Role of VPAC2 receptor in monocrotaline-induced pulmonary hypertension in rats

Motokazu Koga, Yusuke Mizuno, Itaru Watanabe, Hiromasa Kawakami, and Takahisa Goto

Department of Anesthesiology and Critical Care Medicine, Division of Bio-Functional Medicine, Yokohama City University Graduate School of Medicine, Yokohama, Japan

Submitted 25 July 2013; accepted in final form 17 June 2014

Koga M, Mizuno Y, Watanabe I, Kawakami H, Goto T. Role of VPAC2 receptor in monocrotaline-induced pulmonary hypertension in rats. J Appl Physiol 117: 383–391, 2014. First published June 19, 2014; doi:10.1152/japplphysiol.00861.2013.—Pulmonary hypertension (PH) is associated with significant morbidity and mortality. Vasoactive intestinal peptide (VIP) and pituitary adenyl cyclase activating peptide (PACAP) have pulmonary vasodilatory and positive inotropic effects via receptors VPAC1 and VPAC2, which possess a similar affinity for both peptides, and PAC1, a PACAP-prefering receptor. VIP is a promising option for PH treatment; however, various physiological effects of VIP have limited its clinical use. We investigated the effects of VPAC1 and VPAC2 selective agonists VIP and PACAP to explore more appropriate means of treatment for PH. We examined hemodynamic changes in right ventricular systolic pressure (RVSP), systemic blood pressure (SBP), total pulmonary resistance index (TPRI), total systemic resistance index, and cardiac index (CI) in response to their agonists with monocrotaline (MCT)-induced PH and explored involvement of VIP/PACAP expression and receptors in PH. Sprague-Dawley rats were divided into the MCT group (administered MCT 60 mg/kg) and control group. In MCT-induced PH, decreased VIP and PACAP were associated with upregulation of VPAC1, VPAC2, and PAC1 in lung tissues. Intravenous injection of VPAC2-selective agonist BAY 55–9837 and VIP, but not [Ala^{11,22,28}]VIP, improved the CI. The decrease in SBP with VPAC2 agonist was significantly less than that in the control. Although they decreased SBP, these agonists hardly affected RVSP in the control. Activation of VPAC2 receptor with BAY 55–9837 effectively improved RVSP, TPRI, and CI in MCT-induced PH, suggesting a VPAC2 agonist as a possible promising treatment for PH.

PULMONARY HYPERTENSION (PH) may cause serious complications such as pulmonary arterial hypertensive crisis and cardiac arrest (26). Although pulmonary vasodilators are important therapeutic options for PH, they carry a risk of cardiovascular collapse (26) because many patients with PH have relatively fixed pulmonary vascular resistance (30, 40) with normal systemic vasculature. Therefore, more selective pulmonary vasodilators are badly needed for the treatment of PH.

Vasoactive intestinal peptide (VIP) and pituitary adenyl cyclase activating peptide (PACAP) have both a potent pulmonary vasodilatory effect (1, 17) and positive inotropic and chronotropic effects (46). VIP and PACAP share 68% homology in their amino acid sequences and exert biological effects by acting on their three receptors: VIP receptor type 1 (VPAC1), VPAC receptor type 2 (VPAC2), and PACAP type 1 (PAC1). VPAC1 and VPAC2 possess similar affinity for VIP and PACAP, whereas PAC1 is a PACAP-prefering receptor. VIP has been expected as a promising treatment option for PH (9, 24, 36). Inhaled (24, 36) and intravenously administered (20) VIP has been tested for PH treatment in patients and in an animal model. However, various physiological functions of VIP in a variety of tissues have limited its clinical use. VIP injection caused bronchoconstriction in asthma sufferers, tachycardia, hypotension, and cutaneous flushing (45) and changed energy metabolism (44).

Recently, differential roles of VPAC1, VPAC2, and PAC1 in the homeostasis of the pulmonary circulation have emerged. VIP knockout mice developed PH (36). In addition, VIP content in lungs was decreased in PH patients (36) and in animal models (47), suggesting that VIP may play an important role in pulmonary circulation.

