Vasodilation elicited by liposomal VIP is unimpeded by anti-VIP antibody in hamster cheek pouch

HIROYUKI IKEZAKI, SUDHIR PAUL, HAYAT ALKAN-ÖNYÜKSEL, MANISHA PATEL, XIAO-PEI GAO, AND ISRAEL RUBINSTEIN
Departments of Medicine and Pharmaceutics and Pharmacodynamics, University of Illinois at Chicago, and West Side Department of Veterans Affairs Medical Center, Chicago, Illinois 60612; and Department of Anesthesiology, University of Nebraska Medical Center, Omaha, Nebraska 68198

Vasoactive Intestinal Peptide (VIP) is a 28-amino acid peptide localized in perivascular nerves (7, 11, 27). On its release, VIP elicits potent endothelium-dependent and -independent vasodilation in the peripheral microcirculation (1, 5, 6, 12, 15). However, this response is short-lived due, most likely, to proteolytic inactivation of the peptide (32, 36). The role this catabolic pathway plays in regulating VIP vasoreactivity has been addressed in previous studies from our laboratories (4, 8, 18, 29, 32, 34, 35, 37).

To this end, Vishwanatha et al. (37) showed that tissue neutral endopeptidase 24.11 activity, an ectoenzyme widely distributed in the peripheral circulation that cleaves and inactivates VIP very effectively (32), is increased in spontaneously hypertensive hamsters in which VIP vasoreactivity is blunted (34). Importantly, VIP susceptibility to trypsin- and human plasma-catalyzed cleavage is curtailed when the peptide is inserted on the surface of liposomes (4, 8). This, in turn, is associated with an almost 10-fold increase in liposomal VIP-induced vasodilation in the in situ cheek pouch of normotensive hamsters relative to equimolar concentrations of aqueous VIP (18, 30, 35). In addition, liposomal VIP restores vasoreactivity and normalizes systemic arterial pressure for a prolonged period of time in spontaneously hypertensive hamsters (29, 34).

Although these data suggest that proteolytic inactivation regulates, in part, VIP vasoreactivity in vivo, it is uncertain whether it is the sole extracellular catabolic pathway involved. To this end, there is a growing body of experimental evidence that autoantibodies from healthy individuals and patients with autoimmune diseases, such as systemic lupus erythematosus and thyroiditis, express protease and DNase activity (20, 28). In addition, an increased prevalence of circulating high affinity and specific VIP autoantibodies displaying protease-like activity has been reported in healthy individuals who exercise regularly, and in patients with asthma and spontaneously hypertensive hamsters, diseases associated with smooth muscle dysfunction (19, 21–24, 31, 33; S. Paul and G. Gololobov, unpublished observations). Moreover, Michalkiewicz et al. (16) showed that passive immunization of rats against VIP is associated with significant reduction in blood flow to the duodenum, stomach, and lungs. Collectively, these data suggest that VIP autoantibodies modulate VIP vasoreactivity in vivo. However, the mechanisms underlying this process are uncertain.

Hence, the purpose of this study was to begin to address this issue by determining whether a monoclonal anti-VIP antibody, which has been previously shown to bind VIP with high affinity and specificity and catalyzes cleavage of the peptide in vitro (23), attenuates VIP vasorelaxation in vivo and, if so, whether insertion of VIP on the surface of sterically stabilized liposomes (SSL), which protects the peptide from trypsin- and plasma-catalyzed cleavage in vitro, curtails this response. Using intravital microscopy, we found that suffusion of monoclonal anti-VIP antibody (clone c23.5, IgG2ak), but not of nonimmune antibody (myeloma cell line UPC10, IgG2ak) or empty SSL, significantly attenuates VIP-induced vasodilation in the in situ hamster cheek pouch (P < 0.05). By contrast, anti-VIP antibody has no significant effects on vasodilation elicited by isoproterenol, nitroglycerin, and calcium ionophore A-23187, agonists that mediate, in part, VIP vasoreactivity. Suffusion of VIP on SSL, but not of empty SSL, restores the vasorelaxant effects of VIP in the presence of anti-VIP antibody. Collectively, these data suggest that VIP catalysis by high affinity and specific VIP autoantibodies displaying protease-like activity constitutes a novel mechanism whereby VIP vasoreactivity is regulated in vivo.

