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Vascular pro-oxidant effects related to the autoxidation of dopamine

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Abstract

Dopamine autoxidation in an oxygenated physiological salt solution (37°C, pH = 7.4) mostly occurred in a 2.5 h time period. H_2O_2 and dopamine quinones were produced during dopamine autoxidation. In partially pre-contracted rat aortic rings, 10–100 μM dopamine induced endothelium-independent contractions and 0.3–1 mM dopamine induced complete, slow-developing endothelium-independent relaxations. Indomethacin and catalase suppressed the endothelium-independent dopamine contractions. Catalase strongly reduced the endothelium-independent dopamine relaxations. Furthermore, 1 mM dopamine for 60 min followed by a 90 min washout period induced the release of lactate dehydrogenase and the complete impairment of ring reactivity to phenylephrine and KCl. Pre-treatment with catalase or glutathione prevented dopamine-induced deleterious effects so that further concentration-response curves to phenylephrine and KCl could be obtained. The phenylephrine potency was maintained in rings pre-treated with glutathione but not in rings pre-treated with catalase. In conclusion: (1) dopamine is rapidly and non-enzymatically oxidized in physiological solutions, generating H_2O_2 and quinones; (2) low H_2O_2 levels increase vascular tone by activating cyclooxygenase; (3) high H_2O_2 levels cause irreversible relaxations due to unspecific cellular damage; and (4) dopamine quinones cause a specific alteration in the phenylephrine response.

Keywords: *Dopamine, autoxidation, hydrogen peroxide, quinones, rat aorta*

Abbreviations: BSA, bovine serum albumin; COMT, catechol-*O*-methyltransferase; Emax, maximum response; LDH, lactate dehydrogenase; L-NA, N^G-nitro-L-arginine; NAD, nicotinamide adenine dinucleotide; MAO, monoamine oxidase

Introduction

Dopamine (3,4-dihydroxyphenethylamine) is a catecholamine with human relevance: it is a central neurotransmitter particularly important in the regulation of movement; it is synthesized in epithelial cells of the proximal tubule and is thought to exert local diuretic and natriuretic effects. It is also an intermediate in the synthesis of both norepinephrine and epinephrine and is used as a drug, principally in the treatment of some cases of severe congestive heart failure and cardiogenic and septic shock. The effects of dopamine are mediated by several distinct types of receptors: dopamine receptors (D_1, D_2, D_3, D_4, D_5) at low concentrations and adrenergic receptors (α_1 and β_1) at somewhat higher concentrations.

In humans, enzymatic metabolism of dopamine occurs by action of the enzymes monoamine oxidase (MAO; in which hydrogen peroxide and 3,4-dihydroxyphenylacetic acid are generated) and catechol-*O*-methyltransferase (COMT; in which 3-methoxy-4-hydroxy-phenylacetic acid is generated). Also, non-enzymatic oxidation of dopamine occurs by molecular oxygen even in the absence of metal ions (autoxidation) due to the unstable nature of the catechol ring [1–3]. It has been proposed that neuromelanin (a hybrid of eumelanin and pheomelanin) in the substantia nigra is formed by autoxidation of dopamine

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[4–6]. The autoxidative pathway for dopamine is thought to involve several steps [1–3,6,7]: (1) dopamine oxidation to dopamine *o*-quinone; (2) cyclization of the quinone through an addition reaction to produce leukoaminochrome and its subsequent oxidation to dopaminochrome; (3) dopaminochrome rearrangement to 5,6-dihydroxyindole, which can be oxidized to indole-5,6-quinone; and (4) polymerization of indole-5,6-quinone to neuromelanin. Autoxidation of dopamine leads to the formation of reactive oxygen species, including superoxide anions, hydrogen peroxide, hydroxyl radicals and reactive quinones [1,2,8,9]. The reactive oxygen species formed can damage cellular components such as proteins, lipids and DNA [10–12]. Specifically, reactive quinones have been shown to bind covalently to cysteinyl residues of proteins *in vitro* and *in vivo*, resulting in an alteration of protein function [8,10,13]. A variety of *in vitro* and *in vivo* studies have demonstrated that dopamine causes toxic effects associated with oxidative stress [12,14,15]; thus, it is generally accepted that autoxidation of dopamine may contribute to neurodegenerative disorders such as Parkinson's disease [3,8,10,12]. However, other studies have highlighted that dopamine is an effective superoxide scavenger [16], displays antioxidant properties in relation to both the Fenton reaction and lipid peroxidation [9] and might have some antioxidant effects in Parkinson's disease patients [17].

