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Vasopressin increases vascular endothelial growth factor secretion from human vascular smooth muscle cells

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Abstract

Vascular endothelial growth factor (VEGF) is a potent and specific mitogen of vascular endothelial cells which promotes neovascularization in vitro. To determine whether vasopressin induces VEGF secretion in human vascular smooth muscle cells, we performed enzyme-linked immunosorbent assays. Vasopressin potently induced a time-dependent and concentration-dependent (maximal, 10^{-7} M) increase in VEGF secretion by human vascular smooth muscle cells that was maximal after 24 h. Furthermore, vasopressin also concentration-dependently caused mitogenic effect, as reflected by total protein content of cells per culture well. These vasopressin-induced VEGF secretion increase and mitogenic effect of these cells were potently inhibited by vasopressin V_{1A} receptor antagonists, confirming this is a vasopressin V_{1A} receptor-mediated event. These results indicate that vasopressin increases VEGF secretion in human vascular smooth muscle cells, the magnitude of VEGF secretion being temporally related to the mitogenic effect of vascular smooth muscle cells and the potency of the growth-promoting stimulus. Vasopressin-induced VEGF secretion by proliferating vascular smooth muscle cells could act as a paracrine hormone to powerfully influence the permeability and growth of the overlying vascular endothelium, vasopressin play a more fundamental role in the regulation of vascular function than has previously been recognized. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Vasopressin; VEGF (vascular endothelial growth factor); Smooth muscle cell, vascular

1. Introduction

Vascular smooth muscle cells proliferation and endothelial dysfunction are key events in the pathogenesis of atherosclerosis (Ross, 1993). Vasopressin exerts potent mitogenic and hypertrophic effects on vascular smooth muscle cells, however, whether vasopressin can also influence endothelial function is largely unknown. Disturbed endothelial function during the initiation and early propagation of atherosclerotic lesions is characterized by increased permeability of vessels to circulating macromolecules. Although, considerable attention has focused on the pathogenesis of endothelial dysfunction, the factors and mechanisms responsible remain poorly defined. Recently, vascular endothelial growth factor (VEGF), also referred to as vascular permeability factor (VPF), has been identified that is produced predominantly in vascular smooth muscle cells (Leung et al., 1989). VEGF is a 34–42 kDa heparin binding mitogen and hyperpermeability-inducing growth factor, which exhibits low but significant homology to the A and B chain of platelet-derived growth factor (Keck et al., 1989). The mRNAs of several isoforms of the VEGF family, $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$, are generated by alternative splicing from the same gene (Tischer et al., 1991). $VEGF_{165}$ is the predominant isoform secreted by a variety of normal and transformed cells (Ferrara et al., 1992). Furthermore, VEGF is among the most potent vascular permeability-enhancing factors so far identified, some 50,000 times more potent than histamine (Senger et al., 1990). This potent action of VEGF makes it an attractive candidate as a mediator of normal and pathological changes in vascular

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permeability. The biologic effects induced by VEGF are transmitted by at least two specific transmembrane receptors, FLT-1 (*fms*-like tyrosine kinase) and KDR (kinase insert-domain-containing receptor) (De Vries et al., 1992; Terman et al., 1992), which belong to the class III tyrosine kinase receptor family.

Vasopressin is involved in the regulation of vascular tone by inducting vascular smooth muscle cell contraction (Caramelo et al., 1989). Vasopressin also exerts potent mitogenic and hypertrophic effects on vascular smooth muscle cells (Geisterfer and Owens, 1989; Serradeil-Le Gal et al., 1995; Tahara et al., 1997a). Almost all studies that have examined the cardiovascular actions of vasopressin have focused on vascular smooth muscle cells. Whether vasopressin can also influence endothelial function is unknown, despite the fact that the vascular endothelium plays a critical role in regulating various aspects of vascular function, including vascular tone, growth and permeability. It has previously been reported that the expression of VEGF mRNA by human vascular smooth muscle cells is markedly upregulated upon exposure to angiotensin II (Williams et al., 1995). It has also been established that vasopressin stimulates a similar signaling cascade in vascular smooth muscle cells as angiotensin II (Caramelo et al., 1989; Tahara et al., 1997a). In the present study, the ability of vasopressin to stimulate secretion of VEGF protein, and the effects of vasopressin receptor antagonists on vasopressin-induced VEGF secretion were investigated in cultured human vascular smooth muscle cells.

