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European Journal of Medicinal Chemistry 38 (2003) 501–511
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
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Laboratory note

Opposite vascular activity of (R)-apomorphine and its oxidised derivatives. Endothelium-dependent vasoconstriction induced by the auto-oxidation metabolite

Belén Abarca [a,*], Rafael Ballesteros [a], Patricia Bielsa [a], Juan Moragues [a], Pilar D'Ocon [b,*], Eugenia García-Zaragozá [b], M. Antonia Noguera [b]

[a] *Departamento de Química Orgánica, Facultad de Farmacia, Universidad de Valencia, Avda. Vicente Andrés Estellés s/n, 460100 Burjassot, (Valencia), Spain*
[b] *Departamento de Farmacología, Facultad de Farmacia, Universidad de Valencia, Avda. Vicente Andrés Estellés s/n, 460100 Burjassot, (Valencia), Spain*

Received 9 August 2002; received in revised form 10 February 2003; accepted 10 February 2003

Abstract

We have synthetised a series of oxidised apomorphine derivatives (*orto* and *para* quinones **2–5**), in order to analyse their vascular activity. We have performed radioligand binding assays on rat cortical membranes and functional studies on rat aortic rings. Instead the relaxant activity exhibited by (R)-apomorphine, *o*-quinones **2**, **4**, show contractile activity dependent on endothelium in rat aortic rings. Compound **2**, the main metabolite of (R)-apomorphine auto-oxidation, was the product which showed enhanced contractile activity by a complex mechanism related to activation of Ca^{2+} channels through release and/or inhibition of endothelial factors. Moreover, this compound disrupts the endothelial function as shows the lack of response to acetylcholine observed in vessels pretreated with it.

Keywords: Apomorphine derivatives; Oxidation; Rat aorta; Endothelium; Vasoconstriction

1. Introduction

(R)-Apomorphine is a well known therapeutic agent with emetic properties, useful in Parkinson's treatment and pharmacological management of male erectile dysfunction. In vascular tissues, (R)-apomorphine acts as a vasodilatation agent and contractions evoked by noradrenaline (NA) or KCl were concentration-dependently depressed by (R)-apomorphine and derivatives in rat aorta [1,2] but, paradoxically, a concentration-dependent contractile response was also observed [1] which was prevented by ascorbic acid. It is interesting to note that the contraction appeared at the same time as an intensive green colour formed spontaneously in the carbogenated organ bath, which indicates auto-oxidation of apomorphine [3]. Ascorbic acid prevents both, the contractile response and the (R)-apomorphine oxidation.

Knowing that auto-oxidation is the main metabolic route of (R)-apomorphine [4] and taking into account the complex pharmacological behaviour of this compound depending on its oxidation, we have synthetised and studied a series of oxidised apomorphine derivatives in order to clarify the vascular activity of the oxidised metabolites of (R)-apomorphine. The compounds were three *o*-quinones: compounds **2** (the metabolite formed in the organ bath), **4** and **5**; and one *p*-quinone: compound **3** (Fig. 1). We analysed the interaction of the compounds with Ca^{2+} channels and α_1-adrenoceptors by examining their effects on [^{3}H]-nitrendipine or [^{3}H]-(+)-*cis*-diltiazem and [^{3}H]-prazosin binding to rat

Abbreviations: NA, noradrenaline; DMSO, dimethyl sulphoxide; SOD, superoxide dismutase; NOS, nitric oxide synthetase; EDTA, ethylenediaminetetraacetic acid; L-NAME, N^G-nitro-L-arginine methyl esther.

* Corresponding authors.
E-mail address: belen.abarca@uv.es (B. Abarca).

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doi:10.1016/S0223-5234(03)00057-6

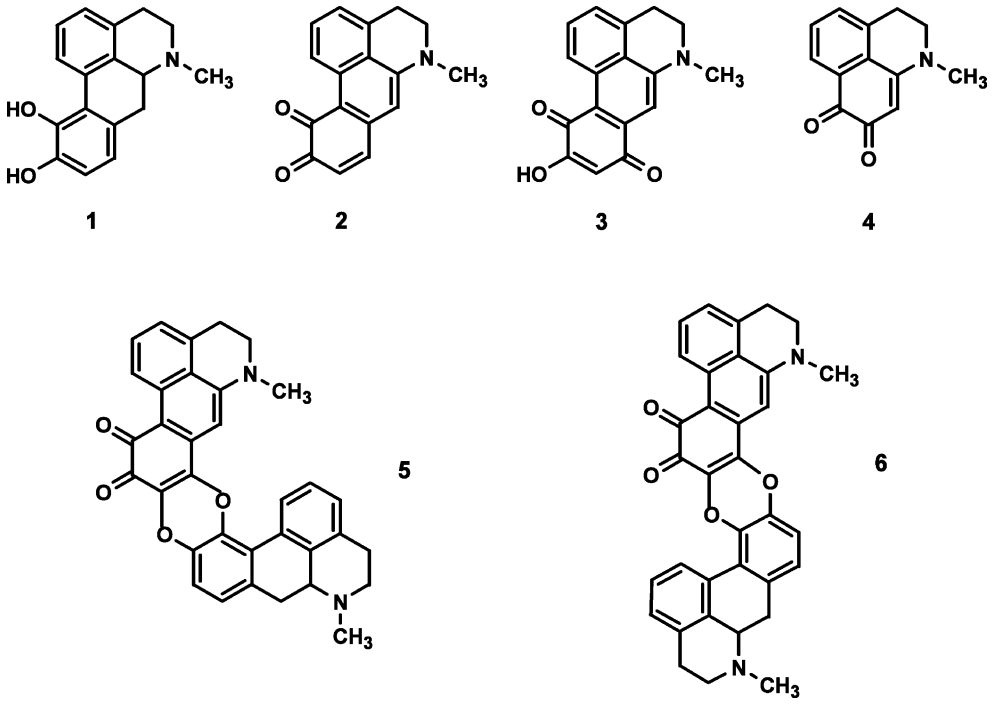

Fig. 1. Structures of compounds **1** to **6**.

cerebral cortical membranes. We also studied the activity of these compounds on vascular smooth muscle performing functional studies in isolated rat aorta, and a possible direct action of compounds on nitric oxide synthetase (NOS) enzyme activity.

