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Article

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Vasopressin-induced von Willebrand factor secretion from endothelial cells involves V2 receptors and cAMP

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Vasopressin and its analogue 1-deamino-8-D-arginine vasopressin (DDAVP) are known to raise plasma von Willebrand factor (vWF) levels. DDAVP is used as a hemostatic agent for the treatment of von Willebrand's disease. However, its cellular mechanisms of action have not been elucidated. DDAVP, a specific agonist for the vasopressin V2 receptor (V2R), exerts its antidiuretic effect via a rise in cAMP in kidney collecting ducts. We tested the hypothesis that DDAVP induces vWF secretion by binding to V2R and activating cAMP-mediated signaling in endothelial cells. vWF secretion from human umbilical vein endothelial cells (HUVECs) can be mediated by cAMP, but DDAVP is ineffective, presumably due to the absence of V2R. We report that DDAVP stimulates vWF secretion in a cAMP-dependent manner in HUVECs after transfection of the V2R. In addition, vasopressin and DDAVP induce vWF secretion in human lung microvascular endothelial cells (HMVEC-L). These cells (but not HUVECs) express endogenous V2R, as shown by RT-PCR. Vasopressin-induced vWF secretion is mimicked by DDAVP and inhibited by the selective V2R antagonist SR121463B. It is mediated by cAMP, since it is inhibited by the protein kinase A inhibitor Rp-8CPT-cAMPS. These results indicate that vasopressin induces cAMP-mediated vWF secretion by a direct effect on endothelial cells. They also demonstrate functional expression of V2R in endothelial cells, and provide a cellular mechanism for the hemostatic effects of DDAVP.

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Introduction

von Willebrand factor (vWF) is an adhesive glycoprotein that plays a key role in primary hemostasis, i.e., the adhesion of platelets to the vascular subendothelium (1, 2). It is also a plasma-carrier protein for coagulation factor VIII (FVIII). Circulating vWF is produced by the vascular endothelium, with a minor contribution from platelets/megakaryocytes (3). In endothelial cells (ECs), vWF is synthesized, stored, and released from specialized secretory granules called Weibel-Palade (WP) bodies (4). Quantitative or qualitative vWF deficiency causes von Willebrand's disease (vWD), a common inherited bleeding disorder (1, 2).

It is known that 8-arginine vasopressin (AVP) and its analogue 1-deamino-8-D-arginine vasopressin (DDAVP) raise circulating vWF and FVIII levels. Indeed, they are widely used for the treatment of vWD and other bleeding disorders (5). However, after more than 20 years of clinical use, the cellular mechanisms for DDAVP's hemostatic effects remain poorly understood (6). It is known that, in contrast to AVP, which binds to three cellular receptors (V1a, V1b, V2), DDAVP is a selective agonist for the V2 receptor (V2R). This vasopressin receptor subtype is expressed in the principal cells of renal collecting ducts and mediates the antidiuretic effect of the hor-

mone (7, 8). DDAVP fails to cause an increase in circulating vWF in patients with X-linked diabetes insipidus who possess a mutation in the V2R (9–11). However, in patients with chronic renal failure, DDAVP raises plasma vWF even after bilateral nephrectomy (12). These clinical observations suggest the involvement of extrarenal V2R. The simplest hypothesis then becomes that DDAVP binds directly to endothelial V2R, inducing rapid vWF secretion from WP bodies. However, extrarenal expression of a functional V2R has not yet been demonstrated. Further, several groups have failed to demonstrate DDAVP-induced vWF secretion in cultured ECs (13–15). An alternative hypothesis is that DDAVP acts on an intermediate cell that releases a "vWF-releasing hormone," which then acts on ECs (16). However, the existence and identity of such a hormone remain quite elusive.

Acute vWF release from WP bodies in cultured human umbilical vein ECs (HUVECs) is induced by receptor agonists, which include thrombin and histamine, acting by way of a rise in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) (17–19). Their effect is blocked by intracellular calcium chelators and mimicked by calcium ionophores, which suggest that the rise in $[Ca^{2+}]_i$ indeed mediates vWF secretion (17). A second group

of agonists, acting by way of an increase in intracellular cAMP ($[cAMP]_i$), has also been reported. Forskolin, an activator of adenylyl cyclase (AC), causes an increase in vWF secretion in HUVECs, independent of a rise in $[Ca^{2+}]_i$. This effect is potentiated by IBMX, a phosphodiesterase inhibitor added to prevent cAMP degradation (13). Adenosine, epinephrine, and prostacyclin induce vWF secretion in a cAMP-dependent manner (13, 20–22).

In principal cells of renal collecting ducts, AVP or DDAVP activation of the V2R causes water retention ("antidiuresis") by inducing the translocation of the water channel aquaporin-2 from intracellular stores to the apical plasma membrane. This mechanism represents an example of cAMP-mediated exocytosis (23). Our previous work has shown that HUVECs express all the molecular machinery for cAMP-mediated secretion: only the V2R is missing to allow DDAVP-induced vWF release. However, HUVECs may not be representative, either because of dedifferentiation in culture or because of phenotypic differences among ECs. In this study we tested the hypothesis that V2R-dependent, cAMP-mediated vWF secretion occurs in ECs from vascular beds other than HUVECs. We first undertook to reconstitute DDAVP-induced vWF secretion by transfection of the V2R in HUVECs and by investigation of the second messengers involved. Subsequently, we identified DDAVP-induced vWF secretion and V2R expression in lung microvascular ECs.

Methods
Materials. AVP, DDAVP, 3-isobutyl-1-methyl-xanthine (IBMX), and forskolin were from Sigma (St. Louis, Missouri, USA). Rp-8CPT-cAMPS (24), was from BIOLOG Life Science Institute (Bremen, Germany). SR121463B was kindly provided by C. Serradeil-Le Gal (Sanofi Recherche, Toulouse, France) (25). Anti-vWF antibodies were purchased from DAKO (Glostrup, Denmark) and rhodamine-conjugated goat anti-rabbit antibodies from Jackson ImmunoResearch Laboratories Inc. (West Grove, Pennsylvania, USA). Cell culture reagents were described previously (20).