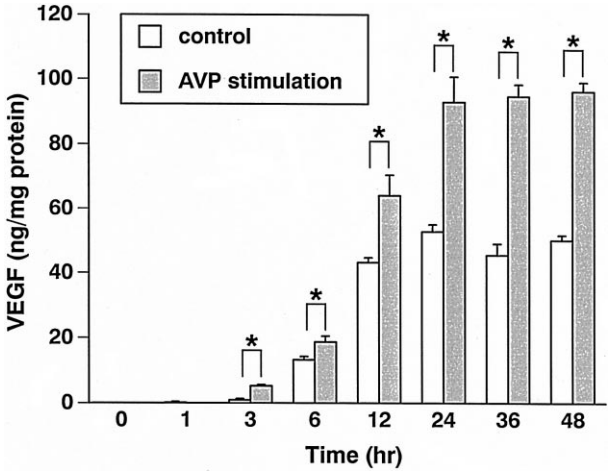

Fig. 1. Time-dependent secretion of vascular endothelial growth factor (VEGF) by human vascular smooth muscle cells stimulated by vasopressin. Cells were serum-starved for 48 h and stimulated by the addition of vehicle or 100 nM vasopressin in conditioned medium, SmGM-2 culture medium supplemented with 0.5 μg/ml human epidermal growth factor, 5 mg/ml insulin, 1 μg/ml human fibroblast growth factor, 0.5% fetal calf serum and 0.1% bovine serum albumin, for the indicated times. Each point represents the means ± S.E.M. of 6 independent experiments. $* P < 0.05$ indicate statistically significant differences from the corresponding values for vehicle treatment (control).

2. Materials and methods

2.1. Materials

Vasopressin was obtained from Peptide Institute (Osaka, Japan). YM087 (4′-[(2-methyl-1,4,5,6-tetrahydroimidazo[4,5-*d*][1]benzazepin-6-yl)carbonyl]-2-phenylbenzanilide monohydrochloride, vasopressin V_{1A}/V_2 receptor antagonist) (Tahara et al., 1997b), SR 49059 ((2*S*) 1-[(2*R* 3*S*)-(5-chloro-3-(2-chlorophenyl)-1-(3,4-dimethoxybenzene-sulfonyl)-3-hydroxy-2,3-dihydro-1*H*-indole-2-carbonyl]-pyrrolidine-2-carboxamide, vasopressin V_{1A} receptor selective antagonist) (Serradeil-Le Gal et al., 1993) and SR 121463A (1-[4-(*N-tert*-butyl-carbamoyl)-2-methoxybenzene sulfonyl]-5-ethoxy-3-spiro-[4-(2-morpholinoethoxy)-cyclohexane]indol-2-one; equatorial isomer, vasopressin V_2 receptor selective antagonist) (Serradeil-Le Gal et al., 1996) were synthesized at Yamanouchi Pharmaceutical (Ibaraki, Japan). The structures of these compounds were determined by ^{1}H-nuclear magnetic resonance, mass spectrometry and elemental analysis. Their purity was measured by high-pressure liquid chromatography and was > 98%. These nonpeptide antagonists were initially dissolved in dimethyl sulfoxide (DMSO) at 10^{-2} M and diluted to the desired concentration with the assay buffer. Fetal calf serum and trypsin–EDTA were from Gibco (Grand Island, NY, USA). Bovine serum albumin was from Nacalai Tesque (Kyoto, Japan).

Table 1
Time dependence of vasopressin-induced vascular endothelial growth factor (VEGF) secretion and mitogenic effect in human vascular smooth muscle cells

		1 h	3 h	6 h	12 h	24 h	48 h
VEGF concentration (ng/ml)	control	< 0.01	0.03 ± 0.01	0.41 ± 0.02	1.30 ± 0.03	1.82 ± 0.06	2.29 ± 0.10
	vasopressin 10^{-7} M	< 0.01	0.18 ± 0.01[a]	0.71 ± 0.04[a]	2.45 ± 0.29[a]	4.08 ± 0.29[a]	4.72 ± 0.22[a]
Protein content (μg/well)	control	7.64 ± 0.35	8.37 ± 0.58	7.81 ± 0.40	7.53 ± 0.29	8.66 ± 0.34	11.5 ± 0.52
	vasopressin 10^{-7} M	7.15 ± 0.39	8.97 ± 0.79	9.78 ± 0.82[a]	9.65 ± 0.65[a]	11.2 ± 0.64[a]	12.3 ± 0.39
VEGF secretion (ng/mg cell protein)	control	< 0.3	0.93 ± 0.35	13.2 ± 1.06	43.3 ± 1.38	52.70 ± 2.29	50.1 ± 1.68
	vasopressin 10^{-7} M	< 0.3	5.20 ± 0.41[a]	18.7 ± 1.83[a]	64.1 ± 6.43[a]	92.9 ± 7.81[a]	96.2 ± 2.83[a]

Values are the means ± S.E.M. obtained from 6 independent experiments. [a] $P < 0.05$ indicate statistically significant differences from the corresponding values for vehicle treatment (control).