2. Chemistry

Apomorphine **1** is well known in chemical literature, its autoxidation reaction is an inherent chemical property which generates an *o*-quinone **2** by dissolved oxygen at physiological pH [5]. Oxidation of apomorphine is also described as a pH dependent reaction [6], in acidic solution yields again compound **2**, in alkaline medium a *p*-quinone **3** and a different *o*-quinone **4** are formed (Fig. 1). We have found that proportions between the reagents and the way they are added, are oxidation reaction conditions which can modify the results.

Apomorphine is a very labile compound in solution, its stability in plasma has been investigated and a half-life of 38.9 min was found [7], in our hands a Krebs-bicarbonate solution (KBS) of apomorphine, maintained at room temperature and gassed with 95% O_2 and 5% CO_2, oxidise rapidly turning into green, a blue compound was isolated from the solution and identified as *o*-quinone **2**. High-performance liquid chromatography analysis does not show another oxidation derivative. Reaction with potassium dicromate in acetic acid using the described conditions [6] gave compound **2** in 41% yield. Nevertheless, with a big excess of potassium dicromate a mixture of compounds **2** and **4** are formed, the yield of each one depends on the reaction time. The orange compound **4** is also formed, in quantitative yield, by oxidation of **2** with potassium dicromate in acid medium. When the oxidant reactive is added very slowly to the apomorphine solution in stoichiometric quantities, an interesting new violet compound is formed. Shown by HRMS to have formula $C_{34}H_{26}N_2O_4$. From a careful analysis of the ^1H and ^{13}C-NMR data we deduced that an apomorphine structure (without protons from OH) is present, as well as, an *o*-quinone **2** structure lacking H_8 and H_9 proton signals. Two alternative structures **5** or **6** are possible. The observed fragmentation pattern in the mass spectrum, COSY and HSQC experiments are consistent with both (Fig. 2 show data for **5**). If the mechanism of the reactions is as we believe (see below) we propose structure **5** for it. We are waiting for crystal structure determination.

When apomorphine is treated in basic medium and air is bubbled, blue hydroxy-*p*-quinone **3** as major, and *o*-quinone **4** as minor compound, are formed. Blue quinone **3** is stable to further oxidation under acid or basic conditions.

To account for the formation of different compounds in oxidation reactions, depending on the conditions used, we propose the following mechanisms.

When autoxidation is produced, an initial step involves the transfer of two electrons and two protons to O_2 with concomitant formation of quinone **7** and H_2O_2, as Dryhurst and co-workers [8] have proposed for

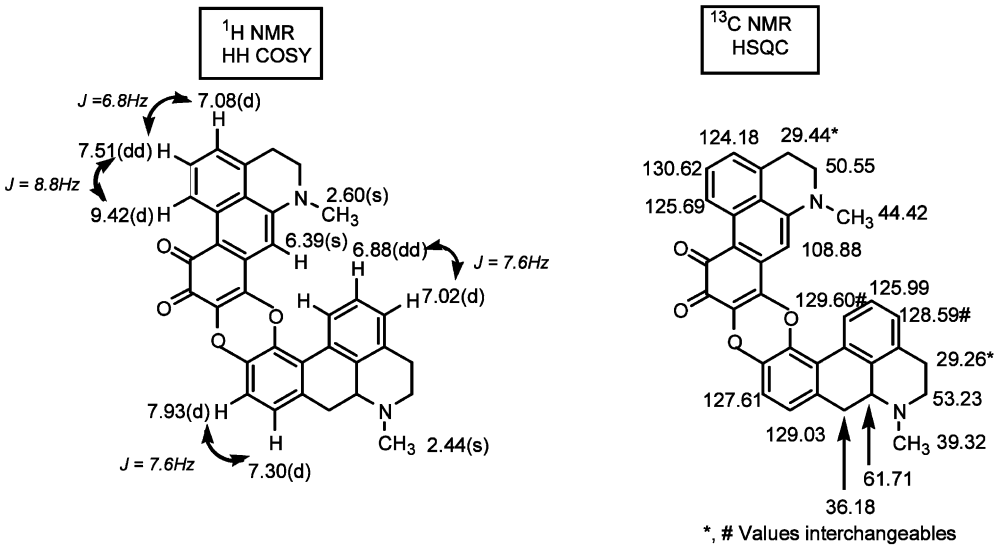

Fig. 2. COSY and HSQC data for compound **5**.

the autoxidation of 5,6-dihydroxytriptamine. In a second step the H_2O_2 oxidise compound **7** to *o*-quinone **2** generating hydroxyl radical (Fig. 3).

When the oxidation is complete with chromic acid, a single electron transfer leads to a semiquinone intermediate [**8**↔**9**] as it is known. The formation of oxygen radical in position 11 is more favourable than in position 10 due to its bigger stabilisation by resonance. A second electron transfer gives compound **7** which is oxidised to **2**. If the oxidant agent is added slowly, the intermediate [**8**↔**9**] could first 'dimerizes' to give a new intermediate **10** which suffers an oxidative cyclisation to give **11**, forerunner of **5** (Fig. 4).

