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The vasorelaxant effect of gallic acid involves endothelium-dependent and -independent mechanisms

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ABSTRACT

The mechanisms of action involved in the vasorelaxant effect of gallic acid (GA) were examined in the isolated rat thoracic aorta. GA exerted a relaxant effect in the highest concentrations (0.4–10 mM) in both endothelium-intact and endothelium-denuded aortic rings. Pre-incubation with L-NAME, ODQ, calmidazolium, TEA, 4-aminopyridine, and barium chloride significantly reduced the pEC_{50} values. Moreover, this effect was not modified by indomethacin, wortmannin, PP2, glibenclamide, or paxillin. Pre-incubation of GA (1, 3, and 10 mM) in a Ca^{2+}-free Krebs solution attenuated $CaCl_2$-induced contractions and blocked BAY K8644-induced vascular contractions, but it did not inhibit a contraction induced by the release of Ca^{2+} from the sarcoplasmatic reticulum stores. In addition, a Western blot analysis showed that GA induces phosphorylation of eNOS in rat thoracic aorta. These results suggest that GA induces relaxation in rat aortic rings through an endothelium-dependent pathway, resulting in eNOS phosphorylation and opening potassium channels. Additionally, the relaxant effect by an endothelium-independent pathway involves the blockade of the Ca^{2+} influx via L-type Ca^{2+} channels.

1. Introduction

Gallic acid (GA) is a polyphenol found in green algae [1], fruits, and plants, and it is one of the phenolic components present in foodstuffs, such as tea and wine [2]. GA is well known as an antioxidant [3–6] and has been reported to have anti-cancer [7–9], antibacterial [10], antiulcer [11], antiviral [12], and anti-inflammatory effects [13]. In addition, antiparkinson activity and beneficial effects in vascular dementia were described [14,15].

Due the beneficial effects of polyphenols to numerous disease states, including the cardiovascular diseases, studies have been performed to determine the vascular effects of gallic acid. They have shown that GA inhibits vascular calcification [16] and has beneficial activity against carotid artery occlusion induced cognitive deficits [15]. A study performed on rat aorta showed that GA failed to induce the vasorelaxant activity [17]. The potentiation of the vasoconstrictor response and inhibition of endothelium-dependent vasorelaxation were also observed [18], as was the endothelium-dependent contraction induced by GA in rat aorta [19]. Posteriorly, GA-produced endothelium-dependent and -independent aorta contractions were shown in lower concentrations (0.1–3 μM and 10–30 μM, respectively), while the endothelium-independent relaxations were observed in higher concentrations (0.1–0.3 mM) [20]. Recently, it was shown that GA isolated from *Spirogyra* sp. algae exerted a vasorelaxant effect by inducing the production of NO in human umbilical vein endothelial cells and reduced blood pressure in spontaneously hypertensive rats [21].

Considering the diversity of GA vascular effects and the fact that a few studies have accessed the mechanisms involved in these effects, this study was performed, aiming to characterize the mechanisms of action involved with the GA vasorelaxant effect.

2. Materials and methods

2.1. Materials

Phenylephrine (Phe), acetylcholine (ACh), calmidazolium, N-[2-[N-(4-chlorocinnamyl)-N-methylaminomethyl]phenyl]-N-(2-hydroxyethyl)-4 methoxybenzenesulfonamide phosphate salt (KN-93), Wortmannin, 4-amino-3-(4-chlorophenyl)-1-(t-butyl)-1

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H-pyrazolo[3,4-d]pyrimidine (PP2), Nω-nitro-L-arginine methyl ester (L-NAME), tetraethylammonium (TEA), indomethacin, 1 H- [1, 2, 4]oxadiazolo[4,3-alpha]quinoxalin-1-one (ODQ), paxillin, glibenclamide, calmidazolium, 4-amynopiridine (4-AP), barium chloride ($BaCl_2$), caffeine, S(−)-Bay K 8644, N,N′,N′-tetraacetic acid (EGTA), calcium chloride, gallic acid, 4- (2-Aminoethyl) benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, aprotinin, sodium orthovanadate, phenylmethyl-sulfonyl fluoride, and dithiothreitol were purchased from Sigma (St. Louis, MO, USA). The GA was prepared as stock solutions in EtOH. The caffeine was soluble in boiling water. The glibenclamide, paxillin, and wortmannin were prepared as stock solutions in DMSO. The indomethacin was dissolved at EtOH, and the other drugs were dissolved in distilled water. The final DMSO and EtOH concentrations did not exceed 1% (*v*/*v*).

2.2. Animals

All experimental protocols were performed in accordance with the Ethical Principles in Animal Experimentation, adopted by the Brazilian College of Animal Experimentation (COBEA), and were approved by the Institutional Ethics in Research Committee at the Federal University of Goiás, Goiás, Brazil (Protocol CEP/UFG 22/2011). Female Wistar rats, 10–12 weeks of age (from the Federal University of Goiás, Goiás, Brazil), were used. The rats were maintained on a 12 h light/dark cycle under a controlled temperature (22 ± 1 °C) with ad libitum access to food and water.