Table 2
Concentration dependence of vasopressin-induced vascular endothelial growth factor (VEGF) secretion and mitogenic effect in human vascular smooth muscle cells

	Control	Vasopressin					
		10^{-11} M	10^{-10} M	10^{-9} M	10^{-8} M	10^{-7} M	10^{-6} M
VEGF concentration (ng/ml)	1.70 ± 0.07	1.74 ± 0.07	1.82 ± 0.12	2.38 ± 0.14[a]	3.00 ± 0.19[a]	4.11 ± 0.24[a]	4.42 ± 0.11[a]
Protein content (μg/well)	6.86 ± 0.28	7.16 ± 0.24	7.08 ± 0.25	7.15 ± 0.33	7.33 ± 0.20	8.55 ± 0.27[a]	8.99 ± 0.22[a]
VEGF secretion (ng/mg cell protein)	62.4 ± 4.02	61.4 ± 3.87	64.1 ± 3.09	84.0 ± 5.46[a]	102 ± 4.98[a]	121 ± 7.07[a]	123 ± 2.63[a]

Values are the means ± S.E.M. obtained from 6 independent experiments. [a] $P < 0.05$ indicate statistically significant differences from the corresponding values for vehicle treatment (control).

2.2. Cell culture

Human vascular smooth muscle cells imported from Clonetics (San Diego, CA, USA) were purchased from IWAKI (Tokyo, Japan). The cells were grown at 37°C in SmGM-2 culture medium (Clonetics) supplemented with 0.5 μg/ml human epidermal growth factor, 5 mg/ml insulin, 1 μg/ml human fibroblast growth factor, 5% fetal calf serum and antibiotics (GA-1000) in a humidified atmosphere of 5% CO_2 in air. The cultures were subcultured every 7 days in a 150 cm^2 culture dish, using 0.05% trypsin–0.53 mM EDTA, with culture medium changed every 3 days. Cells from passage 5–8 were used for the experiment and were identified histochemically by anti-α-actin and factor VIII antibody.

2.3. VEGF secretion by vascular smooth muscle cells

Vascular smooth muscle cells were seeded into 24-well culture plates at 70–80% confluence ($0.8–1.2 \times 10^5$ cells/well), washed with phosphate-buffered salines (PBS) and incubated in serum-free culture medium for 48 h. The cultures were then incubated in conditioned medium, SmGM-2 culture medium supplemented with 0.5 μg/ml human epidermal growth factor, 5 mg/ml insulin, 1 μg/ml human fibroblast growth factor, 0.5% fetal calf serum and 0.1% bovine serum albumin, containing vehicle alone or various concentrations of vasopressin, antagonists, or both. After incubation, the conditioned media (250 μl/well) were collected and centrifuged. These supernatant was stored at −80°C until assay. To determined total protein of cells per culture well, the cell layers were then washed three times with ice-cold PBS and scraped from the plates. The suspensions were homogenized and protein was determined by the Coomassie blue method (Bio-Rad, Hercules, CA, USA), with bovine serum albumin as a standard.