Microcirculation; neuropeptide; autoimmunity; catalysis; sterically stabilized liposomes

General Procedures

Preparation of VIP on SSL. VIP on SSL was prepared as previously described in our laboratory (18, 29, 30). Briefly, egg yolk phosphatidylcholine, egg yolk phosphatidylglycerol, cholesterol, and polyethylene glycol (molecular mass, 1,900 Da) linked to distearoyl-phosphatidylethanolamine (molar ratio, 5:1:3:5:0.5; phospholipid content, 17 mmol) were dissolved and mixed in chloroform. The solvent was evaporated at 45°C in a rotary evaporator under vacuum overnight. The result-
ing lipid film was rehydrated in 250 µl saline, vortexed, bath-sonicated for 5 min, and extruded through stacked polycarbonate filters using the LiposoFast apparatus (pore size: 200, 100, and 50 nm; Avestin, Ottawa, ON, Canada). Human VIP (0.4 mg) and trehalose (30 mg), a cryoprotectant, were added to the extruded suspension, which was then frozen in acetone-dry ice bath and lyophilized overnight at −46°C under constant pressure (Freezone 6; Labconco, Kansas City, MO). Thereafter, the lyophilized “cake” was resuspended in 250 µl deionized water. VIP on SSL was separated from free VIP by column chromatography (Bio-Gel A-5m; Bio-Rad Laboratories, Richmond, CA) and stored under argon for 5 days. SSL were added to the extruded suspension, which was then frozen in acetone-dry ice bath and lyophilized overnight at 46°C under constant pressure (Freezone 6; Labconco, Kansas City, MO). Thereafter, the lyophilized “cake” was resuspended in 250 µl deionized water. VIP on SSL was separated from free VIP by column chromatography (Bio-Gel A-5m; Bio-Rad Laboratories, Richmond, CA) and stored under argon for 5 days.

Preparation of monoclonal anti-VIP antibody and nonimmune antibody. Monoclonal anti-VIP and nonimmune antibodies were purified as IgG preparations from mouse ascites fluids (anti-VIP antibody clone c23.5, IgG2ak; nonimmune antibody, myeloma cell line UPC10. IgG2ak) by affinity chromatography on protein G-Sepharose as previously described (23). This antibody binds VIP with high affinity and specificity and catalyzes cleavage of the peptide. The reaction rate constant is $8 \times 10^{-4}$ min$^{-1}$ and the Michaelis constant is 0.34 $\pm$ 0.04 nM. The latter is consistent with a dissociation constant estimate of 2.6 nM for the binding reaction (22). The antibody does not cleave A-chain and B-chain of insulin (23).

The final preparations were composed of >95% IgG evident as a 150-kDa band on nonreducing SDS-electrophoresis gels contaminated with small amounts of IgG subunits and aggregates thereof known to be formed by disulfide exchange reactions. Total protein concentrations were estimated based on values of absorbency at 280 nm assuming 1.2 A280 unit to be equivalent to 1.0 mg/ml IgG, 1 cm path length.

Preparation of animals. Adult male golden Syrian hamsters (n = 17) weighing 128 ± 6 g were anesthetized with pentobarbital sodium (6 mg/100 g body wt ip). A tracheostomy was performed to facilitate spontaneous breathing. A femoral artery was cannulated to monitor and record systemic arterial pressure. Body temperature was monitored and maintained constant (37–38°C) throughout the experiment by using a heating pad.

To visualize the microcirculation of the cheek pouch, we used an established method in our laboratory (18, 30, 32, 34, 35). Briefly, the left cheek pouch was spread over a small plastic base plate, and an incision was made in the outer skin to expose the cheek pouch membrane. The avascular connective tissue layer of the membrane was removed and a plastic chamber was positioned over the base plate and secured in place by suturing the skin around the upper chamber. This chamber was connected to a reservoir containing warmed bicarbonate buffer (37–38°C) for continuous suffusion of the cheek pouch. The bicarbonate buffer was bubbled continuously with 95% N2:5% CO2 (pH 7.4). The chamber was also connected via a three-way valve to an infusion pump (Sage Instruments, Boston, MA) for constant administration of drugs into the suffusate.