Cell culture. Primary cultures of ECs (HUVECs) were obtained from pools of individual human umbilical veins by collagenase digestion and grown in RPMI-1640 medium supplemented with 10% FCS, 90 μg/mL heparin, and 15 μg/mL ECGS, as described previously (26). Cells were used during passages 1 or 2.

Primary cultures of human microvascular ECs from lung (HMVEC-L), purchased from Clonetics (Walkersville, Maryland, USA), were grown according to the manufacturer's instructions and used in passages 5 to 8. Five batches from three donors were used. These cells have a typical cobblestone-like morphology and stain positive for acetylated LDL and CD31. More than 90% of the cells show typical WP bodies (see Figure 4).

Transfection of HUVEC. The fusion construct pV2R.EGFP of the wild-type V2R to the NH_2-terminus of the autofluorescent red-shifted variant of the green fluorescent protein (EGFP) has been described previously (27). A control plasmid, pA295.EGFP, encoding the V2R truncated after the sixth transmembrane domain (A295, a naturally occurring V2R mutation found in a patient with X-linked diabetes insipidus, our unpublished observations) and fused to EGFP, was generated by PCR. The V2R.EGFP plasmid was amplified with the primer STV2 (5′-CCCCAGCCCCAC-CATGCTCATGGCGTCC-3′, corresponding to nucleotides –13 to +15 of cDNA sequence as reported by ref. 7) and the antisense primer A295 *Bam*HI (5´-TATG-GATCCGCGGCCCACAGCTGCACCAGG-3′, nucleotides 896–917 plus eight nucleotides introducing a *Bam*HI restriction site). The resulting PCR fragment was cut with *Pst*I/*Bam*HI and cloned into the pV2R.EGFP plasmid. The resulting plasmid was verified by automated DNA sequencing. In COS cells the transfected fusion protein was expressed but was functionally inactive (unpublished observations).

The pV2R.EGFP or pA295.EGFP were transiently transfected into nearly confluent HUVECs using the polyamine transfection reagent TransIT-LT2 (Panvera, Madison, Wisconsin, USA). Cells were split 1 day before transfection into six-well plates, and each well was transfected with 1 μg of DNA and 6 μL of TransIT-LT2 diluted in RPMI-1640. The transfection mixture was incubated with the cells in RPMI-1640 supplemented with 10% decomplemented FCS and 15 μg/mL ECGS (but no heparin) for 4 hours at 37°C. It was then replaced by fresh complete culture medium.

FACS sorting of the EGFP-positive cells. Forty-eight hours after transfection, cells were trypsinized and resuspended in Krebs-Ringer bicarbonate buffer (KRBH; pH 7.4, containing 25 mM HEPES, 5.5 mM glucose, and 0.1% BSA) to a concentration of $0.5–1 \times 10^6$ cells/mL. EGFP-positive and negative cells, maintained at 4°C, were sorted using a FACStar-Plus (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) with an argon laser beam tuned to 488 nm at approximately 40-mW output. The sorting parameters were the EGFP fluorescence measured with a 530 plus or minus 25–nm filter and the cellular size measured as the forward scatter. Sorted cells were seeded into 48-well plates at a minimum density of 40×10^3 cells/well.

Studies of vWF secretion in ECs. Secretion studies were performed in KRBH and vWF was measured by ELISA, as described previously (26).

In HMVEC-L cells, basal release over 30 minutes ranged from 0.3 to 1.7 ng/cm². These values are comparable to those observed for HUVECs in earlier studies (13, 20). Because of the variations in basal release, vWF secretion is reported in relative values, with vWF release from unstimulated cells defined as 100%.

Measurements of cAMP. Cell monolayers were extracted in ice-cold 70% ethanol, and proteins were separated from the extracts by centrifugation. The extracts were dried in a Speedvac (Savant Instruments Inc., Holbrook, New York, USA) and resuspended in 0.05

M acetate buffer. The cAMP was quantified using a commercial radioimmunoassay (Amersham, Little Chalfont, United Kingdom).

Immunofluorescence. HUVECs grown and transfected on glass coverslips were fixed in 3.7% formaldehyde and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline. The vWF was visualized by sequential incubation with purified anti-vWF antibodies (2 μg/mL) and with rhodamine-conjugated goat anti-rabbit antibodies diluted 1:400. The slides were examined with an inverted Zeiss-Axiovert 100 fluorescent microscope (Carl Zeiss, Jena, Germany). Pictures were acquired with a Hamamatsu C4742-95-10 digital charge-coupled device (CCD) camera (Hamamatsu Photonics, Osaka, Japan) controlled by the Openlab software (Improvision, Oxford, United Kingdom).

Isolation of mRNA. Total RNA was isolated from HMVEC-L, HUVECs, or lung tissue specimens using TRIZOL reagent (GIBCO BRL, Gaithersburg, Maryland, USA). Lung tissue was collected from the tumor-free area of pneumonectomy specimens resected for lung carcinoma, kindly provided by L. Nicod and S. Kantengwa (Department of Medicine, University Hospital, Geneva, Switzerland). Normal adult human kidney total RNA was obtained from CLONTECH Laboratories Inc. (Palo Alto, California, USA). Isolation of polyA$^+$ RNA (from 120 to 150 μg of total RNA) was performed using the Oligotex mRNA kit from QIAGEN (Hilden, Germany).

