Vasorelaxing effect of 6β-hydroxybetulinic acid

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Research Article

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Abstract

Cardiovascular diseases are currently the main causes of morbidity and mortality in the world. The available medications have undesirable side effects or lose effectiveness due to pharmacological tolerance. For this reason, it is necessary to look for new molecules and therapeutic alternatives for its treatment. 6-Hydroxybetulinic acid is a triterpene isolated from the leaves of *Licania cruegeriana* that demonstrated a hypotensive effect in hypertensive rats. In the present work, we evaluated the vasorelaxing effect of this triterpene in rat aortic rings (*ex vivo*) and its possible action mechanism. 6β-hydroxybetulinic acid develops its vasorelaxing effect in a concentration-dependent manner, and not dependent on the vascular endothelium (n:6, IC50: 9.98 µM) and induced by phenylephrine or KCl in rat aortic rings in a non-competitive manner. The 6HB-induced vasorelaxation was related to the inhibition of Ca$^{2+}$ inflow from the extracellular medium and the inhibition of NO/GMPc pathway. Since vascular tone is a determinant of arterial pressure in mammals, these results could partially explain the hypotensive effect demonstrated in *in vivo* experiments.

INTRODUCTION

Cardiovascular diseases are the first cause of mortality in the world (WHO 2021). The most common manifestation that leads to these deaths of cardiovascular origin is arterial hypertension, which becomes a major public health problem associated with high morbidity and high costs for health systems (Seccia et al. 2017). Despite the wide range of molecules used for the treatment of hypertension, there is partial success in the pharmacological control of the disease, so it will continue to be a risk factor with a high impact on cardiovascular morbidity and mortality in the coming times. Current pharmacological resources for the treatment of hypertension may have problems related to drug tolerance, adverse events and in some cases low efficacy (Williams et al. 2018). added to the risks of polypharmacy, has led to an active search for molecules with a hypotensive effect (Tabares-Guevara et al. 2021) potentially useful in hypertensive patients.

The search for new bioactive molecules in plants is a current alternative, due to the wide use of traditional medicine (Mashour et al. 1998). Evidence suggests that molecules such as the polyphenol curcumin, from Curcuma longa, are atheroprotective (Tabares-Guevara et al. 2021), the sesquiterpene farnesol, of different natural origins, is cardioprotective (Souza et al. 2019). Reserpine, an antihypertensive agent that is no longer used, is extracted from the roots of Rauwolfia serpentine; and verapamil, a papaverine derivative (Zhao et al. 2020) with antiarrhythmic and antihypertensive properties, is now available for commercial use. 6β-Hydroxybetulinic acid (6HB), see Fig. 1, is a triterpenoid isolated from the *Licania cruegeriana*, which demonstrated a hypotensive effect in hypertensive rats and also an antiaggregant effect in human platelets (Estrada et al. 2014). In order to demonstrate that 6HB has a direct effect on smooth muscle contraction that could explain its effect on blood pressure, we decided to evaluate its vasorelaxing effect on isolated rat aortic rings.

MATERIALS AND METHODS
Animals

Male Sprague-Dawley rats weighing 240–350 g used for vascular reactivity assays were obtained from the animal care service of IVIC. They were housed at room temperature (21 ± 2°C) and light-dark (12:00–12:00 h) cycles, with a maximum of four rats per acrylic, transparent, rectangular cage, with wood-derived bedding. Food and tap water were freely available at the conventional animal facilities of the IVIC.

Reagents

The following drugs used for vascular reactivity experiments were purchased from Sigma-Aldrich: D-glucose: phenylephrine hydrochloride, acetylcholine hydrochloride, nifedipine, SNP, glibenclamide, 4-amynopiridine, potassium chloride and calcium chloride, L-NAME, tetraethylammonium, indomethacin (Calbiochem). Nifedipine, glibenclamide, SNP, indomethacin and 6HB acid were prepared as stock solutions in dimethyl sulfoxide (DMSO). The other drugs were dissolved in distilled water. The bath concentration of DMSO did not exceed 0.5%, which was shown to have no effect on the basal tension of the aortic rings preparations or on the agonist mediated contraction or relaxation.

