0007$). As a positive control for receptor-mediated NO stimulation, bradykinin stimulation of HUVECs increased NO release 13-fold ($P < 0.002$). Stimulation of the OT receptor of HUVECs led to NO production (17-fold rise; $P < 0.02$). The effect of OT on NO production was dramatically reduced by chelation of intra- and extracellular calcium.

Absence of stimulation of the cAMP and PG pathways. We also investigated other intracellular pathways, besides the NO pathway, that may have mediated the vasodilating effect of OT in human ECs. Whereas 10 μM forskolin produced a dramatic release of cAMP, no effect of OT was noted. Similarly, stimulation of HUVECs by OT did not produce the release of PGE_2 and 6-keto-$PGF_{1\alpha}$ (data not shown).

Stimulation of cell proliferation. We have shown that human AVP and OT receptor subtypes modulate cellular proliferation (4). Therefore, we studied the effect of OT stimulation on human vascular endothelial cell growth. As shown in Fig. 6, OT stimulation of HUVECs resulted in a dose-dependent proliferative response (EC_{50} = 0.42 nM). The effect of OT on EC proliferation was not altered by the addition of the NO donor sodium nitroprusside, but was slightly reduced in the presence of the NO synthase inhibitor L-NAME (Fig. 7). The protein kinase C inhibitor bisindolylmeleimide significantly

TABLE 3. Effect of OT on nitric oxide production in human ECs

Condition	NO_2 + NO_3 conc. (μM)
Control	2.42 ± 1.08
1 mM sodium nitroprusside	115.65 ± 17.63
100 nM bradykinin	32.18 ± 5.60
100 nM oxytocin	42.04 ± 3.01
100 nM oxytocin + 4 mM EGTA + 10 μM BAPTAAM	7.77 ± 1.30

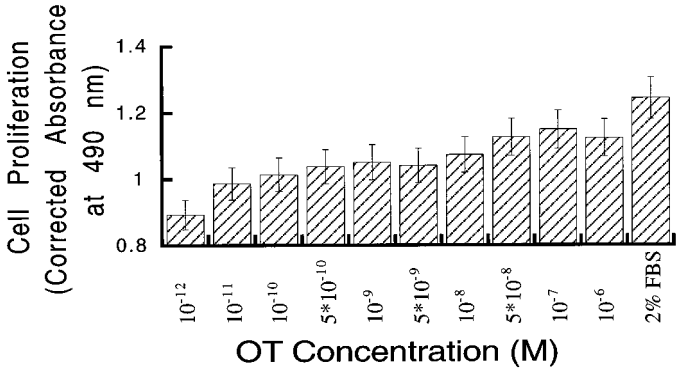

FIG. 6. OT stimulation of human vascular endothelial cell proliferation. HUVECs were grown in 96-well plates as described in *Materials and Methods*. They were stimulated by increasing concentrations of OT for 24 h before addition of the dye solution.

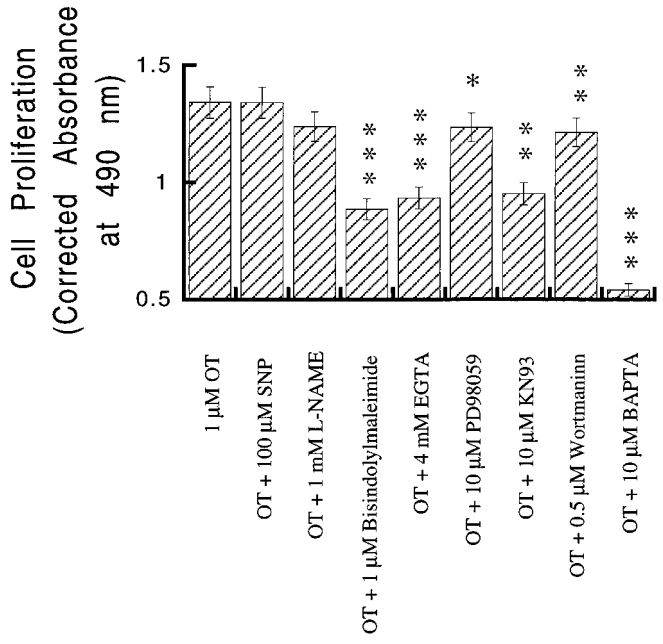

FIG. 7. Effects of various inhibitors on OT stimulation of human vascular endothelial cell proliferation. HUVECs were grown in 96-well plates as described in *Materials and Methods*. They were stimulated for 24 h by OT alone or in the presence of the various compounds before addition of the dye solution (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$).