In spite of many published studies, much remains unknown: (1) the relevance of the dopamine autoxidation process in physiological conditions; (2) the biological effects associated with dopamine autoxidation; and (3) the cause–effect relationship between reactive species formed during the dopamine autoxidation process and its biological effects. Therefore, the aims of this study are: (1) to monitor the dopamine autoxidation process in an oxygenated physiological salt solution; (2) to give an overall picture of effects associated with dopamine autoxidation in rat aorta (vessel tone is very sensitive to reactive species); and (3) to characterize the reactive species responsible for the effects associated with dopamine autoxidation, describing the functional relevance of each effect according to the dopamine concentration. Increasing concentrations of dopamine will be used to elicit different effects.

Materials and methods

Materials

The following drugs were used: acetylcholine chloride, bovine serum albumin (BSA), catalase, dopamine hydrochloride, glutathione, hydrogen peroxide, indomethacin, KCl, N^G-nitro-L-arginine (L-NA), phenoxybenxamine hydrochloride, propranolol hydrochloride and L-phenylephrine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO).

The drugs utilized were prepared daily in deionized water from stock solutions kept at -20°C. The stock solutions were prepared in water with the exception of indomethacin, which was prepared in absolute ethanol. All reagents used in the preparation of physiological solutions were of analytical grade.

Monitoring of dopamine autoxidation

The rate of dopamine autoxidation was determined spectrophotometrically and visually. Ultraviolet and visible absorption spectra were obtained with an Ultrospec 4000 spectrophotometer (Pharmacia Biotech) at wavelengths ranging from 200–800 nm in 1 nm increments and with a scan speed of 6200 nm/s. Dopamine autoxidation solution (1 mM; 30 ml) was freshly prepared, adding the appropriate volume of a dopamine 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) or glutathione (10 mM). At pre-determined times (0, 15, 30, 60, 120 and 180 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 dopamine. Changes in colour of the autoxidation solution were also monitored by direct visual inspection at the pre-determined times. In other experiments, the precipitate formed after autoxidation of a dopamine solution (1 mM) for 120 min, was centrifuged, dried and weighed.

In a sub-set of experiments, 5 ml of a dopamine autoxidation solution (1 mM) in absence (control) or presence of catalase (2000 U/ml) or bovine serum albumin (0.8 mg/ml) taken after 40 min of autoxidation were dialysed against a phosphate buffered saline solution (PBS; 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 dialysed autoxidation solutions were spectrophotometrically scanned. Reference cuvettes also contained all components except dopamine.

H_2O_2 measurements in dopamine autoxidation solutions

H_2O_2 production was quantified using the Amplex Red Hydrogen Peroxide Assay Kit (Invitrogen; Paisley, UK). Dopamine autoxidation solutions (1 mM; 2.5 ml each) were freshly prepared, adding the appropriate volume of a dopamine 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, H_2O_2 standard curve

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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 were 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.

Studies on rat isolated thoracic aortic rings

Animals. Experiments were conducted on 6–8 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 mM = 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.

General procedure of contraction studies. 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 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 previously described [18].

Once appropriately stretched, the rings were equilibrated for at least a 60 min 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.

Studies on rings partially precontracted with KCl. Aortic rings were pre-treated with 0.3 µM phenoxybenzamine plus 5 µM propranolol and pre-contracted with KCl (10–15 mM, depending on the reactivity of the preparation) to achieve a submaximal tone of ~ 20 mN (this tension represents ~ 40% of the maximal contraction induced by 50 mM KCl). After the KCl contraction reached a plateau, cumulative concentrations (0.1 µM–1 mM) of dopamine were added to the tissue bath and isometric tension was monitored. In a sub-set of experiments carried out on rubbed rings, the tissues were also incubated with indomethacin (20 µM), catalase (100 U/ml) or N^G-nitro-L-arginine (L-NA; 30 µM) 15 min before the addition of KCl.

Assessment of irreversible effects of dopamine. Some equilibrated aortic rings were incubated with 1 mM dopamine for 60 min. After a washout period of 90 min, a contraction was achieved with cumulative concentrations of KCl (10–50 mM) or phenylephrine (0.001–3 µM). In a sub-set of experiments, the tissues were pre-incubated with catalase (100 U/ml) or glutathione (10 mM) 15 min before the addition of dopamine.

Assessment of cytotoxicity induced by dopamine in rat isolated thoracic aortic rings. The cytotoxic effect of dopamine 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 KCl (15 mM) for 60 min; another set was incubated with KCl (15 mM) and dopamine (1 mM) also for 60 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

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(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 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.