Vascular reactivity assays

The rats were anaesthetized with sodium pentobarbital (40 mg/kg, intraperitoneal) and the chest was opened by a midline incision to isolate the descending thoracic aorta. After removal of the superficial fat and connective tissue, 10 mm long rings with intact endothelium (E+) were placed in a 10 mL organ chamber containing physiological salt solution, constantly bubbled with a mixture of 95% O2/5% CO2 and maintained at 37°C. The composition of physiological salt solution was as follows (mM): NaCl, 118; KCl, 4.7; KH₂PO₄, 1.2; MgSO₄, 1.2; NaHCO₃, 15; glucose, 5.5; CaCl₂, 2.5. The rings were stretched until an optimal basal tension of 2 g was reached, previously determined by length-tension relationship experiments, then they were allowed to equilibrate for 2 h with the bath fluid being changed every 15–20 min. Mechanical stability of the system was tested by adding 50 mM KCl, three times. Aortic rings were repeatedly washed and allowed to reequilibrate for an additional 30 min period. Endothelial integrity was assessed qualitatively by the degree of relaxation caused by ACh (10 µM) in the presence of the contractile tone induced by 1 µM phenylephrine (PE). In some rings (E-), the endothelial layer was removed immediately after dissection by gently rubbing the luminal surface with a small wooden stick. Only one experiment was carried out in each aorta ring. Isometric tensions were recorded on a polygraph (PowerLab 4/26; PanLab) by means of a force displacement transducer ML201. Data were fed to a computer and a LabChart system (PanLab) was used to convert acquired data into digital form. For 6HB assays, 1 µM PE precontrated E + and E- rings were exposed to increasing doses of (0.01–100 µM) acetylcholine (Ach) to evaluate vasorelaxation.

Statistics

Values are expressed as mean and standard error of the mean (SEM). Statistical analysis was performed applying one-way ANOVA using GraphPad Prism 7.0 (Microcal Software Inc., Northampton, USA). Statistical comparisons between two data mean were made using an unpaired t test with Welch’s
correction, and p values less than 0.05 were considered statistically significant. The vasorelaxing response was expressed in terms of percent decrease of the maximal contraction caused by PE. The IC_{50} value was defined as the concentration of the compound that reduced the maximum contraction elicited by PE by 50% and was calculated from a concentration-response curve.

RESULTS

Effect of 6HB on basal tension in rat aortic rings:

The addition of 6HB to rat aortic rings preparation with functional endothelium (E+) or without functional endothelium (E-) modifies the basal tension in a dose dependent manner (n = 6), additionally we observed that its vasorelaxant effect was greater in rings with functional endothelium, for the control experiments increasing concentrations of DMSO was used and did not modified the basal tension (data do not show), see Fig. 2A.

Effect of 6HB on aortic rings contractions elicited by phenylephrine or KCl

In order to investigate the mechanism through which 6HB develops its vasorelaxant effect, experiments were performed with functional endothelium (E+) or without functional endothelium (E-) at increasing concentrations of 6HB, after precontraction induced with PE (Fig. 2B) or 50 mM KCl (Fig. 2C), in both cases 6HB induces a vasorelaxant effect in a dose dependent manner. Additionally, concentration-response curves were performed against PE (10 pM- 100 µM) or KCl (5–50 mM), figure D and E respectively, in the absence of 6HB (control) and after a 10 minutes incubation period with 6HB (10 µM).

Effect of 6HB on CaCl\textsubscript{2} and Bay K 8644-induced contractions

As can be seen in Fig. 3A, pretreatment with 6HB attenuated CaCl\textsubscript{2}-induced contraction of endothelium-denuded rat aorta exposed to Ca\textsuperscript{2+}-free medium containing KCl. CaCl\textsubscript{2} induced a concentration-dependent contraction of rat aortic rings (n = 6). Preincubation of the rings with 6HB at 3, 10 or 30 µM significantly reduced the Emax values for CaCl\textsubscript{2} (Emax = 87% ±11, 78% ± 9 and 68 ± 8, respectively; n : 6 for each group). On the other hand, with the purpose to investigate if 6HB inhibits Ca\textsuperscript{2+} influx, we evaluated the posible effect of 6HB on Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channels. Contractions induced by Bay K8644 (0.01–10 µM), an L-type Ca\textsuperscript{2+} channels agonist, were reduced by the addition of 6HB (10 µM), as seen in Fig. 3B.

Evaluation of the effect of nifedipine, tetracaine, phorbol-12-myristate-13-acetate (PMA) and caffeine on the vasorelaxant effect of 6HB.
In order to evaluate if intracellular calcium movements mediate the vasorelaxing effect of 6HB, we use antagonists such as nifedipine, tetracaine, phorbol-12-myristate-13-acetate (PMA) and caffeine; the results can be observed in Fig. 4. 6HB vasorelaxant effect was not affected in aortic rings pretreated with nifedipine; while it was almost completely inhibited by tetracaine, (PMA), and caffeine (10mM).

