Mechanisms of relaxation induced by flavonoid ayanin in isolated aorta rings from Wistar rats

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SUMMARY

Introduction: This study shows the relaxant effect induced by ayanin in aorta rings from Wistar rats linked to nitric oxide/cyclic-GMP pathway. This flavonoid is the prevalent compound obtained from Croton schiedeanus Schlecht (Euphorbiaceae), specie used in Colombian folk medicine for the treatment of arterial hypertension.

Objectives: To identify possible action mechanisms of vascular relaxation induced by ayanin (quercetin 3,4',7-trimethyl ether).

Methodology: Isolated aorta rings from Wistar rats obtained at the Animal House of the University of Salamanca were contracted with KCl (80 mM) or phenylephrine (PE, 10⁻⁶ M) and exposed to ayanin (10⁻⁶-10⁻⁴ M). Then, the effect of ayanin was assessed in deendothelized rings contracted with PE and in intact rings contracted with PE previously incubated with: ODQ (10⁻⁶ M), L-NAME (10⁻⁴ M), L-NAME plus D- and L-arginine (10⁻⁴ M), indomethacin (5x10⁻⁶ M), dipyridamole (3x10⁻⁷ M), glibenclamide (10⁻⁶ M), propranolol (10⁻⁶ M), verapamil (10⁻⁷ M) or atropine (3x10⁻⁵ M). In addition, the relaxant effect of acetylcholine (Ach, 10⁻⁸-3x10⁻⁴ M), and sodium nitroprusside (SNP, 10⁻⁹-3x10⁻⁵ M) was assessed in the presence and absence of ayanin (10⁻⁶ M).

Results: Ayanin induced a greater concentration-dependent relaxation in vessels contracted with phenylephrine (pEC₅₀: 5.84±0.05), an effect significantly reduced by deendothelization and by both ODQ and L-NAME. L-arginine was able to reverse the effect of L-NAME. Indomethacin weakly inhibited ayanin response. Dipyridamole, glibenclamide, propranolol, verapamil, and atropine did not affect ayanin relaxation. Ayanin did not have any effect on the relaxation elicited by acetylcholine (ACh), while weakly decreasing the relaxation induced by sodium nitroprusside (SNP).

Conclusion: Ayanin induces endothelium-dependent relaxation in the rat aorta mainly related to nitric oxide/cGMP pathway, according to the response observed in the presence of L-NAME, L-arginine and ODQ.

Keywords: Ayanin; Croton; Endothelium-dependent relaxing factors; Flavonoids; Aorta.

Mecanismos de relajación inducidos por el flavonoide ayanina en la aorta de ratas Wistar

RESUMEN

Introducción: Este estudio muestra el efecto vasodilatador inducido por ayanina en anillos de aorta de ratas Wistar vinculado con la vía óxido nítrico/GMP-cíclico. Este flavonoid es el compuesto mayoritario aislado de Croton schiedeanus Schlecht (Euphorbiaceae), especie utilizada en la medicina popular colombiana para el tratamiento de la hipertensión arterial.

Objetivos: Identificar los posibles mecanismos vasodilatadores inducidos por la ayanina (queeretin 3,4',7-trimetileter).

Métodología: Se adicionó ayanina (10⁻⁶ - 10⁻⁴ M) a anillos aislados de aorta procedentes de ratas Wistar contraídos con KCl (80 mM) o fenilefrina (10⁻⁶ M). Luego se evaluó el efecto de la ayanina en anillos sin endotelio contraídos con fenilefrina y en anillos íntegros, contraídos con fenilefrina, previamente incubados con: ODQ (10⁻⁴ M), L-NAME (10⁻⁴ M), L-NAME más

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La ayanina produjo una mayor relajación en los anillos contraídos con fenilefrina (pEC50: 5.84±0.05), efecto que se redujo en anillos sin endotelio o en anillos íntegros preincubados con ODQ y L-NAME. L-arginina fue capaz de revertir la respuesta inducida por L-NAME. La indometacina inhibió discretamente la relajación generada por la ayanina. El dipyridamol, la glibenclamida, el propranolol, el verapamilo y la atropina no modificaron el efecto de la ayanina. La ayanina no afectó la relajación inducida por la acetilcolina y débilmente disminuyó la inducida por el nitroprusiato de sodio.

