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Endothelium-Dependent Vasorelaxant Effect of a Bioactive Tripeptide, Valine-Proline-Proline, on Rat Aortic Rings

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Abstract The aim of this study was to gain further insights into the mechanisms responsible for the Val-Pro-Pro-induced vasodilation of rat aortic rings. The vasorelaxant effect was found at 10^{-9}-10^{-4} M of the tripeptide on phenylephrine-precontracted rings. It was endothelium-dependent and concentration-dependent at the higher concentrations. The vasodilator response was not modified by preincubation with 3.1 x 10^{-7} M glibenclamide, 10^{-3} M 4-aminopyridine (4-AP), 10^{-5} M indomethacin or 10^{-5} M cycloheximide. However, this response was significantly diminished by preincubation with 10^{-5} M L-NG-Nitroarginine methyl ester (L-NAME), 10^{-7} M 1H-(1,2,4)oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), 10^{-6} M (9S,10R,12R)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-i] [1,6] benzodiazocine-10-carboxylic acid, methyl ester (KT 5823), 10^{-2} M tetraethylammonium (TEA) and 10^{-7} M apamin plus 10^{-7} M charybdotoxin, as well as by the removal of the vascular endothelium. Apparently, Val-Pro-Pro directly produced vasorelaxation of phenylephrine-precontracted rat aortic rings by stimulating the vascular endothelium and activating the NO/cGMP/PKG/Ca^{2+}-activated K^+ channel pathway.

Keywords: *bioactive peptides, nitric oxide, potassium channels, rat aorta, vascular endothelium.*

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1. Introduction

Hypertension is the most common chronic disease and the most important risk factor for cardiovascular disease. It affects nearly 1 billion adults, accounts for about 9% of global disability-adjusted life years, and is associated with over 9 million deaths annually [1]

Hypertension is treated non-pharmacologically and pharmacologically. The former method involves lifestyle modifications such as losing weight in cases of obesity, adopting a diet rich in fruits and vegetables, consuming sodium below the recommended threshold, avoiding a high level of alcohol consumption, and refraining from smoking. These strategies have been effective for preventing or controlling stage 1 hypertension [2]. Pharmacological treatment with angiotensin-converting enzyme inhibitors (ACEIs), AT1 receptor blockers (ARBs), calcium channel blockers (CCBs) is necessary for more severe cases.

Different clinical studies have demonstrated that the consumption of macronutrients can play a key role in the management of high blood pressure. According to clinical trials, the partial replacement of carbohydrates with either protein or monounsaturated fat can reduce high blood pressure, and as a consequence, the risk of coronary heart disease [3].

The active peptide fragments (bioactive peptides) encrypted in the structure of food proteins exert beneficial effects on human health above and beyond their expected nutritional value. They are released from their parent

proteins by gastrointestinal digestion, fermentation, or food processing [4]. Food-derived bioactive peptides have a wide range of potential applications as functional foods and nutraceuticals for the prevention and management of hypertension.

Hence, an evaluation has been made of various bioactive peptides in the proteins of distinct types of foods. Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) peptides are known to inhibit the angiotensin-converting enzyme [5], increase nitric oxide production in human umbilical vein endothelial cells (HUVECs), and generate an endothelial-dependent vasodilation in *ex vivo* and *in vitro* studies [6]. There is evidence that mechanisms of VPP-induced vasodilation involve nitric oxide and the endothelium-derived hyperpolarizing factor (EDHF) via the activation of the bradykinin B2 receptor [7]. The aim of the current contribution was to carry out a more in-depth examination of the mechanisms of VPP-induced vasodilation, especially the possible participation of the NO/cGMP/PKG/Ca^{2+} -activated K^+ channel pathway.

2. Material and Methods

2.1. Animals

Experiments were performed on thoracic aortic rings isolated from adult male Wistar rats (body weight 250-300 g), which were purchased (*n*=52) from the bioterium of the Escuela Superior de Medicina. Animals were housed in plastic cages in a special temperature-controlled room (22±2 °C, 50% humidity) on a 12:12 h light/dark cycle (lights on at 7 am). The protocol was approved by the Animal Care Committee of the Escuela Superior de Medicina (Mexico City) and is in agreement with the 1986 Animals (Scientific Procedures) Act of the British Parliament:
http://www.legislation.gov.uk/ukpga/1986/14/contents, accessed on January 12, 2023.