Statistical analysis and data presentation

Results shown in the text and figures are expressed as means ± SEM; 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 $p < 0.05$.

For phenylephrine and KCl, the pD_2 values (the negative logarithm of the molar concentration of the contractile agent required to elicit 50% of maximal response) were calculated. The contractile response to phenylephrine and KCl is expressed in mN. The response to dopamine is expressed as the percentage increase or reduction of tension in the pre-contracted state of the rings.

Results

Monitoring of dopamine autoxidation

The absorption spectrum of 1 mM dopamine at 0 time in an oxygenated buffered aqueous solution at pH 7.4 exhibited two bands in the UV range, one ~220 nm and the other ~280 nm. Within minutes the oxygenated dopamine solution developed a bronze colour and displayed two additional absorption bands (Figure 1A): a UV band (~305 nm) and a broad visible band (~470 nm). The bronze colour began to change to bronze-grey at ~40 min and then evolved to a grey colour by ~90 min. Coinciding with the colour changes, the absorbance of the oxygenated dopamine solution progressively increased in the entire visible region and stabilized within 120 min. Centrifugation (23 000 g; 10 min) of dopamine autoxidation mixture consistently removed the grey colour if it was present; in particular, centrifugation of a 1 mM dopamine solution autoxidized for 120 min allowed 52 ± 4% ($n = 3$) of the original dopamine present in the mixture to be recovered as a precipitate.

Catalase (100 U/ml) weakly accelerated the time-dependent spectral and colour transformations of the dopamine autoxidation solution, but it did not induce any qualitative change in them. Glutathione (10 mM) prevented the colour transformations and all the absorbance increases observed in the visible region of the dopamine autoxidation solution, but favoured the development of a new band ~250 nm.

As shown in Figure 1B, a dialysed dopamine solution autoxidized in the presence of either catalase (2000 U/ml) or bovine serum albumin (BSA; 0.8 mg/ml) exhibited some bands (~245, 305 and 550 nm) which were not present in the control solution (absence of proteins).

H_2O_2 production during dopamine autoxidation

The Amplex Red H_2O_2 assay showed that H_2O_2 is extensively produced during dopamine autoxidation,

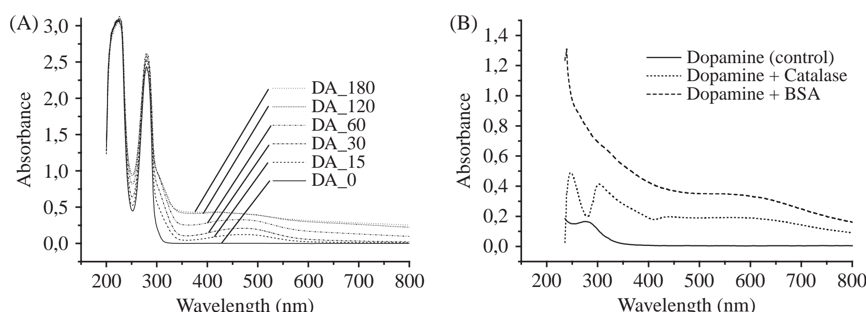

Figure 1. UV and visible absorption spectra of (A) dopamine (DA; 1 mM) autoxidized for 0, 15, 30, 60, 120 and 180 min in an oxygenated buffered solution (pH = 7.4; 37°C); (B) dialysed dopamine (1 mM) autoxidized for 40 min in an oxygenated buffered solution (pH = 7.4; 37°C) in the absence (control) or presence of catalase (2000 U/ml) or bovine serum albumin (BSA; 0.8 mg/ml). In panel B, no data were recorded in the 200–245 nm region because of interference with the characteristic absorption peak of proteins.

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reaching its maximum concentration at ~60 min (Table I). Specifically, 1 mM dopamine originated 0.23 ± 0.026 mM ($n = 3$) H_2O_2 at 30 min and 0.33 ± 0.025 mM ($n = 3$) H_2O_2 at 60 min. Our results also show that catalase (100 U/ml) blocked the presence of H_2O_2 in a 1 mM dopamine autoxidation solution at 30 and 60 min.