RT-PCR analyses of V2R expression. RT-PCR was performed using the one-tube, two-enzyme Access RT-PCR system from Promega (Madison, Wisconsin, USA) following instructions from the manufacturer. Primers for amplification of the V2R sequence (modified from ref. 28) were obtained from Microsynth (Balgach, Switzerland): V2Rc 5′-CCGCTTCCGTGGGCCAGATGC-3′ (sense strand, positions 309–329) and V2Rd 5′-GGCAGCTGAGCTTCTCAAAGCC-3′ (antisense, positions 1144–1165). These primer sequences are localized in exon 2 and in the 3′-UTR of the *V2R* gene, respectively. The RT-PCR program involved a 45-minute incubation at 48°C (first-strand DNA synthesis), followed by a 2-minute denaturation step at 94°C, 40 cycles of 30-second denaturation at 94°C, 1 minute of annealing at 65°C, 2 minutes of elongation at 68°C, and by a final 7-minute elongation at 68°C. Primers for actin mRNA amplification were described previously (29). PCR products were resolved on 1.5% agarose gels, isolated using the Qiaquick PCR purification kit (QIAGEN) and directly sequenced using primers V2Rc and V2Rd.

In a second set of experiments, the full-length V2R sequence was amplified from human lung polyA$^+$ RNA by RT-PCR using the sense primer STV2 (described above) and the antisense primer V2R*Bam*HI (5′-GACACCCAACGGATCCTCACGATGAAGTG-3′, nucleotides 1104 to 1132 of the cDNA, with introduction of a *Bam*HI restriction site). RT was performed with the Super-Script Preamplification System (GIBCO BRL), using oligo(dT)$_{12-18}$ (without or with prior treatment of RNase-free DNaseI). RNA was removed from RNA/cDNA hybrids with RNase H. PCR was performed with AmpliTaq polymerase. The reaction mixes were submitted to 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute, followed by a final elongation step of 7 minutes. The PCR fragments were subcloned into the pCR 2.1 vector followed by transformation into One Shot competent cells (TA Cloning Kit; Invitrogen, Leek, the Netherlands). The resulting plasmids, harboring the different PCR fragments, were completely sequenced.

Southern blot analysis of the RT-PCR products was performed as described in reference 30. The samples (representing 0.1% of the RT-PCR reaction for HMVEC-L and HUVEC and 0.02% for kidney and lung samples, respectively), transferred to a Nytran membrane (Amersham Pharmacia Biotech), were hybridized with a [^{32}P]-dCTP–labeled probe corresponding to nucleotides 357–1011 of the V2R cDNA, obtained by digestion of pV2R.EGFP with *Pst*I and *Hin*dIII.
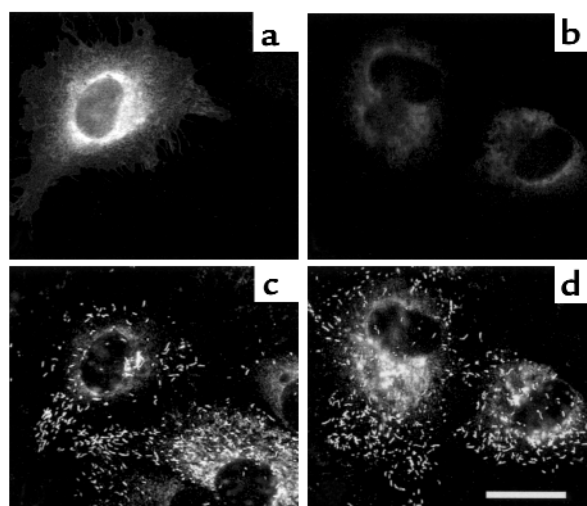
Results

Effect of DDAVP on vWF release from HUVECs expressing V2R.EGFP. To reconstitute DDAVP responsiveness, pV2R.EGFP or pA295.EGFP (coding for the expression of wild-type V2R or the inactive truncation mutant A295, respectively) were transiently transfected into HUVECs. Cells expressing V2R.EGFP, but not those expressing A295.EGFP, showed an expression pattern typical for plasma membrane labeling (Figure 1). In addition, cells expressing each of the two constructs showed intracellular labeling, likely to reflect retention of the protein in the RER and/or in the Golgi apparatus (27). The transfection did not result in depletion of WP bodies, as revealed by immunostaining for vWF (Figure 1).

The transfection efficiency reached up to 25% and 10% of cells expressing the pV2R.EGFP and pA295.EGFP constructs, respectively, measured as the EGFP-positive cells by FACS (not shown). The lower efficiency obtained with the mutant receptor is likely to be due to degradation in the RER. Transfected cells expressing V2R or the A295 mutant were sorted by FACS using EGFP fluorescence. EGFP-positive and -negative cells were returned to culture for 72 hours. These cell populations were then incubated for 1 hour in presence of 100 μM IBMX, either alone or in presence of either 0.1 μM DDAVP or 10 μM forskolin (an activator of AC) as a positive control. The release of vWF into the media was measured by ELISA (Figure 2).

HUVECs expressing V2R.EGFP showed a significant increase in vWF secretion after incubation with DDAVP (from 100 to 243%; $n = 6$, $P < 0.001$). The response to DDAVP was quantitatively similar to that obtained upon forskolin treatment (243 ± 22% vs. 193 ± 20% of the basal release, respectively; $n = 6$, $P < 0.01$ for both substances). DDAVP-induced vWF secretion from cells expressing V2R was still observed upon

Figure 1

Expression of V2R.EGFP or A295.EGFP in HUVECs and immuno-fluorescence staining of the cells for vWF. HUVECs were transfected with pV2R.EGFP or pA295.EGFP. Twenty-four hours later they were fixed, permeabilized, and stained using anti-vWF antibodies followed by rhodamine-conjugated goat anti-rabbit antibodies. Images show EGFP fluorescence from HUVECs expressing V2R.EGFP (**a**) or A295.EGFP (**b**) and distribution of vWF in WP bodies in the same cells (**c**) and (**d**), respectively. Membrane labeling with EGFP was seen only in cells expressing V2R.EGFP, whereas perinuclear labeling was seen with both constructs. Bar, 20 μm.

incubation with lower concentrations of this agonist. Incubation with 0.1 nM DDAVP induced a half-maximal stimulation of vWF release (data not shown). DDAVP alone also stimulated vWF secretion from cells expressing V2R.EGFP, although at lower levels than in the presence of IBMX (to about 150%), but not from untransfected cells (not shown). Incubation with IBMX alone did not significantly affect vWF secretion, as observed previously (13). DDAVP did not induce any significant release of vWF from untransfected HUVECs or from cells expressing the mutant receptor construct A295.EGFP, confirming that DDAVP-induced vWF secretion is the result of the functional expression of transfected V2R.

