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Vascular pro-oxidant effects secondary to the autoxidation of gallic acid in rat aorta☆

José Gil-Longo*, Cristina González-Vázquez

Department of Pharmacology, Faculty of Pharmacy, Universidade de Santiago de Compostela, E-15782 Santiago de Compostela, Spain

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Abstract

Gallic acid autoxidation was monitored by absorption spectroscopy and H_2O_2 production; vascular effects related to the autoxidation process were studied on intact and rubbed aortic rings from WKY rats. Gallic acid autoxidation in an oxygenated physiological salt solution (37°C, pH=7.4) mostly occurred in a 2-h time period. Superoxide anions, H_2O_2 and gallic acid quinones were produced during gallic acid autoxidation. In rings partially precontracted with phenylephrine, 0.1–3 μM gallic acid induced marked and largely endothelium-dependent contractions, 10–30 μM gallic acid induced endothelium-independent contractions and 0.1–0.3 mM gallic acid induced complete, fast-developing, endothelium-independent relaxations. Superoxide dismutase (SOD) shifted the endothelium-dependent gallic acid contractions to the right, and N^G-nitro-L-arginine abolished them. Indomethacin suppressed the endothelium-independent gallic acid contractions, and catalase abolished the endothelium-independent contractions and relaxations. Gallic acid (30 μM) inhibited the relaxant effects of acetylcholine and sodium nitroprusside. In rings maximally precontracted with KCl, 0.1–100 μM gallic acid did not modify the tone, whereas 0.3 mM induced complete, slow-developing, endothelium-independent relaxations. Moreover, 0.3 mM gallic acid induced an irreversible impairment of ring reactivity and the release of lactate dehydrogenase. Catalase and *N*-acetyl cysteine suppressed the deleterious effects induced by gallic acid in the rings. In conclusion: (a) gallic acid is rapidly and nonenzymatically oxidized in physiological solutions, generating superoxide anions, H_2O_2 and quinones; (b) superoxide anions (by destroying NO) and low H_2O_2 levels (by activating cyclooxygenase) both increase vascular tone; (c) moderate H_2O_2 levels decrease vascular tone; (d) high H_2O_2 and quinone levels cause irreversible relaxations due to cellular damage.

Keywords: Gallic acid; Superoxide anions; Hydrogen peroxide; Quinones; Rat aorta; Necrosis cell death

1. Introduction

Gallic acid (3,4,5-trihydroxibenzoic acid) is a naturally occurring polyphenol present in many fruits, vegetables and derivative products (tea, wines, etc.). Gallic acid is very well absorbed in humans; in fact, micromolar concentrations of free and glucuronidated forms of gallic acid and its main metabolite 4-*O*-methylgallic have been found in human blood plasma after ingestion of gallic acid-rich foods [1,2].

Gallic acid has been described as a strong natural antioxidant, which is able to scavenge reactive oxygen species (ROS), e.g., superoxide anions, hydrogen peroxide, hydroxyl radicals and hypochlorous acid [3,4]. This antioxidant effect could prove beneficial to numerous disease states, including cardiovascular disease. However, other studies have highlighted the cytotoxic activity of gallic acid: (a) antitumoral effects via apoptosis in certain tumor cell lines [5,6]; (b) cytotoxicity in vascular smooth muscle cells [7] and hepatocytes [8]. The cytotoxic activity seems to be mediated by intracellular Ca^{2+} and reactive species such as superoxide anions, hydrogen peroxide, hydroxyl radicals and quinones [5,6,8–10]. Moreover, gallic acid has been reported to have both pro-oxidant and antioxidant properties [10,11]. Apart from this, an endothelium-dependent contraction of rat thoracic aorta induced by gallic acid has been described [12].

Gallic acid autoxidation has been studied very little, although Tulyathan et al. [13] offer a good paper on the subject. In any case, an overall picture of the process can be drawn. In oxygenated aqueous solutions, gallic acid spontaneously autooxidizes to its semiquinone free radical. Two semiquinones may then go on to form a quinone with the regeneration of a phenol from the other semiquinone. Also, two semiquinones — or a quinone and a new gallic acid molecule— can dimerize, regenerating the hydroquinone. The dimer can then react with other quinones or reoxidize to form its own semiquinone or quinone, which can further react with other units, leading to a greater degree of polymerization and to the formation of new easily oxidized polymers. Under alkaline conditions, quinones of gallic acid and quinones of oligomers can also oxidize to open ring products. During gallic acid autoxidation, oxygen is consumed and superoxide anions and hydrogen peroxide are produced (two molecules of hydrogen peroxide/molecule of gallic acid). It has been reported that polyphenols do not react directly with oxygen and that the catalytic intervention of transition metals is essential [14]. Since iron is the

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* Corresponding author. Departamento de Farmacoloxía, Facultade de Farmacia, Universidade de Santiago de Compostela, Campus Universitario Sur, E-15782 Santiago de Compostela, Spain. Tel.: +34 981 563 100x14897; fax: +34 981 594 595.