**Conclusion of endothelium-derived relaxing factors on the vasorelaxant effect of 6HB**

In order to demonstrate if the main vasorelaxant effect of 6HB is dependent on the vascular endothelium, the role of ATP-dependent potassium channels (KATP), the cyclooxygenase-nitric oxide (NO) pathway, the voltage-dependent potassium channels (Kv) and the participation of different G-protein coupled receptors (muscarinic, α-adrenergic or purinergic) we performed experiments with selective and non-selective antagonists as we will describe next. 6HB effect was not affected in aortic rings pretreated with glibenclamide or indomethacin, see Fig. 5A; while the effect of 6HB was inhibited by L-NAME and methylene blue as well as by 4-aminopyridine. On the other hand, the non-selective beta-blocker propranolol, the non-selective Ach receptor antagonist atropine nor suramine could block the vasorelaxant effect of 6HB, Fig. 5B. Finally, the contribution of nitric oxide (NO) by an exogenous NO donor, such as sodium nitro prussiate (SNP) was evaluated through SNP concentration-response curves, constructed as control experiments and then after exposure to 6HB (10 µM), incubated for 10 minutes. When the SNP curve was repeated, it was observed that 6HB can increase the vasorelaxing effect induced by SNPs, see Fig. 5C.

In previous works we demonstrated that 6HB has a hypotensive effect *in vivo* experiment [8]. This study was carried out with the aim of contributing to the understanding of the mechanism through which this compound causes its cardiovascular effect. Our results suggest that 6HB promoted vasorelaxation for both endothelium-dependent and -independent pathways. The contribution of endothelium-derived relaxing factors such as NO and prostanoids in the vasorelaxant response to 6HB was studied in PE-precontracted aortic rings (E+) previously exposed to the NO synthase inhibitor L-NAME or to the cyclooxygenase inhibitor indomethacin, respectively. As showed in Fig. 5A, 6HB response was inhibited by L-NAME and not affected by indomethacin, which suggests that NO could be the mean endothelial factor involved in its vascular action. In smooth vascular cells NO stimulates cGMP production by activating soluble guanylyl cyclase (sGC) and that elevated production of cGMP is correlated with the NO-mediated vasodilatation (Nakatsu and Diamond 1989). Therefore, to evaluate the involvement of NO in the relaxant effect of 6HB, we examined, the role of the NOS-sGC signaling pathway, using the non-selective soluble guanyl cyclase inhibitor methylene blue (Bolotina et al. 1994), on the 6HB response. As also shown in Fig. 5A, methylene blue completely blocked the relaxant effect of 6HB, suggesting that cGMP could participate in its mechanism of action.

In addition to the involvement of the NO/GMPc pathway in the relaxant effect of 6HB, the role of potassium channels in this effect was also evaluated. Multiple K⁺ channels are present in vascular
smooth muscle cells, and those different K⁺ channels play unique roles in regulating vascular tone (Brayden 1996). The 6HB vasorelaxant effect was attenuated by 4-aminopyridine, but not by glibenclamide, Fig. 5A, indicating that only the voltage-dependent K⁺ (KV) and inward rectifier K⁺ (KIR) channels are involved in the vascular effect of 6HB. The possible involvement of cGMP-dependent protein kinases on the relaxant effect of 6HB was examined in the presence of SNP, an activator of guanylate cyclase. The relaxation elicited by sodium nitroprusside on PE-contracted aorta was significantly potentiated by 6HB in terms of potency and efficacy (Fig. 5C). According to our results, the removal of functional endothelium did not abolish the effect of 6HB, suggesting that its vasorelaxing effect also involves the endothelium-independent pathway. Among the mechanisms involved in the endothelium-dependent vasorelaxant effect on vascular smooth muscles cells are the decrease of calcium influx by cell membrane calcium channels and Ca²⁺ release inhibition from intracellular stores (Matthew et al. 2004). Considering that 6HB inhibited the vasoconstriction induced by calcium supplementation in the aortic rings precontracted with KCl (50 mM) in the Ca²⁺-free solution, see Fig. 4, it suggests that 6HB behaves as a noncompetitive calcium antagonist. As it is well known, L-type calcium channels (Ca L) channels regulate the calcium influx in vascular muscle cells, leading to vasoconstriction (Moosmang et al. 2003). Therefore, some experiments were conducted to verify whether 6HB could inhibit calcium influx by a blockade of Ca²⁺ L channels. Aortic ring precontraction induced by Bay K 8644, a selective activator of Ca²⁺ L channels, was significantly reduced by 6HB, indicating that the relaxing effect of the 6HB involves the blockade of the Ca²⁺ L channels. On the other hand, the influence of 6HB on the release of Ca²⁺ from intracellular stores was also analyzed, and our experiments demonstrate that 6HB did not alter the contraction induced by caffeine or tetracaine, see Fig. 5. Therefore, it seems unlikely that the vascular effects of 6HB acid involve a reduction of Ca²⁺ release from intracellular stores. Protein kinase C (PKC) has been proposed to play a key role in the maintenance of tonic contractions of vascular smooth muscle (Rasmussen et al. 1987). Phorbol esters (ie, PMA) activate PKC and induce slow-onset sustained contraction in rat aorta. In the present study, 6HB did not inhibit the tonic contraction induced by the PKC activator PMA.