Resumen: La ayanina induce una relajación dependiente del endotelio en la aorta de rata, efecto vinculado principalmente con la vía de óxido nítrico/GMP cíclico, de acuerdo con la respuesta observada en presencia de L-NAME, ODQ y L-arginina.

Palabras clave: Ayanina; Croton; Factores relajantes endotelio-dependientes; Flavonoides; Aorta.

Materiales y Métodos

Extracción e Isolación de Ayanina. Ayanina (quercetin 3,4',7-trimethyl ether) (Figura 1) fue aislado de C. schiedeanus Schlecht (Euphorbiaceae), una planta recolectada de la región de Tocaima, Cundinamarca, Colombia en noviembre 1998 (voucher nº 432164). El aeroal plano de esta especie fue exhaustivamente extraído con 96% EtOH a temperatura ambiente. Los extractos combinados fueron concentrados bajo presión reducida y se partieron para obtener un extracto acuoso y CHCl3. El residuo del extracto CHCl3 fue fraccionado con 4% NaOH. Luego, ayanina se aisló de la fracción neutral por cromatografía en polvo de silice gel con hexano-EtOAc (1:1). Ayanina fue identificada por comparar m.p., 1H-NMR, 13C-NMR valores con los reportados.

Preparación de anillos de aorta de rata. Los experimentos se realizaron en el laboratorio de Farmacología de la Universidad de Salamanca. Ratas Wistar macho (320-400 g) de la colonia (PAE-001) fueron anestesiadas con etanol y decapitadas. La aorta descendente fue desgarrada y colocada en un tubo de ensayo con la siguiente composición (en mM): NaCl, 118.0; KCl, 4.75; CaCl2, 1.8; MgSO4, 1.2; KH2PO4, 1.2; NaHCO3, 25; glucosa, 11 y ácido ascórbico 0.1.
Rings of thoracic aorta (4–6 mm long) were carefully excised and submerged in tissue bath chambers containing 5 ml of Krebs solution of bathing medium maintained at 37°C and bubbled with 95% O2 and 5% CO2 gas mixture (pH=7.4). Rings were mounted by means of two parallel L-shaped stainless-steel holders inserted into the lumen. One holder served as an anchor, while the other was connected to a force-displacement transducer (Harvard UF1) to measure isometric contractile force recorded by a MacLab/8-computer system (AD InstruMents Ltd, London, UK). The rings were stretched progressively to an optimal basal tension of 2 g, determined by length-tension relationship experiments and, then, allowed to equilibrate for 60 min., and changing the bath fluid every 15–20 min. When necessary, the endothelium was removed by rubbing the intimal surface with a wooden stick. The presence of functional endothelium was assessed by the ability of ACh (10^–6 M) to induce more than 50% relaxation of rings pre-contracted with phenylephrine (PE, 10^–6 M)^19. Each preparation was allowed to equilibrate for at least 60 min. prior to initiation of experimental procedures and during this period, the incubation media were changed every 20 min. before the addition of drugs.

**Ethical issues.** In all of these experiments, the provisions regarding the protection of animals used for experimental purposes in current Spanish legislation (Real Decreto 1201/2005), European Community (EEC 86/609) and specifications were adhered to.

**Experimental procedures.** To observe if ayanin induces relaxant effects, intact aorta rings incubated in Krebs solution were stimulated with PE (10^–6 M) or high KCl (80 mM) until the contractile response reached a steady tension. Then ayanin was added in progressively cumulative concentrations (10^–7–10^–4 M) at 30 min. intervals.