2.2. Preparation of Aortic Rings

Animals were euthanized by decapitation and the aortas were immediately excised and placed in cold buffer before being cleaned and freed from surrounding connective tissue. The isolated arteries were cut into rings (4-5 mm long) and placed in 10 ml tissue chambers filled with Krebs-Henseleit bicarbonate buffer (118 mM NaCl ; 4.7 mM KCl; 1.2 mM KH_2PO_4; 1.2 mM $MgSO_4.7H_2O$; 2.5 mM $CaCl_2•2H_2O$; 25 mM $NaHCO_3$; 11.7 mM dextrose; and 0.026 mM calcium disodium EDTA). Tissue baths, maintained at 37 °C and pH 7.4, were bubbled with a mixture of 95% O_2 and 5% CO_2.

Aortic rings were mounted on two stainless steel hooks, one fixed to the bottom of the chamber and the other to a BIOPAC TSD125C-50g pressure transducer connected to a BIOPAC MP100A-CE data acquisition system (BIOPAC Systems, Inc., Santa Barbara, CA, USA) in order to record the isometric tension. Optimal tension, selected from preliminary experiments, was that which afforded the greatest response to phenylephrine (10^{-6} M). The rings were given 2 g (100%) of initial tension and allowed to equilibrate for 2 h. Thirty minutes after setting up the organ bath, tissues were first contracted with 10^{-6} M phenylephrine to test their contractile responses. Aortic were denuded of endothelium by turning the rings gently several times on the distal portion of small forceps. Endothelial integrity was pharmacologically assessed with acetylcholine-induced vasodilatation (10^{-6} M). Segments showing no relaxation to acetylcholine were considered to be endothelium-denuded. After applying 10^{-6} M phenylephrine or 10^{-6} M acetylcholine, tissues were rinsed three times with Krebs solution to restore basal tension.

2.3. Drugs

All drugs except VPP were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The lactotripeptide was acquired from Productos y Equipos Biotecnológicos S.A. de C.V laboratories. All compounds were dissolved in distilled water. Fresh solutions were elaborated for each experiment.

2.4. Experimental Protocols

Two sets of experiments were conducted to establish the mechanism responsible for the VPP-induced relaxant effect on phenylephrine-precontracted rat aortic rings.

First set of experiments: Thirty minutes after the restoration of basal tension (see preparation of aortic rings), 10^{-6} M phenylephrine was added to endothelium-intact and -denuded rat aortic rings, and 20 min later a steady contraction began. Thirty minutes after adding phenylephrine, 10^{-9} - 10^{-4} M VPP or 10^{-9} - 10^{-5} M acetylcholine (a positive control of vasorelaxation) were cumulatively added in intervals of ~10 min. Tension was expressed as a percentage of the aortic contraction resulting from the application of phenylephrine alone (3.54 ± 0.25 g), considered as 100%.

Second set of experiments: Since VPP produced moderate concentration-dependent vasorelaxation on phenylephrine-precontracted rat aortic segments, an attempt was made to determine the mechanism involved. Thirty minutes before adding 10^{-6} M phenylephrine (see first set of experiments), endothelium-intact aortic rings were preincubated for 30 min with various compounds: (i) the vehicle (distilled water); (ii) 10^{-5} M L-NAME (a direct inhibitor of NO synthase); (iii) 10^{-7} M ODQ (an inhibitor of nitric oxide-sensitive guanylyl cyclase); (iv) 10^{-6} M KT 5823 (an inhibitor of protein kinase G); (v) 10^{-2} M TEA (a Ca^{2+}-activated K^+ channel blocker and nonspecific voltage-activated K^+ channel blocker); (vi) 10^{-7} M apamin plus 10^{-7} M charybdotoxin (blockers of small- and large-conductance Ca^{2+}-activated K^+ channels, respectively); (vii) 10^{-3} M 4-AP (a voltage-activated K^+ channel blocker); (viii) 3.1 X 10^{-7} M glibenclamide (an ATP-sensitive K^+ channel blocker); (ix) 10^{-5} M indomethacin (a prostaglandin synthesis inhibitor) and (x) 10^{-5} M cycloheximide (a general protein synthesis inhibitor). Following preincubation with each compound, evaluation of the VPP-induced vasorelaxant response (with 10^{-9} – 10^{-4} M of the peptide) indicated whether the effect was significantly different. Accordingly, it was possible to gain insights into the mechanism of action of VPP.