E-mail address: jose.gil.longo@usc.es (J. Gil-Longo).

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most abundant transition metal in the human body, and since gallic acid is a strong chelating agent that forms complexes of high stability with iron, the interaction between iron and gallic acid is of primary importance; the complex reaction system containing iron and gallic acid in the presence of O_2 forms blue-colored complexes [15]. Humic-like substances could result from the gallic acid autoxidation process [16].

In spite of published studies, much remains unknown: (1) the relevance of the gallic autoxidation process in physiological conditions; (2) the biological effects associated with gallic acid autoxidation; (3) the range of gallic acid concentrations responsible for a particular effect; and (4) the cause–effect relationship between reactive species formed during the gallic acid autoxidation process and its biological effects. Therefore, the aims of this study were (1) to monitor the gallic acid autoxidation process in an oxygenated physiological salt solution; (2) to give an overall picture of gallic acid effects in rat aorta; and (3) to characterize the more relevant mechanisms responsible for gallic acid effects, describing the functional relevance of each effect according to the gallic acid concentration. Rat aorta was used because, under appropriate experimental conditions, changes in vessel tension allow us to detect easily the presence of some reactive species (e.g., superoxide anions and H_2O_2) or cytotoxic effects. Increasing concentrations of gallic acid will be used to elicit different effects.

2. Materials and methods

2.1. Monitoring of gallic acid autoxidation

The rate of gallic acid autoxidation was determined spectrophotometrically and visually. Ultraviolet and visible absorption spectra were obtained with an Ultrospec 4000 spectrophotometer (Pharmacia Biotech) at wavelengths ranging from 200 to 800 nm in 1-nm increments and with a scan speed of 6200 nm/s. Gallic acid autoxidation solution (0.3 mM; 30 ml) was freshly prepared, adding the appropriate volume of a gallic acid stock solution (0.1 M) to a Krebs bicarbonate solution (pH=7.4; 37°C; oxygenated with carbogen). In some experiments, the autoxidation solution also contained catalase (100 U/ml), SOD (200 U/ml), *N*-acetyl cysteine (NAC; 3 mM) or ethylenediaminetetraacetic acid (EDTA; 0.03, 0.3, 3 and 30 μM, and 0.3, 3 and 30 mM). At predetermined times (0, 15, 30, 60, 120 and 300 min), 3-ml aliquots of the autoxidation solution were transferred into a 1-cm quartz cuvette, and UV and visible absorption spectra were immediately obtained. Reference cuvettes contained all components except gallic acid. Changes in color of the autoxidation solution were also monitored by direct visual inspection at the predetermined times.

In a subset of experiments, 5 ml of a gallic acid autoxidation solution (0.6 mM) in the absence (control) or presence of catalase (3000 U/ml) or bovine serum albumin (0.8 mg/ml) taken after 40 min of autoxidation was dialyzed against a phosphate buffered saline solution (Na_2HPO_4 20 mM; NaCl 119 mM) adjusted at pH=6.0 to stop the autoxidation process. After readjusting the pH to 7.4, the dialyzed autoxidation solutions were spectrophotometrically scanned. Reference cuvettes also contained all components except gallic acid.

2.2. H_2O_2 Measurements in gallic acid autoxidation solutions

H_2O_2 production was quantified using the Amplex Red Hydrogen Peroxide Assay Kit (Invitrogen; Paisley, UK). Gallic acid autoxidation solutions (0.03, 0.1 and 1 mM; 2.5 ml each) were freshly prepared, adding the appropriate volume of a gallic acid stock solution (0.1 M) to a Krebs bicarbonate solution (pH=7.4; 37°C; oxygenated with carbogen). In some experiments, the autoxidation solution also contained catalase (100 U/ml). A pipette was used to place 50 μl of autoxidation solutions (diluted if necessary), H_2O_2 standard curve samples and controls into individual wells of a microplate. Then, 50 μl of the Amplex Red reagent/0.2 U/ml horseradish peroxidase working solution was added to each microplate well and incubated for 3 min at room temperature. Fluorescence as indicator of H_2O_2 presence was then measured with a spectrofluorimeter (FLx 800 Microplate Reader, Bio-Tek Instruments) using excitation at 545 nm and fluorescence detection at 590 nm. Triplicate measurements were obtained for each data point.