Effect of DDAVP on [cAMP]$_i$ of HUVECs expressing V2R.EGFP. We next determined whether DDAVP-induced vWF release in V2R-expressing HUVECs is mediated by cAMP. We started by measuring the [cAMP]$_i$ by radioimmunoassay in cells expressing V2R or control cells (Figure 3a). In cells expressing V2R.EGFP incubated with 0.1 μM DDAVP (together with 100 μM IBMX), [cAMP]$_i$ increased to 375 plus or minus 83% compared with cells incubated with IBMX alone ($n = 4$, $P < 0.05$). No increase in [cAMP]$_i$ could be detected in untransfected cells. As a positive control, incubation with 10 μM forskolin and IBMX increased the [cAMP]$_i$ of cells expressing V2R.EGFP or untransfected cells, to 389 plus or minus 81% and 367 plus or minus 78% of their basal [cAMP]$_i$, respectively ($n = 4$, $P < 0.05$ for each cell population). Thus DDAVP-induced vWF secretion is paralleled by a rise in [cAMP]$_i$ in HUVECs expressing V2R.

We next tested the effect of Rp-8CPT-cAMPS (24), which competes with cAMP for its binding site on protein kinase A (PKA). In the presence of Rp-8CPT-cAMPS (0.5 mM), the secretory response to forskolin was reduced by 51 plus or minus 19% in cells expressing V2R ($n = 5$, $P < 0.05$; Figure 3b), an inhibition similar to that observed in untransfected cells (not shown). Similarly, Rp-8CPT-cAMPS inhibited DDAVP-stimu-

lated vWF release by 44 plus or minus 17% in V2R.EGFP-expressing HUVECs ($n = 5$, $P < 0.01$). No significant effect of the inhibitor could be detected on cells incubated with IBMX alone or on untransfected cells incubated with DDAVP (not shown). Taken together, these results indicate that DDAVP-induced vWF secretion is mediated by a rise in [cAMP]$_i$, as it is for the forskolin-mediated vWF secretion.

DDAVP-induced, cAMP-dependent vWF secretion from HMVEC-L cells. The experiments just described show that V2R expression is sufficient to reconstitute DDAVP-induced vWF secretion in HUVECs. These observations raise the possibility that vasopressin-induced vWF secretion, mediated by the V2R and
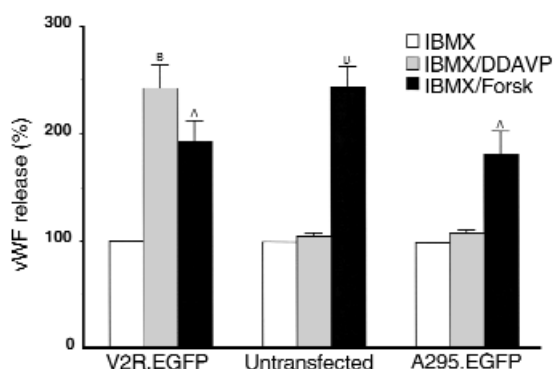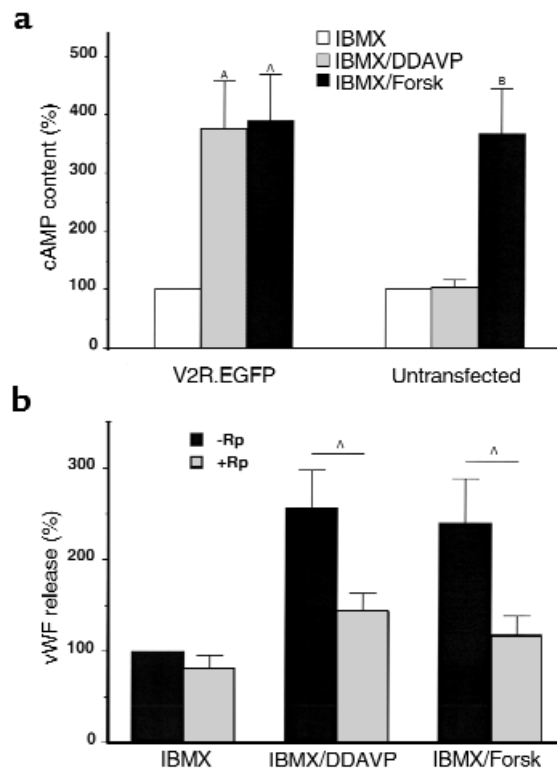

Figure 2

DDAVP-mediated vWF secretion from cells expressing V2R.EGFP. HUVECs were transfected with pV2R.EGFP or pA295.EGFP. Forty-eight hours later, EGFP-positive and -negative cells were sorted by FACS and seeded into 48-well plates. Seventy-two hours later they were incubated for 1 hour in KRBH containing 100 μM IBMX alone (open bars) or in the presence of either 0.1 μM DDAVP (shaded bars), or 10 μM forskolin (filled bars). Then, vWF in the media was measured by ELISA. The results are shown as a percentage of the vWF secreted in basal conditions (IBMX alone). The data are means ± SEM for six independent experiments. ([A]$P < 0.01$; [B]$P < 0.001$ by Student's paired t test).