2.5. Data Analysis and Statistics

Data are expressed as the mean SEM. In all experiments, the aortic segments were obtained from 6 animals. The vasorelaxant effect of VPP or acetylcholine on endothelium-intact aortic rings precontracted with phenylephrine was examined with one-way analysis of variance (ANOVA) of repeated measurements. In relation to the influence of the endothelium, the antagonist, inhibitors, and blockers on the VPP-induced relaxation of phenylephrine-precontracted aortic segments, significant differences were assessed with two-way ANOVA of repeated measurements. Each ANOVA was followed by the Student-Newman-Keuls post hoc test. The statistical analysis was performed on the SigmaPlot 12 program (Systat Software Inc., San Jose, CA, USA), with significance considered at $P<0.05$ [8].

3. Results

Effect of the Val-Pro-Pro tripeptide on phenylephrine-precontracted rat aortic rings.

The effects of cumulative concentrations of VPP on phenylephrine-precontracted aortic rings of rats (endothelium-intact or -denuded) were recorded (Figure 1). The concentration-dependent relaxation observed when applying 10^{-9}-10^{-4} M VPP to intact aortic rings was not found with denuded aortic rings. The maximum relaxation (E_{max}) of phenylephrine-precontracted aortic rings was 80.05 ± 0.95 % (EC50 $10^{-4.94}$ M) for those with intact endothelium and 1.54 ± 0.46 % (EC50 $10^{-6.67}$ M) for those with the endothelium denuded.

Effect of L-NAME or TEA on the Val-Pro-Pro-induced relaxation of phenylephrine-precontracted rat aortic rings.

Preincubation of phenylephrine-precontracted aortic rings with 10^{-5} M L-NAME or 10^{-2} M TEA was examined (Figure 2). The maximum vasorelaxant effect produced by 10^{-9} - 10^{-4} M VPP was decreased as a result of preincubation with 10^{-5} M L-NAME (Figure 2A; 80.20 ± 1.27 % vs 29.65 ± 0.50 %, respectively) and 10^{-2} M TEA (Figure 2B; 88.20 ± 4.88 % vs 24.70 ± 1.35 %, respectively).

Effect of apamin plus charybdotoxin, glibenclamide, and 4-AP on the Val-Pro-Pro-induced relaxation of phenylephrine-precontracted rat aortic rings.

Preincubation of phenylephrine-precontracted aortic rings with glibenclamide, 4-AP, or 10^{-7} M apamin plus 10^{-7} M charybdotoxin was evaluated (Figure 3). The maximum vasorelaxant effect elicited by 10^{-9} - 10^{-4} M VPP was significantly diminished following preincubation with apamin plus charybdotoxin (Figure 3A; 84.35 ± 1.47 % vs 19.80 ± 1.32 %, respectively), but was unaffected by glibenclamide (Figure 3B; 79.20 ± 5.00 % vs 79.32 ± 4.61 %, respectively) or 4-AP (Figure 3C; 81.26 ± 4.68 % vs 80.15 ± 4.09 %, respectively).

Effect of ODQ or KT 5823 on Val-Pro-Pro-induced vasorelaxation of phenylephrine-precontracted rat aortic rings.

Pretreatment of phenylephrine-precontracted aortic rings with 10^{-7} M ODQ or 10^{-6} M KT 5823 was assessed (Figure 4). The maximum vasorelaxation provided by 10^{-9} - 10^{-4} M VPP was significantly reduced after pretreatment with ODQ (Figure 4A; 85.11 ± 2.97 % vs 11.66 ± 1.74 %, respectively) and KT 5823 (Figure 4B; 78.60 ± 1.16 % vs 17.59 ± 0.05 %, respectively).

Effect of indomethacin or cycloheximide on the Val-Pro-Pro-induced vasorelaxation of phenylephrine-precontracted rat aortic rings.

Pretreatment of phenylephrine-precontracted aortic rings with 10^{-5} M indomethacin or cycloheximide was examined (Figure 5). The maximum vasorelaxant effect afforded by 10^{-9} - 10^{-4} M VPP was unchanged following preincubation with indomethacin (Figure 5A; 91.19 ± 4.31 vs. 89.77 ± 4.20, respectively) or cycloheximide (Figure 5B; 97.11 ± 5.56 % vs. 98.66 ± 9.15, respectively).

