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Adenoviral Transfer of Vasoactive Intestinal Peptide (VIP) Gene Inhibits Rat Aortic and Pulmonary Artery Smooth Muscle Cell Proliferation

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Abstract

Vasoactive intestinal peptide (VIP), a 28 amino acid peptide, has been shown to inhibit proliferation of vascular smooth muscle cells. In previous studies VIP and VIP analogues have been used to study the effects of the peptide on vascular smooth muscle cell function. In this study an adenovirus encoding the VIP gene was used to investigate the mechanism of the antiproliferative action of VIP in vascular smooth muscle cells. Primary cultures of aortic and pulmonary artery smooth muscle cells from male Sprague-Dawley rats were transfected with varying concentrations of serotype 5 adenovirus encoding human VIP (Ad5CMVhVIP). Transfection efficiency and subsequently VIP gene expression were confirmed by western blot analysis and immunohistochemistry. In this study a decrease in vascular smooth muscle cell proliferation at vector concentrations of 150, 300 and 600 MOI (multiplicity of infection) was observed. In addition, there was increased production of cAMP in pulmonary artery and aortic smooth muscle cells transfected with VIP. Treatment of cells with a PKA inhibitor (Rp-8-BrcAMPs) restored proliferation to about 80% of control whereas treatment with the PKG inhibitor Rp-8-BrcGMPs had no significant effect suggesting the involvement of the PKA pathway in the antiproliferative actions of VIP.

Keywords

pulmonary hypertension; vascular smooth muscle cells; vasodilators; cAMP

1. Introduction

Under physiologic conditions vascular smooth muscle cells are usually in a contractile state, however, these cells may convert to a synthetic or proliferative phenotype in disease states as well as in culture [7]. It has been reported that vascular smooth muscle cells undergo a phenotypic change in disease states such as atherosclerosis, coronary artery restenosis,

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systemic and pulmonary hypertension (PH)[32]. Research is ongoing to find methods of attenuating this increase in vascular smooth muscle cell proliferation. The effects of vasodilators such as nitric oxide (NO), calcitonin gene related peptide (CGRP) and adrenomedullin (ADM) on vascular smooth muscle cell proliferation have been examined [5, 6,15]. Vasoactive intestinal peptide (VIP) which also has vasodilator actions has been shown to have antiproliferative effects on vascular smooth muscle cells [13]. In addition VIP has also been reported to inhibit growth of airway smooth muscle cells [21]. These observations have lead to the suggestion that VIP could be used as a therapeutic agent for the treatment of PAH [27]. Decreased levels of VIP in the serum of patients with pulmonary artery hypertension has been reported [27]. There is also an absence of VIP containing nerve fibers in the wall of pulmonary arteries from patients with PAH[27]. It has been reported that there is evidence of VIP gene alterations in the coding and non-coding regions in patients with PAH however the evidence was not conclusively linked to idiopathic pulmonary arterial hypertension (IPAH) [11]. VIP gene knockout mice have been reported to develop moderate pulmonary hypertension with pulmonary vascular remodeling [28]. Treatment with VIP has been reported to have a beneficial effect in patients and animal models of PH [10,27].

VIP is a 28 amino acid peptide which was first isolated from the porcine duodenum by Said and Mutt in 1970[29]. VIP is a member of the secretin-glucagon and PACAP family of peptides and is formed from cleavage of the 170 residue prepro-VIP. Circulating VIP originates from VIP containing nerve endings distributed throughout the body [29]. Among other actions, the biological effects of VIP include vasodilation and inhibition of smooth muscle cell proliferation [8,13,25]. These biological effects are mediated by two type II G-protein coupled receptors VIP/pituitary adenylate cyclase activating peptide (PACAP) receptor 1 (VPAC1) and VIP/PACAP receptor 2 (VPAC2)[17]. The downstream targets for these receptors in vascular smooth muscle cells are unknown and are believed to vary in different cell types. The vasodilatory effects of VIP are mediated by a PKA dependent pathway [8,30]. The involvement of the PKG/cGMP pathway in the VIP signaling pathway has not been fully explored in vascular smooth muscle cells. Experiments in isolated pulmonary arteries suggests that the vasodilator response to VIP in the pulmonary vascular bed may be dependent on nitric oxide release from the endothelium[2].

The purpose of the present study was to investigate the effects of transfection of vascular smooth muscle cells with an adenovirus encoding VIP. Previous studies have used either synthetic peptides or analogs of VIP that may not be stable under cell culture conditions [14]. The use of an adenoviral vector may provide continuous VIP release and therefore be advantageous in the study of the response and signaling pathways involved in the actions of this peptide.