Effect of dopamine in rat aortic rings partially pre-contracted with KCl

As shown in Figure 2, in aortic rings pre-treated with 0.3 μM phenoxybenzamine plus 5 μM propranolol and partially pre-contracted with KCl (~20 mN), moderate concentrations of dopamine (10–100 μM), added cumulatively, induced an increase of tone in intact and rubbed rings (~100% of previous tone; endothelium-independent contractions). A higher concentration of dopamine (0.3 mM) produced a biphasic response in intact and rubbed rings, with an initial transient contraction and then an endothelium-independent relaxation. The highest concentration of dopamine (1 mM) induced a complete, rather slow-developing (~60 min to baseline) relaxant effect in intact and rubbed rings (endothelium-independent relaxation); after a 90 min washout period, the responsiveness of aortic rings to 10–50 mM KCl was completely impaired.

Influence of indomethacin, catalase and L-NA on the dopamine contractions in rubbed rings. Either indomethacin (20 μM) or catalase (100 U/ml) suppressed the dopamine (10–300 μM) contractions, whereas L-NA (30 μM) did not modify them (Figure 2).

Influence of indomethacin, catalase and L-NA on the dopamine relaxations in rubbed rings. Catalase (100 U/ml) strongly reduced the 1 mM dopamine-induced relaxations (the relaxant effect of dopamine was only $34.6 \pm 4.7\%$, $n = 6$, after acting for 60 min; Figure 2), whereas indomethacin (20 μM) and L-NA (30 μM) did not modify them.

Irreversible effect of dopamine on aortic ring responsiveness

As shown in Figure 3, dopamine at 1 mM for 60 min followed by a 90 min washout period completely impaired the responsiveness of the rings to cumulative concentrations of phenylephrine (0.001–3 μM) or KCl (10–50 mM).

Influence of catalase and glutathione on the persistent impairment of aorta responsiveness induced by dopamine. Further concentration-response curves could be obtained for phenylephrine or KCl in the presence of either catalase (100 U/ml) or glutathione (10 mM), after treatment with dopamine and the washout period. Compared to control rings, the maximum response (E_{max}) of phenylephrine was preserved in rings pre-treated with either catalase or glutathione, but its potency was preserved only in rings pre-treated with glutathione (Figure 3A). The pD2 of phenylephrine was significantly lower ($p < 0.05$) in rings treated with dopamine in the presence of catalase (6.5 ± 0.23, $n = 6$) than in control rings (7.4 ± 0.27, $n = 6$). The E_{max} and the potency of KCl were preserved in rings pre-treated with either catalase or glutathione (Figure 3B).

Effect of dopamine in aortic ring LDH release

Treatment of rubbed aortic rings with 1 mM dopamine caused a significant increase in LDH activity in the incubation solution (no liberation of LDH was observed in control rings). LDH activity in the incubation solution reached $7.6 \pm 0.26\%$ ($n = 4$; $p < 0.05$) in rings pre-treated with dopamine for 60 min and $11.5 \pm 0.62\%$ ($n = 4$; $p < 0.05$) in rings pre-treated with dopamine for 120 min.

Discussion

In an oxygenated buffered solution at 0 time, dopamine was characterized by two bands, one ~220 and another ~280 nm. Based on previous reports, the observed effect of time on the absorption spectrum of dopamine would be associated with the autoxidative degradation of dopamine. Specifically, the appearance of bands at 305 and 470 nm would be associated with orange dopaminochrome [2,6,19] and the overall increases of absorbance in the visible region would be associated with the formation of black insoluble pigment neuromelanin [6,19,20]. Our studies also indicate that the degradation of

Table I. H_2O_2 concentration in different dopamine autoxidation solutions at pre-determined times. Data are expressed as the mean ± SEM.

		Autoxidation time (min)	H_2O_2 concentration
Dopamine (mM)	0.1	30	49 ± 4.5 μM ($n = 3$)
	1	30	0.23 ± 0.026 mM ($n = 3$)
	1	60	0.33 ± 0.025 mM ($n = 3$)
	1	90	0.30 ± 0.028 mM ($n = 3$)
Dopamine (mM) + catalase (100 U/ml)	1	30	< 0.5 μM ($n = 3$)
	1	60	< 0.5 μM ($n = 3$)

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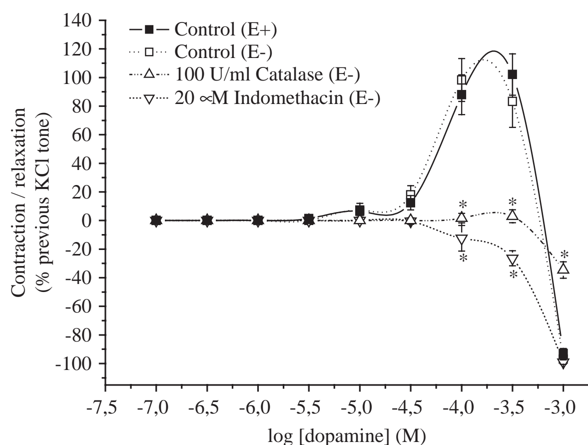