Regarding these receptors, VPAC1 (11, 49) and VPAC2 knockout mice (2) did not develop PH, whereas those with PAC1 did (49). Therefore, it is possible that VPAC1 and VPAC2 could interact in a compensatory manner in the knockout mice, thus resulting in preservation of pulmonary circulation because both VPAC1 and VPAC2 receptors showed similar immunohistochemical patterns appearing in smooth muscle cells in the wall of human lungs (29). In addition, expression of both VPAC1 and VPAC2 is upregulated in PH, possibly reflecting a response to decreased VIP in PH patients and animal models (36, 50). On the other hand, PACAP-deficient pups had a high mortality rate until weaning (21), and their hemodynamics are not known. The role of PACAP/PAC1 in the pulmonary circulation is unclear.

Although insights into VIP/PACAP signaling including ligands and receptors in PH could provide an alternative treatment for PH, the precise mechanism of the signaling is not fully understood. Furthermore, no selective agonist of the receptors for PH treatment has been tested yet. In the present study, we investigated the roles of VPAC1, VPAC2, and PAC1 to explore more appropriate therapy for pulmonary vasodilatory therapy. We examined various hemodynamic changes in response to their selective agonists in rats with monocrotaline (MCT)-induced PH. We also examined expression of VIP/PACAP ligands and the receptors.

MATERIALS AND METHODS

Animal experiments and chemicals. Eight-week-old male Sprague-Dawley rats (Japan SLC, Shizuoka, Japan) were divided into two groups: the MCT group, which received 60 mg/kg of MCT subcutaneously once, and the control group, which received the same volume of physiologic saline subcutaneously once. A total of 110 rats was used in this study. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Yokohama City
University School of Medicine. All chemicals used were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise stated. **Measurement of right ventricular pressure and systemic blood pressure.** Four weeks after injection with either MCT or saline, the rats were anesthetized with isoflurane, and their tracheas were cannulated with 16-gauge catheters. The rats were mechanically ventilated with 1% isoflurane with positive pressure using a ventilator (SN-480–7; Shinano, Tokyo, Japan). A polyethylene tube (PE-50 Intramedic PE tubing; Becton Dickinson, Sparks, MD) was advanced into the right ventricle via the right jugular vein to measure the right ventricular systolic pressure (RVSP). The position of the catheter was confirmed by the pressure waveform. The right femoral artery was cannulated with a 24-gauge catheter to record systemic blood pressure (SBP). These tubes were connected to a pressure transducer (PowerLab system, ADInstruments, Colorado Springs, CO).

**Measurements of cardiac output, total pulmonary resistance, and total systemic resistance.** Cardiac output was measured by the thermodilution technique. Changes in blood temperature were measured by an IT-23 thermocouple probe (Physiometrics Instruments, Clifton, NJ) positioned in the aortic arch via the left carotid artery. A 0.2-ml volume of 0.9% NaCl at 0°C temperature as the indicator was injected into the jugular vein catheter with its tip near the right atrium. The data were recorded and analyzed with a PowerLab cardiac output module (ML313; ADInstruments). Cardiac index (CI) was calculated as the ratio of cardiac output to body weight. Total pulmonary resistance index (TPRI) was defined as RVSP divided by CI (7). Total systemic resistance index (TSRI) was defined as mean SBP divided by CI (7).