To study possible mechanisms related to the relaxant effects induced by ayanin, the following protocols were performed: First, the role of endothelium was studied adding the flavonoid in similar way in deendothelized PE contracted rings. To assess whether nitric oxide, cGMP, phosphodiesterase-5, K^+ATP channels, prostanoid, beta-receptor, L-type calcium channels, or muscarinic receptors are related to the relaxant mechanisms induced by ayanin, this flavonoid was added to rings contracted with PE treated 30 min. previously with one of the following substances, respectively: L-NAME (10^–4 M [Nw-nitro-L-arginine methyl ester hydrochloride]), a NO synthase inhibitor; ODQ (10^–6 M [1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one]), a guanylate cyclase inhibitor; dipyridamole (3x10^–7 M), a cGMP-phosphodiesterase5 inhibitor; glibenclamide (10^–6 M), a K^+ATP channel blocker; indomethacin (5x10^–6 M), a cyclooxygenase (COX) inhibitor; propranolol (10^–6 M), a β-adrenoceptor blocking agent; verapamil (10^–7 M), a L-type calcium channels blocker and atropine (3x10^–5 M), a muscarinic receptor blocking agent.

To advance in possible interactions with NO/cGMP pathway the inactive α-amino acid D-arginine or the active L-arginine (10^–4 M, substrate for NO synthesis) were added (10 min. before) to rings incubated with L-NAME (10^–4 M). In addition, ayanin (10^–6 M) was added (30 min. before) to rings contracted with PE (10^–6 M) exposed to Ach (10^–5–3x10^–4 M) or SNP (10^–5–3x10^–5 M), added at 30 s intervals.

**Experiment design and data analysis.** In all instances, the dependent variable was the relaxant response obtained as a percentage of the maximal contraction induced by PE (10^–6 M) or KCl (80 mM). Sigmoidal regression to lead pEC50 values (- log of the molar concentration of agonist to produce 50% of the maximal relaxation in contracted rings) were obtained from concentration-response curves. Each point represents the means ± SEM of at least five experiments. Analysis of variance with Dunnett’s post test were applied to compare three or more groups and Student t test to compare two groups. p<0.05 was considered significant. The GraphPad Sofware (version 4.0; San Diego, CA, USA) was used for data analysis.

**Drugs and chemicals.** The following salts and drugs were used: NaCl, CaCl2, NaHPO4, NaHCO3, glucose (Panreac), KCl, MgCl2, KH2PO4, and MgSO4 (Probus), ascorbic acid (Prolabo), phenylephrine hydrochloride, acetylcholine chloride, indomethacin, L-NAME, L-arginine, D-arginine, ODQ, dipyridamole, glibenclamide, dimethylsulfoxide, verapamil hydrochloride, sodium nitroprusside, atropine sulfate, and propranolol hydrochloride (Sigma-Aldrich). Ayanin, glibenclamide and dipyridamole was dissolved in DMSO. All other substances were dissolved in physiological saline solution. The final DMSO concentrations in the bathing media were always less than 0.1%. Control tests demonstrated that this amount of DMSO failed to exhibit any response.
RESULTS

The maximal contractile response obtained with KCl and PE in intact aorta rings was 3335 ± 197 mg (n=26) and 3439±165 (n=52), respectively. In de-endothelized rings PE elicited a contraction of 3126 ±161 (n=33) (Table 1). Ayanin induced a greater relaxant effect against PE (pEC_{50}=5.84±0.05) than KCl (Table 1, Figure 2).

Pretreatment of the endothelium-intact rings with L-NAME (Figure 3) or ODQ (Figure 4) significantly reduced ayanin vaso-dilatation, support a NO/cGMPc dependent mechanism.

The relaxant response to ayanin was not affected by pretreatment with dipyridamole, atropine, verapamil or propranolol (Table 1), and slightly by indomethacin (Figure 4). Finally, ayanin unchanged the ACh relaxing effect (pEC50=7.55±0.04) and slightly modified SNP relaxation (pEC50=7, Table 2, Figure 5).