In summary, the results presented in the present work demonstrate that 6HB relaxes rat thoracic aorta by both an endothelium-dependent and endothelium-independent mechanism. The vasorelaxant effect of 6HB involves: 1. The NO/cGMP signaling pathway, 2. A K⁺ channel opening causing membrane hyperpolarization of vascular smooth muscle and 3. Blockade of extracellular calcium influx through L-type Ca²⁺ channels. Therefore, these findings contribute to a better understanding of the mechanism of action involved in the vasorelaxant effect of 6HB.

**Declarations**

**Conflicts of Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
Author Contribution

OE conceived the study and revised the final manuscript. OE wrote the manuscript. OE, AC prepared figures and tables and wrote the manuscript. OE, AC helped with the performance of experiments. The final version of the manuscript was read and approved by all authors.

Ethical approval

The authors declare that the experiments with animals were performed in compliance with international rules on the care and use of laboratory animals. The animal studies were evaluated and approved by the Bioethics Commission for Investigations in Animals at the IVIC (Protocol 201417, approval on November 2017), in accordance with the Code on Bioethics and Biosecurity (2008) established by the Bioethics Commission National Fund on Science and Technology (FONACIT), under the national legislation.

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References


**Figures**
Figure 1

Structure of 6β-hydroxybetulinic acid
Figure 2

Vasorelaxant effect induced by 6HB on isolated rat aortic rings. Cumulative concentration–response curves to 6HB (1 µM to 300 µM) were constructed (E+, ■ and E−, □) in, panel A : Effect of 6HB on the basal tension of rat aortic rings. DMSO was used as a control, in panel B, PE (0.1 µM) precontracted aortic rings or panel C using KCl (50 mM) precontracted aortic rings. In panels D and E, PE or KCl cumulative concentration–effect curves were constructed in the absence or in the presence of 6HB (10 µM). Unpaired t-test with Welch’s correction (*p < 0.05, **p < 0.01, and ***p < 0.001 versus □).

Figure 3
Effect of 6HB on CaCl$_2$-induced contractile response in E- aortic rings, panel A. Concentration– response curves for CaCl$_2$ were determined in Ca$^{2+}$-free solution containing KCl (50 mM). The curves were determined in the absence (control, ○), after a 10-min period of incubation with 6HB (3 □, 10 △ or 30 ◊ µM). Panel B: Effect of 6HB on the Bay K8644-induced contractile response in endothelium denuded rat aortic rings. The curves were constructed in the absence of 6HB (control) or after 10 min of incubation of 6HB (10 µM) prior to the cumulative addition of Bay K8644 (0.01–10 µM). Data are presented as the mean ± SEM of 6 experiments performed on preparations obtained from different animals. *P < 0.05 denotes a significant difference between the E max values and the control.

Figure 4

Effect of nifedipine (60 nM), tetracaine (60 nM), PMA (20 nM) and caffeine (10 mM) on the vasorelaxant effect of 6HB (10 µM). ** There are statistically significant differences between treatment and control with 99% confidence.
Figure 5

Experiments of the vasorelaxing effect of 6HB in the presence of: panel A: L-NAME (100 µM), B: Methylene Blue (10 nM), C: Glibenclamide (0.1 µM), D: 4-Aminopyridine [1 mM], and E: Indomethacin [0.1 µM], panel B: Atropine (10 µM), Propanolol (10 µM) and Suramin (10 µM), panel C Cumulative concentration–response curves to SNP (0.1 pM to 10 µM) was constructed in the absence (■) or in the presence (▲) of 6HB (10 µM). For all the experiments Control = IC50 relaxation of 6HB (10 µM) in a phenylephrine-induced contraction. ** There are statistically significant differences between treatment and control with 99% confidence.

Supplementary Files

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