Figure 2. Concentration-response curves of dopamine in intact (E+; full symbols) and rubbed (E −; open symbols) rings pre-treated with 0.3 μM phenoxybenzamine plus 5 μM propranolol and partially pre-contracted (∼20 mN) with KCl, in the absence (controls) or presence (only in rubbed rings) of the drugs under study. Each point represents the mean response of at least six aortic rings; SEM is represented by vertical lines. *$p < 0.05$ vs control rubbed rings.

dopamine by autoxidation is a rather rapid process in an oxygenated solution: dopaminochrome peaked at ∼60 min, insoluble end-product neuromelanin started to appear at ∼40 min and more than 50% of the dopamine present in the autoxidation mixture could be recovered as neuromelanin precipitate after a 2 h autoxidation period. Unfortunately, the quantitative analysis of dopamine autoxidation by absorption spectroscopy is unfeasible because of the black neuromelanin precipitate that appears as the autoxidation proceeds.

Additionally, our findings show that catalase does not prevent the dopamine autoxidation process or the formation of neuromelanin, ruling out a significant role for H_2O_2 in these processes. However, the results confirm that glutathione is a good tool for preventing the presence of dopaminochrome and black insoluble pigment neuromelanin. The new band that formed ∼250 nm seems to indicate that dopamine autoxidizes to dopamine *o*-quinone and then the quinone rapidly reacts with glutathione to give 5-S-glutathionyldopamine [21]. Furthermore, the dialysed dopamine solution autoxidized in the presence of catalase or BSA showed some bands that were not present in the control solution, indicating the binding of dopamine semiquinones or dopamine quinones to the proteins. This result agrees with previous reports [8,10,13] and serves as a warning about the potential toxicity of autoxidized dopamine, due to the covalent modification of endogenous proteins. In general, the reaction of quinones with catalase, BSA and glutathione are not surprising, because it is well-established that quinones react directly with nucleophilic groups, e.g. with non-protein and protein sulphydryls.

According to our measurements, dopamine autoxidation generates large quantities of hydrogen peroxide, enough to produce all the vascular effects—including cellular damage—described elsewhere for the peroxide [22]. Therefore, hydrogen peroxide must be taken into account when seeking to understand the vascular effects of dopamine that are unrelated to its adrenergic agonist action. The hydrogen peroxide assay also indicated that catalase (100 U/mL) completely prevented the presence of hydrogen peroxide in the dopamine autoxidation solutions.

The present study shows that dopamine can induce two different effects in isolated rat aorta. In sub-maximally-KCl pre-contracted rings pre-treated with phenoxybenzamine and propranolol, dopamine induces: (i) an endothelium-independent contraction that becomes noticeable between 0.01–0.1 mM; and (ii) a complete, rather slow-developing endothelium-independent relaxation at 0.3–1 mM. The contractile and relaxant effects of dopamine do not seem to be receptor-mediated, since adrenergic receptors are blocked by phenoxybenzamine and propranolol and

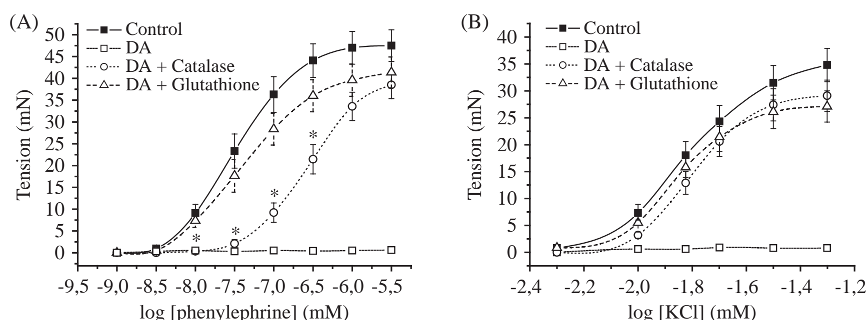

Figure 3. Concentration-response curves of (A) phenylephrine and (B) KCl in rubbed rat aortic rings. Before obtaining the concentration-response curves, the rings were incubated with dopamine (DA; 1 mM) for 60 min (open squares), dopamine (1 mM) for 60 min in the presence of catalase (100 U/ml; open circles), dopamine (1 mM) for 60 min in the presence of glutathione (10 mM; open triangles), or catalase (100 U/ml) plus glutathione (10 mM) for 60 min (full squares; controls) and then washed for 90 min. Each point represents the mean response of at least six aortic rings; SEM is represented by vertical lines. * $p < 0.05$ vs control.