DISCUSSION

The results of ayanin in KCl- and PE-intact rings and in PE de-endothelized rings show that this flavonoid exerts anti-adrenergic effect of endothelium-dependent origin. Subsequent experiments with the NO synthase inhibitor L-NAME and ODQ, a guanylly cyclase inhibitor, which were able to reduce markedly ayanin vaso-dilatation, support a NO/cGMPc dependent mechanism.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PE-contraction (mg)</th>
<th>pEC_{50}</th>
<th>Maximal response (E_{max})</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl (80 mM)</td>
<td>3335 ± 197</td>
<td>ND</td>
<td>35.47 ± 3.65*</td>
</tr>
<tr>
<td>PE (10^{-4} M)</td>
<td>3439 ± 165</td>
<td>5.84 ± 0.05</td>
<td>92.99 ± 1.30</td>
</tr>
<tr>
<td>L-NAME (10^{-4} M) + PE</td>
<td>4882 ± 189*</td>
<td>ND</td>
<td>32.99 ± 7.14*</td>
</tr>
<tr>
<td>ODQ (10^{-4} M) + PE</td>
<td>5242 ± 101*</td>
<td>ND</td>
<td>40.88 ± 5.50*</td>
</tr>
<tr>
<td>L-ARG (10^{-4} M) + L-NAME (10^{-4} M) + PE</td>
<td>3834 ± 201</td>
<td>5.61 ± 0.04</td>
<td>93.02 ± 2.66</td>
</tr>
<tr>
<td>D-ARG (10^{-4} M) + L-NAME (10^{-4} M) + PE</td>
<td>4972 ± 158*</td>
<td>ND</td>
<td>30.12 ± 6.07*</td>
</tr>
<tr>
<td>Indomethacin (5x10^{-4} M) + PE</td>
<td>3876 ± 182*</td>
<td>5.43 ± 0.04*</td>
<td>90.70 ± 2.27</td>
</tr>
<tr>
<td>Dipyridamole (3x10^{-7} M) + PE</td>
<td>2300 ± 205*</td>
<td>6.05 ± 0.16</td>
<td>92.29 ± 2.49</td>
</tr>
<tr>
<td>Verapamil (10^{-7} M) + PE</td>
<td>1694 ± 108*</td>
<td>5.99 ± 0.11</td>
<td>95.10 ± 1.54</td>
</tr>
<tr>
<td>Glibenclamide (10^{-4} M) + PE</td>
<td>2818 ± 159</td>
<td>5.90 ± 0.01</td>
<td>96.61 ± 3.61</td>
</tr>
<tr>
<td>Propranolol (10^{-4} M) + PE</td>
<td>3587 ± 186</td>
<td>5.92 ± 0.09</td>
<td>87.97 ± 3.21</td>
</tr>
<tr>
<td>Atropine (3x10^{-5} M) + PE</td>
<td>2945 ± 205</td>
<td>5.82 ± 0.07</td>
<td>90.98 ± 4.02</td>
</tr>
</tbody>
</table>

Data are the mean ± SEM. n>5 experiments. pEC_{50} represents -log molar concentration of ayanin to produce 50% of the maximal relaxation. E_{max}, maximal relaxation (%). *p<0.05 compared with PE (10^{-6} M). ND, not determined.
It is noticeable that L-arginine (the substrate for NO synthesis), but not D-arginine, was able to reverse the inhibitory effects induced by L-NAME against ayanin. These findings suggest that ayanin enhances the production of NO from L-arginine in the vascular endothelium. It is known that nitric oxide (the endothelium derived relaxing factor, EDRF) activates soluble guanylyl cyclase, causing the formation of cyclic GMP and subsequently vascular relaxation. The cellular accumulation of cGMP depends upon the rate of their synthesis and their breakdown. The latter is achieved by cyclic nucleotide phosphodiesterase (PDE) isoforms PDE1 and/or PDE5 isolated in vascular smooth muscle cells. The potentiating of vasodilator effects of agents that increase cGMP has become a simple test to study the possible role of PDE isoforms. relaxation induced by ayanin may be related to the production of NO, resulting in an increase of the cGMP in the endothelium.