Figure 3

DDAVP mediates vWF secretion by an increase in $[cAMP]_i$ in cells expressing V2R.EGFP. (**a**) DDAVP-mediated increase in $[cAMP]_i$ in cells expressing V2R.EGFP. HUVECs transfected with V2R were handled as in Figure 2. Sorted EGFP-negative cells were used as controls. At the end of the incubations (with 100 µM IBMX, alone [open bars], in the presence of 0.1 µM DDAVP [shaded bars], or of 10 µM forskolin [filled bars]) cAMP was extracted and quantified by radioimmunoassay. The results are shown as a percentage of the $[cAMP]_i$ in basal conditions (IBMX alone). The data are means ± SEM for four independent experiments. ($^A P < 0.05$; $^B P < 0.01$ by Student's paired t test). (**b**) Inhibition of DDAVP-mediated vWF secretion from cells expressing V2R.EGFP by the PKA inhibitor Rp-8CPT-cAMPS. Seventy-two hours after FACS sorting, V2R.EGFP-positive cells were preincubated for 30 minutes in the absence (filled bars) or presence (shaded bars) of 0.5 mM Rp-8CPT-cAMPS in KRBH. They were then incubated for 1 hour in the presence of 100 µM IBMX alone or together with either 0.1 µM DDAVP or 10 µM forskolin (as in Figure 2). Then, vWF in the media was measured by ELISA. The results are shown as a percentage of the vWF secreted in basal conditions (IBMX alone). The data are means ± SEM for five independent experiments. ($^A P < 0.05$ by Student's paired t test).

cAMP-dependent signaling, is present in ECs from vascular beds other than HUVECs. To address this hypothesis, we tested microvascular ECs from human lung, HMVEC-L. Using indirect immunofluorescence with both anti-vWF (Figure 4) and anti-propeptide (vWF:AgII, not shown) antibodies, HMVEC-L were found to contain WP bodies in numbers comparable to those seen in HUVECs.

HMVEC-L grown in confluent monolayers were incubated for 30 minutes at 37°C with forskolin, AVP (1 µM), or DDAVP (1 µM), in the absence or the presence of IBMX (Figure 5a). The doses of AVP and of DDAVP were selected to obtain maximal stimulation of AC (7). Forskolin, used as a positive control, induced a greater than ninefold increase in vWF secretion, whether in the presence or absence of IBMX. Both AVP and DDAVP caused a small but significant increase in vWF secretion (from 100% to 187 ± 26%, $n = 4$, and 147 ± 21%, $n = 10$, respectively; $P < 0.05$ for both substances). In the presence of IBMX (100 µM), the secretory response was more robust, with increases from 179 plus or minus 43% (IBMX alone) to 340 plus or minus 72% after DDAVP ($n = 10$, $P < 0.05$) and to 490 plus or minus 149% ($n = 4$, $P < 0.05$) after AVP. This represents a 1.9- and 2.7-fold increase in response to DDAVP and AVP, respectively. The small increase in response to IBMX alone was only marginally significant ($P = 0.1$) because it was clearly observed in only one of the cell batches tested.

To address further the involvement of cAMP-dependent signaling in DDAVP-induced secretion, the secretion experiments were repeated in the presence of the PKA inhibitor Rp-8CPT-cAMPS (Figure 5b).

HMVEC-L were preincubated with Rp-8CPT-cAMPS at a concentration of 0.2 mM for 30 minutes before incubation with forskolin, thrombin, or DDAVP for 30 minutes. Rp-8CPT-cAMPS completely blocked the response to forskolin but not to thrombin used as a control $[Ca^{2+}]_i$-raising agent. Thus, Rp-8CPT-cAMPS selectively inhibits cAMP-mediated but not $[Ca^{2+}]_i$-mediated vWF secretion. DDAVP-induced vWF secretion was completely inhibited by this agent, which confirms that cAMP-dependent signaling is involved in the response to DDAVP. We also performed direct measurements of cellular cAMP content (not shown). Although 10 µM forskolin (together with 100 µM IBMX) induced a 5.0 plus or minus 1.8–fold increase in $[cAMP]_i$ ($P = 0.006$, $n = 5$), no significant increase in response to either DDAVP or IBMX added alone was measured. However, DDAVP (1 µM) added together with IBMX caused a significant 20% increase in $[cAMP]_i$ when compared with IBMX alone (from 745 ± 192 to 887 ± 239 fmol/well; $P = 0.01$, $n = 5$). This only modest increase can be explained by the small secretory effect and the variability in cAMP extraction and measurements. Taken together, these results indicate that DDAVP-induced vWF secretion in HMVEC-L is mediated by cAMP-dependent signaling.

AVP-induced vWF secretion in HMVEC-L cells: involvement of V2R. The involvement of V2R in DDAVP-induced cAMP-mediated vWF secretion was addressed in experiments using the selective V2R antagonist SR121463B (25). HMVEC-L cells were incubated with SR121463B (100 µM) together with IBMX (100 µM). After 5 minutes, AVP (1 µM) or adenosine (100 µM, used as a control cAMP-
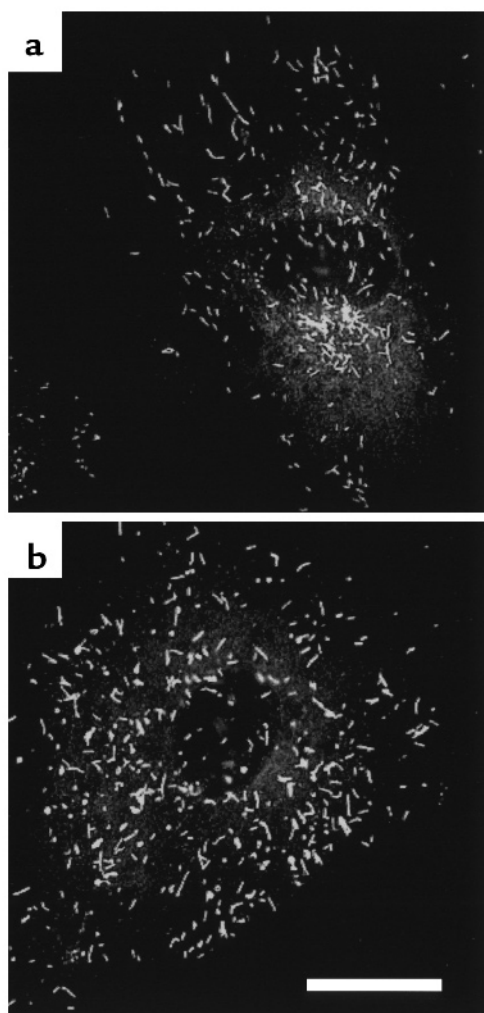
Figure 4
Immunofluorescence staining for vWF in HUVECs (**a**) and HMVEC-L cells (**b**). Cells were fixed, permeabilized, and stained using anti-vWF antibodies as described in Figure 1. Both cell types contain similar numbers of typical WP bodies. Bar, 20 μm.

raising secretion agonist) was added for 30 minutes, and released vWF was measured by ELISA. SR121463B had no significant effect on vWF basal release. However, this antagonist totally inhibited AVP-induced vWF secretion. We also observed a significant increase in vWF secretion in response to adenosine (100 μM), which was not inhibited by SR121463B pretreatment (not shown). Thus, SR121463B specifically inhibited AVP-induced vWF secretion, indicating that the effect of AVP is mediated by V2R.