**Intravenous injection of VIP and PACAP analogs.** The changes in RVSP and SBP in response to intravenous bolus injections of graded doses of [Ala]1,22,28VIP, a VPAC1 agonist with >1,000-fold selectivity compared with that of VIP (32) (PolyPeptide Laboratories, Strasbourg, France); BAY 55–9837, a VPAC2 agonist with >100-fold selectivity over that of VPAC1 (44) (Tocris Bioscience, Bristol, UK); VIP (Bachem, Bubendorf, Switzerland); and PACAP-38 (Peptide Institute, Osaka, Japan) were investigated 4 weeks after injection with either MCT or saline. The measurements were performed once for each agonist dose. One animal was given an agonist in graded doses. Maximum changes in RVSP and SBP were recorded. Each dose of the agonists was injected intravenously with 50 μl of saline. After each injection, sufficient time (20–60 min) was allowed for return of the hemodynamic parameters to their basal levels. Cardiac output was also measured in some animals, and the measurement was performed twice for each dose. The [Ala]1,22,28VIP was dissolved in 20% acetonitrile and diluted in saline before use. BAY 55–9837, VIP, and PACAP were dissolved in saline.

**Assessment of right ventricular hypertrophy.** Four weeks after injection with either MCT or saline, the rats were killed with an intravenous injection of 100 mg/kg of pentobarbital. The heart was then isolated to evaluate right ventricular hypertrophy. The lung tissues were isolated and processed for quantitative PCR and Western blot analyses. Blood was drawn to collect a serum sample. The serum samples were frozen in liquid nitrogen and stored at −80°C until use. The heart was isolated to weigh the right ventricle (RV) and the left ventricle with the intraventricular septum (LV+ISP). The weight ratio of RV to LV+ISP, RV/(LV+ISP), was calculated as an indicator of right ventricular hypertrophy (6).

**Quantitative reverse transcription PCR.** Total RNA was extracted from the rat lung tissues using Sepasol RNA I Super G (Nacalai Tesque, Kyoto, Japan), and 1 μg of total RNA was treated with DNase (Promega, Madison, WI) and reverse-transcribed using a PrimeScript cDNA Synthesis kit (Takara Bio, Shiga, Japan) with random hexaoligonucleotides as a primer. Quantitative reverse transcription PCR (qRT-PCR) was performed with a real-time PCR system (Bio-Rad Laboratory, Hercules, CA). The reaction mixture consisted of the following components: 1 μl each of forward and reverse primers, 10 μl of 2 × SYBR Premix Ex Taq (Takara Bio), 2 μl of distilled water, and 6 μl of diluted cDNA. The primers used were the previously reported oligonucleotides for all primers (22, 38): PACAP (forward: ACAGCGTCTCCTGTTCACT; reverse: CCTGGCGCTGGGTGTA; product size: 179 bp), VIP (forward: CACCGCCTTAAGACAAATGG; reverse: GCTCTTCTGAGAAGAAAGTCTGGAG; product size: 101 bp), and GAPDH (forward: CAACCTCTCAAGATTGTGACGAA; reverse: GCCATGGACTGTGTGATGA; product size: 118 bp). Relative RNA expression was calculated using the comparative threshold cycle method ([ΔΔCT] method), with GAPDH as an internal standard (25). The normalized value in the control group was expressed as 1 arbitrary unit for quantitative comparison.

**Western blot analysis.** The lung tissues were homogenized with a buffer containing 125 mM Tris (pH 6.8), 20% glycerol, 4% SDS, and 1% protease inhibitor cocktail (Nacalai Tesque). Protein concentrations were measured with a BCA Protein Assay kit (Thermo Scientific, Rockford, IL). Ten micrograms of protein was subjected to SDS-PAGE. Proteins were transferred onto a PVDF membrane and visualized by immunoblotting using the following primary antibodies: anti-PAC1, anti-VPAC1, anti-VIP, anti-PACAP (Santa Cruz Biotechnology, Santa Cruz, CA), anti-VPAC2 (Abcam, Cambridge, UK), and anti-B-actin antibodies (Sigma Aldrich). After incubation with secondary antibodies conjugated to horseradish peroxidase, expression of proteins was analyzed by an enhanced chemiluminescence detection system (GE Healthcare Biosciences, Piscataway, NJ). The images were obtained with an Image Analyzer LAS-3000 (Fujiﬁlm, Tokyo, Japan). The expressions of the proteins were normalized to the corresponding B-actin. The normalized value in the control group was expressed as 1 arbitrary unit for quantitative comparison.