Determination of arteriolar diameter. The cheek pouch microcirculation was visualized with a microscope (Nikon, Tokyo, Japan) coupled to a 100-W mercury light source at a magnification of $\times 40$. The microscope image was projected through a low-light TV camera (Panasonic TR-124 MA, Matsushita Communication Industrial, Yokohama, Japan) onto a video screen (Panasonic). The inner diameter of second-order arterioles (48–55 µm) was determined from the video display of the microscope image using a videomicrometer (VIA 100; Boeckler Instruments, Tucson, AZ) as previously described in our laboratory (18, 30, 32, 34, 35). In each animal, the same arteriolar segment was used to measure vessel diameter during the experiment.

Experimental Protocols

Effects of anti-VIP antibody on VIP-induced vasodilation. The purpose of these studies was to determine whether anti-VIP antibody attenuates vasodilation elicited by aqueous VIP in the cheek pouch. After suffusing buffer for 45 min (equilibration period), two concentrations of human VIP (0.5 and 1.0 nmol) were suffused on the cheek pouch for 7 min each in an arbitrary fashion. At least 45 min elapsed between subsequent suffusions of VIP. Once suffusion of VIP was stopped and arteriolar diameter returned to baseline, anti-VIP antibody (0.02 and 0.04 mg) was suffused together with VIP (0.5 and 1.0 nmol) for 7 min. At least 45 min elapsed between subsequent suffusions of anti-VIP antibody together with VIP. Arteriolar diameter was measured before and every minute during and after each intervention. In preliminary studies, we determined that repeated suffusions of VIP (0.5 and 1.0 nmol) were associated with reproducible results. In addition, suffusion of anti-VIP antibody (0.02 and 0.04 mg) alone for 7 min was not associated with significant changes in arteriolar diameter (1 ± 1 and 1 ± 1% increase from baseline, respectively; each group, n = 4; P > 0.5). Likewise, suffusion of saline (vehicle) for the entire duration of the experiment was not associated with significant changes in arteriolar diameter. The concentrations of VIP and anti-VIP antibody used in these studies are based on previous and preliminary studies in our laboratories (23, 31, 32, 34, 35).

Mechanisms underlying anti-VIP antibody-induced responses. To begin to elucidate the mechanisms by which anti-VIP antibody mitigates VIP-induced vasodilation, we tested the hypothesis that adsorption of VIP on SSL curtails the effects of anti-VIP antibody in the cheek pouch, and that this response is specific.