inhibited the mitogenic effect of OT, whereas the MEK inhibitor PD98059 and the PI3 kinase inhibitor wortmaninn had a limited effect on OT action. Chelation of extracellular calcium by EGTA, chelation of intracellular calcium by BAPTA, and inhibition of calmodulin kinase II by KN-93 dramatically hampered the mitogenic action of OT.

Discussion

The presence of specific AVP/OT receptors in vascular ECs of several species has long been suspected, but the subtype of receptor(s) expressed in ECs remains controversial because of contradictory findings derived from pharmacological studies. Some studies suggested that a AVP V_2 receptor subtype was involved (15, 16). Other studies demonstrated that AVP activated endothelial V_1 receptors (2, 17, 18). Vanner *et al.* reported that 20 nM DDAVP did not alter the vessel diameter of resting or preconstricted submucosal arterioles, and these researchers concluded that they have found no evidence of V_2 receptors in submucosal arterioles (19). These discrepancies are presumably related to several factors, including 1) the administration of large and consequently no longer receptor subtype-specific concentrations of agonists and antagonists, 2) the narrow receptor subtype selectivity of some AVP analogs used in these studies, 3) the interspecies differences between AVP/OT analogs in term of receptor subtype selectivity, 4) the use of various vascular preparations, and 5) the type of vascular bed studied.

Because of these shortcomings, we decided to address this issue in human ECs by combining pharmacological and molecular biological techniques. All radioligand binding data performed in our studies with the natural ligands AVP and OT as well as with two AVP and OT antagonists suggest that human vascular ECs express a single class of high affinity binding sites that belong to the OT receptor subtype. The same binding profile was observed in the three endothelial territories studied, *i.e.* umbilical vein, aorta, and pulmonary artery. Previous pharmacological studies performed in the rat and pig suggested the existence of two subtypes of OT receptors, at least in the uterus (20, 21). Two OT antagonists were found to have different effects on the PG-releasing action of OT in rat endometrial/decidual cells, whereas they were equally effective in blocking the uterotonic action of OT in rat myometrial cells (20). Similarly, the OT antagonist D$d(CH_2)_5$[Tyr(Me)^2]OVT blocked the effect of OT in longitudinal strips of rat myometrium, but did not inhibit the effect of OT in circular strips. We compared the binding affinity constants of the nine AVP/OT analogs used in the present study of human ECs and in our previous study of Chinese hamster ovary (CHO) cells stably expressing the human uterine OT receptor (4). A high level of correlation was found ($r = 0.986$; slope = 1.15; $P < 0.0001$), thus suggesting that the ligand binding profile of the human EC OT receptor is similar to that of the classical uterine OT receptor (Fig. 8). Recent work from Fahrenholz's laboratory indicates that alterations in the myometrial plasma membrane cholesterol content modulates the binding affinity of the OT receptor (22). Moreover, progesterone was found recently to directly alter the uterine sensitivity to OT by interfering with receptor binding and inositol phosphate production (23). All of these observations could be reconciled by considering the existence of a single type of OT receptor whose ligand binding profile and signal transduction pathways are modulated by the receptor phenotypic environment.