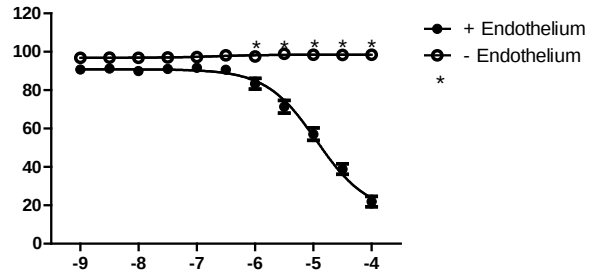

Figure 1. Concentration-dependent relaxation of 10^{-9} - 10^{-4} M Val-Pro-Pro on endothelium-intact (closed circles) and endothelium-denuded (open circles) phenylephrine-precontracted rat aortic rings. Data are expressed as the mean ± SEM of n observations (n= 6). (*) P<0.001 vs (+) Endothelium.

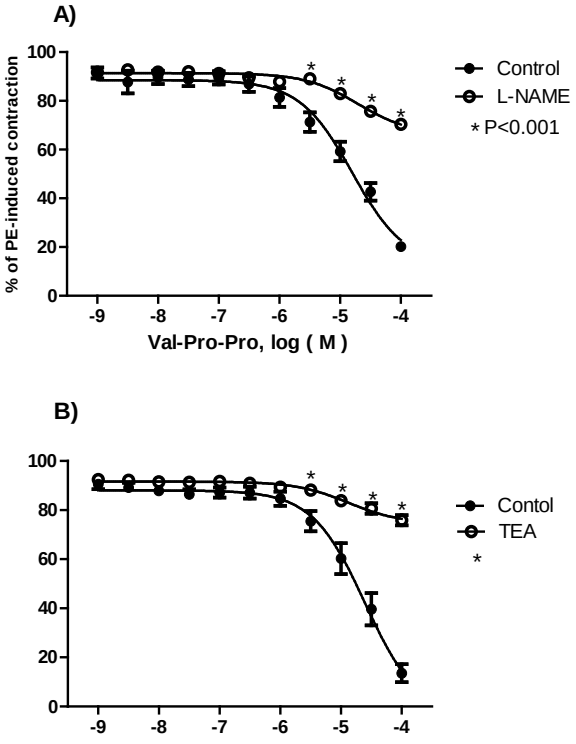

Figure 2. Effects of 10^{-5} M L-NG-Nitroarginine Methyl Ester (L-NAME) (A) and 10^{-2} M tetraethylammonium (TEA) (B) on Val-Pro-Pro-induced vasorelaxation in functional endothelium rat aortic rings precontracted with 10^{-6} M phenylephrine (PE). Data are expressed as the mean ± SEM of n observations. (*) P<0.001 vs control.

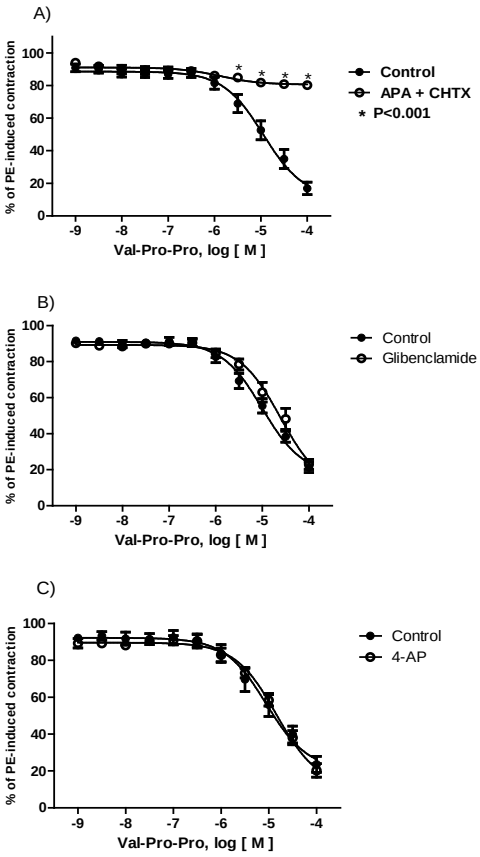

Figure 3. Effects of: (A) 10^{-7} M apamin plus 10^{-7} M charybdotoxin, (B) 3.1 x 10^{-7} M glibenclamide and (C) 10^{-3} M 4-aminopyridine (4-AP) on Val-Pro-Pro-induced vasorelaxation in functional endothelium rat aortic rings precontracted with 10^{-6} M phenylephrine (PE). Data are expressed as the mean ± SEM of n observations (n=6). * P<0.001 vs control (two-way ANOVA).