2.3. Preparation of rat aortic rings

Vascular reactivity was performed in an organ bath setting, as previously described [22]. After euthanasia, the thoracic aorta was quickly removed and cleaned in a physiological solution, containing (mM) NaCl—130, $NaHCO_3$—14.9, KCl—4.7, KH_2PO_4—1.18, $MgSO_4$•$7H_2O$—1.17, $CaCl_2$•$2H_2O$—1.6, and glucose—5.5. The aorta was cut into 4-mm rings, which were then mounted in a muscle bath, containing a Krebs solution at 37 °C and bubbled with 95% O_2–5% CO_2. Isometric force generation was recorded with an isometric force transducer (AQCAD, AVS Projetos, São Carlos, Brazil). A resting tension of 1.5 g was imposed on each ring, and the rings were allowed to equilibrate for 1 h. Endothelium integrity was assessed by measuring the dilatory response to ACh (10 μM) in Phe-contracted vessels (1 μM). For studies of endothelium-intact vessels, a ring was discarded if relaxation with ACh (10 μM) was not 90% or greater. In some experiments, the endothelium of the aortic rings was mechanically removed by gently rolling the lumen vessel on a thin wire. For studies of endothelium-denuded vessels, the ring was discarded if there was any degree of relaxation by acetylcholine (10 μM). After assessing the presence of functional endothelium, vascular tissues were allowed to recuperate for at least 1 h, during which time, the Krebs–Henseleit solution was replaced every 15 min, before any experiment protocol to start.

2.3.1. Characterization of GA-induced vasorelaxation

A cumulative concentration–response curve to GA (0.001–10 mM) was constructed for aortic rings precontracted with Phe (1 μM) with intact endothelium. In a second set of experiments, once the contractile response to Phe (1 mM) was obtained, cumulative concentration–response curves to GA (0.4–10 mM) were constructed in rat aortic rings with intact or denuded endothelium. To address any residual or nonreversible effects of GA on contractile or relaxation events, aortic rings (exposed to cumulative concentrations of GA) were washed before receiving a new exposure to Phe (1 μM) and ACh (10 μM).

2.3.2. The signal transduction pathways of GA-induced vasorelaxation

Further studies were carried out to identify the signal transduction pathways involved in the vasorelaxant effect of GA. To determine if NO, guanylyl cyclase soluble (sGC), calcium calmodulin complex (Ca^{2+}/CaM), phosphatidylinositol3-kinase (PI_3K), and Src-kinase were involved in the relaxant effect of GA, endothelium-intact rings were incubated with L-NAME (a NO synthase inhibitor, 100 μM), ODQ (a sGC inhibitor, 10 μM), calmidazolium (a Ca^{2+}/CaM complex inhibitor, 30 μM), wortmannin (a PI_3K inhibitor, 1 μM), or PP2 (a Src-kinase inhibitor, 10 μM) for 30 min prior to pre-contraction with Phe (1 μM). The cumulative concentration–response curves of GA were then constructed and compared with those obtained with untreated rings.

To determine if prostanoids were involved in the relaxant effect of GA, endothelium-intact rings were incubated with indomethacin (a non-selective COX inhibitor, 10 μM) for 30 min prior to precontraction with Phe (1 μM).

To verify the involvement of K^+ channels in GA-induced relaxation, endothelium-intact rings were incubated for 30 min prior to precontraction with Phe (1 μM) with several K^+ channel inhibitors: glibenclamide (a selective blocker of ATP-sensitive K^+ channels, 10 μM), 4-aminopyridine (a selective blocker of voltage-dependent K^+ channels, 4-AP, 10 μM), TEA (a nonselective K^+ channel blocker, 1 mM), paxillin (as a large-conductance Ca^{2+}-activated K^+ channel inhibitor, 10 μM), and barium chloride (a rectifier K^+ (K_{IR}) channels inhibitor, 100 μM).

2.3.3. Effect of different concentrations of GA on $CaCl_2$-induced contraction-dependent on extracellular Ca^{2+}

To investigate the inhibitory effects of GA on Ca^{2+} influx through voltage-operated calcium channels (VOCC), endothelium-denuded aortic rings were exposed to a Ca^{2+}-free Krebs solution in the presence of K^+ (60 mM). The Ca^{2+}-free Krebs solution had the same composition as a normal Krebs solution, except that $CaCl_2$ was omitted. Cumulative concentration–response curves for $CaCl_2$ (0.01–10 mM) were obtained. Three different concentrations of GA (1, 3, and 10 mM) or vehicle were added to the bath and allowed to act for 30 min before the cumulative concentration–response curve was recorded for $CaCl_2$. Each preparation was exposed to only one concentration of GA. The results were expressed as percentages of the maximal response for KCl (120 mM), and the curves were statistically compared.

2.3.4. Investigation of the GA effect on S(−)-bay K 8644-induced precontractions

To determine whether the inhibition of the L-type calcium channel could contribute to the vasorelaxant effect of GA, endothelium-denuded aorta ring preparations were pre-incubated with a depolarizing solution of KCl 20 mM for 10 min, a procedure performed to obtain a better response for the contractile agent. Then, after 30 min of incubation in the presence of GA (1, 3, and 10 mM) or vehicle, the agonist by S(−)-Bay K 8644 (0.01–1 μM), an L-type Ca^{2+} channel activator, was added to induce a sustained contraction. Responses to S(−)-Bay K 8644 were expressed as increased amounts from the baseline, which was precontracted by KCl (120 mM), and the curves were statistically compared.

2.3.5. Effect of GA pretreatment on the Ca^{2+} release from intracellular stores

To investigate whether GA could interfere with the Ca^{2+} release from intracellular stores, the inhibitory effects of GA on caffeine-induced contractions in the absence of extracellular Ca^{2+} were determined in endothelium-denuded aortic rings. A normal Krebs solution was replaced with a Ca^{2+}-free solution containing EGTA (1 mM) for 15 min and then was washed with a Ca^{2+}-free solution. The rings were stimulated with caffeine (20 mM). The contraction induced by agonist was obtained after 30 min of incubation in the presence of GA (1, 3, and 10 mM) or vehicle.

2.4. Western blotting analysis

Endothelium-intact aortic segments, incubated with GA (1, 3, and 10 mM) or vehicle for 15 min, were subsequently frozen in liquid