Expression of V2R in HMVEC-L. To confirm the involvement of V2R in AVP-induced secretion, we verified the expression of this receptor in HMVEC-L by RT-PCR. For this purpose we isolated polyA⁺ RNA from these cells. PolyA⁺ RNA from kidney and from HUVECs was also isolated as positive and negative controls. Presence and integrity of the mRNA samples was performed using actin primers for the RT-PCR. The expected 236-bp band was obtained in all samples as expected (not shown).

Demonstration of V2R mRNA expression was achieved using primers V2Rc and V2Rd, designed to amplify a 857-bp sequence spanning exons 2 and 3. A band of the expected size was obtained from both kidney and HMVEC-L RNA (Figure 6a). Direct sequencing of both bands revealed a 100% homology with the published sequence of the human V2R (7). This band was not observed when the reaction was performed in the absence of RT. It was also not amplified from HUVEC RNA. These results strongly suggest that V2R mRNA is present in HMVEC-L but not in HUVECs, as predicted from the secretion experiments. A larger band of approximately 960 bp was amplified from all mRNA samples and was also detected when the reaction was performed in absence of RT. Sequencing of this band amplified from the HUVEC sample identified the V2R sequence, including the whole second intron. This band thus represents contaminant genomic DNA. Additional RT-dependent bands of lower molecular weight were also obtained for all samples. A Southern blot analysis using a probe derived from V2R cDNA ascertained that these bands were unrelated to the V2R sequence (Figure 6b). It also confirmed the presence of a genomic contaminant in kidney, lung, and HUVEC samples. More importantly, it confirmed the expression of V2R in kidney, lung, and HMVEC-L, but not in HUVECs.

Identification of full-length and alternatively spliced V2R transcripts from human lung polyA⁺ RNA by RT-PCR. Finally, we looked for the expression of V2R in whole human lung. We could, indeed, detect an approximately 860-bp band from lung polyA⁺ mRNA (Figure 6a), which was shown to be V2R by DNA sequencing.

Using other primers (STV2 and V2R*Bam*HI) allowing for amplification of the whole sequence of V2R, we obtained four fragments of different sizes (at about 1,100 bp, 700 bp, 440 bp, and 240 bp) from human lung polyA⁺ RNA of two different donors (Figure 6c, lane 3 and 4). After pretreatment of human lung polyA⁺ RNA with RNase-free DNaseI before RT-PCR (lane 5), only three fragments were identified (at about 1,100 bp, 700 bp, and 240 bp), indicating that the band at 440 bp resulted from genomic contamination. Cloning and sequencing of the PCR fragments revealed that the band at about 700 bp did not harbor any V2R-specific sequence. The 1,100-bp fragment represented the full-length V2R transcript, whereas the band at 240 bp resulted from alternative splicing within the *V2R* gene (unpublished observations). Thus, V2R mRNA is found in whole lung as well as in HMVEC-L.

Discussion
DDAVP is thought to raise plasma vWF levels by increased exocytosis from WP bodies. This is suggested by the rapid effect of the drug (less than 1 hour) and by the appearance of high-molecular-weight vWF multimers typically released from WP bodies (31). The vWF is stored in WP bodies together with its propeptide (vWF:AgII) in a 1:1 molar ratio. DDAVP causes a simultaneous equimolar increase in vWF and propeptide,
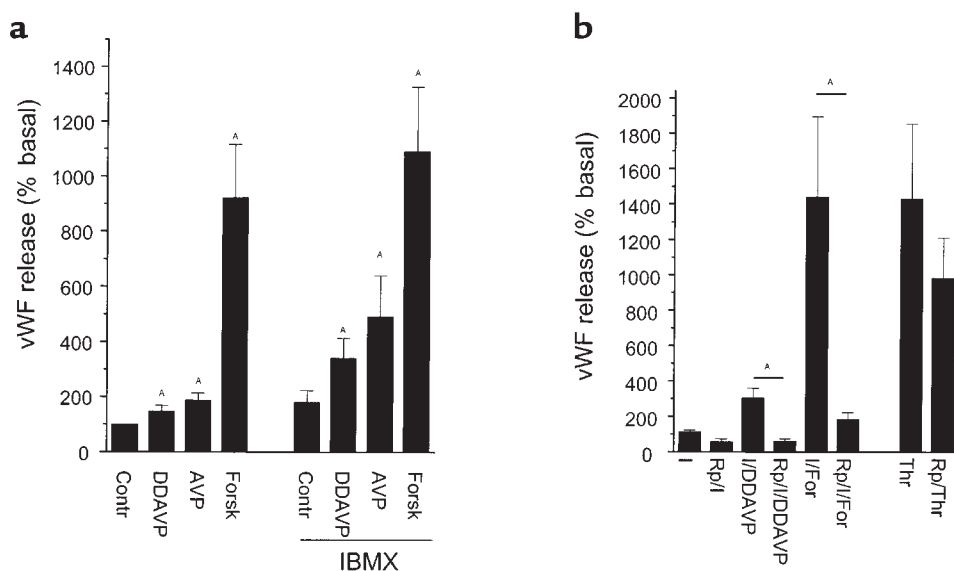
Figure 5