2.3. Studies in rat isolated thoracic aortic rings

2.3.1. Animals

Experiments were conducted on 6- to 8-month-old female Wistar Kyoto (WKY) rats, obtained from the rat colony maintained at the animal facilities of our department. The care and the use of these animals were in accordance with the European Community guidelines for the use of experimental animals. The rats were killed by stunning and exsanguination. The thoracic aorta was rapidly removed and placed in a Petri dish with Krebs bicarbonate solution (composition in millimolars: NaCl, 119; $CaCl_2 \cdot 2H_2O$, 1.5; $NaHCO_3$, 25; KCl, 4.7; $MgSO_4 \cdot 7H_2O$, 1.2; KH_2PO_4, 1.2; glucose, 11; pH, 7.4; 37°C), oxygenated with carbogen (95% O_2+5% CO_2), cleaned of adherent connective tissue, stripped (when necessary) of endothelium by gentle rubbing of the intimal surface with a wide cotton thread, and cut into 3- or 12-mm-long (depending on the studies) cylindrical rings.

2.3.2. Contraction studies

2.3.2.1. General procedure. The aortic rings (3 mm long) were immediately transferred to an organ bath containing 10 ml of the Krebs solution thermoregulated at 37°C and bubbled with carbogen. Two stainless steel pins were inserted through the lumen of each arterial segment: one pin was fixed to the organ bath and the other was connected to a force-displacement transducer to record the isometric tension using a computerized system. The distance between the wires was accurately controlled using a micrometer attached to the force transducer.

Before initiating specific experimental protocols, each ring segment was stretched until it reached an internal circumference equal to the in vivo internal circumference of the thoracic aorta during the diastole phase. To calculate the average value of this circumference, in a previous study we determined the points of intersection of the isobars (based on the Law of Laplace) for 90 mmHg (diastolic arterial pressure in our WKY rats) with plots of passive wall tension against the internal circumference of rings with endothelium. The value obtained was 6.83±0.094 mm (n=13). Bearing in mind that the rings are flat when stretched between the wires, and that we know the wire diameter, it was possible to calculate the distance needed between the wires in order to achieve the diastolic circumference of the rings. This procedure was employed to obtain more physiological contraction and relaxation responses and it is similar to that described in detail by Angus et al. [17].

Once appropriately stretched, the rings were equilibrated for at least a 1-h period, during which the physiological solution was replaced every 20 min. Endothelial integrity was determined by the relaxation in response to acetylcholine (0.1 μM) after a contraction by phenylephrine (0.03 μM). The preparations were then equilibrated again.

2.3.2.2. Studies on rings partially precontracted with phenylephrine. Phenylephrine (1–30 nM, depending on the reactivity of the preparation) was used to achieve a submaximal tone of approximately 15 mN (this tension represents 51% and 43% of the maximal contraction induced by 60 mM KCl in intact and rubbed rings, respectively). After the phenylephrine contraction reached a plateau, cumulative concentrations (0.1 μM–0.3 mM) of gallic acid were added to the tissue bath and isometric tension was monitored. In a subset of experiments carried out on intact rings, the tissues were incubated with N^G-nitro-L-arginine (L-NA; 30 μM) or SOD (200 U/ml) 15 min before the addition of phenylephrine. In a second subset of experiments carried out on rubbed rings, the rings were incubated with indomethacin (20 μM) or catalase (100 U/ml) or L-NA (30 μM) 15 min before the addition of phenylephrine.

2.3.2.3. Studies on maximally precontracted rubbed rings. A stable contraction was obtained using KCl (60 mM) and then cumulative concentrations of gallic acid (0.1 μM–1 mM) were added to the tissue bath and isometric tension was monitored. In a subset of experiments, the tissues were incubated with catalase (100 U/ml) or NAC (3 mM) 15 min before the addition of gallic acid. Control rings were incubated with 3 mM hydrogen peroxide instead of gallic acid.

In a second set of experiments, once the contractile response to KCl (60 mM) was obtained, the tissues were incubated with gallic acid (0.3 or 1 mM) for 120 min. After a washout period of 90 min, a second contraction with KCl (60 mM) was achieved. In a subset of experiments, the tissues were incubated with catalase (100 U/ml) 15 min before the addition of gallic acid.

In a third set of experiments, once a contractile response to phenylephrine (10 μM) was obtained, acetylcholine (3 nM to 10 μM) or sodium nitroprusside (3 pM to 0.3 μM) relaxation responses were studied. In a subset of experiments, the tissues were incubated with 30 μM gallic acid for 15 min before the addition of acetylcholine or sodium nitroprusside.