**Determination of VIP serum concentration.** VIP serum concentration was determined by enzyme immunoassay (Phoenix Pharmaceuticals, Burlingame, CA). For each assay, 250 μl of serum was used.

**Data analysis.** All values are shown as mean ± SE. Comparisons of two independent data sets were performed by a two-tailed Student t-test. The hemodynamic data were analyzed using ANOVA followed by Dunnett’s post hoc test. Statistical signiﬁcance was set at P < 0.05.

### RESULTS

**MCT-induced PH and right ventricular hypertrophy.** SBP, RVSP, weight ratio of RV/(LV+ISP), CI, heart rate, TPRI, and TSRI were measured 4 wk after MCT or vehicle injection (Table 1). SBP in the MCT and control groups was similar (133 ± 5 vs. 106 ± 5 mmHg, not significant). We examined

### Table 1. Effects of monocrotaline treatment on cardiovascular measurements

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Monocrotaline</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right ventricular systolic pressure, mmHg (n = 13–17)</td>
<td>26 ± 2</td>
<td>74 ± 5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Systemic blood pressure, mmHg (n = 13–17)</td>
<td>106 ± 5</td>
<td>113 ± 5</td>
<td>NS</td>
</tr>
<tr>
<td>Weight ratio, RV/(LV+ISP) (n = 8)</td>
<td>0.31 ± 0.01</td>
<td>0.45 ± 0.02</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Cardiac index, ml·min⁻¹·100 g⁻¹ (n = 6–9)</td>
<td>41.4 ± 2.4</td>
<td>32.5 ± 0.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total pulmonary resistance index, mmHg·min⁻¹·100 g⁻¹ (n = 6–9)</td>
<td>0.74 ± 0.15</td>
<td>2.53 ± 0.36</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Total systemic resistance index, mmHg·min⁻¹·100 g⁻¹ (n = 6–9)</td>
<td>2.98 ± 0.18</td>
<td>3.43 ± 0.18</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are given as mean ± SE. RV, right ventricle; LV+ISP, left ventricle and intraventricular septum; NS, not significant.
RVSP as an indicator of pulmonary arterial pressure (5). RVSP in the MCT group was significantly higher than that in the control group (74 ± 5 vs. 26 ± 2 mmHg, \( P < 0.0001 \)). Similarly, the weight ratio of RV/(LV+ISP) as a marker of right ventricular hypertrophy was significantly higher in the MCT group than that in the control group (0.45 ± 0.02 vs. 0.31 ± 0.01, \( P < 0.0001 \)). These results confirmed that MCT successfully produced PH and consequent right ventricular hypertrophy. CI in the MCT group was significantly lower than that in the control (32.5 ± 0.9 vs. 41.4 ± 2.4 ml·min\(^{-1}\)·100 g\(^{-1}\), \( P < 0.01 \)), TPRI in the MCT group was significantly higher than that in the control (32.5 ± 0.8 vs. 0.9 vs. 41.4 ± 2.4 ml·min\(^{-1}\)·100 g\(^{-1}\), \( P < 0.05 \)). In contrast, TSRI in the MCT and control group was similar (3.43 ± 0.18 vs. 2.99 ± 0.18 mmHg·min·100 g·ml\(^{-1}\), not significant).

Changes in messenger RNA and protein expression of VIP and PACAP in lung tissues. Messenger RNA expression in the lung tissues was measured by qRT-PCR analysis and is presented as one arbitrary unit in the control group. The expressions of VPAC1, VPAC2, and PAC1 receptors in lung tissues was measured by Western blot analysis (Fig. 1) and is presented as one arbitrary unit in the control group. The expressions of VPAC1, VPAC2, and PAC1 receptors in the lung tissue was measured by qRT-PCR analysis and is presented as one arbitrary unit in the control group. The expressions of VIP and PACAP in the lung tissues was measured by Western blot analysis (Fig. 1) and is presented as one arbitrary unit in the control group. The expressions of VIP and PACAP in the MCT group were 0.37 ± 0.06, 4.0 ± 0.8, 2.8 ± 0.8, and 0.05, respectively (Fig. 1, A and B). These values were significantly lower than those of their respective controls (\( P < 0.05 \) and \( P < 0.005 \), respectively). Protein expression of VIP and PACAP precursors in the lung tissue was measured by Western blot analysis (Fig. 1C) and is presented as one arbitrary unit in the control group. The expressions of VIP precursor (0.32 ± 0.04; \( P < 0.005 \)) and PACAP precursor (0.42 ± 0.14; \( P < 0.05 \)) in the MCT group were significantly lower than those of their respective controls (Fig. 1, D and E).