Vasopressin-induced, cAMP-dependent vWF secretion from HMVEC-L cells. (**a**) Confluent HMVEC-L grown in 12-well plates were incubated in KRBH for 30 minutes in the presence of DDAVP (1 µM), AVP (1 µM), forskolin (Forsk; 10 µM), or KRBH alone (Contr). The experiments were performed in the absence or the presence of 100 µM IBMX, and vWF released in the incubation medium was assayed by ELISA. Results are shown as relative values, release from control cells being defined as 100%. The data are the mean ± SEM of 10 (DDAVP and forskolin) or four (AVP) experiments. ([A]$P < 0.05$ by Student's paired t test). (**b**) Confluent HMVEC-L grown in 12-well plates were incubated in KRBH for 30 minutes at 37ºC in the presence or absence of 0.2 mM Rp-8CPT-cAMPS (Rp). Then, 100 µM IBMX (I), 1 µM DDAVP, 10 µM forskolin (Forsk), or 0.5 U/mL thrombin (Thr) were added and the incubation continued for 30 minutes. Results are expressed in relative values, release from control cells being defined as 100%. The vWF released during the preincubation was subtracted, assuming continuous basal release over 1 hour. Data are the mean ± SEM of five experiments. ([A]$P < 0.02$ by Student's paired t test).

again suggesting exocytosis of the two moieties from WP bodies (32, 33). The most obvious explanation is a direct effect of vasopressin or DDAVP on ECs. However, several groups have failed to demonstrate vasopressin-induced vWF secretion from cultured HUVECs (13–15), a finding confirmed in the present study. Further, several reports have shown that in HUVECs exocytosis from WP bodies is mediated by a rise in $[Ca^{2+}]_i$ (17–19). These findings were hard to reconcile with the features of the vasopressin V2R, which is known to activate cAMP-mediated signaling in principal cells of renal collecting ducts.
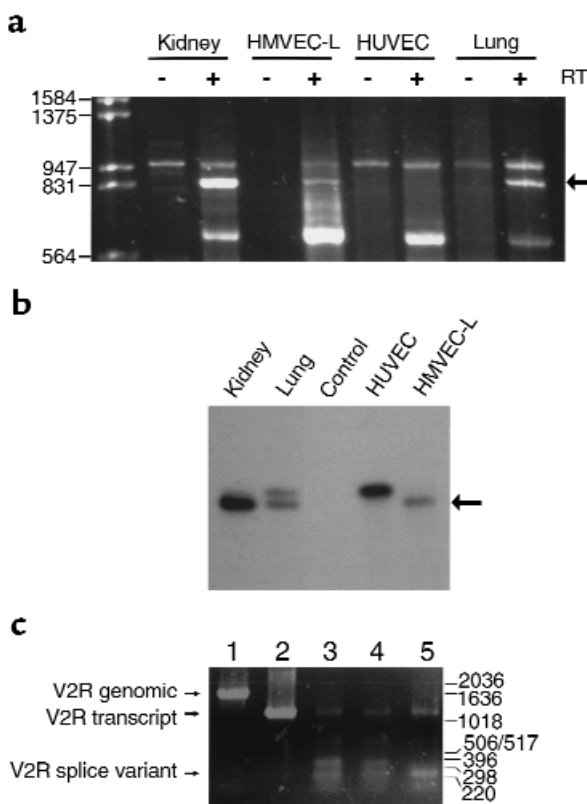
The present data demonstrate that vasopressin and DDAVP can directly induce vWF secretion from ECs by activation of V2 receptors. This effect appears to be mediated by a rise in $[cAMP]_i$. Previous work has shown that a rise in $[cAMP]_i$ is sufficient to induce vWF secretion from WP bodies in HUVECs (13, 20–22). We therefore predicted that expression of V2R in HUVECs would be sufficient to reconstitute DDAVP-induced vWF release from these cells. Indeed, in HUVECs transfected with V2R (but not with the truncated, nonfunctional A295 receptor), DDAVP induced a striking increase in vWF secretion. DDAVP was as effective as forskolin, whereas activation of endogenous AC-coupled receptors (e.g., epinephrine and adenosine receptors) had a relatively smaller effect, as observed in our previous studies (13, 20). This difference is likely explained by the relative over-

expression of the transfected V2R receptor. DDAVP-induced vWF secretion was accompanied by an increase in $[cAMP]_i$ and was inhibited by the PKA inhibitor Rp-8CPT-cAMPS. These data indicate that DDAVP-induced vWF secretion from V2R-transfected HUVECs is mediated by cAMP-dependent signaling. These results strongly encouraged us to test the hypothesis that ECs from other vascular beds express V2R in addition to the same postreceptor molecular machinery for cAMP-dependent exocytosis.

In HMVEC-L cells, both vasopressin and DDAVP induced vWF secretion. This secretory response was observed within 30 minutes, suggesting release from preformed stores (i.e., WP bodies) rather than increased synthesis and constitutive release. The DDAVP response was potentiated by IBMX (added to inhibit endogenous phosphodiesterases) and was associated with a small but significant rise in $[cAMP]_i$. More importantly, the PKA inhibitor Rp-8CPT-cAMPS inhibited vWF secretion in response to DDAVP and forskolin, but not to thrombin. Taken together these results indicate that DDAVP-induced vWF secretion in HMVEC-L cells is mediated by cAMP-dependent signaling. Vasopressin-induced vWF secretion in HMVEC-L cells is due to activation of V2 receptors. This is indicated by the response to DDAVP and by the inhibitory effect of SR121463B, a selective inhibitor of the renal V2 receptors (25). Finally, V2R expression was identified by RT-PCR in HMVEC-L cells but not in HUVECs,