2. Materials and Methods

2.1. Isolation and culture of aortic and pulmonary artery smooth muscle cells

Aortic and pulmonary artery smooth muscle cells were isolated as previously described [5]. All Procedures were approved by the animal use and care committee (IACUC) at the Tulane University School of Medicine. Briefly male Sprague-Dawley rats weighing between 350-500g were anesthetized with thiobutabarbital sodium (Inactin®, Sigma Chemical Co., St Louis MO) 100mg/kg ip. The chest cavity was opened by a midline incision and the aorta and pulmonary artery were isolated. The aorta and pulmonary artery were placed in medium M199 (Sigma Chemical Co., St Louis MO). The vessels were cleaned to remove residual blood and fat tissue. The vessels were incubated in collagenase solution (200U/ml, collagenase, type 1; 0.4 mg/ml trypsin inhibitor) for 30 minutes at 37°C. Following this incubation the endothelium and the adventitia were carefully removed. The vessels were cut into smaller segments and placed in collagenase/elastase solution (200U/ml, collagenase, and type 1, 15 U/ml elastase)

for 2 hours at 37°C. The vessel segments were washed three times in Medium M199 supplemented with 10% fetal bovine serum and penicillin, streptomycin, and amphotericin B (10000 units/ml, 10000 µg/ml, 25 µg/ml) (Atlanta Biologicals, Atlanta, GA). The tissue was placed in a 25 cm² cell culture flask and placed in a humidified incubator (95% air, 5% CO₂) at 37°C. The tissue was left undisturbed for 5-7 days to allow attachment and growth. Fresh complete media was added and the cells allowed to grow to about 70% confluency. Cells were then placed into 75 cm² cell culture plates. The cells were confirmed to be smooth muscle cells by the characteristic “hill and valley” pattern of growth and by western blot analysis and immunohistochemistry for smooth muscle cells specific α -actin. Passage II and passage III cells were used for all experiments.

2.2 Adenoviral Vectors

Adenoviruses are replication-deficient recombinant viruses encoding nuclear targeted β -galactosidase or VIP. The VIP adenovirus was obtained from B.M. Lodde at NIH and amplified using HEK293 cells to give a final stock of approximately 1×10^{12} pfu/mL. Ad5CMVntlacZ was purchased from University of Iowa gene transfer vector core (Iowa City, IA) and the titer was 2×10^{10} pfu/mL.

2.3. Proliferation studies

Aortic smooth muscle cells were plated at a density of 6×10^4 cells/well and pulmonary artery cells were plated at 3×10^4 cells/well, in 6 well cell culture plates (BD Falcon). On day 2 the cells were transfected with 150, 300 and 600 MOI of Ad5CMVhVIP. MOI was calculated as the number of pfu per cell. Transfection with AdCMVntlacZ (600 MOI) was used as a reporter gene and as control for the adenoviral vector. Following the treatment, cell counts were obtained microscopically on a daily basis for 4 days. All assays and measurements from hereon are based on transfection with 300 MOI as this dose was effective for inhibiting the proliferation without causing any toxicity.

2.4. Western Blot for VIP and β -Tubulin

Cells were plated and treated as described in 2.1. The media from each treatment group was collected on day 4 post treatment. Cells were harvested by trypsinization using 0.25% trypsin-EDTA (Invitrogen, Carlsbad, CA). The harvested cells were placed in a 2 mL Eppendorf tube and lysed using hypotonic buffer (10 mM Tris, 1.5 mM MgCl₂, protease cocktail, Roche, Sigma Aldrich, St. Louis, MO). The BCA protein assay (Pierce, Rockford, IL) was used to quantify protein in each group. Equal quantities of proteins were electrophoresed on 4-20% gradient gels (Jule Inc, Milford CT, USA); Cruz-Marker® (Santa Cruz) was used as a molecular weight ladder in these experiments. The protein was transferred to a nitrocellulose membrane (GE Healthcare, Piscataway, NJ). The membrane was blocked for 1 hour using 5% milk in Tris buffered saline (TBS) + 0.1% Tween (TBST). The membrane was then washed twice for 10 minutes each and incubated in polyclonal goat anti-VIP antibody for 1 hour (Santa Cruz biotechnology, Santa Cruz, Ca) at a dilution of 1: 500. The membrane was washed twice with TBS and then the bound antibody was detected using anti-goat IgG-HRP secondary antibody (Santa Cruz biotechnology, Santa Cruz, Ca) at a dilution of 1:1000 for 30 minutes. ECL chemiluminescence method of analysis was used to detect and quantitate VIP.