In basic medium we believe that quinone **7** is formed firstly, in a similar way as we have described for autoxidation procedure, the H_2O_2 also formed is ionised in the medium and the hydroperoxide anion, as good

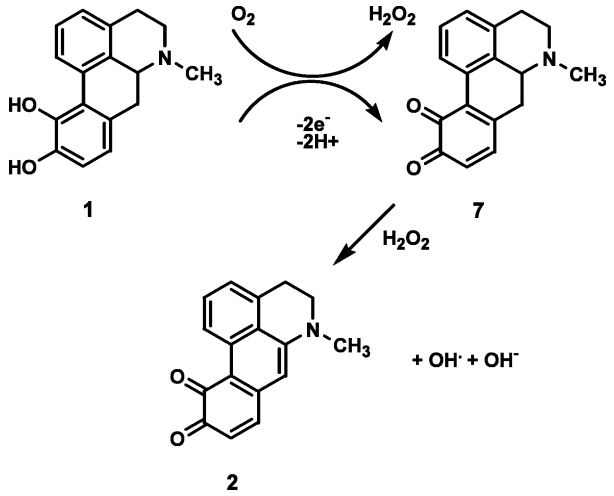

Fig. 3. Mechanism for formation of o-quinone **2** by autoxidation of apomorphine **1**.

nucleophile, could attack position 8 as is summarised in Fig. 5. The intermediate **12** formed is stabilised loosing water to give **13**, its oxidation and neutralisation produce compound **3**.

Compound **4** is formed by oxidative degradation from compounds **2** or **7**.

3. Pharmacology

3.1. Results

3.1.1. Binding assays

The affinity of the oxidised derivatives **2**, **3** and **4** for dihydropiridine, benzothiazepine and α_1-adrenoceptor binding sites in rat cerebral cortical membranes was investigated using the selective ligands [^{3}H]-nitrendipine, [^{3}H]-diltiazem and [^{3}H]-prazosin, respectively.

The results obtained indicate that compound **4** weakly inhibited [^{3}H]-prazosin binding ($46.19 \pm 12.73\%$, $n = 6$) at the highest concentration tested (100 μM) and compounds **2** and **3** had no affinity for α_1-adrenoceptors (Table 1).

Binding of [^{3}H]-nitrendipine (0.3 nM) to their respective sites at the Ca^{2+} channels was not significantly modified by the presence of compounds **2**, **3** and **4** in concentrations up to 100 μM in the incubating medium ($n = 3$) (Table 1). Compounds **2** and **4**, at the highest concentration tested (100 μM), partially inhibited the binding of [^{3}H]-diltiazem ($31.3 \pm 10.4\%$, $n = 3$ and $49.12 \pm 11.10\%$, $n = 4$, respectively).

3.1.2. Functional studies

*3.1.2.1. Effect of compounds **2**, **3** and **4** on KCl- and NA-induced contractile response.* Addition of 1 μM NA

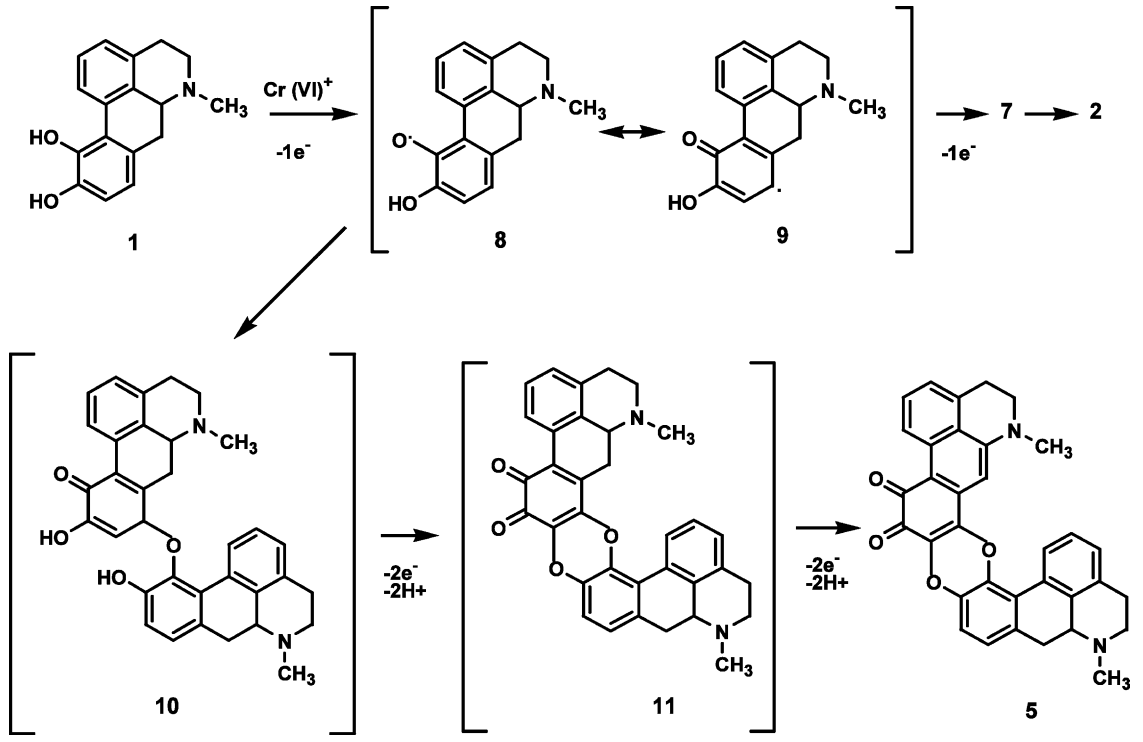

Fig. 4. Mechanisms for formation of compounds **2** and **5** by potassium.