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dopamine does not induce relaxant effects via D_1 receptors in rat aorta [23]. Nor does nitric oxide seem to be involved because the contractile and relaxant effects of dopamine are observed in rubbed rings pretreated with the nitric oxide synthase inhibitor L-NA.

The endothelium-independent contractile effect of dopamine was practically abolished by the hydrogen peroxide breakdown enzyme catalase and by the cyclo-oxygenase inhibitor indomethacin, suggesting that hydrogen peroxide and a product of the cyclo-oxygenase pathway are involved in the dopamine-induced contraction. These conclusions are in agreement with our results, which show that dopamine produces large amounts of hydrogen peroxide during its autoxidation and with the fact that hydrogen peroxide contracts the rat aorta by direct activation of cyclo-oxygenase [22,24].

Since autoxidation of dopamine proceeds via 1-electron transfer reactions, dopamine initially reacts with oxygen to give semiquinone radicals and superoxide anions [1,2]. Unexpectedly, in our study dopamine does not induce an endothelium-dependent contractile effect (superoxide anions formed should rapidly inactivate endothelial resting NO tone and should induce endothelium-dependent contractions). Therefore, superoxide anions would not be involved in the effects secondary to dopamine autoxidation, suggesting that dopamine or dopamine autoxidation products very effectively reduce superoxide anions to peroxides. Supporting this idea, it has been reported that dopamine is an effective superoxide scavenger [16].

The slow-developing endothelium-independent relaxation that appears at 1 mM dopamine is associated with an irreversible impairment of ring responsiveness to contractile agents. These results may indicate that dopamine 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 dopamine on LDH release from rings. Since 1 mM dopamine induced a significant release of intracellular LDH, it is very probable that the amine alters the integrity of cell membranes and that the slow-developing relaxation and the impairment of ring responsiveness are related to necrotic cell death rather than to specific damage to the contractile machinery. Moreover, because phenoxybenzamine is a potent and competitive inhibitor of extraneuronal monoamine transport system 'uptake$_2$' [25], the deleterious effect of dopamine must be related to vascular smooth muscle cell membrane damage.

Although the mechanism involved in the cytotoxic effect of dopamine is unknown, hydrogen peroxide and dopamine quinones are the main candidates thought to be the responsible for it, because (a) hydrogen peroxide and dopamine quinones have been associated with cytotoxic effects [8,10,13,22,26]; and (b) our studies confirmed that hydrogen peroxide and quinones are produced during dopamine autoxidation. That hydrogen peroxide is largely responsible for the cytotoxic effect of dopamine is supported by our results, which show that, in the presence of 100 U/ml catalase, 1 mM dopamine does not alter the E_{max} of phenylephrine or KCl. However, catalase does not prevent all alterations of aortic ring responsiveness to phenylephrine (the potency of the drug remains altered) induced by dopamine; therefore, hydrogen peroxide is probably not the only factor responsible for aortic ring alterations secondary to dopamine autoxidation.

In our study, the quinone and hydrogen peroxide scavenger glutathione (10 mM) completely normalized the aortic ring responsiveness to phenylephrine after 1 mM dopamine treatment, suggesting that dopamine semiquinones or dopamine quinones are responsible for the dopamine-induced alteration of the potency of phenylephrine in the presence of catalase. Since in our experiments catalase completely prevented the alterations induced by 1 mM dopamine on aortic ring responsiveness to KCl but not to phenylephrine, any dopamine quinones or semiquinones formed should alter a specific step in the phenylephrine response. Keeping in mind that dopamine has a rather high affinity for adrenergic receptors, our findings support the hypothesis that dopamine semiquinones or dopamine quinones specifically impair α-adrenergic receptor function. The identity of these quinones is not known. Dopamine *o*-quinone is thought to react with the sulphydryl groups of proteins and glutathione [13,21], but if cysteinyl residues are absent or sterically protected, indole-5,6-quinone would be more reactive than dopamine *o*-quinone or dopaminochrome [6].

In conclusion, our results show that dopamine is rather rapidly and non-enzymatically oxidized in physiological solutions, generating hydrogen peroxide and quinones. In consequence, dopamine produces different vascular effects: (a) an endothelium-independent contractile effect related to the generation of low levels of hydrogen peroxide and the activation of cyclooxygenase; (b) an irreversible, slow-developing endothelium-independent relaxation due to high hydrogen peroxide levels, which cause cellular damage; and (c) a specific alteration in the phenylephrine response related to semiquinones or quinones that could impair α-adrenergic receptor function.