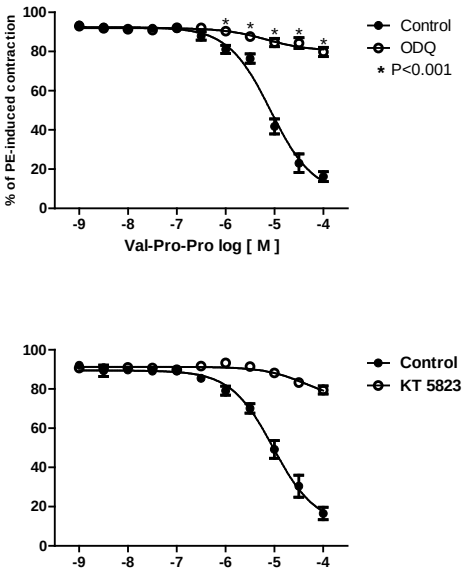

Figure 4. Effects of 10^{-7} M ODQ (A) and 10^{-6} M KT 5823 (B) on Val-Pro-Pro-induced vasorelaxation in functional endothelium rat aortic rings precontracted with 10^{-6} M phenylephrine (PE). Data are expressed as the mean ± SEM of n observations. (*) P<0.001 vs control (two-way ANOVA).

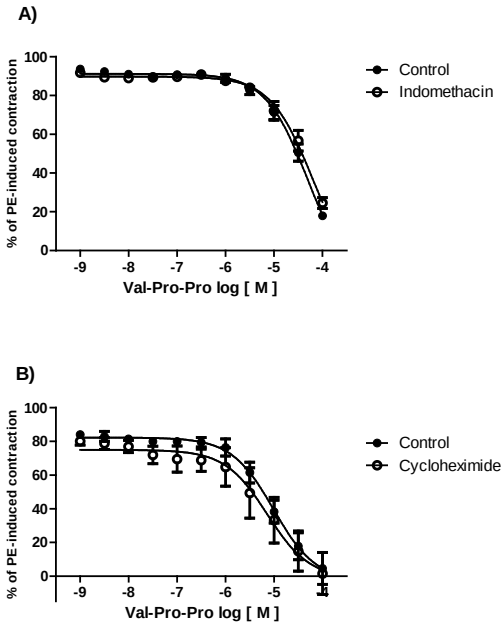

Figure 5. Effects of 10^{-5} M Indomethacin (A) and 10^{-5} M cycloheximide (B) on Val-Pro-Pro-induced vasorelaxation in functional endothelium rat aortic rings precontracted with 10^{-6} M phenylephrine (PE). Data are expressed as the mean ± SEM of n observations.

4. Discussion

The results of the present study clearly show that the VPP tripeptide causes vasodilation of endothelium-intact rat aortic rings precontracted with phenylephrine, which is in agreement with other studies that have evaluated the vascular effects of valyl prolyl proline (VPP) and isoleucyl prolyl proline (IPP). [7]

According to previous reports, peptides have diverse types of activity, including antimicrobial [9,10], antidiabetic [11,12], immunomodulatory [13,14], and antihypertensive [15,16]. With regard to the antihypertensive effect, the vasodilatory properties of bioactive peptides (e.g., VPP and IPP) are known to involve the inhibition of the angiotensin-converting enzyme [17], a decrease in the level of endothelin-1 [18], and an increase in the production of nitric oxide in human umbilical vein endothelial cells [6].

The vasorelaxant effect of VPP on phenylephrine-precontracted, endothelium-intact rat aortic rings is consistent with such activity described in the literature [7]. Nevertheless, to our knowledge, there are no studies on the possible role of the NO / cGMP / PKG / Ca^{2+} - activated K^{+} channel pathway in the vasodilation generated by VPP.

Precontraction of aortic rings with phenylephrine makes vasodilator responses to different agents (e.g., acetylcholine, bradykinin and statins) more evident [19,20]. Thus, the present experimental protocols were carried out on phenylephrine-precontracted rat aortic rings, finding that the application of 10^{-9} to 10^{-4} M of VPP caused a concentration-dependent vasodilation.

Interestingly, we found that the vascular endothelium plays a very important role in the relaxant effect of VPP. The above is due to the absence of endothelium or in the

presence of L-NAME, the relaxant effect of this peptide was strongly inhibited, which clearly demonstrates that the endothelium participates in this relaxation through the liberation of NO.