2.3.3. Assessment of cytotoxicity induced by gallic acid in rat isolated thoracic aortic rings

The cytotoxic effect of gallic acid was assessed by measuring lactate dehydrogenase (LDH) release. The aortic rings (12 mm in length) were put in an organ chamber containing 1 ml Krebs solution at 37°C and oxygenated with carbogen. One set of rings was incubated with phenylephrine (30 nM) for 90 min; another set was incubated with phenylephrine (30 nM) and gallic acid (0.3 mM) also for 90 min. After the incubation period, 100 μl of the incubation solution was removed and the LDH released into the solution was determined. To study the total LDH activity, the aortic rings themselves and the remaining incubation solution supplemented with 100 μl Krebs were also removed. After a manual homogenization of the aortic rings, the tissue suspension obtained was treated with 1% MTO-Triton X-100 for 30 min. The suspension was then centrifuged at 2000×g for 10 min and the supernatant was used. LDH activity was measured using a commercially available method (Sigma Diagnostics Lactate dehydrogenase LD-L), which measures the enzyme activity based on the

oxidation of lactate to pyruvate with simultaneous reduction of nicotinamide adenine dinucleotide (NAD). Formation of reduced NAD results in an increase in absorbance at 340 nm. Measurement of LDH activity was initiated by adding 50 μl of the sample solution to a 1-ml commercial phosphate buffer (pH 8.9) containing 50 mM lactate and 7 mM NAD. The absorbance change to 340 nm was then time monitored in a spectrophotometer. The rate of increase in absorbance at 340 nm is directly proportional to LDH activity in the sample. The percentage of LDH released into the incubation solution was evaluated by comparing the absorbance change per minute of the incubation solution with that of the tissue homogenate supernatant. The percentage of LDH released is thus an estimate of the percentage of the cells necrosed by a particular treatment.

2.4. Statistical analysis and data presentation

Except where otherwise specified, results shown in the text and figures were expressed as means±S.E.M.; *n* indicates the number of observations, one for each animal. Student's two-tailed *t* test for unpaired data was used to compare the differences between two groups. A variance analysis was used for comparisons among three or more experimental data groups; if significant differences were found, the Bonferroni test was used to make specific comparisons. Results were considered to be significant at $P<.05$.

The contractile response of phenylephrine, KCl and gallic acid was expressed in millinewton. For drugs that elicit constriction or relaxation of previously constricted isolated aortic rings, the response was also expressed as the percentage increase or reduction of tension in the precontracted state. Moreover, for acetylcholine and sodium nitroprusside, the pIC_{50} values (the negative logarithm of the molar concentration of sodium nitroprusside required to elicit 50% inhibition of maximum) were calculated.

2.5. Drugs

The following drugs were used: acetylcholine chloride, NAC, bovine serum albumin (BSA), catalase, EDTA, hydrogen peroxide, indomethacin, KCl, L-NA, L-phenylephrine hydrochloride, sodium nitroprusside and SOD were purchased from Sigma-Aldrich (St. Louis, MO, USA); gallic acid monohydrate was purchased from Fluka (Buchs, Switzerland).

The drugs utilised were prepared daily in de-ionized water from stock solutions kept at −20°C. The stock solutions were prepared in water with the exception of gallic acid (which was prepared in a 20:80 ethanol/water solution) and indomethacin (which was prepared in absolute ethanol). Hydrogen peroxide was purchased as 30% w/w concentrated solution and was subsequently diluted in distilled water. All reagents used in the preparation of physiological solutions were of analytical grade.

3. Results

3.1. Monitoring of gallic acid autoxidation

The absorption spectrum of 0.3 mM gallic acid at 0 time in an oxygenated buffered aqueous solution at pH 7.4 exhibited two bands, both in the UV range, one around 220 nm and the other around 260 nm. Within minutes, the oxygenated gallic acid solution developed a blue color and at the same time displayed two additional absorption bands (Fig. 1A): a UV band (around 320 nm) and a visible band (around 630 nm). The blue color progressively changed to blue-green and then to green, coinciding with the development of a new band around 400 nm. In the meantime, the initial UV bands rapidly decreased and almost stabilized in a 2-h time period.

Catalase (100 U/ml) or SOD (200 U/ml) had little influence on the time-dependent spectral and color transformations of gallic acid autoxidation solution; in particular, the intensity of the 400-nm band after a 2-h period was similar in the presence or absence of the enzymes. However, NAC (3 mM) impeded the development of both the blue color and the spectral bands described (320, 400 and 630 nm), but favored the development of a new band around 470 nm. Furthermore, EDTA at 30, 3 and 0.3 mM influenced the time-dependent spectral transformations of the gallic acid autoxidation solution: (a) inhibiting the decrease of the band at 260 nm (Fig. 2); (b) inhibiting the development of bands at 320, 400 and 630 nm. No effects were observed with EDTA at 30, 3, 0.3 and 0.03 μM.

As shown in Fig. 1B, a dialyzed gallic acid solution autoxidized in the presence of either catalase (3000 U/ml) or BSA (0.8 mg/ml) exhibited a much greater absorption than the control solution (absence of proteins), especially around 260 nm.

3.2. H_2O_2 production during gallic acid autoxidation

The Amplex Red H_2O_2 assay showed that H_2O_2 is largely produced during gallic acid autoxidation, reaching its maximum concentration at approximately 1 h (Table 1). Our results also showed that catalase (100 U/ml) blocked the presence of H_2O_2 in a 1 mM gallic acid autoxidation solution.