Changes in protein expression of VPAC1, VPAC2, and PAC1 receptors in lung tissues. Protein expression of VPAC1, VPAC2, and PAC1 receptors in lung tissue was measured by Western blot analysis (Fig. 2A) and is presented as one arbitrary unit in the control group. The expressions of VPAC1 (6.7 ± 2.1; \( P < 0.05 \)), VPAC2 (4.0 ± 0.8; \( P < 0.005 \)), and PAC1 (2.8 ± 0.8; \( P < 0.05 \)) in the MCT group were significantly higher than those of their respective controls (Fig. 2, B–D).

Effect of intravenous injection of VPAC1- and VPAC2-selective agonists VIP and PACAP on RVSP and SBP. We tested hemodynamic changes of SBP and RVSP in response to their agonists. The maximum injection doses of BAY 55–9837, VIP, and [Ala\(^{11,22,28}\)]VIP were chosen to produce similar and close to 50% decreases in SBP in the control rats because the decreases in SBP with the analogs at maximum dose were less than 80–90 mmHg, and analogs administered at over the maximum dose could induce circulatory collapse. The maximum injection doses of BAY 55–9837, VIP, and [Ala\(^{11,22,28}\)]VIP were determined to be 10, 3, and 3 \( \mu \)g/kg, respectively. The maximum dose of PACAP was determined to be 3 \( \mu \)g/kg, where PACAP produced maximum decrease in SBP in the
control group. The responses of decreases in RVSP and SBP were obtained within 1 min after administration of the agonists. The injection of BAY 55–9837 at dosages of 0.3–10 μg/kg decreased RVSP in a dose-dependent manner in the MCT group (Fig. 3A). RVSP with 10 μg/kg injection of BAY 55–9837 was significantly lower than that at basal level. However, RVSP with BAY 55–9837 injection in the MCT group was significantly higher than that in the control group at each dose. BAY 55–9837 also decreased SBP in a dose-dependent manner in both groups. SBPs with 3 and 10 μg/kg injections of BAY 55–9837 were significantly lower than those at basal level in both groups, whereas SBP with BAY 55–9837 injection in the MCT group was higher than that in the control group at each dose. The difference in SBP
at 3 μg/kg of BAY 55–9837 between the groups was significant (P < 0.05) (Fig. 3E).

The injection of VIP at dosages of 0.1–3.0 μg/kg also resulted in small dose-dependent decreases in RVSP in the MCT group. RVSP with VIP injection at each dose in the MCT group was significantly higher than that in the control group (Fig. 3B). VIP decreased SBP in both groups almost equally (Fig. 3F). SBPs with 1 and 3 μg/kg injections of VIP were significantly lower than those at basal level in both groups.

[Ala11,22,28]VIP at dosages of 0.1–3.0 μg/kg hardly affected RVSP in the MCT and control groups (Fig. 3C), whereas it decreased SBP in a dose-dependent manner in both groups (Fig. 3G). SBPs with 1 and 3 μg/kg of [Ala11,22,28]VIP injection were significantly lower than those at basal level in both groups.