Effects of anti-VIP antibody on VIP on SSL-induced vasodilation. After the equilibration period, two concentrations of VIP on SSL (0.05 and 0.1 nmol) that elicited vasodilation similar to that of VIP alone (0.5 and 1.0 nmol) were suffused on the cheek pouch for 7 min each in an arbitrary fashion. At least 45 min elapsed between subsequent suffusions of VIP on SSL. Once suffusion of VIP on SSL was stopped and arteriolar diameter returned to baseline, anti-VIP antibody (0.02 and 0.04 mg) was suffused together with VIP on SSL (0.05 and 0.1 nmol) for 7 min. At least 45 min elapsed between subsequent suffusions of anti-VIP antibody together with VIP on SSL. In another series of experiments, VIP alone (0.5 nmol), empty SSL, and anti-VIP antibody (0.04 mg) were suffused together on the cheek pouch for 7 min. Arteriolar diameter was measured during each intervention as outlined above. In preliminary studies, we determined that repeated suffusions of VIP on SSL (0.05 and 0.1 nmol) were associated with reproducible results. In addition, suffusion of empty SSL for 7 min was not associated with significant changes in arteriolar diameter. The concentrations of VIP on SSL used in these studies are based on previous studies in our laboratory (18, 30).
Specificity of anti-VIP antibody-induced responses. We used two strategies to address this issue. First, we determined the effects of nonimmune antibody on aqueous VIP and VIP on SSL-induced vasodilation in the cheek pouch. The experimental design was similar to that outlined above except that VIP alone (0.5 and 1.0 nmol) or VIP on SSL (0.05 and 0.1 nmol) were suffused before and together with nonimmune antibody (0.04 mg) for 7 min. Arteriolar diameter was measured during each intervention. In preliminary studies, we determined that suffusion of nonimmune antibody (0.04 mg) alone for 7 min had no significant effects on arteriolar diameter. In another series of experiments, we determined the effects of anti-VIP antibody on vasodilation elicited by three structurally and functionally distinct agonists, isoproterenol, an endothelium-independent, cAMP-dependent vasodilator; nitroglycerin, an endothelium-independent, cGMP-dependent vasodilator; and calcium ionophore A-23187, a receptor-independent, endothelium- and cGMP-dependent vasodilator, in the cheek pouch. The experimental design was similar to that outlined above except that isoproterenol (0.01 µM), nitroglycerin (0.1 µM), and calcium ionophore A-23187 (1.0 µM), at concentrations shown to elicit vasodilation similar to that of VIP alone (1.0 nmol) and VIP on SSL (0.1 nmol), were suffused before and together with anti-VIP antibody (0.04 mg) for 7 min. Arteriolar diameter was measured during each intervention. The concentrations of isoproterenol, nitroglycerin, and calcium ionophore A-23187 used in these studies are based on previous and preliminary studies in our laboratory (32, 34).

Chemicals and Drugs

Egg yolk phosphatidylcholine, egg yolk phosphatidylglycerol, cholesterol, trehalose, nonimmune antibody, isoproterenol, and calcium ionophore A-23187 hemimagnesium salt were obtained from Sigma Chemical (St. Louis, MO). Human VIP was obtained from American Peptide (Sunnyvale, CA). Polyethylene glycol (molecular mass 1,900 kDa) linked to distearoylphosphatidyl-ethanolamine was obtained from Avanti Polar Lipids (Alabaster, AL). Nitroglycerin was obtained from American Regent Laboratories (Shirley, NY). All drugs were dissolved in saline to the desired concentrations on the day of the experiment.

Data and Statistical Analyses

When a compound was suffused on the cheek pouch, we determined the maximal change in arteriolar diameter and used it as the response to that compound in each animal. Arteriolar diameter was expressed as the ratio of experimental to control diameter, with control diameter normalized to 100% to account for intra- and interanimal variability. Data are expressed as means ± SE except for the size of VIP on SSL and body weight, which were expressed as means ± SD because these data are not used for comparison between experimental groups. Statistical analysis was performed on actual values using repeated-measures ANOVA with Newman-Keuls multiple-range post hoc test to detect values that were different from control values. A value of $P < 0.05$ was considered significant. $n$ is given as the number of experiments, with each experiment representing a separate animal.

RESULTS

Mean systemic arterial pressure was 97 ± 2 mmHg at the start and 95 ± 2 mmHg at the conclusion of the experiments ($n = 17; P > 0.4$).

Effects of Anti-VIP Antibody on VIP-Induced Vasodilation

Suffusion of VIP alone elicited a significant, concentration-dependent increase in arteriolar diameter in the cheek pouch (Fig. 1; each group, $n = 4; P < 0.05$). These effects were significantly attenuated by suffusion of anti-VIP antibody in a concentration-dependent fashion (Fig. 1; each group, $n = 4; P < 0.05$). Arteriolar diameter increased by 19 ± 1% from baseline during suffusion of VIP (1.0 nmol) alone, and by 21 ± 1 and 7 ± 1% during suffusion of 1.0 nmol VIP together with 0.02 and 0.04 mg anti-VIP antibody, respectively (Fig. 1; each group, $n = 4; P < 0.05$).