Following this, the membrane was reprobed for β -tubulin to substantiate that the same quantity of protein was loaded in all lanes. The membrane was washed twice for 10 minute periods in excess TBST. The membrane was then blocked for 1 hour in 5% milk in TBST. Western blot analysis was then carried out as previously described using rabbit monoclonal anti- β -tubulin (Santa Cruz) 1:1000 dilution and anti-rabbit IgG-HRP 1:2000. (Santa Cruz) .

2.5 Determination of Cell Viability

Cell viability was determined using the trypan blue exclusion method. After harvesting the cells were placed in trypan blue at 1:1 ratio. A hemocytometer was used to determine the percentage of viable cells. In addition, Western blots for Bax and Bcl-2 was performed as previously described using mouse monoclonal anti-Bax IgG (Santa Cruz) 1:500 and mouse monoclonal anti-Bcl-2 (Santa Cruz) 1:500.

2.6. Immunohistochemistry for VIP

Aortic smooth muscle cells were plated at 6×10^4 cells/well and pulmonary artery smooth muscle cells were plated at 3×10^4 cells/well on chamber slides (Fisher Scientific). On the following day the cells were transfected with VIP adenovirus at a dose of 300MOI. Cells were allowed to grow for 4 days and then the post transfection cells were stained for VIP using immunohistochemistry staining kit (Peninsula Labs LLC, San Carlos, CA). In order to facilitate the attachment of cells, they were rinsed in PBS and placed in ice cold 100% methanol for 5 minutes at 4°C. Endogenous peroxidase activity was blocked by incubating cells in 3% hydrogen peroxide for 7 minutes. This was followed by incubation in normal donkey serum for 30 minutes. Subsequently the cells were washed three times in TBS for 2 minutes each on table top shaker. Primary antibody (goat anti-VIP) at a dilution of 1:200 was added to cells and they were incubated overnight at 4°C. Following this incubation the cells were washed twice in TBS for 2 minutes each on the shaker. To this, prediluted secondary antibody (biotinylated anti- goat IgG) was added and allowed to stand for 1 hour at room temperature. At the end the 1 hour, cells were washed twice in TBS for 2 minutes each. Streptavidin-HRP conjugate solution was added to the cells and they were incubated for 30 minutes and then they were washed twice in TBS for 2 minutes each. Cells were incubated in DAB substrate and monitored for development of a brown reaction product at which point the reaction was terminated by rinsing with distilled water. Slides were then counterstained with Mayer's hematoxylin for about 2 minutes. Subsequently they were washed in tap water and then immersed in PBS and rinsed in distilled water. A few drops of mounting solution were added and the cover slip was placed on the slides and allowed to dry. The slides were viewed and images were captured using the Nikon Eclipse E800 microscope equipped with Nikon DXM1200 digital camera and ACT-1 software.

2.7. Measurement of cyclic nucleotides

Cells were plated on 96 well plates at a density of 10^5 cells per well. Cells were allowed to attach overnight and were then treated with VIP at MOI 150, 300 and 600, transfection with ntLacZ was used as a control. Cells were allowed to grow for 4 days and assayed for total of cAMP and cGMP levels. The non-acetylation protocol of Biotrak cAMP EIA and the acetylation protocol of Biotrak cGMP (GE-health care, Arlington Height, IL) were used for analysis. Plates were read using a microplate reader and analyzed using Softmax Pro software (Molecular Devices Corp, Sunnyvale, CA).

2.8. Inhibitor studies

The effect of PKA and PKG inhibitors on proliferation of VIP transfected cells was determined. Cells were plated as previously described and on day two cells were treated with VIP and Rp-8-BrcGMPs or Rp-8-BrcAMPs, (Axxora, San Diego, Ca) PKA and PKG inhibitors respectively. The effect on cell growth was determined by every day cell counting.

2.9. Statistical Analysis

The data is expressed as Mean $\pm$ SE and was analyzed by a 1-way ANOVA followed by a post hoc analysis with Tukey's test using GraphPad Prism® software.