PACAP at dosages of 0.1–3.0 μg/kg caused small decreases in RVSP in the MCT group (Fig. 3D). After an initial period of small decrease in RVSP in the 1st minute, the RVSP increased to above basal level over the next 1–5 min. RVSP then returned to the basal level 20 min after PACAP administration. PACAP decreased SBP in both groups almost equally (Fig. 3H). SBPs with 1 and 3 μg/kg of PACAP injection were significantly lower than those at basal level in both groups.

Relation between decreases in RVSP and SBP in response to BAY 55–9837, VIP, and [Ala11,22,28]VIP. The decreases in RVSP and SBP in response to BAY 55–9837, VIP, and [Ala11,22,28]VIP in the MCT and control groups were plotted. For a similar decrease in SBP, BAY 55–9837 produced greater decrease in RVSP than did VIP and [Ala11,22,28]VIP in the MCT group (Fig. 4A). In the control group, these agonists decreased SBP with little effect on RVSP. The results showed the specific effect of BAY 55–9837 on decreasing RVSP compared with that of VIP and [Ala11,22,28]VIP.

Effect of intravenous injection of VPAC2 selective agonist and VIP on CI, TPRI, and TSRI. We examined the effects of intravenous injection of BAY 55–9837 and VIP on CI, TPRI, and TSRI, because BAY 55–9837 and VIP, but not PACAP and [Ala11,22,28]VIP, decreased RVSP in the MCT group. Injection of BAY 55–9837 at dosages of 0.3–10 μg/kg increased CI and decreased TPRI and TSRI in a dose-dependent manner in the MCT group (Fig. 5, A, C, and E). The increases in CI with 3 and 10.0 μg/kg injections of BAY 55–9837 were significantly larger than the CI at basal level. However, VIP at dosages of 0.1–3.0 μg/kg showed little effect on CI (Fig. 5B) and caused a slight but not significant decrease in TPRI (Fig. 5D). In contrast, VIP decreased TSRI in a dose-dependent manner in the MCT group (Fig. 5F).

In the control group, BAY 55–9837 and VIP did not change CI and TPRI significantly, similar to the results obtained from RVSP measurements in this study (Fig. 5, A–D). CIs at the basal level and with 0.3 μg/kg injections of BAY 55–9837 in the MCT group were significantly lower than those in the control group, respectively, whereas a significant difference in CI was observed at the basal level and at every dosage of VIP. BAY 55–9837 and VIP also decreased TSRI in a dose-dependent manner in the control group (Fig. 5, E and F). During these hemodynamic experiments lasting for 3–4 h, right ventricular end-diastolic pressure was measured as an index of cardiac filling and was not affected by intravenous injection of the agonists.

VIP concentration in serum. VIP concentration in the serum was determined to be 107 ± 14 pg/ml in the MCT group and 111 ± 16 pg/ml in the control group. There was no significant difference between the groups (Fig. 6).

DISCUSSION

In this study, we found that expressions of VIP and PACAP were decreased concomitantly with upregulated expression of their three receptors, VPAC1, VPAC2, and PAC1, in the lung tissues of rats with MCT-induced PH. These results are consistent with previous reports demonstrating upregulated expression of the receptors, reflecting decreased VIP both in patients and in an animals with PH (36).

We also demonstrated that Bay 55–9837, a VPAC2 selective agonist, and VIP, but not [Ala11,22,28]VIP, a VPAC1 selective agonist, dose dependently decreased RVSP in PH, and for a similar decrease in SBP, Bay 55–9837 produced greater reduction in RVSP than did VIP. Bay 55–9837, but not VIP, also decreased TPRI and improved CI in the rats with PH. In addition, the decrease in SBP with Bay 55–9837 in PH was significantly less than that in the control. All of these agonists decreased SBP but hardly affected RVSP in the control. Based on these findings, it appears that selective agonist for VPAC2, but not VPAC1 and PAC1, affects pulmonary resistance and consequent improvement of cardiac output in MCT-induced PH.