Mechanisms Underlying Anti-VIP Antibody-Induced Responses

Effects of anti-VIP antibody on VIP on SSL-induced vasodilation. Suffusion of VIP on SSL elicited a significant, concentration-dependent increase in arteriolar diameter in the cheek pouch (Fig. 2; each group, $n = 4; P < 0.05$). Suffusion of anti-VIP antibody had no significant effects on VIP on SSL-induced responses (Fig. 2; each group, $n = 4; P < 0.05$). Arteriolar diameter increased by 22 ± 1% from baseline during suffusion of 0.1 nmol VIP on SSL alone, and by 21 ± 1 and 19 ± 1% during suffusion of 0.1 nmol VIP together with 0.02 and 0.04 mg anti-VIP antibody, respectively (Fig. 2; each group, $n = 4; P < 0.05$). By contrast, suffusion of VIP (0.5 nmol) together with empty SSL and anti-VIP antibody (0.04 mg) was associated with significant attenuation of vasodilation relative to that elicited by VIP (0.5 nmol) alone (5 ± 1% increase from baseline vs. 11 ± 1% increase from baseline, respectively; each group, $n = 4; P < 0.05$).

Specificity of anti-VIP antibody-induced responses. Suffusion of nonimmune antibody (0.02 and 0.04 mg) had no significant effects on vasodilation elicited by VIP (0.5 and 1.0 nmol) alone and VIP on SSL (0.05 and 0.1 nmol; Fig. 3; each group, $n = 4; P > 0.5$). Suffusion of anti-VIP antibody (0.02 and 0.04 mg) had no significant effects on vasodilation elicited by isoproterenol (0.01 µM), nitroglycerin (0.1 µM), and calcium ionophore A-23187 (1.0 µM; Fig. 4; each group, $n = 4; P > 0.5$).

DISCUSSION

There are three new findings of this study. First, we found that an anti-VIP monoclonal antibody that binds VIP with high affinity and specificity and catalyzes cleavage of the peptide attenuates VIP-induced vasodilation in the in situ hamster cheek pouch. These effects are specific and unrelated to vascular smooth muscle dysfunction because nonimmune antibody has no significant effects on VIP-induced responses and because anti-VIP antibody has no significant effects on vasodilation elicited by isoproterenol, nitroglycerin, and calcium ionophore A-23187, three structurally and functionally distinct agonists that activate intracellular effector systems that mediate, in part, VIP vasoreactivity (1, 11, 12, 30). The relatively bulky anti-VIP antibody may not have gained sufficient access to the cheek.
pouch microcirculation to curtail VIP-induced vasodilation at higher concentrations of peptide used in this study (22, 23). Likewise, residual vasodilation observed after suffusing VIP together with anti-VIP antibody may reflect incomplete degradation and inactivation of the peptide.

Second, suffusion of anti-VIP antibody alone, at concentrations used in this study, has no significant effects on baseline arteriolar diameter in the cheek pouch of normal hamsters. These data suggest that endogenous VIP does not contribute appreciably to resting vasomotor tone in this organ. This phenomenon

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**Fig. 1.** Effects of suffusion of human vasoactive intestinal peptide (VIP) on the in situ hamster cheek pouch for 7 min on arteriolar diameter in the absence and presence of anti-VIP antibody. Values are means ± SE; each group, n = 4. *P < 0.05 in comparison to VIP alone. †P < 0.05 in comparison to 1.0 nmol VIP and 0.02 mg anti-VIP antibody.

**Fig. 2.** Effects of suffusion of human VIP on sterically stabilized liposomes (SSL) on the in situ hamster cheek pouch for 7 min on arteriolar diameter in the absence and presence of anti-VIP antibody. Values are means ± SE; each group, n = 4.
may also be related, in part, to regional variability in
VIP vasoreactivity reported in hamsters and other
species (1, 5, 6). Whether VIP autoantibodies modulate
resting arteriolar diameter in cardiovascular disorders
associated with vascular smooth muscle dysfunction,
such as essential hypertension (34), remains to be
determined.
Third, insertion of VIP on the surface of SSL curtails
anti-VIP antibody-induced responses. This process could
not be ascribed to the SSL moiety because anti-VIP
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antibody still attenuates VIP-induced vasodilation in the presence of empty SSL. Conceivably, association of VIP with SSL confers resistance to VIP molecule from anti-VIP antibody catalysis. Collectively, these data suggest that VIP catalysis by high affinity and specific VIP autoantibodies displaying protease-like activity constitutes a novel mechanism whereby VIP vasoreactivity is regulated in vivo.