3.3. Effect of gallic acid in rat aortic rings

3.3.1. Effect of gallic acid in rings partially precontracted with phenylephrine

In aortic rings precontracted with phenylephrine to achieve a submaximal tone of approximately 15 mN, relatively low concentrations of gallic acid (0.1–3 μM), added cumulatively, induced an important concentration-dependent increase of tone in intact rings (endothelium-dependent contractions; 93±7.8%, $n=6$, of the previous tone), but not in rubbed rings (Fig. 3). Higher concentrations of gallic acid (10–30 μM), also added cumulatively, induced an increase of tone in both intact and rubbed rings (approximately 25% of the previous tone; endothelium-independent contractions). The highest range of gallic acid concentrations (0.1–0.3 mM), added cumulatively, reverted the contractile effect and induced a complete, rather fast-developing endothelium-independent relaxant effect (Fig. 3).

3.3.1.1. Influence of L-NA and SOD on the endothelium-dependent gallic acid contractions. N^G-nitro-L-arginine (30 μM) abolished the contractile response induced by gallic acid (0.1–3 μM). For its part, SOD (200 U/ml) significantly reduced the gallic acid contractile effect up to 36±7.1% ($n=6$) of the previous tone.

3.3.1.2. Influence of indomethacin, catalase and L-NA on the endothelium-independent gallic acid contractions. Both indomethacin (20 μM) and catalase (200 U/ml) suppressed the endothelium-independent gallic acid (10–300 μM) contractions, whereas L-NA (30 μM) did not modify it.

3.3.2. Effect of gallic acid in rubbed rings maximally precontracted with KCl

In rubbed aortic rings maximally precontracted with KCl (60 mM), the cumulative addition of gallic acid (0.1–100 μM) did not significantly modify the ring tone. However, 0.3 mM gallic acid induced a complete, slow-developing (90 min to baseline) relaxation.

3.3.2.1. Influence of catalase and NAC on the gallic acid effect. In the presence of catalase (100 U/ml), neither 0.3 mM gallic acid for 120 min nor 1 mM gallic acid for 120 min induced any significant relaxant effect. However, recovery of basal tone was not complete (84.0±1.6%, $n=6$, in comparison to 100.9±0.96%, $n=6$, in control rings; $P<.05$) after the gallic acid treatment in the presence of catalase, followed by a 90-min washout period. The incomplete recovery was not observed in rings pretreated with catalase plus NAC (3 mM).

3.3.3. Irreversible effect of gallic acid on aortic ring responsiveness

Gallic acid at 0.3 mM for 90 min followed by a 1-h washout period completely impaired the responsiveness of the rings to 60 mM KCl.

3.3.3.1. Influence of catalase on the persistent impairment of aorta responsiveness. In the presence of catalase (100 U/ml), neither 0.3 mM gallic acid for 120 min nor 1 mM gallic acid for 120 min

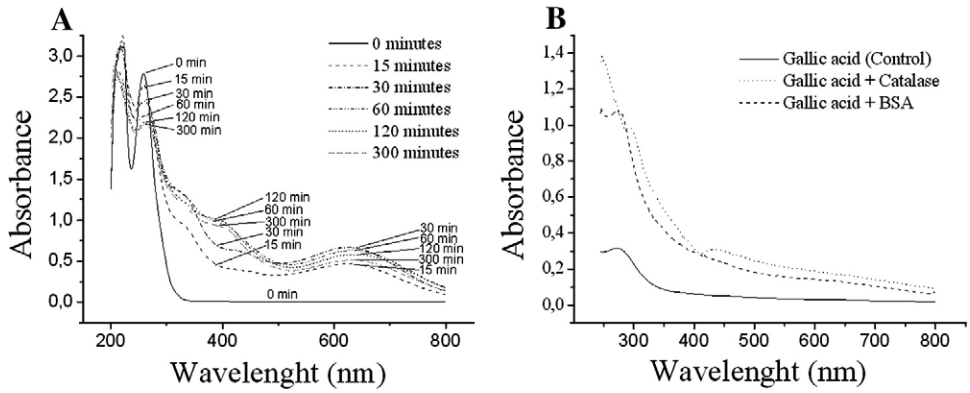

Fig. 1. UV and visible absorption spectra of: gallic acid (0.3 mM) autoxidized during 0, 15, 30, 60, 120 and 300 min in an oxygenated buffered solution (A) and dialyzed gallic acid (0.6 mM) autoxidized during 40 min in an oxygenated buffered solution in the absence (control) or presence of catalase (3000 U/ml) or BSA (0.8 mg/ml). In Panel (B), no data were recorded in the 200- to 245-nm region because of interference with the characteristic absorption peak of proteins.

significantly impaired the responsiveness of the rings to 60 mM KCl, compared to control rings.