2.4. VEGF protein assay

The VEGF concentrations in the conditioned medium were measured by a highly sensitive sandwich enzyme-linked immunosorbent assay (ELISA). Each well of 96-well microtiter plates was coated with the monoclonal antibody MA-851-5 (AUSTRAL Biologicals, CA, USA) (1 μg/ml, 100 μl/well) in PBS and incubated overnight at 4°C. The plates were washed with PBS containing 0.1% gelatin and 0.05% Tween 20 (gelatin/Tween buffer), blocked with PBS containing 0.1% gelatin for 1 h at 37°C and rinsed twice with gelatin/Tween buffer. Between washes in the following steps, wells were rinsed three times with gelatin/Tween buffer. Standard solutions were freshly prepared by dilution of recombinant human $VEGF_{165}$ (R&D Systems, MN, USA) in gelatin/Tween buffer. Diluted standards and samples were dispensed into coated plates (100 μl/well) and the plates were incubated at 37°C for 1 h. Wells were washed and rabbit anti-human VEGF polyclonal antibody A-20 (Santa Cruz Biotechnology, CA, USA) was added (diluted 1:1000 in gelatin/Tween buffer, 100 μl/well) and incubated at 37°C for 1 h. The wells were again washed, horseradish peroxidase-labeled anti-rabbit immunoglobulin G (diluted 1:1000 in gelatin/Tween buffer, 100 μl/well) was added, and the cells incubated at 37°C for 1 h. After final wash, substrate for horseradish peroxidase (*o*-phenylenediamine 2HCl, 4 mg in 10 ml of 0.1 M citrate-$NaHPO_4$ buffer, pH 4.5, containing 4 μl of 35% H_2O_2) was added (100 μl/well) and color developed at room temperature. The reaction was stopped by adding 50 μl 2 N H_2SO_4 and absorbance at 492 nm was mea-

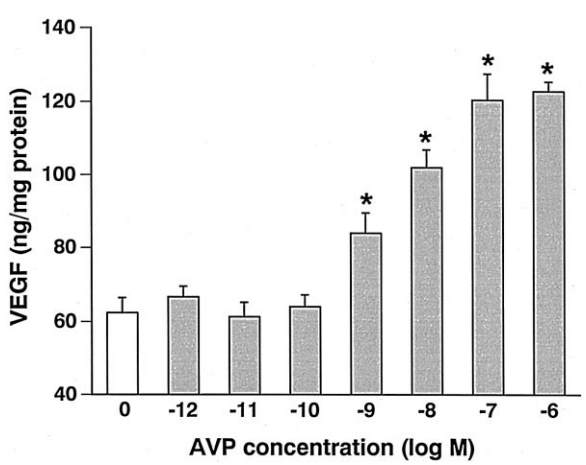

Fig. 2. Concentration-dependent secretion of vascular endothelial growth factor (VEGF) by human vascular smooth muscle cells stimulated by vasopressin. Cells were incubated in conditioned medium with various concentrations of vasopressin for 24 h. Values are expressed as the means ± S.E.M. of 6 independent experiment. * $P < 0.05$ indicate statistically significant differences from the corresponding values for vehicle treatment.

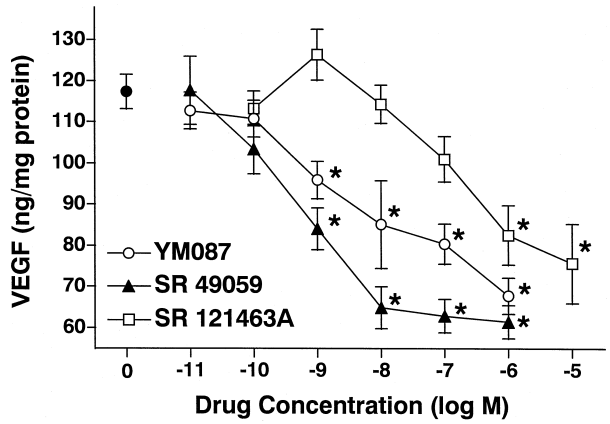

Fig. 3. Inhibitory effects of vasopressin receptor antagonists on vasopressin-stimulated vascular endothelial growth factor (VEGF) secretion in human vascular smooth muscle cells. Cells were incubated in conditioned medium with various concentrations of vasopressin receptor antagonists and vasopressin (100 nM) for 24 h. Each point represents the means ± S.E.M. for 6 independent experiments. $* P < 0.05$ indicate statistically significant differences from the corresponding values for 100 nM vasopressin treatment.

sured. A standard curve was generated by plotting absorbance vs. VEGF concentration using nonlinear regression fitting and detection limits were 10 pg/ml. Concentrations of VEGF in the samples were calculated by interpolation from the standard curve and results were normalized per cellular protein contents.

2.5. Statistical analysis

Experimental results are expressed as the means ± S.E.M. or the means with 95% confidence limits. Statistical comparison by one-way analysis of variance (ANOVA) followed by Dunnet's multiple-range test. Comparison of two different groups was done by using the unpaired Student's *t*-test. A value of $P < 0.05$ was taken to be significant.