3. Results

3.1 Effect of Ad5CMVhVIP on PASMC and ASMC proliferation

There was a significant decrease in proliferation in both pulmonary artery and aortic smooth muscle cells when treated with Ad5CMVhVIP at vector concentrations of 150, 300 and 600 MOI (Fig 1A and 1B). Treatment with a control viral vector Ad5CMVntlacZ produced no significant change in proliferation in untreated/control cells. These results indicate that the adenovirus itself has no effect on cell proliferation and that growth inhibition was due to the effect of the VIP adenovirus. Figure 1 shows the growth curves of cells over a four day period and summary data on day four after transfection with VIP. Trypan blue exclusion showed greater than 95% cell viability which indicates that the decrease in cell number was due to inhibition of proliferation and not to cell death. This was further confirmed by Western blot analysis for Bax and Bcl-2, two markers of apoptosis (Figure 2). Western blot analysis in figure 2 shows no change in the expression of Bax and Bcl2 and suggests that there was no difference in expression of either protein when compared in transfected cells and control cells.

3.2. Detection of VIP

The ability of the adenovirus to produce VIP in both vascular smooth muscle cell types was determined by Western blot analysis and immunohistochemistry. Figure 3 shows Western blots for the expression of VIP in the media and cell lysate on day 4 after transfection with ad5CMVhVIP. VIP was not detected in control cells or in the cells transfected with AdCMVntlacZ. Figure 4 shows the expression of VIP (in cells stained for VIP) when compared to control/untreated cells. The cells were stained on day 4 after transfection. Figure 4 shows brown staining that indicates the presence of VIP in the cytoplasm of >70% of both cell types.

3.3. Measurement of cyclic nucleotides

Figure 5 shows the levels of cAMP and cGMP in vascular smooth cells transfected with VIP. There was a significant increase in cAMP levels in both vascular smooth muscle cells types. The levels of cGMP were not significantly different in both cell types transfected with VIP when compared to control cells.

3.4. Effect of PKA and PKG inhibitors

The effect of cyclic nucleotide inhibitors (Rp-8-BrcGMPs and Rp-8-BrcAMPs) on VIP transfected cells was determined. As shown in figure 6, cells transfected with VIP and treated with the PKA inhibitor showed a statistically significant restoration of cell proliferation. Cell growth was restored to about 80% of the pretreatment value in both cell types. In contrast treatment with a PKG inhibitor produced a non-significant restoration of proliferation (data not shown).

4. Discussion

Pulmonary arterial hypertension is a fatal disease for which there are few treatment options and no cure. It has been reported that there is a deficiency of VIP in the serum and lung tissue from patients with pulmonary hypertension and that inhaled VIP has a beneficial effect in treating this disease [27]. In addition, it has been reported that VIP knockout mice have a moderate increase in pulmonary artery pressure and develop a moderately severe form of pulmonary arterial hypertension [28]. Since VIP inhibits cell proliferation and has a beneficial effect in patients with pulmonary hypertension, it is our hypothesis that sustained release of VIP using an adenoviral vector encoding the peptide may have a better effect than treatment with the peptide itself.

Adenoviral vectors can be used to study the biological effects of various gene products. These vectors have also been used for gene therapy to deliver peptide products for the treatment of vascular diseases [22,24]. This is the first report of the effect of an adenoviral vector encoding VIP on pulmonary artery and aortic smooth cell proliferation. Previous work has investigated the use of adenoviral vectors in gene therapy in response to vessel injury [26]. Adenoviruses encoding other vasodilators such as NO, CGRP and ADM have been used for the treatment of vascular diseases [1,3,4]. The synthesis of an adenoviral vector encoding VIP was only reported recently [20].

In the present study we show that transfection of vascular smooth muscle cells with an adenovirus encoding VIP (ad5CMVhVIP) resulted in the production of VIP peptide which was detected in the cytoplasm of vascular smooth muscle cells as well as in the cell culture media. The production of VIP was confirmed by both Western blot analysis and immunohistochemistry. As shown in figure 3 there was no measurable VIP detected in either the control or AdCMVntLacZ treated vascular smooth muscle cells from the aorta or pulmonary artery. We also observed a decrease in proliferation in cells transfected with VIP. In cells from the aorta there was a decrease in cell number by about 30% whereas in the cells from the pulmonary artery the decrease was approximately 20%. Treatment with a control adenovirus encoding ntLacZ (AdCMVntLacZ), a reporter gene, had no significant effect on cell proliferation indicating that the virus itself does not affect cell growth. The continuous production of VIP by the adenovirus is advantageous in the study of the downstream signaling events over a period of time. In this study the effect of VIP on both pulmonary artery and aortic smooth muscle cells, the cells were observed to have different growth rates and inhibitory responses to VIP.