(NA_1) in Krebs solution induced a sustained contractile response in intact rat aortic rings (371.45 ± 58.99 mg, $n = 6$). The presence of endothelium was confirmed by addition of 100 μM acetylcholine on the contractile plateau and observation of a relaxant response (88.49 ± 7.52%; $n = 6$). After washing, second addition of NA (NA_2) induced a new contractile response (453.60 ± 88.60 mg; $n = 4$) and each of three oxidised derivatives of apomorphine (compounds **2**, **3** and **4**) were added in cumulative doses on this contractile plateau. No relaxant response was observed in any case. However, compound **2** (0.1–100 μM) induced a concentration-dependent contractile response with a maximal response of 134.96 ± 26.15% ($n = 6$) relative to the previous NA-induced contraction (NA_1) (Fig. 6a). Compounds **3** and **4**, when added in the presence of NA, also promoted contractile responses ($n = 5$–6) but significantly smaller ($P < 0.001$) than those obtained with compound **2** (Fig. 6a). After washing the aortic strips, NA was added for a third time (NA_3) and a complete recovery of response

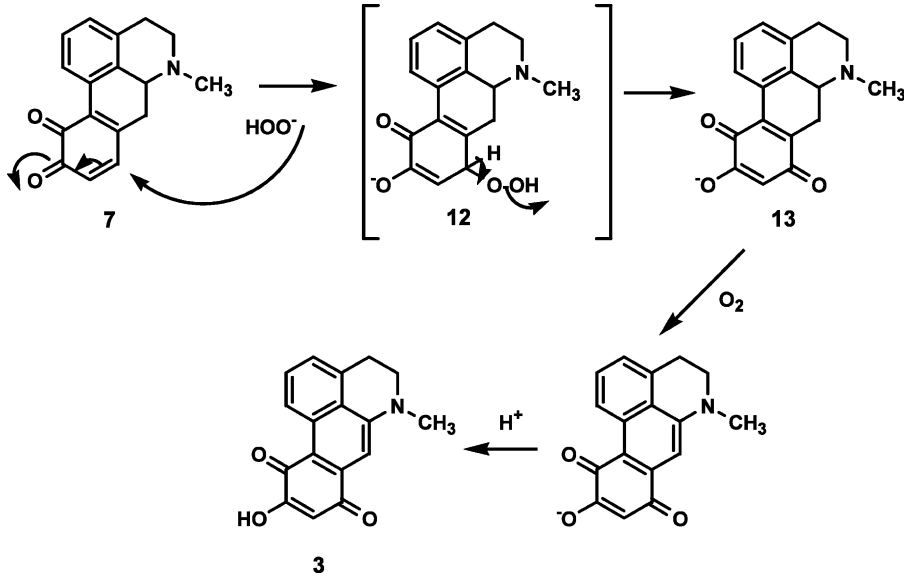

Fig. 5. Mechanism for formation of p-quinone **3** in basic medium.

Table 1
pK_i values of the tested agents on dihydropiridine; benzothiazepine and α_1-adrenoceptor receptor sites in cortical membranes

	Cerebral cortex		
	Dihidropyridine [^{3}H]-nitrendipine	Benzothiazepine [^{3}H]-diltiazem	α_1-Adrenoceptors [^{3}H]-prazosin
1	n.d.	[a] 4.58 ± 0.17	[a] 5.67 ± 0.05
2	n.d.	> 3.5	n.d.
3	n.d.	n.d.	n.d.
4	n.d.	> 4	> 4

$n = 3$ experiments; n.d. = not displacement at 100 μM.
[a] Data obtained from Ivorra et al. [1].

was obtained in all cases (181.02 ± 26.37%, $n = 6$; 109.67 ± 17.34%, $n = 5$ and 144.48 ± 10.43%, $n = 6$, respectively for each compound, and relative to NA_2).

Conversely, addition of compounds **2**, **3** and **4** (0.1–100 μM) on NA-induced contractile plateau (982.21 ± 125.06 mg; $n = 3$) in denuded aortic rings did not modify the contractile tone (Fig. 6b). The absence of endothelium was previously assessed by addition of acetylcholine to denuded rings and only a small relaxant activity was observed 9.97 ± 4.12% ($n = 6$).

In another set of experiments, addition of KCl (80 mM) to aortic rings induced a sustained contractile response either in the presence (637.54 ± 57.62 mg, $n = 4$) or in the absence of endothelium (607.18 ± 62.27 mg, $n = 3$). Subsequent addition of the three oxidised derivatives of apomorphine (compounds **2**, **3** and **4)** on the contractile plateau induced by depolarising solution did not modify it either in intact or denuded aortic rings.

*3.1.2.2. Effect of compounds **2**, **3**, **4** and **5** on basal tone of aortic rings.* NA 1 μM (NA_1) induced a maximal contractile response of 541.87 ± 107.93 mg ($n = 7$) in intact rings or 600.04 ± 72.57 mg ($n = 6$) in denuded rings which was used as a control of the contractile activity of the vessel. Acetylcholine 100 μM was added to assess the presence or absence of endothelium. After washing, addition of cumulative doses of compound **2** (1–100 μM) to the organ bath induced a concentration-dependent contractile response in intact rings (Fig. 6c) with a maximal contraction of 117.94 ± 9.82% ($n = 6$) relative to previous NA-induced response (NA_1). Addition of compounds **3** or **4** (1–100 μM) evoked contractile responses only at the highest concentration tested ($n = 6$–9) and they were significantly smaller ($P < 0.01$) than those obtained with compound **2**. Compound **5** was also tested at the same concentrations and no contractile response was observed with this product ($n = 4$) (Fig. 6c).

In denuded aortic rings, only 100 μM compound **2** induced a contractile response ($n = 6$) but it was