3.3.4. Effect of gallic acid in aortic ring LDH release

Treatment of rubbed aortic rings with 0.3 mM gallic acid for 90 min caused a significant increase in LDH activity in the incubation solution. In relation to the total LDH activity in the aortic rings, LDH activity in the incubation solution reached 3.5±0.13% (n=4; P<.05). No liberation of LDH was observed in control rings.

3.3.5. Effect of gallic acid on the relaxation induced by both acetylcholine and sodium nitroprusside

In intact rat aortic rings precontracted with phenylephrine (10 μM), the administration of acetylcholine (3 nM to 10 μM) induced an important dose-dependent relaxation (Fig. 4), with an E_{max} of 91.3±3.5% (n=5) and a pIC_{50} of 6.69±0.43. This acetylcholine-induced relaxation decreased significantly (P<.05) and shifted to the right in rings pretreated with 30 μM gallic acid (E_{max}=52.5±2.2%, n=5; pIC_{50}=5.1±0.32).

In rubbed rat aortic rings also precontracted with phenylephrine (10 μM), the administration of sodium nitroprusside (3 pM to 0.3 μM) induced a complete dose-dependent relaxation (Fig. 4), with an E_{max} of 99.4±0.3% (n=5) and a pIC_{50} of 9.30±0.21. This sodium nitroprusside-induced relaxation was clearly shifted to the right (P<.05) in rings pretreated with 30 μM gallic acid (E_{max}=90.3±4.8%, n=5; pIC_{50}=7.5±0.25).

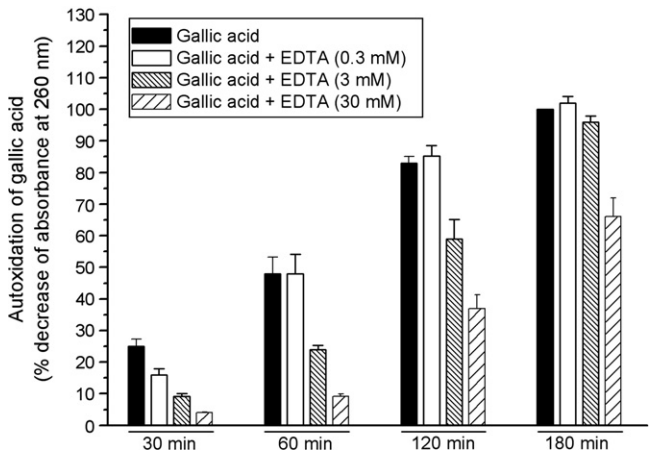

Fig. 2. Effects of EDTA on the autoxidation of gallic acid (0.3 mM) in an oxygenated buffered solution at different times. The percentage decrease of absorbance at 260 nm was considered as indicative of gallic acid autoxidation. Decrease in the absorbance of gallic acid solutions at 260 nm in the absence of EDTA after 180 min of autoxidation was taken as 100% (control). Data represents the mean of three experiments; S.E.M. is represented by vertical lines.

4. Discussion

Gallic acid was characterized in an oxygenated buffered solution at 0 time by two bands, one around 220 and another around 260 nm. Considering previous reports [13,15,18], the observed effect of time on the absorption spectrum of gallic acid would be associated with the rapid autoxidative degradation of gallic acid (decrease in the UV peaks), with the formation of blue chemical complexes between oxidized forms of gallic acid and iron (630-nm band) and with the formation of quinones and polymers that incorporate quinone groups (specially the 400-nm band). Altogether, our spectrophotometric studies also indicate that the degradation of gallic acid by autoxidation is a rapid process in an oxygenated solution, since gallic acid was largely transformed into its quinone forms in a 2-h time period. Moreover, our results indicate that (a) catalase and SOD does not prevent the gallic acid autoxidation process or the formation of gallic acid quinones, ruling out a significant role for H_2O_2 and superoxide anions in these processes; (b) NAC is a good tool to avoid the presence of quinones (absence of 400-nm band); (c) EDTA inhibits the oxidation of gallic acid by molecular oxygen, suggesting that metal ions present as contaminants in the Krebs bicarbonate solution play a significant role in catalyzing gallic acid oxidation by molecular oxygen. Furthermore, the dialyzed gallic acid solution autoxidized in the presence of catalase or BSA showed a much greater absorption than the control solution, indicating the binding of gallic acid semiquinones or quinones to proteins. This important result serves as a warning about the potential toxicity of autoxidized gallic acid

Table 1
H_2O_2 concentration in different gallic acid autoxidation solutions at predetermined times

	Autoxidation time (min)	H_2O_2 concentration
Gallic acid (mM)		
0.03	60	32±0.22 μM (n=3)
0.1	60	0.27±0.025 mM (n=3)
1	30	0.46±0.033 mM (n=3)
1	60	0.95±0.11 mM (n=3)
1	120	1.02±0.21 mM (n=3)
Gallic acid (mM)+catalase (100 U/ml)		
1	60	<0.5 μM (n=3)

Data are expressed as the mean±S.E.M.

