Augmentation of the effects of vasoactive intestinal peptide aerosol on pulmonary hypertension via coapplication of a neutral endopeptidase 24.11 inhibitor

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PULMONARY HYPERTENSION (PH) is characterized by an imbalance between pulmonary vasoconstrictive/proliferative and vasodilative/antiproliferative mediators leading to sustained pulmonary vasoconstriction and remodeling of pulmonary arteries and consequently increased pulmonary vascular resistance. The vasoactive intestinal peptide (VIP) is abundantly expressed in the pulmonary vasculature (4), and VIP deficiency has been suggested to be involved in the development of PH (18, 23). VIP is a neuuropeptide leading to relaxation of pulmonary vascular smooth muscle cells (8, 21). A knockout of the VIP gene leads to hemodynamic and histomorphological features of pulmonary arterial hypertension in mice (23), whereas supplementation of VIP by subcutaneous application partially reverses these changes. In addition, several patients with idiopathic pulmonary arterial hypertension were deficient for VIP but had increased VIP receptor expression and binding affinity. Treatment with inhaled VIP led to hemodynamic and functional improvement (18). However, because VIP is prone to rapid degradation, e.g., by neutral endopeptidases (NEP) 24.11 (31), the assumed effect of inhaled VIP is short. Nevertheless, because a deficiency of VIP has also been suggested to be involved in other lung diseases the aerosol approach is of special interest (19, 22, 24). In terms of PH, the inhalative approach has been beneficial in acute (13, 26, 27) and chronic (28) experimental studies as well as in human PH (16). The present study aimed to characterize the biological activity of inhaled VIP alone and during coapplication with thiorphan, an inhibitor of NEP 24.11 in a model of acute PH.

MATERIALS AND METHODS

The model and the experimental protocol were approved by the governmental animal care committee. All experiments were performed according to the Helsinki convention for the use and care of animals. The isolated ventilated and buffer-perfused lung model. This model and our modifications have been described elsewhere (13, 14) and have been used to characterize pulmonary vasodilators during intravascular and aerosol application before. This lung model facilitates a focused investigation of substances that mediate pulmonary vasodilation in a quasi in vivo setting without interference of central or humoral influences (e.g., circulating blood cells). Under constant flow condition, elevation of the pulmonary arterial pressure (PAP) directly reflected an increase in pulmonary vascular resistance.

Briefly, rabbits (New Zealand white bastards) of either sex, weighing 2.2–2.5 kg, were anesthetized using ketamine (Ketamin Inresa; Inresa, Freiburg, Germany) and xylazine (Rompun; Bayer, Leverkusen, Germany). Animals were anticoagulated with heparin (1,000 U/kg body wt). After tracheotomy, artificial ventilation was started with room air using a Harvard respirator (tidal volume 8–10 ml/kg, frequency 30 breaths/min). Midsternal thoracotomy followed and the whole system was heated to 38°C. Room air, supplemented with Krebs-Henseleit hydroxyethylamyllopectine buffer (120 mM NaCl, 4.3 mM KCl, 1.1 mM KH2PO4, 25 mM NaHCO3, 2.4 mM CaCl2, 1.3 mM MgPO4, and 2.4 g/l glucose) and 2.5% hydroxyethylamyllopectine (molecular weight 200 kDa) as an oncotic agent (Serag Wiesner, Naila, Germany). All concentrations are given as the final concentration in the buffer fluid.

The isolated lungs were excised from the chest and freely suspended from an electronic force transducer (Transducer U1; Hottinger Baldwin Messtechnik, Darmstadt, Germany) in a humidified and tempered glass thorax. Perfusion was performed at a constant rate of 120 ml/min in a recirculating manner (total perfusate volume 300 ml), and the whole system was heated to 38°C. Room air, supplemented with ~4% carbon dioxide was used for artificial ventilation to reach...
a pH of 7.34 to 7.38, and a positive-end expiratory pressure (PEEP) was set at 1.0–1.3 mmHg (reference point hilum). Pressure in left atrium was set at 1.2 mmHg. To improve comparability, time was set as a stable pulmonary vasoconstriction (PAP plateau). The experiments were stopped at time $t = 120$ min.