Table 2
Effect of ayanin (10^-6 M) on the relaxant responses of acetylcholine (ACh, 10^-6-3x10^-4 M) and sodium nitroprusside (SNP, 10^-9-3x10^-5 M) in endothelium-intact aorta rings precontracted with phenylephrine (PE, 10^-6 M)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PE-contraction (mg)</th>
<th>pEC_{50}</th>
<th>Maximal response(E_{max})</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh (10^-6-3x10^-4 M)</td>
<td>3439 ± 165</td>
<td>7.82 ± 0.02</td>
<td>64.41 ± 3.25</td>
</tr>
<tr>
<td>Ayanin (10^-6 M)+ ACh (10^-6-3x10^-4 M)</td>
<td>3192 ± 202</td>
<td>7.55 ± 0.04</td>
<td>71.73 ± 3.15</td>
</tr>
<tr>
<td>SNP (10^-9-3x10^-5 M)</td>
<td>3439 ± 165</td>
<td>8.38 ± 0.006</td>
<td>100.00 ± 0.00</td>
</tr>
<tr>
<td>Ayanin (10^-6 M)+ SNP (10^-9-3x10^-5 M)</td>
<td>3192 ± 202</td>
<td>8.08 ± 0.01*</td>
<td>99.92 ± 0.08</td>
</tr>
</tbody>
</table>

Data are the mean ± SEM of >5 experiments. pEC_{50} represents -log molar concentration of Ach or SNP to produce 50% of the maximal relaxation. E_{max}, maximal relaxation (%). *p<0.05 compared with PE (10^-6 M) (E+). ND, not determined.

Figure 3. Concentration response induced by ayanin in endothelium-intact rat aorta rings contracted with phenylephrine (PE, 10^-6 M), in absence and presence of: L-NAME (10^-4 M), L-NAME plus L-arginine (10^-4 M) and L-NAME plus D-arginine (10^-4 M), *p<0.05 (Anova and Dunnett tests).

Figure 4. Concentration response induced by ayanin in endothelium-intact rat aorta rings contracted with phenylephrine (PE, 10^-6 M) in absence and presence of ODQ (10^-6 M) or indomethacin (5x10^-6M), *p<0.05 (Anova and Dunnett tests).
inhibition in the relaxant effect of vasodilators. In these experiments, ayanin slightly increased the relaxant effect of SNP, a NO donor. Furthermore, dipyridamole, a cGMP-PDE5 inhibitor did not modify the relaxant responses to ayanin. Hence, these results suggest that inhibition of the GMP-dependent PDE is not mainly linked to the vasodilator effect of ayanin.

Blockage of cyclo-oxygenase by indomethacin (5×10⁻⁶ M) produced a rightward shift in the concentration-response curve for ayanin. The pEC₅₀ value of ayanin in the presence of the indomethacin was significantly decreased from 5.84±0.05 to 5.43±0.04 (p<0.05, Figure 4, Table 1). Therefore, it is possible in some degree that endothelial vasorelaxant factors derived from cyclooxygenase participated in the relaxant effect of ayanin.

Also, according to experiments with: atropine, a muscarinic receptor antagonist; verapamil, a L-type calcium channel blocking agent; propranolol, a β-adrenoeceptor blocking agent; and glibenclamide, a K⁺<sub>ATP</sub> channel blocker (Table 1), it can be presumed that muscarinic agonism, beta agonism, voltage-dependent calcium channel activation, and K⁺<sub>ATP</sub> channel activation do not seem important action mechanisms related to the ayanin relaxant profile. However, these observations do not rule out the possibility that other types of pharmaco-dynamic interactions may mediate ayanin-induced relaxation.

In conclusion, the ayanin flavonoid induces endothelium-dependent relaxation in Wistar rat aorta mainly related to NO/cGMP pathway. Further work is necessary to clarify the mechanisms involved in this effect. Since ayanin is the predominant compound isolated from *C. schiedeanus* («almizclillo»), it is highly probable that it plays an important role in the cardiovascular effects described for this specie. These results support the traditional use of *C. schiedeanus* for the treatment of arterial hypertension.
Conflict of interest. None of the authors has conflicts of interest related to this study.

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