3. Results

Time-dependent secretion of VEGF by human vascular smooth muscle cells stimulated by vasopressin is shown in Table 1. When vascular smooth muscle cell monolayers were incubated with conditioned medium, SmGM-2 culture medium supplemented with 0.5 μg/ml human epidermal growth factor, 5 mg/ml insulin, 1 μg/ml human fibroblast growth factor, 0.5% fetal calf serum and 0.1% bovine serum albumin, VEGF secretion by vascular smooth muscle cells was increased in a time-dependent manner. Vasopressin (100 nM) significantly enhanced VEGF secretion in a time-dependent manner compared to conditioned medium treated cells, and this increase in VEGF secretion was not observed up to 1 h after stimulation by vasopressin, but was clearly detected at 3 h and thereafter (Fig. 1). Vasopressin-induced VEGF secretion increased almost linearly over the first 24 h and then gradually for up to 48 h. Table 2 shows the concentration-dependent increases in VEGF secretion from vascular smooth muscle cells stimulated with various concentrations of vasopressin. The minimal stimulatory effect of vasopressin on VEGF secretion from vascular smooth muscle cells was observed at a concentration of 1 nM and its maximal effect was observed at a concentration of 100 nM (Fig. 2). The EC_{50} value of vasopressin was 2.57 (1.08–6.16) nM and maximal stimulation was 190% over that of conditioned medium-treated cells. Furthermore, vasopressin significantly and concentration-dependently increased total protein content of cells per culture well with a maximal increase of 130% over that of conditioned medium-treated cells (Table 2). The effects of various vasopressin receptor antagonists were then studied in preparations stimulated with 100 nM vasopressin (Fig. 3). SR 49059, a selective vasopressin V_{1A} receptor antagonist, potently and concentration-dependently inhibited vasopressin-stimulated VEGF secretion with an IC_{50} value of 0.49 (0.13–1.90) nM. In contrast, SR 121463A, a selective vasopressin V_2 receptor antagonist, did not potently inhibit vasopressin-stimulated VEGF secretion, showing an IC_{50} value of 266 (67.8–1040) nM. When tested under the same experimental conditions, YM087, a vasopressin V_{1A}/V_2 receptor antagonist, potently inhibited vasopressin-stimulated VEGF secretion (Fig. 3 and Table 3) with an IC_{50} value of 1.21 (0.37–3.92) nM. Furthermore, YM087 inhibited AVP-induced mitogenic effect in a concentration-dependent manner. Under the same experimental conditions, SR 49059 also inhibited AVP-induced mitogenic effect, however,

Table 3
Effect of YM087 on vasopressin-induced vascular endothelial growth factor (VEGF) secretion and mitogenic effect in human vascular smooth muscle cells

	Control	Vasopressin 10^{-7} M	+ YM087				
			10^{-10} M	10^{-9} M	10^{-8} M	10^{-7} M	10^{-6} M
VEGF concentration (ng/ml)	1.80 ± 0.08	4.39 ± 0.14[a]	4.36 ± 0.11	3.51 ± 0.10[b]	2.48 ± 0.09[b]	2.02 ± 0.13[b]	1.79 ± 0.12[b]
Protein content (μg/well)	6.19 ± 0.40	9.33 ± 0.20[a]	9.89 ± 0.27	9.22 ± 0.37[b]	7.67 ± 0.66[b]	6.31 ± 0.31[b]	6.64 ± 0.27[b]
VEGF secretion (ng/mg cell protein)	73.9 ± 3.71	117 ± 1.60[a]	111 ± 4.45	95.9 ± 4.53[b]	85.1 ± 10.7[b]	80.4 ± 4.93[b]	67.7 ± 4.40[b]

Values are the means ± S.E.M. obtained from 6 independent experiments. [a]$P < 0.05$ indicate statistically significant differences from the corresponding values for vehicle treatment (control). [b]$P < 0.05$ indicate statistically significant differences from the corresponding values for 10^{-7} M vasopressin treatment.

SR 121463A did not potently inhibited AVP-induced mitogenic effect (data not shown). Each antagonist, up to concentrations of 1 μM, had no effect on VEGF secretion and hyperplasia in the absence of vasopressin (data not shown).

4. Discussion

Accelerated vascular smooth muscle cell growth plays a fundamental role in the formation and propagation of atherosclerotic lesions in man (Schwartz et al., 1986; Ross, 1993). The present study demonstrates that AVP-induced vascular smooth muscle cell growth potently causes the secretion of VEGF from human vascular smooth muscle cells, the magnitude of the response being directly related to the concentration of the growth-inducing stimulus. These observations demonstrate that the secretion of VEGF from human vascular smooth muscle cells is temporally related to the growth phase of the cell and proportional to the potency of the growth-promoting stimulus.