Physiological concentrations of dopamine range from nanomolar levels in plasma [27] to estimated millimolar levels in vesicles and synaptic clefts in the striatum [28]. Pharmacological treatments that increase the concentration of dopamine typically generate micromolar levels in plasma [29,30]. It is therefore unlikely that the autoxidation of dopamine in humans produces hydrogen peroxide concentrations able to cause irreparable cellular damage. However, any hydrogen peroxide formed

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will certainly contribute to oxidative stress. Similarly, although the concentrations produced by incubating 1 mM dopamine for 60 min are probably not reached in humans, chronic exposure to the concentrations that are generated may be sufficient to harm α-adrenergic receptors *in vivo*. Indeed, it is plausible that aggravation of the adverse effects of dopamine by treatments that increase its concentration may be responsible for the dysfunctions of autonomic cardiovascular control that are commonly observed in Parkinson's disease patients receiving L-dopa [31], a problem that is not yet well understood. *In vivo* antioxidant mechanisms may certainly mitigate the toxic effects described in our study, but their inherent limitations do not guarantee full effectiveness. Taken together, our findings support the hypothesis that dopamine behaves as a pro-oxidant of tissues in physiological solutions and point to the possibility of its having toxic effects *in vivo*, at least in the long-term and under appropriate conditions.

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References

[1] Graham DG, Tiffany SM, Bell WR Jr, Gutknecht WF. Autoxidation versus covalent binding of quinones as the mechanism of toxicity of dopamine, 6-hydroxydopamine, and related compounds toward C1300 neuroblastoma cells *in vitro*. Mol Pharmacol 1978;14:644–653.
[2] Herlinger E, Jameson RF, Linert W. Spontaneous autoxidation of dopamine. J Chem Soc [Perkin II] 1995;2:259–263.
[3] Graumann R, Paris I, Martínez P, Rumanque P, Marín P, Díaz-Grez F, Caviedes R, Caviedes P, Segura-Aguilar J. Oxidation of dopamine to aminochrome as a mechanism for neurodegeneration of dopaminergic systems in Parkinson's disease. Possible neuroprotective role of DT–diaphorase. Pol J Pharmacol 2002;54:573–579.
[4] Ikemoto K, Nagatsu I, Ito S, King RA, Nishimura A, Nagatsu T. Does tyrosinase exist in neuromelanin-pigmented neurons in the human substantia nigra? Neurosci Lett 1998;253: 198–200.
[5] Zecca L, Tampellini D, Gerlach M, Riederer M, Fariello RG, Sulzer D. Substantia nigra neuromelanin: structure, synthesis, and molecular behaviour. J Clin Pathol Mol Pathol 2001;54:414–418.
[6] Bisaglia M, Mammi S, Bubacco L. Kinetic and structural analysis of the early oxidation products of dopamine. Analysis of the interactions with α-synuclein. J Biol Chem 2007;282:15597–15605.
[7] Costa C, Bertazzo A, Allegri G, Toffano G, Curcuruto O, Traldi P. Melanin biosynthesis from dopamine. II. A mass spectrometric and collisional spectroscopic investigation. Pigment Cell Res 1992;5:122–131.
[8] Asanuma M, Miyazaki I, Diaz-Corrales FJ, Ogawa N. Quinone formation as dopaminergic neuron-specific oxidative stress in pathogenesis of sporadic Parkinson's disease and neurotoxin-induced parkinsonism. Acta Med Okayama 2004;58:221–233.
[9] Hermida-Ameijeiras A, Méndez-Álvarez E, Sánchez-Iglesias S, Sanmartín-Suárez C, Soto-Otero R. Autoxidation and MAO-mediated metabolism of dopamine as a potential cause of oxidative stress: role of ferrous and ferric ions. Neurochem Inter 2004;45:103–116.
[10] Stokes AH, Hastings TG, Vrana KE. Cytotoxic and genotoxic potential of dopamine. J Neurosci Res 1999;55:659–665.
[11] Pattison DI, Dean RT, Davies MJ. Oxidation of DNA, proteins and lipids by DOPA, protein-bound DOPA, and related catechol(amine)s. Toxicology 2002;177:23–37.
[12] Chen L, Ding Y, Cagniard B, Van Laar AD, Mortimer A, Chi A, Hastings TG, Kang UJ, Zhuang X. Unregulated cytosolic dopamine causes neurodegeneration associated with oxidative stress in mice. J Neurosci 2008;28:425–433.
[13] Ito S, Kato T, Fujita K. Covalent binding of catechols to proteins through the sulphydryl group. Biochem Pharmacol 1988;37:1707–1710.
[14] Haque ME, Asanuma M, Higashi Y, Miyazaki I, Tanaka K, Ogawa N. Apoptosis-inducing neurotoxicity of dopamine and its metabolites via reactive quinone generation in neuroblastoma cells. Biochim Biophys Acta 2003;1619: 39–52.
[15] Pedrosa R, Soares-Da-Silva P. Oxidative and non-oxidative mechanisms of neuronal cell death and apoptosis by L-3,4-dihydroxyphenylalanine (L-DOPA) and dopamine. Br J Pharmacol 2002;137:1305–1313.
[16] Siraki AG, Smythies J, O'Brien PJ. Superoxide radical scavenging and attenuation of hypoxia-reoxygenation injury by neurotransmitter ferric complexes in isolated rat hepatocytes. Neurosci Lett 2000;296:37–40.
[17] Buhmann C, Arlt S, Kontush A, Moller-Bertram T, Sperber S, Oechsner M, Stuerenburg HJ, Beisiegel U. Plasma and CSF markers of oxidative stress are increased in Parkinson's disease and influenced by antiparkinsonian medication. Neurobiol Dis 2004;15:160–170.
[18] Angus JA, Cocks TM, Satoh K. α2-adrenoceptors and endothelium-dependent relaxation in canine large arteries. Br J Pharmacol 1986;88:767–777.
[19] Graham DG. Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones. Mol Pharmacol 1978;14:633–643.
[20] Meredith P, Powell BJ, Riesz J, Nighswander-Rempel SP, Pederson MR, Moore EG. Towards structure–property–function relationships for eumelanin. Soft Matter 2006;2:37–44.
[21] Dagnino-Subiabre A, Cassels BK, Baez S, Johansson A-S, Mannervik B, Segura-Aguilar J. Glutathione transferase M2-2 catalyzes conjugation of dopamine and dopa o-quinones. Biochem Biophys Res Commun 2000;274:32–36.
[22] Gil-Longo J, González-Vázquez C. Characterization of four different effects elicited by H_2O_2 in rat aorta. Vascul Pharmacol 2005;43:128–138.
[23] Varma DR, Chemtob S. Endothelium- and *beta*-2 adrenoceptor-independent relaxation of rat aorta by tyramine and certain other phenylethylamines. J Pharmacol Exp Ther 1993;265:1096–1104.
[24] Gao YJ, Hirota S, Zhang DW, Janssen LJ, Lee RMKW. Mechanisms of hydrogen-peroxide-induced biphasic response in rat mesenteric artery. Br J Pharmacol 2003;138: 1085–1092.
[25] Hayer-Zillgen M, Brüss M, Bönisch H. Expression and pharmacological profile of the human organic cation