**Intravascular application of VIP and thiorphan.** When applied intravascularly, VIP and thiorphan were injected in the reservoir of the recirculating buffer fluid. Dose response curves for bolus intravascularly injected VIP ($n = 4$ each dose) were performed (Fig. 1A). In the subsequent experiments, 0.142 μM VIP was given at $t = 0$, and all parameters were registered for another 120 min.

Thiorphan inhibits the metalloprotease neprilysin (neutral endopeptidase 24.11) ($K_1 = 4$ nM) and neprilysin 2 ($K_1 \sim 200$ nM) (20) and exerts its biological effects in a time-delayed manner after its application (34). Therefore, thiorphan was applied into the buffer fluid after a stable baseline period of 20 min and before U46619 start (each dose $n = 4$). To characterize its effects on pulmonary vasoconstriction, thiorphan was also applied intravascularly in separate experiments at $t = 0$ (each dose $n = 4$). In the subsequent experiments, thiorphan was injected or aerosolized (3 μM) as pretreatment 20 min after a stable baseline period.

**Aerosolization.** Our modification of this aerosolization system has been described before (13, 14). Placebo (NaCl 0.9%), VIP, and thiorphan were aerosolized with an ultrasonic device (Optineb, Nebutec, Elsenfeld, Germany). This nebulizer produces an aerosol with a mass median aerodynamic diameter of 4 μm and a geometric SD of 1.5. The nebulizer was placed into the inspiratory limb of the ventilatory system. A comparable nebulization system was evaluated by Schmehl and coworkers (29) in an isolated lung model, and an absolute deposition fraction of 0.25 ± 0.02 was determined. In separate dose-finding experiments, 3.7 μM of VIP aerosol (Fig. 1B) was found to cause a similar pulmonary vasodilation compared with 0.142 μM VIP when applied as an intravascular bolus.

**Control groups.** During baseline experiments, no intervention was performed over a time period of up to 4 h. In the sham groups, a stable pulmonary vasoconstriction was followed by a bolus of 1 ml of NaCl 0.9% either given into the recirculating buffer fluid (intravascularly) or as an aerosol over 15 min starting at $t = 0$.

**VIP interventional groups.** After establishing a stable pulmonary vasoconstriction over 15 min ($t = 0$), VIP was either given intravascularly ($n = 4$) or as an aerosol ($n = 5$).

**Combined intervention with VIP and thiorphan.** After a baseline period of 20 min, thiorphan was applied either intravascularly ($n = 4$) or as an aerosol ($n = 6$), followed by the administration of U46619. VIP was consecutively administered intravascularly ($n = 4$) or as an aerosol ($n = 6$) at $t = 0$.

**Data analysis.** All data are shown as means ± SE. For primary data analysis, comparison was performed between the U46619 groups and intravascular VIP. Second, the VIP aerosol group was compared with the respective placebo inhalation group in U46619-induced PH.

**Comparison between groups.** For all parameters at the end of inhalation ($t = 15$ min) and the end of the experiment. For comparison of statistical difference between groups, we performed either an unpaired Student’s $t$-test or a one-factorial ANOVA with the Bonferroni correction. Significance was assumed when $P < 0.05$.

**RESULTS**

**Baseline conditions and sham groups.** After completion of a 20-min steady-state period, PAP was between 6 and 8 mmHg, and VP was between 2 and 4 mmHg (Fig. 2). Over a total time period of up to 4 h, no significant changes of PAP, VP, or lung weight were registered (not shown in detail).

Titrated U46619 provoked a rapid pulmonary vasoconstriction, leveling off in a plateau phase of 15 min with a PAP of 25.5 ± 0.7 mmHg ($t = 0$). This plateau was stable even in the intravascular or aerosol sham group until the experiment was stopped (PAP 26.4 ± 1.3 mmHg at $t = 120$ min). VP and lung weight were also stable during the whole experiment.