According to clinical guidelines and consensus (14, 29), pulmonary vasodilators such as PGI2 analogs are recommended for treatments in patients with mild to severe (WHO

![Fig. 4. Relation between change in right ventricular systolic pressure (RVSP) and that in systolic blood pressure (SBP) in response to BAY 55–9837, VIP, and [Ala11,22,28]VIP. A: changes in RVSP and SBP in response to intravenous injection of BAY 55–9837 (Bay) at 0.3, 1, 3, and 10 μg/kg, VIP at 0.1, 0.3, 1, and 3 μg/kg, and [Ala11,22,28]VIP (Ala) at 0.1, 0.3, 1, and 3 μg/kg in the MCT group are shown in A. Changes in RVSP and SBP in response to the agonists in the control group are shown in B. Values are given as mean ± SE (n = 8–12).](http://jap.physiology.org)
class II to IV) PH. However, despite clear progress and improvements in outcomes (15), because of the insufficient efficacy of the present therapies, novel treatments including pulmonary vasodilator and combined treatments are still needed (15, 42). The relative importance of VPAC2 in PH has also been supported by a previous report that another VPAC2 selective agonist, Ro 25–1553, decrease isometric tension of the human pulmonary arterial rings precontracted with PGF2\(\alpha\) (41). The VPAC2 receptor is expressed in vascular smooth muscle and induces vasodilation in response to VIP released from the intramural nerves. In contrast, vasodilation via VPAC1 is dependent on nitric oxide generated in the vascular endothelium (19). This may be why a VPAC1 agonist did not decrease RVSP in our study because the function of endothelial nitric oxide synthesis in pulmonary vessels is likely impaired in PH (26, 30, 40).

Bay 55–9837 increased CI probably because decreased pulmonary resistance leads to increased left ventricular preload. This increase in CI also contributed to less decrease in SBP in PH than in the control. However, CI can also be affected by cardiac contractility, and the VIP analog could affect cardiac contractility via VPAC1 and VPAC2 receptors (46). In this study, BAY 55–9837 administration improved CI up to normal values in the MCT group, whereas improvement of TPRI did not reach normal. Recently, Vasomera, a novel VPAC2 agonist, was reported to enhance cardiac contractility in normal and cardiomyopathic hearts in dogs (Carlos L. unpublished data). Therefore, it is possible that the discrepancy in the improvement between CI and TPRI with BAY 55–9837 administration might be due to an increase in cardiac contractility. Moreover, increased expression of VPAC2 in the cardiac ventricles was observed in MCT-induced PH (Koga, unpublished data), which may suggest involvement of the VPAC2 receptor in cardiac function in PH.

In this study, VIP did not significantly improve CI in PH despite its known inotropic and chronotropic effects (46). The amount of decrease in RVSP even with the maximum dose (3 \(\mu\)g/kg) of VIP tested was small (−5 mmHg) and was similar to those at BAY 55–9837 doses of 0.3 and 1 \(\mu\)g/kg. These doses did not produce significant improvement of CI or TPRI. There-
fore, it is likely that the decrease in RVSP with VIP may be not adequate to increase CI significantly in this model.

The expression of VPAC1 was increased in lung tissues in PH, but the VPAC1 agonist did not affect RVSP. Therefore, the role of VPAC1 in PH remains unclear. VPAC1 has been reported to have anti-inflammatory (18) and neuroprotective effects (10). Recently, it was reported that both VPAC1 and VPAC2 receptors are necessary for the optimal anti-inflammatory effect of VIP in lung tissues and that high VPAC1 expression in alveolar macrophages reflects chronic inflammation in the lung of COPD patients (3). Thus upregulation of VPAC1 expression might be an anti-inflammatory response to the development of PH in cooperation with upregulation of VPAC2.