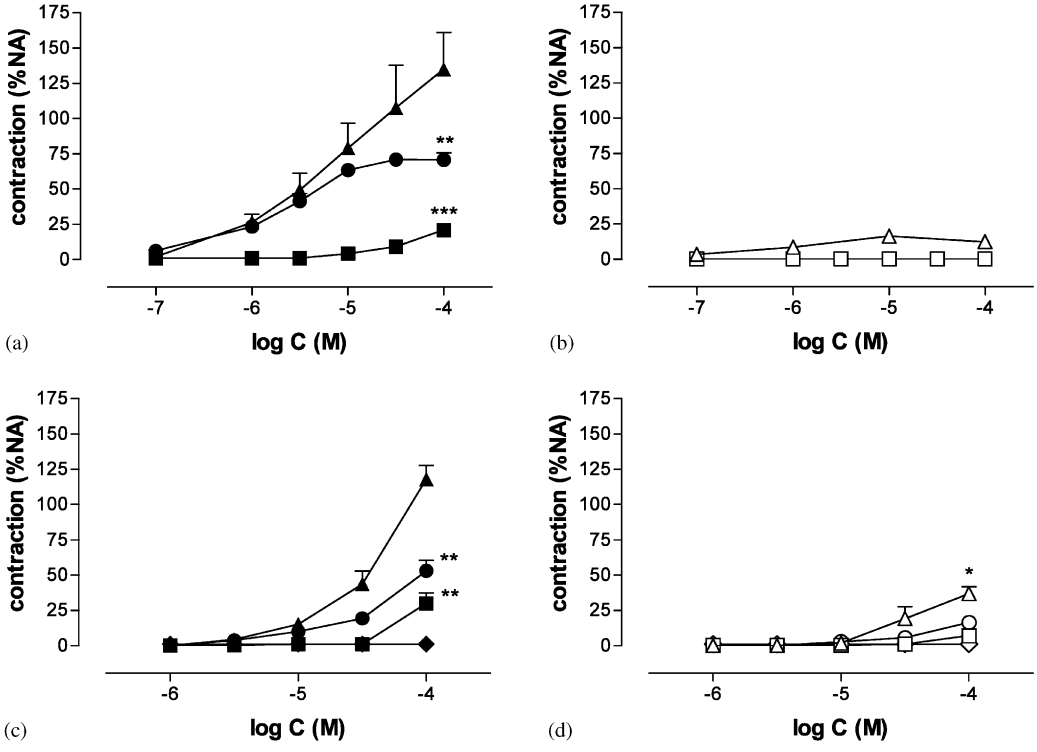

Fig. 6. Concentration-dependent contractile responses of compounds **2** (▲), **3** (■), **4** (●) or **5** (◆) in endothelium intact (solid symbols) or endothelium-denuded (open symbols) aortic rings. (a) and (b) contractile responses on NA-induced contraction. (c) and (d) contractile responses on basal tone in Krebs solution. Responses are expressed in percentage of NA-induced contractile response. Values are means ± S.E.M. of 3–9 experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ with respect compound **2**-induced contractile response in intact aortic rings.

significantly smaller than that obtained with endothelium ($P < 0.05$; Fig. 6d). Upon washing the preparation a complete recovery of NA-induced contractile response was observed (99.7 ± 9.4%, $n = 5$ relative to NA_1).

In order to know the dependence of the observed contractile response on the extracellular calcium entry, in another set of experiments the compounds which promoted a contractile response in the presence of Ca^{2+} (**2**, **3** and **4**) were added to intact aortic rings loaded in Ca^{2+}-free medium at the concentration which induced the maximal response in Krebs solution (100 μM). Only compound **2** induced a contractile response (17.77 ± 1.29%, $n = 4$ relative to NA-induced contraction in the presence of Ca^{2+}), but no response was observed in denuded aortic rings.

*3.1.2.3. Effect of compounds **2**, **3**, **4** and **5** on endothelium-dependent relaxant activity of acetylcholine.* In order to study the effect of these compounds on endothelium functionality, we tested the relaxant properties of acetylcholine after pre-treatment for 20 min with the highest concentration of each compound. In these experiments, NA-induced contractile response was similar to that obtained before pre-treatment, but addition of acetylcholine (100 μM) did not reproduce the relaxant activity observed in untreated rings. In fact, compound **2** abolished the relaxant response to acetylcholine ($n = 6$), compound **4** diminished it ($n = 7$) and compounds **3** and **5** did not modify the acetylcholine-induced relaxation ($n = 3$–5; Fig. 7).

The same results were observed when maximal concentration of each compound was added on NA-induced contraction instead of on the basal tone. Addition of NA after treatment with compounds reproduced the contractile response observed previously. However, acetylcholine 100 μM did not induce a relaxant activity in aortic rings treated with compound **2** ($n = 6$) and induced a decreased relaxant response after compound **4** treatment ($n = 6$). Pre-treatment with compound **3** did not modify the relaxant response to acetylcholine ($n = 5$; Fig. 7).

Moreover, as in experiments realised on NA-induced contraction, when compound **2** was added on KCl-induced contraction and washed, subsequent acetylcholine-induced relaxation of the contractile response elicited by posterior NA addition was also abolished ($n = 3$). Compound **4** partially decreased acetylcholine-induced relaxation and compound **3** did not modify it ($n = 4$; Fig. 7).

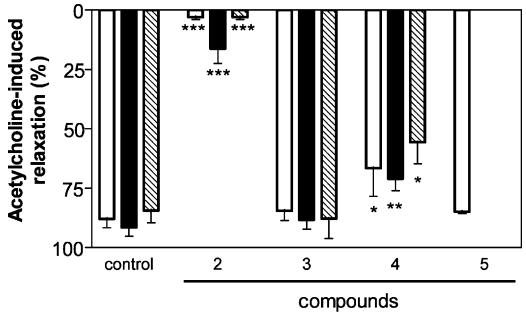

Fig. 7. Acetylcholine-induced relaxation of contractile responses elicited by NA in intact aortic rings before (control experiments) and after pre-treatment of aortic rings with compounds **2**, **3** or **4**. Compounds were added on basal tone (white bars), on NA-induced contraction (black bars) or on KCl-induced contraction (stripped bars). Responses are expressed as percentage relaxation evoked by acetylcholine on NA-induced contraction and represent mean ± S.E.M. of 3–6 experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ with respect to control experiments.

*3.1.2.4. Pharmacological analysis of the contractile response to compound **2**.* As contractile responses induced by compound **2** are strongly dependent on the presence of endothelium and we observed an endothelial dysfunction promoted by this compound, we analyse the intervention of NO in this response. In order to do so, the compound was added in the presence of 300 μM L-NAME and a decreased contractile response was obtained ($P < 0.05$; $n = 5$), although it was not abolished (Fig. 8).