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transporters hOCT1, hOCT2 and hOCT3. Br J Pharmacol 2002;136:829–836.
[26] Li J, Li W, Su J, Liu W, Altura BT, Altura BM. Hydrogen peroxide induces apoptosis in cerebral vascular smooth muscle cells: possible relation to neurodegenerative diseases and strokes. Brain Res Bull 2003;62:101–106.
[27] LeBlanc J, Ducharme MB. Plasma dopamine and noradrenaline variations in response to stress. Physiol Behav 2007;91:208–211.
[28] Garris PA, Ciolkowski EL, Pastore P, Wightman RM. Efflux of dopamine from the synaptic cleft in the nucleus accumbens of the rat brain. J Neurosci 1994;14: 6084–6093.
[29] Mac Gregor DA, Smith TE, Prielipp RC, Butterworth JF, James RL, Scuderi PE. Pharmacokinetics of dopamine in healthy male subjects. Anesthesiology 2000;92:338–346.
[30] Dethy S, Laute MA, Van Blercom N, Damhaut P, Goldman S, Hildebrand J. Microdialysis-HPLC for plasma levodopa and metabolites monitoring in parkinsonian patients. Clin Chem 1997;43:740–744.
[31] Bouhaddi M, Vuillier F, Fortrat JO, Cappelle S, Henriet MT, Rumbach L, Regnard J. Impaired cardiovascular autonomic control in newly and long-term-treated patients with Parkinson's disease: involvement of L-dopa therapy. Auton Neurosci Basic Clin 2004;116:30–38.

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