The synthesis of the endothelial cell growth factor VEGF is stimulated by a variety of activators, however, the effects of important vasoactive peptides on VEGF synthesis are not well understood. Previous studies have shown that growth factors including platelet-derived growth factor (PDGF), transforming growth factor-β1, basic-fibroblast growth factor, prostaglandins, angiotensin II and endothelin-1 can stimulate the production/secretion of VEGF (Tsai et al., 1995; Williams et al., 1995; Pedram et al., 1997). The present study has demonstrated that vasopressin also stimulates the secretion of VEGF from cultured human vascular smooth muscle cells. Vasopressin-induced VEGF secretion in vascular smooth muscle cells was rapid, which was detected at 3 h and maximal within 24 to 36 h. The rapidly of this response is consistent with previous report of PDGF-induced VEGF secretion in lung fibroblasts and pulmonary vascular smooth muscle cells, which is detected at 6 h and maximal within 12 to 15 h (Nauck et al., 1997). The vasopressin concentration required to maximally induce VEGF secretion is 100 nM and this concentration of vasopressin is considerably higher than normal circulating levels of vasopressin. Simon et al. (1992) reported that rat and cow blood vessels contain vasopressin which is of local rather than hypothalamopituitary origin. It has also reported that vasopressin immunoreactivity exists in cultured vascular endothelial cells derived from rat and rabbit aorta (Lincoln et al., 1990; Loesch et al., 1991). Therefore locally produced vasopressin may elicit physiologic effects of vascular smooth muscle cells including secretion of VEGF in either an autocrine or paracrine fashion.

The accumulation of neointimal smooth-muscle cells resulting from media smooth-muscle proliferation and migration in response to vascular injury is believed to be one of the main events involved in the initiation of atherosclerosis (Schwartz et al., 1986). Arterial smooth muscle cell proliferation also contributes to the long-term changes in vascular structure that develop as a result of chronic cardiovascular diseases such as hypertension and atherosclerosis (Bobik and Campbell, 1993). Growth factors responsible for vascular injury-induced intimal smooth muscle cell replication have only begun to be identified. It was previously reported that vasopressin exerts potent mitogenic effect via vasopressin V_{1A} receptors in cultured rat vascular smooth muscle cells (Tahara et al., 1997a). The present study has also demonstrated that vasopressin induced mitogenic effect, as reflected by total protein content of cells, in cultured human vascular smooth muscle cells. In addition to this mitogenic effect, the present study demonstrates a novel action of vasopressin on human vascular smooth muscle cells, notably a direct and potent regulation of VEGF secretion. Furthermore, SR 49059, a vasopressin V_{1A} receptor selective antagonist, and YM087, a vasopressin V_{1A}/V_2 receptor antagonist, potently inhibited vasopressin-stimulated secretion of VEGF from vascular smooth muscle cells. In contrast, SR 121463A, a vasopressin V_2 receptor selective antagonist, did not potently inhibit vasopressin-induced secretion of VEGF. The ability of vasopressin V_{1A} receptor antagonists to block vasopressin-induced secretion of VEGF indicates that vasopressin is acting through vasopressin V_{1A} receptors. It is conceivable that during vascular smooth muscle cell proliferation in the development of the early atherosclerotic lesion, VEGF production/secretion would be increased. This would allow VEGF to act as a paracrine hormone and thereby directly influence the cell growth and permeability of the overlying endothelium, and the capacity of vasopressin to increase the secretion of VEGF by human vascular smooth muscle cells suggests a mechanism whereby vasopressin could play an important role in the normal growth and repair of the overlying vascular endothelium in vivo.

In conclusion, vasopressin caused an increase in VEGF secretion from human vascular smooth muscle cells through the vasopressin V_{1A} receptor. Through this action, vasopressin may have novel physiologic functions on the endothelial cell that suggests it could play a key role in regulating normal endothelial functions in health and the pathogenesis of endothelial dysfunction during growth-promoting injury to the vascular smooth muscle cells of blood vessels in vascular diseases. Vasopressin V_{1A} receptor antagonists, including YM087, may have clinical application in vascular remodeling and angiogenesis in a variety of disease states.

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