In another group of experiments we try to analyse the intervention of oxygen free radicals in the contraction elicited by compound **2**. A concentration of 100 μM was added in Krebs solution in presence of 5 mM DMSO or 125, 250 and 500 U mL^{-1} SOD; all of them were added 15 min prior to the addition of compound **2**. A similar contractile response was obtained in presence of 5 mM DMSO ($n = 8$) and 125 U mL^{-1} SOD ($n = 3$), but significantly smaller contractile responses were induced by compound **2** in the presence of 250 or 500 U mL^{-1} SOD ($P < 0.05$; $n = 3$–5) (Fig. 8). At the end of the experiment, contractile response to NA was restored by the addition of NA 1 μM but the relaxant response to acetylcholine was not observed in any case ($n = 3$–8).

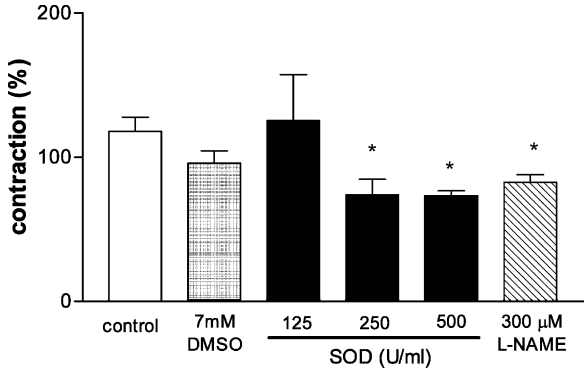

Fig. 8. Contractile responses induced by compound **2** (100 μM) in presence of 5 mM DMSO, 125, 250 or 500 U mL^{-1} SOD or 300 μM L-NAME. Responses are expressed in percentage of NA-induced contractile response. Values are the means ± S.E.M. of 3–8 experiments. *$P < 0.05$ with respect to control experiments.

3.1.3. Inhibition of NOS activity

We studied a possible direct action of compounds on NOS enzyme as a mechanism involved in the increment on basal tone and the impairment observed in the vasorelaxant response elicited by acetylcholine. Synthetase activity was estimated in the presence of three concentrations of the products. These experiments require large amounts of tissue, and in our experimental conditions NO was only produced by constitutive endothelial NOS. Therefore, we performed the experiments in stomach homogenate as biological substrate.

Ca^{2+}-dependent NOS activity was obtained in homogenates of gastric mucosa and this activity was inhibited in the presence of 300 μM L-NAME and 1 mM EDTA ($n = 4$; Fig. 9).

Compounds **2** and **3** had no inhibitory effect on the enzyme activity ($n = 3$–4), but compound **4** partially inhibited it at the highest concentrations tested ($P < 0.01$; $n = 4$) (Fig. 9).

3.2. Discussion

We previously reported that (R)-apomorphine showed dual contractile/relaxant properties in smooth muscle [1] and this complex behaviour depends on its autoxidation. Four oxidised derivatives of apomorphine were synthetised and pharmacologically tested but the results obtained differ from those observed with (R)-apomorphine. The main differences can be summarised as follows.

None of the oxidised compounds exhibit affinity as (R)-apomorphine for any of the α_1-adrenoceptor subtypes and benzothiazepine-site at the voltage-dependent calcium channel. According to these results, the two hydroxyl groups in position 9 and 10 in (R)-apomorphine, are essential to interact with the α_1-adrenoceptors or the calcium channel and the oxidation to *o*-quinone leads to a significant loss of affinity for these proteins.

This loss of affinity was accompanied by a loss in the relaxant activity of these compounds with respect to (R)-apomorphine in rat aorta. On the contrary, some of them (compound **2** and **4**), exhibit a contractile activity unrelated to α_1 adrenoceptor activation since it was observed when the maximal response to NA had been reached. Compound **2**, but not **4**, elicited a small contractile response in Ca^{2+}-free medium in intact rings. So, it is probable that compound **2** may provide a pathway for Ca^{2+} entry as well as producing a small Ca^{2+}-release from intracellular stores. However, the influx of extracellular Ca^{2+} appears to play a principle

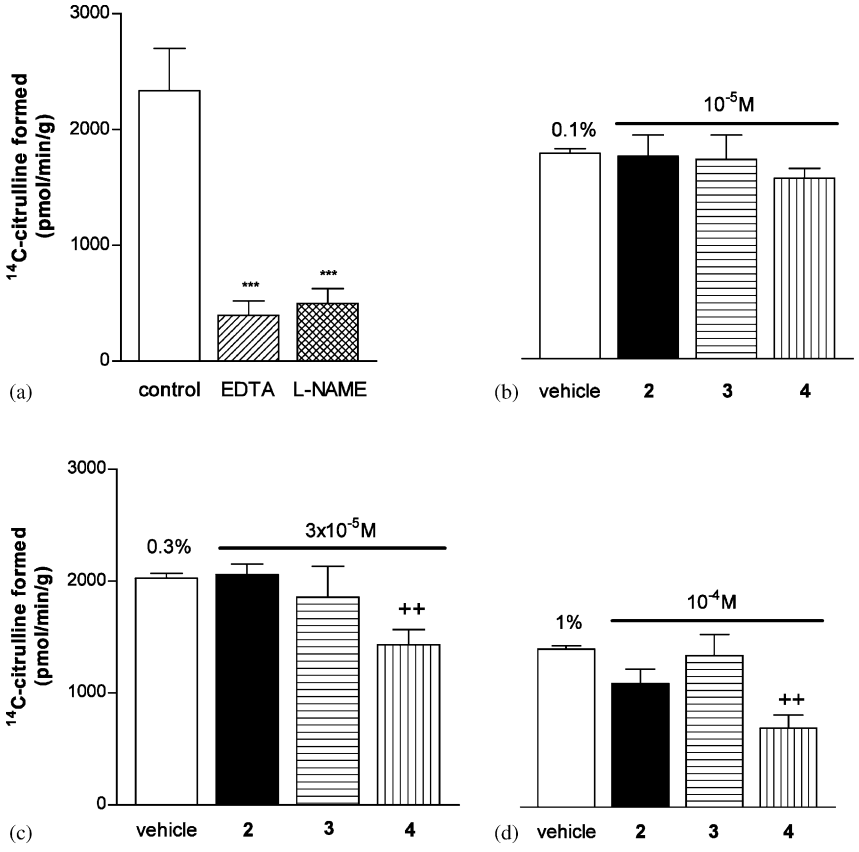

Fig. 9. Inhibition of NOS activity on rat stomach homogenate by 1 mM EDTA or 300 μM L-NAME (a) or by compounds **2**, **3** and **4** at concentration 10 μM (b); 30 μM (c) and 100 μM (d). Responses are expressed as picomol of ^{14}C-citrulline generated per minute per gram of tissue. Values are the means ± S.E.M. of 3–4 experiments. ***$P < 0.001$ with respect to control experiments. $^{++}P < 0.01$ with respect to vehicle.