**Effects of VIP on pulmonary vasoconstriction.** During the dose-finding experiments, VIP either applied intravascularly or as an aerosol caused a dose-dependent vasodilation (data not shown). The VIP intravascularly and aerosol-treated lungs started at the same baseline conditions (PAP 7 ± 1 and 7.5 ± 1.1 mmHg, respectively) and PAP plateau phase (PAP 25.0 ± 0.4 and 25.7 ± 0.3 mmHg, respectively at $t = 0$) as the U46619 sham group (Fig. 3). Intravascular administration of 0.142 μM VIP caused a rapid and significant reduction of PAP after 15 min compared with the sham group (18.9 ± 1.4 mmHg).
Aerosolization of 3.7 μM VIP also led to a reduction of PAP after 15 min compared with sham (20.6 ± 0.8 vs. 26.1 ± 0.8 mmHg, P < 0.01). In this group, the effect on PAP reduction disappeared within 30 min after aerosol stopping. Again, none of the other registered parameters changed in this group.

**Effects of thiorphan on pulmonary vasoconstriction.** Application of thiorphan independently of the dose, route (intravascularly or as an aerosol), or experimental phase (baseline conditions vs. pulmonary vasoconstriction) had no effect on PAP, VP, or lung weight (data not shown in detail).

**Coapplication of VIP and thiorphan.** In addition to the above-mentioned VIP intervention, lungs were pretreated with thiorphan, which was either applied intravascularly or as an aerosol. Pretreatment with intravascular thiorphan in subsequently VIP intravascularly treated lungs caused a significant reduction of PAP, which was comparable to that registered with VIP intravascular treatment alone. Neither in the PAP nor in any other of the registered parameters was a significant difference observed (Fig. 3).

Pretreatment with thiorphan aerosol in subsequently VIP intravascularly treated lungs caused a pronounced reduction of PAP in the VIP intravascularly treated group (t = 15 min, PAP 18.9 ± 1.4 vs. 23 ± 1.3 mmHg, P > 0.05). Besides a tendency to more pronounced effects in the VIP intravascular treatment group, the hemodynamic profile was comparable to the combined treatment group (Fig. 4).

Starting at a comparable level of pulmonary vasoconstriction of the VIP aerosol and VIP aerosol plus thiorphan intravascular group (PAP 25.7 ± 0.3 vs. 25 ± 1 mmHg, respectively P > 0.05), pretreatment with thiorphan intravascularly in subsequently VIP aerosol-treated lungs resulted in a significant augmentation of the VIP effects on PAP. This was true at t = 15 min (PAP 16.2 ± 1.4 vs. 20.6 ± 0.8 mmHg, P < 0.05) and at the end of the experiment at t = 120 min (PAP 20 ± 0.8 vs. 26.3 ± 0.6 mmHg, P < 0.001). All other registered parameters were not affected (Fig. 5).

PAP plateau was also similar in lungs that were pretreated with thiorphan aerosol or not when VIP aerosol was given subsequently (VIP aerosol 25.7 ± 0.3 mmHg and VIP aerosol plus thiorphan aerosol PAP 25.9 ± 0.6 mmHg, P > 0.05). However, pretreatment with thiorphan aerosol augmented the effect of VIP aerosol on PAP alone at t = 15 min (PAP 19.5 ± 0.8 vs. 26.3 ± 0.6 mmHg, P < 0.05).

Fig. 2. Stable pulmonary vasoconstriction during continuous application of the thromboxane mimetic U46619 (n = 4). Effects of NaCl aerosol as the sham (U46619 + NaCl aerosol) group (n = 4).

Fig. 3. Pulmonary vasodilative effects of i.v. (n = 4) or inhaled (n = 5) VIP in a model of U46619-induced pulmonary vasoconstriction.