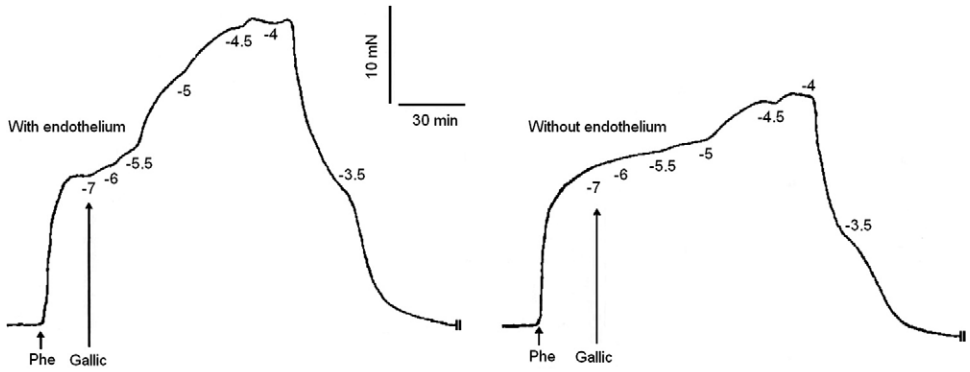

Fig. 3. Rat aortic rings precontracted with phenylephrine to achieve a submaximal tone of approximately 15 mN: typical effects of cumulative concentrations of gallic acid. The different concentrations are expressed as logarithms of molar concentrations.

through covalent modification of endogenous proteins. In general, the reaction of quinones with catalase, BSA and NAC is not surprising, because it is well established that quinones react directly with nucleophilic groups, e.g., with nonprotein and protein sulfhydryls.

According to our measurements, gallic acid autoxidation generates large quantities of hydrogen peroxide, which are enough to produce all the vascular effects — even cellular damage — described elsewhere for the peroxide [19]. Therefore, hydrogen peroxide must be taken into account to understand the vascular effects of gallic acid. The hydrogen peroxide assay also indicated that catalase (100 U/ml) completely prevents the presence of hydrogen peroxide in the gallic acid autoxidation solutions.

The present study shows that gallic acid can induce four different effects in isolated rat aorta. In submaximally phenylephrine precontracted rings, gallic acid induces an endothelium-dependent contraction that predominates at concentrations equal to or lower than 3 μM and an endothelium-independent contraction that becomes noticeable between 10 and 30 μM. Moreover, gallic acid produces a rather fast-developing endothelium-independent relaxation that predominates at 0.1 mM (not present in KCl precontracted rings) and an irreversible slow-developing endothelium-independent relaxation that starts at 0.3 mM.

The endothelium-dependent contractile effect of gallic acid was reduced by SOD (a superoxide scavenger) and abolished by L-NA (a nitric oxide synthase inhibitor). In agreement with a previous study [12], this observation suggests that the superoxide anion and the L-arginine–NO pathway are implicated. Because gallic acid may act as a generator of superoxide anions (see Introduction) and superoxide anions rapidly inactivate NO, the contractile effect seems to be secondary to the inactivation of endothelial resting vasodilator NO tone. This assumption is supported by two more facts: (1) gallic acid reduces and shifts to the right the relaxation induced by acetylcholine; and (2) gallic acid shifts to the right the relaxation induced by sodium nitroprusside. The endothelium-independent contractile effect of gallic acid was practically abolished by the hydrogen peroxide breakdown enzyme catalase and by the COX inhibitor indomethacin, suggesting that hydrogen peroxide and a product of the COX pathway are involved in the gallic acid-induced contraction. These conclusions are in agreement with our results, which show that gallic acid largely produces hydrogen peroxide during its autoxidation (see Introduction), and with the fact that hydrogen peroxide contracts the rat aorta by direct activation of cyclooxygenase [19,20]. The two contractile effects described for gallic acid, especially the endothelium-dependent contractile effect, which is very important at micromolar concentrations, could explain why ingestion of tea (independently of its caffeine content) and grape-seed polyphenols (in combination with vitamin C) induces an acute increase in blood pressure in humans [21,22].

The rather fast-developing endothelium-independent relaxation of gallic acid was abolished by 100 U/ml catalase and was not present in KCl precontracted rings, suggesting that hydrogen peroxide is involved in this gallic acid relaxant effect and that the peroxide acts through the activation of K^+ conductance in the smooth muscle cells (at high K^+ concentrations, the effect of K^+-channel openers is negligible because outward-directed electrochemical gradient for K^+ is reduced). This mechanism for the hydrogen peroxide effect has been previously suggested by pharmacological and electrophysiological studies. Different K^+ channels have been proposed as being responsible for the hydrogen peroxide effect, and different molecular pathways have been involved in the activation of K^+ channels by hydrogen peroxide [19,20,23]. The slow-developing endothelium-independent relaxation noticeable at gallic acid concentrations higher than 0.3 mM is associated with an irreversible impairment of ring