role in the contractions since they almost disappear in Ca^{2+}-free medium. Moreover, the contractions could be related to K^+-channels since they were observed on basal tone and on NA-induced contraction but not on KCl-induced contractile response. We propose that a mechanism related to the blockage of K^+ channels leads to further depolarisation and the opening of voltage-dependent Ca^{2+} channels [9] permitting calcium entry and contraction.

The contractile activity of compounds **2** and **4** is endothelium-dependent since it almost disappears in denuded tissues. The endothelium contributes to the local regulation of vascular tone by releasing relaxing factors (NO as principal mediator in rat aorta [13]) and contracting factors such as endothelins, vasoconstrictor prostanoids and superoxide anions [10–13]. The fact that the contractile responses to quinones **2** and **4** were diminished or abolished in endothelium-denuded rings indicates that these compounds either removed an endothelium-derived vasodilator or released an endothelium-dependent vasoconstrictor.

In the case of compound **4**, the contractile activity could be related to the inhibition of the synthesis of NO through inhibition of endothelial NO synthetase, as present results show. The contractile activity of the compound **2** is more complex, and it is not related to the inhibition of NO synthetase, nor to a direct scavenging of the NO released by the endothelium, because it can be only slightly decreased in presence of L-NAME.

It has been reported that abnormalities of endothelial cells, like changes in their functionality, were manifested by a predominant formation of endothelium-derived contracting factors, being oxygen free radicals some of them [12–16]. Hence, these radicals play a role in endothelium-dependent contractions in hypertension and diabetes, both with endothelial dysfunction [12,16]. In our case, the contraction induced by compound **2** was not affected by a scavenger of hydroxyl anions such as DMSO and only slightly inhibited by a scavenger of superoxide anions such as SOD therefore these free radicals are not primarily responsible for this response.

Afterwards, we also tested the effect of these oxidised compounds on endothelium functionality by examining the relaxant properties of acetylcholine after treatment with the products. The results show that compound **2** abolished the relaxant activity of acetylcholine and, in this way, dramatically disrupts endothelial functionality. This action is observed in different experimental procedures and does not depend on contraction appearing, although compound **2** did not induce any contractile response on KCl-depolarised aorta, it also abolished the relaxant action of acetylcholine. Taken together, these results could relate contractile activity of compound **2** with damage of endothelial cells or disruption in its functionality which subsequently leads to an increment on basal tone by releasing a contracting factor other that superoxide anion, or by inhibiting the release of a vasodilator factor other than NO. It is interesting to note that compound **5**, that includes in its structure a tetracyclic moiety common to compound **2**, does not exhibit contractile activity indicating that, a steric hindrance or a change in the electronic distribution of the *o*-quinone moiety due to the addition of the aporphinic portion, could be responsible for this different activity.

In summary, all our findings indicate that simple oxidised *o*-quinones, **2** and **4**, show contractile activity in intact rat aortic rings instead of the relaxant activity exhibited by apomorphine, so oxidation of alkaloid dramatically changes the pharmacological properties at the vascular level. Each compound has particular mechanisms of action, being compound **2** the product which shows enhanced contractile and a more complex mechanism of action, related to activation of Ca^{2+} channels through release and/or inhibition of endothelial factors. The fact that auto-oxidation of (R)-apomorphine is the main metabolic route of this therapeutical agent [4], and the metabolite resulting from it was the compound **2**, adds new value to the present findings, specially when we consider that, as present results show, compound **2** alters endothelial functionality avoiding the relaxant activity of acetylcholine in vessels.

4. Experimental protocols

4.1. Chemistry

Melting points were determined on a Kofler heated stage and are uncorrected. NMR spectra were recorded on a Bruker AC250 MHz in $CDCl_3$ as solvent. HRMS (EI) determinations were made using a VG Autospec Trio 1000 (Fisons). Infrared spectra were recorded in KBr discs on a Bio-Rad FTS-7. Ultraviolet spectra were recorded on a Shimazdu UV-2101. HPLC analyses were carried out with a chromatographic system consisting of a Model M515 pump, connected to a type Rheodyne 7725i injector, differential detector model M410/PWL from Waters.

4.1.1. 6-Methyl-5,6-dihydro-4H-dibenzo[de,g]quinoline-10,11-diona 2

4.1.1.1. Procedure A. A solution of Apomorphine HCl·1/2H_2O **1** (307 mg) in water (50 mL) and acetic acid (5 mL) was cooled in an ice bath and was stirred for 2 h. Then an aqueous solution of potassium dichlorocromate 0.1 N (40 mL) was added. A green colour developed. Extraction with chloroform gave a deep blue solution that was washed with sodium bicarbonate, dried and