Fig. 4. Pulmonary vasodilative effects of i.v. applied VIP alone (n = 4) or in combination with i.v. neutral endopeptidases (NEP) 24.11 inhibitor thiorphan (n = 4) in a model of U46619-induced pulmonary vasoconstriction.

Fig. 5. Pulmonary vasodilative effects of i.v. applied VIP alone (n = 4) or in combination with inhaled NEP 24.11 inhibitor thiorphan (n = 6) in a model of U46619-induced pulmonary vasoconstriction.
significantly (Figs. 6 and 7). Nevertheless, because a deficiency of VIP has also been associated with pulmonary arterial hypertension in mice, whereas supplementation of VIP gene led to hemodynamic and histomorphological features mimicking PH (10). In men, a group of patients with idiopathic pulmonary arterial hypertension was used to establish a stable pulmonary vasoconstriction. Compared with other publications, this level of pulmonary vasoconstriction was not associated with significant edema formation, potentially compromising pulmonary vasodilative reserve (26).

The link between VIP deficiency and PH is based on experimental and clinical findings. A genetic knockout of the VIP gene led to hemodynamic and histomorphological features of pulmonary arterial hypertension in mice, whereas supplementation of VIP by intraperitoneal injections improved pulmonary vascular and right ventricular remodeling (23). In another model, treatment with intraperitoneal VIP injections either prevented the occurrence of or alleviated established monocrotaline-induced PH in rats (10). In men, a group of patients with idiopathic pulmonary arterial hypertension was deficient for VIP but had increased VIP receptor expression and binding affinity. In this study, chronic supplementation with inhaled VIP led to hemodynamic and functional improvement (18). In an acute setting, inhalation of VIP in patients with PH provoked minor but significant hemodynamic effects (12). Finally, the clinical trial of chronic treatment with VIP aerosol in PH failed (7), so that the future role of VIP aerosol in this setting has been questioned. Although the rationale seems to be clear, the thus far reported hemodynamic and clinical effects of VIP aerosol were only minor, whereupon bioavailability and the overall potential have been questioned (7). Nevertheless, because a deficiency of VIP has also been suggested to be involved in other lung diseases, there is still a good rationale for the molecule as well as for the aerosol approach (19, 22, 24).

Application of VIP either into the recirculating buffer fluid or as an aerosol provoked a pulmonary vasodilation with reduction of PAP by ~25%. This effect was rapid in onset, maximal after 15 min, and ceased within 60 min at the latest.

The vasodilative properties of VIP have been attributed to two type II G protein-coupled membrane-bound (VPAC1 and VPAC2) receptors (8, 11), leading to the formation of cAMP followed by an activation of protein kinases and reduction of intracellular calcium concentrations (6). These receptors have been identified on smooth muscle cells of bronchi and pulmonary arteries in human lungs (4). Relaxation of pulmonary smooth muscle cells and vessels has been shown before in vitro (8, 9, 21). In another isolated lung model intravascular administration of a cyclic stable VIP analog partially antagonized acute hypoxic pulmonary vasoconstriction (33), a mechanism also known as von Euler Liljestrand reflex, which is understood to redirect blood flow from nonventilated to well-ventilated areas in the lung. Therefore, bypassing hypoxic pulmonary vasoconstriction could lead to or aggravate preexisting gas exchange disturbances. To overcome this and other shortcomings of intravascularly administrated vasoactive substances, the inhalative route has been suggested in acute (13, 26, 27) and chronic (28) experimental studies, as well as in human PH (16). However, it is not self-evident that biological properties can be transferred from the intravascular to the inhalational application mode. In our present study, the pulmonary vasodilative properties of VIP were preserved after its inhalation. This is in line with a recent study of Yin et al. (33), who demonstrated that inhalation of a stable analog of VIP antagonizes acute hypoxic pulmonary vasoconstriction in an intact rat model.