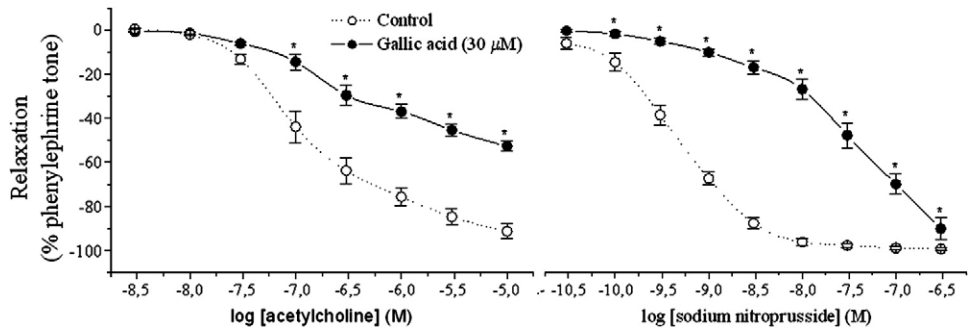

Fig. 4. Concentration–response curves showing the relaxation induced by acetylcholine in intact rings (left) and sodium nitroprusside in rubbed rings (right). Rings were precontracted with phenylephrine (10 μM) in the absence (control) or presence of gallic acid (30 μM). Each point represents the mean response of at least five aortic rings; S.E.M. is represented by vertical lines. *$P<.05$ vs. control.

responsiveness to contractile agents. These results may indicate that gallic acid induces a persistent impairment of contractile machinery or even that it has a cytotoxic effect on the rings. To clarify this point, we studied the effect of gallic acid on LDH release from rings. Since 0.3 mM of gallic acid induced a significant release of intracellular LDH, it is very probable that the slow-developing relaxation and the impairment of ring response are related to necrotic cell death rather than to a specific damage to the contractile machinery.

Although the mechanism involved in the cytotoxic effect of gallic acid is unknown, hydrogen peroxide and quinones are the main candidates thought to be the responsible for it: (a) hydrogen peroxide and quinones have been associated with cytotoxic effects [8,19,24,25]; and (b) our studies confirmed that hydrogen peroxide and quinones are produced during gallic acid autoxidation. The significance of hydrogen peroxide in the cytotoxic effect of gallic acid is supported by our results, which show that the deleterious effect of gallic acid was largely reduced by 100 U/ml catalase (as indicated previously, 1 mM gallic acid autoxidation did not generate detectable levels of hydrogen peroxide in the presence of 100 U/ml catalase). However, because 100 U/ml catalase does not prevent the alteration that 1 mM gallic acid induces on the recovery of basal tone, hydrogen peroxide probably is not the only deleterious factor associated with gallic acid autoxidation. In our study, the quinone scavenger NAC (3 mM) normalises the recovery of basal tone in the presence of 1 mM gallic acid, suggesting that quinones also have a role in gallic acid toxicity. Of course, the presence of nucleophilic groups in the catalase could also scavenge the quinones, but, however, any significant role of this enzyme in scavenging quinones is discarded due to the very low concentration of catalase.

In conclusion, our results show that gallic acid is rapidly and nonenzymatically oxidized in physiological solutions, generating superoxide anions, hydrogen peroxide and quinones. In consequence, gallic acid produces different vascular effects: (a) an endothelium-dependent contraction caused by the superoxide anions and the subsequent destruction of NO; (b) an endothelium-independent contractile effect related to the generation of low levels of hydrogen peroxide and the COX activation; (c) an endothelium-independent relaxation associated with moderate levels of H_2O_2 and the activation of smooth muscle K^+ channels; and (d) an irreversible, slow-developing endothelium-independent relaxation due to high hydrogen peroxide levels and quinones, which cause cellular damage.

As far as we know, this is the first report that describes all four gallic acid effects, their functional relevance and the molecular mechanisms involved in them. Since only micromolar concentrations of gallic acid have been found in human blood plasma after ingestion of foods rich in gallic acid [1,2], it is therefore unlikely that the autoxidation of gallic acid in humans produces hydrogen peroxide and quinone concentrations able to cause acute irreparable cellular damage. However, both ROS will certainly contribute to oxidative stress and suggest the possibility of toxic effects in vivo, at least in the long-term and under appropriate conditions. Indeed, it is plausible that in vivo micromolar concentrations of gallic acid secondary to the generation of superoxide anions may be responsible for some dysfunctions, e.g., increases in blood pressure in humans 30 min after drinking green tea and black tea [21], increases in blood pressure in humans 24 h after taking supplements containing combinations of polyphenols and vitamin C [22]. Therefore, our findings support the hypothesis that gallic acid behaves as a pro-oxidant polyphenol in physiological solutions and provides a warning about their potential toxicity in vivo.

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