Cloning and sequencing of the human vascular EC receptor by RT-PCR clearly indicate that human ECs express an

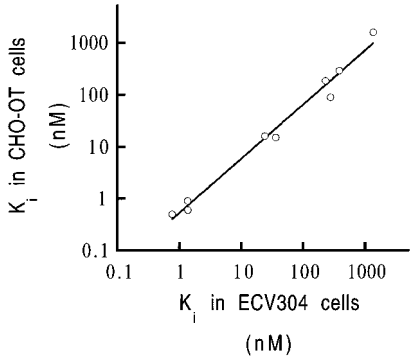

FIG. 8. Correlation between the affinities of AVP/OT analogs for the human vascular endothelial cell receptors and for the human uterine OT receptors stably expressed in CHO cells. The affinity constants of the nine AVP/OT analogs presented in Table 2 were plotted against the affinities of the same compounds tested in CHO cells stably transfected with the human uterine OT receptor.

OT receptor whose open reading frame is similar to that of the uterine OT receptor. Thus, one may conclude that the human OT receptor expressed in these two tissues is the product of the same gene. As a matter of fact, Kimura could not find any additional clone with a different restriction map during his screening process (14). Southern blotting of human genomic DNA with the uterine OT receptor cDNA under low stringency conditions did not detect extra signals (24). Thus, our findings support Kimura's conclusion that there is "little possibility for the presence of OT receptor subtype molecules" (24). Amplification and direct sequencing of the whole coding region of the human EC OT receptor revealed a perfect match with the nucleotide sequence of the uterine OT receptor (14). This homology was important to verify, as heterogeneity in the third intracytoplasmic region of the OT receptor-encoding gene has been observed between species (25). For instance, the sheep OT receptor contains three and two additional amino acids relative to the rat and human receptors. Furthermore, in the rat, two V_2 receptor messenger RNA (mRNA) variants have been identified (26). The long one encodes the adenylyl cyclase-coupled receptor, whereas the short one lacks the nucleotide sequence encoding the putative seventh transmembrane domain and is functionally inactive. From our nucleotide sequencing results, it appears that the human vascular EC OT receptor does not undergo alternative splicing in reference to its uterine homolog. However, this does not preclude the possibility of structural variations in the promoter region and the 3′-untranslated region of the message, an issue that will be addressed in the future.

In terms of signal transduction pathways, OT binding to its uterine receptor is known to produce phospholipase C activation, calcium mobilization, and stimulation of phosphatidyl inositol turnover (24). A recent publication by Ohmichi *et al.* indicates that stimulation of the OT receptor of human uterine myometrial cells induces mitogen-activated protein kinase phosphorylation through a pertussis toxin-sensitive, G protein-coupled mechanism (27). In human myometrial cells, the OT receptor activates phospholipase Cβ by interacting with at least two types of G proteins, a member of the pertussis toxin-sensitive G_i family and a member of the pertussis toxin-insensitive $G_{q/11}$ family (28). The human myometrium OT receptor also couples to the 80-kDa $G_h\alpha$ and can be coimmunoprecipitated with a specific anti-$G_h\alpha$ antibody (29).

The cellular signal transduction pathways linked to the endothelial OT receptor are presently unknown. Our data indicate that the OT receptor of human ECs is coupled to functional intracellular signals. As in the other organs where it is expressed, stimulation of the endothelial OT receptor leads to the mobilization of intracellular calcium. More specific to the vascular endothelial phenotypic environment, the OT receptor stimulates the NO-cGMP vasodilatory pathway, whereas no activation of the cAMP or PG pathways could be elicited. These findings are in agreement with the observation that in rats and dogs, the administration of L-NAME, an antagonist of NO synthase, markedly attenuated the vasodilating effect of DDAVP while not altering its effect on cAMP release (18, 30). A bradykinin antagonist or indomethacin did not affect the hemodynamic effects of DDAVP, suggesting little or no participation of bradykinin or PGs in the hemodynamic responses to DDAVP (18, 30, 31). OT stimulation of calcium mobilization seems to be instrumental in the activation of the NO pathway, as shown by the dramatic reduction of OT-induced NO release when calcium is chelated. Similarly, stimulation of the endothelial $endothelin_b$ receptor induces a transient vasorelaxation through activation of NO via a tyrosine kinase-dependent and calcium-calmodulin-dependent pathway (32).