The significant reduction in the VPP-induced vasorelaxation of rat aortic rings by 10^{-5} M L-NAME (a direct inhibitor of NOS; Figure 3A) [21] , 10^{-2} M TEA (a Ca^{2+}-activated K^+ channel blocker and non-specific voltage-activated K^+ channel blocker; Figure 3B) [22,23] , and 10^{-7} M apamin plus 10^{-7} M charybdotoxin (blockers of small- and large-conductance Ca^{2+}-activated K^+ channels, respectively; Figure 3C) [24,25] suggests the involvement of the release of nitric oxide by the endothelium, Ca^{2+}-activated K^+ channels, and to a lesser extent the voltage-activated K^+ channel blocker. In this sense, VPP has been described to upregulate eNOS gene expression in spontaneously hypertensive rats [26]. On the other hand, the combination of apamin plus charybdotoxin was used because it was previously reported that a complete blockage of Ca^{2+}-activated K^+ channels is necessary to produce a pharmacological response [27]. According to a pilot experiment in our lab, apamin or charybdotoxin alone does not modify the vasorelaxant responses to VPP (data not shown). Hence, the combination of apamin plus charybdotoxin was presently employed. It is possible that VPP produces vascular hyperpolarization attributable to the release of the endothelium-dependent hyperpolarizing factor, as has been described for acetylcholine [28,29]. However, the aforementioned proposal is speculative. Future research, which is beyond the scope of the present study, will be required to further investigate the contribution of this factor to the vascular effects generated by peptides.

The current findings about the participation of NO and K^+ channels in the VPP-induced vasorelaxation of rat aortic rings corroborate several reports suggesting that NO and K^+ channels play an important role in regulating vascular tone [30,31,32,33,34,35].

Since the vasorelaxant response to VPP did not differ significantly after preincubation with 4-AP (a voltage-activated K^+ channel blocker) or glibenclamide (an ATP-sensitive K^+ channel blocker), the voltage-activated and ATP-sensitive K^+ channels were not involved in the mechanism of action. Future research is necessary to gain greater insights into the specific subtype of Ca^{2+}-activated K^+ channels implicated in the vasorelaxant effect produced by the tripeptide.

Given that the VPP-induced vasorelaxation was not modified by indomethacin (a prostaglandin synthesis inhibitor) or cycloheximide (a general protein synthesis inhibitor), neither prostacyclins or protein synthesis contributes to the endothelium-mediated vasodilation. This is in contrast to previous findings obtained with small mesenteric arteries isolated from spontaneously hypertensive rats, in which prostacyclin and the IP receptor played a role in the vasodilation generated by the Arg-Ile-Tyr peptide [36]. A possible explanation for this apparent discrepancy may be at least partially related to the blood vessel and the peptide used. Similar to the current results, the vasodilation produced in small mesenteric arteries by another antihypertensive peptide, Leu-Arg-Ala was not mediated by PGI_2 [37]. Finally, cycloheximide, a general protein synthesis inhibitor, did not alter the VPP-induced vasorelaxation of aortic rings, thus excluding the involvement of protein synthesis.

5. Conclusion

The present study demonstrates that the vasodilator effect of VPP on phenylephrine-precontracted rat aortic rings was unaltered by pretreatment with 4-AP, glibenclamide or indomethacin, but was notably decreased by L-NAME, TEA, ODQ, KT5823, apamin + charybdotoxin and by the removal of the endothelium. Hence, VPP-induced vasodilation seems to depend on the stimulation of the vascular endothelium and involve activation of the NO / cGMP / PKG / Ca^{2+} -activated K^+ channel pathway. The bioactive peptide herein studied could possibly have therapeutic benefit in the high blood pressure management.

List of Abbreviations

Angiotensin-converting enzyme inhibitors	(ACEIs)
AT1 receptor blockers	(ARBs)
Calcium channel blockers	(CCBs)
Val-Pro-Pro	(VPP)
Ile-Pro-Pro	(IPP)
Human umbilical vein endothelial cells	(HUVECs)
Endothelium-derived hyperpolarizing factor	(EDHF)
4-aminopyridine	(4-AP)
L-NG-Nitroarginine methyl ester	(L-NAME)
1H-(1,2,4)oxadiazolo[4,3-a]quinoxalin-1-one	(ODQ)
Tetraethylammonium	(TEA)

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Statement of Competing Interests

The authors declare no conflicts of interest.

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