From comparison of the changes in RVSP with VIP and PACAP injections, PAC1 did not appear to be involved in the decrease in pulmonary resistance, although we did not use a PAC1-selective agonist. Biphasic changes in RVSP with intravenous injection of PACAP in this study were similar to those of a previous report of perfusion pressure in hindquarter vessels (17). These results are consistent with previous studies in which PACAP was shown to stimulate sympathetic nerves (13, 23) and renin secretion via activation of PAC1 (12). However, PAC1 gene deletion leads to the development and/or worsening of PH (34). Furthermore, PACAP plays protective roles in injury in many organs such as acute lung injury (50), cerebral ischemia (8), and cardiac myopathy (16) through its antiproliferative, antiapoptotic (51), and anti-inflammatory (28) effects. Thus it seems possible that PACAP signaling may play such protective roles rather than decrease RVSP in PH. However, further investigation will be necessary to explore the roles of PAC1 in PH.

The VIP concentration in serum did not differ in the MCT and control groups, although its mRNA expression and precursor protein in the lung tissues were reduced in PH. It has been reported that VIP is present in nerves and endocrine cells of the respiratory organs (27). Therefore, VIP content in the lung tissues appeared to be regulated independently of the serum concentration in PH.

We showed the beneficial effects of BAY 55–9837 on pulmonary circulation compared with VIP, [Ala^{11,22,28}]VIP, and PACAP. BAY 55–9837 could have less unintentional physiological effects than VIP, which is known as a potential treatment for PH because of its poor affinity for the VPAC1 receptor. In addition, VPAC2 was reported to play a dominant role in VIP in the inhibition of pulmonary artery smooth muscle proliferation (43). Therefore, BAY 55–9837 could be a more promising treatment than VIP. However, when evaluating the VPAC2 agonist as a therapeutic option, several issues such as the delivery method and the dose should be considered. Most peptide hormones and their analogs such as BAY 55–9837 are unstable in the serum, and their half-lives are short (35). Such properties have limited their clinical utility when administered systemically. In addition, the presence of VPAC2 in a variety of tissues could cause unintentional physiological effects if VPAC2 agonists are administered systemically. Because even treatments for PH in the guidelines (14, 29) such as calcium channel blockers, PGI2 analogs, and phosphodiesterase-5 inhibitors have systemic effects such as hypotension, delivery methods have been optimized through inhaled, oral, subcutaneous, and intravenous administration to reduce the adverse effects.

In fact, inhalation of VIP has been expected as a treatment for various lung diseases including asthma (48), acute airway inflammation (33), sarcoidosis (37), and PH (24, 36). A long-lasting VIP analog has been developed for clinical use (48). These reports support the possibility of the use of an inhaled VPAC2 agonist for PH treatment. Appropriate delivery methods of BAY 55–9837 for PH treatment were not explored in this study. To support the possibility of a VPAC2 agonist for PH treatment, optimization of delivery methods should be investigated in the future.

Although we demonstrated the beneficial effects of activation of VPAC2 with BAY 55–9837 on systemic and pulmonary circulation in MCT-induced PH, the precise roles of the increase in receptor expression are still unclear because we did not investigate the roles in the absence of exogenously administered agonist in PH or protection from PH. Upregulation of VPAC1 and VPAC2 receptors was reported to be a compensatory response to decreased VIP in patients and in animals with PH (36). However, a decrease in pulmonary arterial pressure under normal conditions and an enhanced decrease under conditions of increased pulmonary resistance were observed in previous studies with prostacyclin (31). Acute PH models with no increase in the expression of these receptors induced by acute hypoxia or administration of vasoconstrictors also could provide a more precise understanding of VPAC2 in PH.

The MCT-induced PH model can lead to a limitation of the evaluation of VPAC2 agonist for PH treatment in humans. However, involvement of abnormal VIP/VPAC2 signaling has been reported in primary PH patients (36). These data also support the possibility of a VPAC2 agonist for PH treatment in humans.

In conclusion, the present study provided evidence that activation of VPAC2 receptor with Bay 55–9837, a VPAC2 selective agonist, effectively improved RVSP, TPRI, and CI. These results support a VPAC2 agonist as a promising treatment for PH.

**ACKNOWLEDGMENTS**

We thank the Cardiovascular Research Institute of Yokohama City University Graduate School of Medicine for providing excellent research facilities and Mrs. Yuki Yuba for excellent technical assistance.