Figure 6

Expression of V2R in HMVEC-L and human lung. (**a**) RT-PCR amplification from human kidney, HMVEC-L, HUVECs, and human lung polyA⁺. The expected 857-bp fragment (arrow) was amplified from V2R mRNA using primers V2Rc and V2Rd. The RT-PCR reaction was performed in absence or presence of RT. First lane: *Hin*dIII/*Eco*RI lambda marker. The gel was stained with ethidium bromide. (**b**) Southern blot analysis of the RT-PCR products amplified from kidney, lung, HUVECs, and HMVEC, using a probe derived from V2R cDNA. Negative control: RT-PCR reaction performed in absence of template. The arrow indicates V2R transcript (857 bp); the band visible just above represents genomic V2R DNA. Five times less of the RT-PCR reaction was loaded on the gel for both kidney and lung compared with HUVECs and HMVEC-L. (**c**) RT-PCR amplification of reversed transcribed polyA⁺ RNA from human lung from two different donors (lanes 3 and 4) using primers STV2 and V2R*Bam*HI. The RT-PCR reaction was performed without (lanes 3 and 4) or with prior pretreatment of the samples with RNase-free DNaseI (lane 5). The size of the fragment expected to be amplified from V2R mRNA is 1,144 bp, as verified by amplification of plasmid DNA carrying the full-length coding region of the V2R (lane 2). Lane 1: RT-PCR amplification of plasmid DNA harboring the genomic DNA of the V2R (expected size: 1,611 bp). The gel was stained with ethidium bromide.

as predicted from the secretion studies. Our results firmly establish that vasopressin can directly induce endothelial vWF secretion and demonstrate — to our knowledge for the first time — that V2R are functionally expressed in vascular cells.

Our study identified the V2R in whole human lung tissue, thereby extending the results of Fay et al. (28). Indeed, the full-length receptor was amplified from human lung RNA by RT-PCR with a nucleotide sequence identical to the published sequence of the renal V2R. This result supports our conclusion that vasopressin-induced, V2R-mediated vWF secretion in HMVEC-L cells reflects a physiological phenomenon rather than ectopic V2R expression in cultured ECs. We identified V2R expression in HMVEC-L cells but not in HUVECs, an observation that raises the possibility that V2R expression is restricted to specific vascular beds. It is possible that V2R is functionally expressed in additional tissues. A complete study of cell type- and tissue-specific expression of the V2R remains to be performed.

A cAMP-mediated secretion is most likely to be relevant for the physiological regulation of plasma vWF levels. Indeed, epinephrine infusion raises plasma vWF levels, and physical activity increases plasma vWF levels by catecholamines acting on β2-adrenergic, AC-coupled receptors (34, 35). Most [Ca²⁺]ᵢ-raising agonists are mediators of inflammation and/or thrombosis that also induce cell retraction. In contrast, cAMP-raising agonists preserve cell-cell junctions, a property more compatible with the physiological, systemic regulation

of vWF secretion (36). The effects of vasopressin and DDAVP on vWF secretion in HMVEC-L cells were small and were best demonstrated in the presence of IBMX. In contrast, [Ca²⁺]ᵢ-raising agents such as thrombin induce a much stronger vWF secretory response both in HMVEC-L (Figure 5b) and in HUVECs (20). However, this weak secretory response (as well as the dependence on IBMX) is a general feature of cAMP-raising agents such as adenosine and epinephrine (13, 20). It is possible that V2R expression is low in HMVEC-L compared with ECs in vivo as a result of dedifferentiation in culture. Another possibility is that constitutive vWF secretion is increased in culture, which would lead to an underestimation of the relative increase in regulated secretion after DDAVP. Increased constitutive release is reported for several types of secretory cells, compared with the parent tissue (e.g., ref. 37). Further, it is possible that increased levels of phosphodiesterases in cultured cells blunt the response to cAMP-raising agents, accounting for the potentiating effect of IBMX.

Several authors have proposed an indirect mechanism for DDAVP-induced vWF secretion: DDAVP activates an intermediate cell, which in turn secretes a vWF-releasing hormone that acts on ECs (6, 16). Our data make this hypothesis less attractive, although the existence of two parallel mechanisms cannot be excluded. Hashemi et al. have suggested that platelet-activating factor (PAF) released from macrophages in response to DDAVP induces vWF secretion from ECs (16). However, these authors have not demonstrated

V2R expression in macrophages. The observation that pretreatment with a PAF inhibitor does not suppress DDAVP-induced vWF secretion in dogs also argues against this hypothesis (38).

DDAVP raises not only vWF levels, but also tissue-type plasminogen activator (t-PA) and FVIII levels (39, 40). The t-PA is synthesized, stored, and released from ECs. We and others have reported that t-PA can be stored in WP bodies in cultured HUVECs (41, 42). A distinct storage compartment (consisting of small, round granules) has also been described (43). The endothelial storage compartment for t-PA remains to be confirmed in vivo and may vary between tissues. Colocalization of vWF and t-PA in WP bodies would imply that a single regulatory mechanism accounts for the increase in the plasma levels of the two proteins. Even if t-PA is stored in a distinct granule pool, it appears very likely that this pool also responds to V2R activation with cAMP-mediated secretion (22). Similarly, Rosenberg et al. have shown that heterologous expression of FVIII, transfected into EC, colocalizes with vWF in WP bodies (44). If this colocalization is confirmed in vivo, DDAVP-induced FVIII increase could also be explained by the same regulatory mechanism.

In addition to its hemostatic effects, DDAVP has vasodilator properties that are unlikely to result from renal V2R activation (9). Our finding that V2R is functionally expressed in ECs raises the possibility that DDAVP induces the synthesis of endothelium-dependent vasodilators. One candidate is prostacyclin. However, we have shown that prostacyclin production is inhibited by cAMP (21). DDAVP could induce endothelial nitric oxide (NO) production, but a link between activation of cAMP-dependent signaling pathways and NO production remains to be established.

In summary, we have shown that vasopressin and its analogue DDAVP induce vWF secretion from cultured ECs. This effect is due to V2R activation and is mediated by cAMP-dependent signaling. V2R expression was shown in cultured lung ECs and in whole lungs, providing evidence for the functional extrarenal expression of this receptor. These observations provide a cellular mechanism for the hemostatic effect of DDAVP.

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