This study demonstrates the use of an adenoviral vector approach to investigate the actions of VIP on vascular smooth muscle cell proliferation. VIP has been shown to have vasodilator effects in both pulmonary and systemic vascular beds [9,25,31]. Although VIP has vasodilator actions in both the pulmonary and systemic vascular beds the physiologic role of this peptide in these two circulations may be different. Alterations in VIP gene expression in knockout mice lead to the development of PAH suggesting an important role in the pathogenesis of this disease [12,28]. VIP gene alteration has not been associated with the development of systemic hypertension suggesting different roles for the peptide in the two circulations. It has been reported that the biological actions of VIP are mediated by two G protein coupled receptors VPAC1 and VPAC2 [18]. The vasodilatory effects of the peptide have been reported to be mediated by a cAMP dependent mechanism [8,23]. It has also been shown that release of NO from the endothelium may mediate vasodilatory responses to VIP [16]. The involvement of cAMP and other downstream signals in the antiproliferative effects has not been adequately explored. In the present study an increase in cAMP levels was observed in both aortic and pulmonary artery vascular smooth muscle cells transfected with VIP. In contrast cGMP levels were not significantly increased, suggesting that in cells cultured without the endothelium cGMP may not be involved in the antiproliferative response. The inhibition of protein kinase/cAMP activity using Rp-8-BrcAMPs resulted in restoration of proliferation to about 80% of pretreatment levels. The lack of total restoration may suggest the involvement of alternate pathways in the process.

In conclusion, the results of the present investigation show that an adenoviral vector encoding VIP can be used to transfect pulmonary artery and aortic smooth muscle cells. VIP is produced by the transfected cells and secreted into the culture media resulting in the inhibition of vascular smooth muscle cell proliferation. The use of an adenovirus to transfect cells may represent a novel way of examining the mechanism of the antiproliferative action of VIP in vascular smooth muscle cells and that gene therapy using a VIP adenovirus may be useful in the treatment of pulmonary hypertensive disorders.

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Abbreviations

VIP, vasoactive intestinal peptide; MOI, Multiplicity of infection; ASMC, aortic smooth muscle cell; PASMC, pulmonary artery smooth muscle cell; pfu, plaque forming units; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate.

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A.

B.

Figure 1.

Dose response relationship for ad5CMVhVIP on proliferation of pulmonary artery and aortic smooth muscle cells. Cells were either transfected or untransfected with adCMVntLacZ as a control. Daily cell counts of pulmonary artery smooth muscle cells (A) and aortic smooth muscle cells (B) were made over a 4 day period. Each point represents the mean cell count for 3 wells, $\pm$ SE, $n=3$. The left panel shows the growth curve over a four day period and the right panel shows a comparison on day 4. * $p<0.05$ vs control.

A

B

Figure 2.

Determination of cell viability. Panel A represents cell viability as determined by trypan blue exclusion. n=3. Panel B shows Western blot analysis of cell lysates for Bax and Bcl-2 on day 4 after treatment with ad5CMVhVIP.

Lysate

Media

Fig 3.

Western blot analysis for expression of VIP in the cell lysate and the culture media of ASMC's and PASMC's transfected with ad5CMVhVIP. Western blot analysis for β -tubulin were used as a loading control. n=3.

Figure 4. Immunohistochemical staining for VIP on day four following transfection with adCMVhVIP. Panels A and C represent untreated pulmonary artery smooth muscle cells and aortic smooth muscle cells respectively. Pulmonary artery (B) and aortic smooth muscle cells (D) treated with VIP show positive brown staining. Arrows indicate areas of positive staining. Magnification $\times 200$.

Figure 5.

Enzyme linked immunoassay for cAMP and cGMP production in PASMC and ASMC. Panel A shows cAMP levels in aortic smooth muscle cells on day 4 post treatment and panel B shows levels in pulmonary artery smooth muscle cells. Panel C shows cAMP levels in aortic smooth muscle cells on day 4 post treatment and panel D shows levels in pulmonary artery smooth muscle cells. The data is reported as change in cyclic nucleotide level compared to control in fmol * $p < 0.05$ vs control. $n=4$

A

B

C.

Figure 6.

Effect of Rp-8-bromoadenosine 3', 5'-cyclic monophosphothioate (Rp-6-BrcAMPs) a PKA inhibitor on cell proliferation in VIP transfected pulmonary artery (A) and (B) aortic smooth muscle cells. The curves in the left hand panel show daily cell count over 4 days and the right panel shows the day 4 post treatment comparison. * p < 0.05 vs control and # p < 0.001 vs control. n=3