One may wonder what is the physiological significance of the stimulation of NO production by OT in human vascular ECs. The review of the literature revealed that indeed the administration of OT produces a vasodilating effect (33). There is agreement that these events are related to a peripheral vasodilating effect, not to a direct cardiac effect of OT. The hypotensive effect of large doses of OT has been blamed for severe hypotensive episodes reported in hemorrhagic postpartum situations. In addition to its systemic hemodynamic effect, several studies have suggested that OT influences renal hemodynamics and sodium excretion at physiological concentrations. As reviewed by Conrad *et al.* (34), OT in the rat produces increases in GFR and effective filtration fraction as well as sodium excretion (35). Renal OT receptors are found in the glomeruli, particularly at the vascular pole and the macula densa. The signal transduction pathways of OT receptors include a rise in cytosolic calcium and activation of phospholipase C. The increased cytosolic calcium, in turn, stimulates the constitutive, calcium-calmodulin-dependent NO synthase that activates soluble guanylate cyclase to produce cGMP (36). In conscious male rats, the infusion of OT, producing a plasma concentration of 12.7 $\pm$ 3.3 pmol/liter, led to significant increases in sodium excretion, urine flow, and glomerular filtration rate (37). These alterations were specifically blocked by the coadministration of OT antagonists.

Our studies indicate that the stimulation of endothelial OT receptors leads to cellular proliferation. This finding is in agreement with our observation that the human OT receptor stably transfected in CHO cells is also mitogenic. Numerous mediators have been shown to be involved in the process of endothelial cell migration and proliferation. Obvious candi-

dates include calcium and various kinases that are activated by the OT receptor. However, it is not known what role is played by OT-stimulated NO formation in the mitogenic effect of the endothelial OT receptor. Indeed, NO plays a key role in angiogenesis, although conflicting reports showing both angiogenic and antiangiogenic properties have been presented (38–42). Exogenous NO was shown to inhibit the proliferation of cultured bovine vascular endothelial cells (39), whereas endogenous and exogenous NO production could stimulate the proliferation of ECs at the microvascular level (38). In HUVECs and calf pulmonary artery ECs, NO acts as a crucial signal in the angiogenic response to basic fibroblast growth factor (42). These conflicting results can be explained by several parameters, including the use of different cell lines and cells from different vascular beds, and the experimental conditions of the mitogenic assays (various degrees and duration of serum starvation before agonist stimulation, use of various agonists, stimulation in the presence or absence of FCS, cells plated on plastic, collagen, or fibrin matrix). From our data, one may conclude that the endothelial OT receptor has trophic properties that may participate in the maintenance of the integrity of the vascular endothelial lining. The same property may be shared by other vasoactive peptides, such as angiotensin II, which potentiates vascular endothelial growth factor-induced angiogenic activity (43). The mitogenic action of OT in ECs involves several mediators, including calcium, calmodulin kinase II, and protein kinase C.

In conclusion, this is the first report about the nature and functions of a specific receptor of the AVP/OT family expressed in human vascular endothelial cells. This receptor, which is structurally identical to the uterine OT receptor, activates in a calcium-dependent fashion a vasodilatory pathway and modulates cell proliferation. Our findings, which are related to three vascular territories, do not exclude the possible existence of a second vasoconstricting AVP/OT receptor differentially expressed in other vascular beds. Based on the literature and our present findings, the intracellular pathways coupled to the endothelial OT receptor include the following. OT binding to its endothelial receptor stimulates phospholipase C to produce 1,4,5-trisphosphate, with subsequent influx and internal release of calcium. Calcium activates NOS and NO production. NO stimulates guanylate cyclase to produce cGMP, a potent activator of cGMP-dependent protein kinase G. Also, the formation of diacylglycerol stimulates protein kinase C, which, in turn, modulates the production of NO through activation of NOS. Other possibilities, such as an interaction between protein kinases C and G, may also take place and will be investigated. As a matter of fact, little is known about the interaction of G protein-coupled receptors and NO activation besides the fact that NO negatively modulates the generation of inositol 1,4,5-trisphosphate and diacylglycerol and the ensuing blunting of calcium release (44).

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20th Conference of European Comparative Endocrinologists
Faro, Portugal, 5–9 September 2000

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