The hamster cheek pouch is an established model to investigate mechanisms underlying the vasoactive effects of VIP and liposomal VIP in the in situ peripheral microcirculation (4, 18, 29, 30, 32, 34, 35). This intravital microscopy preparation is stable over time, which enables the use of each animal as its own control, thereby simplifying data analysis. For instance, Séjourné et al. (30) showed that adsorption of VIP on SSL amplifies the vasorelaxant effects of the peptide in the cheek pouch of normotensive hamsters by almost 10-fold relative to aqueous VIP alone. This response is specific, partly receptor-dependent, and transduced by the l-arginine-nitric oxide biosynthetic pathway. The results of the present study support and extend these observations by showing that adsorption of VIP on SSL protects the peptide from binding and catalysis by VIP autoantibodies, thereby promoting vasorelaxation.

Other investigators showed that passive immunization of rats and cats against VIP alters smooth muscle contractility in the peripheral circulation and trachea (10, 16). However, the mechanisms underlying this process were not elucidated. The results of the present study suggest that one mechanism whereby VIP autoantibodies modulate VIP vasoreactivity in vivo is catalytic cleavage of the peptide because vasodilation elicited by VIP on SSL, unlike that evoked by aqueous VIP, is resistant to anti-VIP antibody. This phenomenon may be related, in part, to phospholipid-induced conformational change in VIP molecule from predominantly random coil in aqueous solution to α-helix, which reduces anti-VIP antibody-catalyzed cleavage of VIP in vitro (3, 8, 17, 18, 25).

This hypothesis is supported, in part, by the study of Gololobov et al. (8), who showed recently that liposomal VIP is less susceptible to degradation by catalytic VIP autoantibodies compared with aqueous VIP because of decreased binding affinity of liposomal VIP to autoantibodies. This process could not be ascribed to a barrier effect arising from sequestration of VIP away from autoantibodies because, if present, it would have been expected to decrease the catalytic rate constant rather than binding affinity. Moreover, the half-life of circulating liposomal VIP in mice is increased by almost fivefold relative to aqueous VIP (8). On balance, these data imply that phospholipid-induced conformational change in VIP molecule reduces peptide susceptibility to VIP autoantibodies-catalyzed cleavage.

The above notwithstanding, attenuation of VIP-induced vasodilation by anti-VIP antibody may also reflect diminished ability of antibody-VIP complex to bind VIP receptors in target cells in the cheek pouch (9, 13, 16). Alternatively, binding of anti-VIP antibody to VIP may mask a receptor-reactive epitope(s) of VIP on the surface of these cells (2, 13). Conceivably, failure of anti-VIP antibody to attenuate VIP on SSL-induced vasodilation could be related, in part, to fusion of VIP on SSL with plasma membrane and intracellular delivery of VIP leading to activation of intracellular effector systems and vascular smooth muscle relaxation (8, 13, 17). Further studies using cell biology, biochemical and pharmacological methods are warranted to address these issues.

Perspectives

The results of this study have important implications for our understanding of the mechanisms regulating VIP vasoreactivity in vivo. They suggest that VIP catalysis by high-affinity and specific VIP autoantibodies displaying protease-like activity constitutes a novel mechanism whereby VIP vasoreactivity is regulated in vivo, particularly in conditions associated with high prevalence of circulating VIP autoantibodies, such as exercise, asthma, and essential hypertension (21, 22, 26, 33).

In summary, we found that a monoclonal antibody that binds VIP with high affinity and specificity and catalyzes cleavage of the peptide attenuates VIP-induced vasodilation in vivo in a specific fashion. This response is curtailed when VIP is inserted on the surface of liposomes.

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Address for reprint requests: I. Rubinstein, Dept. of Medicine (M/C 787), Univ. of Illinois at Chicago, 840 South Wood St., Chicago, IL 60612–7323.

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