Besides reaching the lung as the target organ directly, the inhalational application mode also offers the possibility to deliver pharmacological agents to a large surface to save substance or to enhance effects (5). With the use of this isolated lung model, aerosolization of different structured substances (e.g., salts) was applied in equimolar dosages to provoke comparable effects as the intravenously administrated substance (13). In another study, dosages were even lower.
during the inhalational compared with the intravascular route (32). Of note, in the present study, the dosage of VIP given as an aerosol had to be 26-fold above that of the intravascularly administered dose to reach a similar magnitude of vasodilation. This is comparable to previous data using a peptide preparation in this model (14) and discloses the difficulties that have to be taken into account when aerosolizing peptides to the lung. Therefore, stable analogs of VIP or liposomal-packed VIP have been suggested to prolong the biological activity of VIP when used as an aerosol (17, 30, 33).

Besides structural damage during the aerosolization process and its direct hydrolysis (5), the biological activity of VIP is thought to be mainly determined by the presence of the peptides’ degrading enzymes, the neutral endopeptidases, especially NEP 24.11 (31). Therefore, another approach to stabilize and consequently augment the biological effects of VIP could be the coapplication of thiorphan, an inhibitor of NEP 24.11. In this present study, pretreatment with thiorphan, either intravascularly or as an aerosol, clearly boosted the pulmonary vasodilative effects of VIP aerosol. Thiorphan, not only prolonged, but also amplified the maximum effect of VIP aerosol, supporting the recent observation that the majority of VIP-degrading NEP 24.11 enzyme is located on the airway surface (17). Accordingly, there was no effect when both VIP and thiorphan were applied intravascularly. There was a tendency for higher maximal VIP intravascular effects compared with the thiorphan aerosol-pretreated lungs. This effect did not reach statistical significance, and it remains elusive to speculate on the cause of this observation. However, it is a major finding of this study that VIP effects were only amplified when it was applied as an aerosol. This may be of special interest when the clinical potential of NEP inhibition is discussed. NEP degrades several endogenous vasoactive but also bronchoactive peptides, including natriuretic peptides and bradykinin. Therefore NEP inhibition may be ambivalent because also tachykinins are metabolized by NEP, and this could lead to a pronounced bronchoconstriction (3). However, during coapplication, e.g., with the atrial natriuretic peptide (ANP), NEP inhibition with thiorphan augmented the bronchodilative effects of ANP. However, comparable to our present findings, this was only true when thiorphan was aerosolized (2) and not when administered systemically (1). Recently, a combination therapy with the NEP inhibitor sacubitril (AHU377) and the angiotensin receptor blocker valsartan had superior effects in this model (14) and discloses the difficulties that have to be taken into account when aerosolizing peptides to the lung.

In our present study, we did not observe an increase in VPs during the inhalational compared with the intravascular route (32). Of note, in the present study, the dosage of VIP given as an aerosol had to be 26-fold above that of the intravascularly administered dose to reach a similar magnitude of vasodilation. This is comparable to previous data using a peptide preparation in this model (14) and discloses the difficulties that have to be taken into account when aerosolizing peptides to the lung. Therefore, stable analogs of VIP or liposomal-packed VIP have been suggested to prolong the biological activity of VIP when used as an aerosol (17, 30, 33).

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In our present study, we did not observe an increase in VPs and therefore can rule out growth bronchial constriction. However, because our model is blood free and buffer perfused, this could have led to less activity of endogenous peptides, and therefore we may have missed such effects. This is also true for the missing effects of thiorphan on the recirculating NEP during intravascular application in this model. Nevertheless, the biological half-life of VIP aerosol in this model was at least quadrupled (from 30 to 120 min) by the coapplication of thiorphan. Another major limitation of this study is that we cannot conclude on any chronic effect of the coapplication of NEP inhibitors and VIP.

Despite these limitations, this is the first study supporting the concept of cotreatment of VIP and NEP inhibition augmenting the biological effects of VIP aerosol alone and potentially offering a new treatment approach for PH.

DISCLOSURES

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AUTHOR CONTRIBUTIONS


REFERENCES