evaporated, giving a residue which was purified by silica chromatography. Elution with chloroform gave compound **2** as a blue solid (105 mg, 41% yield). Mp 254–258 ($CHCl_3$/hexane). HRMS found for M^+ 263.0946. $C_{17}H_{13}NO_2$ requires 263.0946. λ_{max} (nm) (log ε) 594.0 (3.743), 404.0 (3.721), 334.0 (4.186), 234.0 (4.177). ν_{max} (KBr) (cm^{-1}) 1669(C=O). ^{1}H-NMR δ 9.44 (dd, $J_1 = 8.77$, $J_2 = 1.00$ 1H), 7.54 (dd, $J_1 = 8.77$, $J_2 = 6.95$, 1H), 7.20 (d, $J = 9.87$, 1H), 7.10 (dd, $J_1 = 6.95$, $J_2 = 1.00$, 1H), 6.34 (d, $J = 9.87$, 1H), 6.27 (s, 1H), 3.63 (t, $J = 6.57$, 2H), 3.26 (s, 3H), 3.19 (t, $J = 6.57$, 2H). ^{13}C-NMR δ 177.97 (C), 171.26 (C), 147.01 (C), 141.49 (CH), 133.88 (C), 129.75 (C), 126.84 (C), 125.89 (CH), 123.07 (CH), 120.23 (CH), 118.36 (CH), 108.36 (C), 101.67 (CH), 45.20 (CH_2), 35.03 (CH_3), 23.67 (CH_2). MS (%), 265 (100) $(M+2)^+$, 264 (27) $(M+1)^+$, 263 (15) $(M)^+$, 236 (15), 235 (42), 234 (11).

4.1.1.2. Procedure B. A solution of Apomorphine HCl·1/2H_2O **1** (100 mg) in a KBS (150 mL), was stirred and gassed with 95% O_2 and 5% CO_2 during 24 h at room temperature and a green colour developed. Then it was extracted, washed and dried as in procedure A giving a residue which was purified by silica chromatography. Elution with ethyl acetate/hexane gave blue compound **2** (11 mg, 13% yield).

The composition of Krebs solution was as follows (mM): NaCl 118, KCl 4.75, $CaCl_2$ 1.8, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 11.

4.1.2. 6-Methyl-10-hydroxy-5,6-dihydro-4H-dibenzo[de,g]quinoline-8,11-diona 3 and 6-Methyl-5,6-dihydro-4H-benzo[de]quinoline-8,9-diona 4

Apomorphine HCl·1/2H_2O **1** (1.1 g) was added to a 10% aqueous solution of NaOH (55 mL), was stirred and air bubbled for 24 h. Gas was evolved and the solution became green. Extraction with chloroform gave a brown solution that was washed with water, dried and evaporated, giving a residue which was purified by silica chromatography. Elution with chloroform/methanol (1:1) gave compound **4** as a orange solid (68 mg, 9% yield). Mp 213–214 ($CHCl_3$/hexane). HRMS found for M^+ 213.0879. $C_{13}H_{11}NO_2$ requires 213.0789. λ_{max} (nm) (log ε) 466.0 (3.092), 354.5 (2.957), 282.5 (3.606), 240.5 (3.624). ν_{max} (KBr) (cm^{-1}) 1677 (C=O). ^{1}H-NMR δ 7.99 (d, $J = 6.92$, 1H), 7.46 (dd, $J_1 = 6.92$, $J_2 = 7.30$, 1H), 7.41 (d, $J = 7.30$, 1H), 5.80 (s, 1H), 3.70 (t, $J = 6.57$, 2H), 3.23 (s, 3H), 3.10 (t, $J = 6.57$, 2H). ^{13}C-NMR δ 155.32 (C), 133.79 (CH), 131.12 (CH), 127.36 (CH), 100.19 (CH), 50.23 (CH_2), 40.87 (CH_3), 27.25 (CH_2). MS (%), 215 (21) $(M+2)^+$, 214 (9) $(M+1)^+$, 213 (10) $(M)^+$, 186 (21), 185 (100), 184 (29), 156 (27), 69 (13). Aqueous phase was acidified with 10% HCl, a deep blue colour was developed. Extraction with chloroform, washed with water, dried and evaporated gave compound **3** as a blue solid, almost pure (570 mg, 58% yield). Mp 233–237 ($CHCl_3$/hexane). HRMS found for M^+ 279.0896. $C_{17}H_{13}NO_3$ requires 279.0895. λ_{max} (nm) (log ε) 562.0 (2.863), 310.50 (3.401), 251.0 (3.628). ν_{max} (KBr) (cm^{-1}) 3258 (OH), 1642 (C=O). ^{1}H-NMR δ 9.38 (d, $J = 8.77$, 1H), 7.54 (dd, $J_1 = 8.77$, $J_2 = 6.93$, 1H), 7.21 (s, 1H), 7.19 (s, 1H), 7.13 (d, $J = 6.93$, 1H), 6.07 (s, 1H), 3.57 (t, $J = 6.22$, 2H), 3.24 (s, 3H), 3.16 (t, $J = 6.22$, 2H). ^{13}C-NMR δ 180.01 (CO), 169.98 (CO), 158.42 (C), 151.89 (C), 137.10 (C), 132.06 (C), 130.65 (CH), 125.36 (CH), 124.17 (CH), 121.53 (C), 111.01 (C), 105.54 (CH), 102.29 (CH), 50.29 (CH_2), 40.26 (CH_3), 29.02 (CH_2). MS (%), 279 (100) $(M)^+$, 251 (88), 250 (20).

4.1.3. 1,13-Dimethyl-2,3,7,8,12,12a,14,15-octahydro-1H-benzo[de]-1″H-benzo[d′e′] benzo[g]quinoline-[6″,7″:5′,6′][1,4]dioxino[2′,3′:3,4]benzo[g]quinoline-7,8-diona 5

A solution of Apomorphine HCl·1/2H_2O **1** (300 mg) in water (50 mL) and acetic acid (5 mL) was cooled in an ice bath and was stirred for 2 h. Then an aqueous solution of potassium dicromate 0.1 N (40 mL) was added very slowly (ca. in 2 h). A green colour developed. Extraction with dichloromethane gave a violet solution that was washed with sodium bicarbonate, dried and evaporated, giving a residue which was purified by silica chromatography. Elution with ethyl acetate/methanol (8:2) gave compound **5** as a violet solid (70 mg, 23% yield). Mp 242–244 (MeOH/ethyl acetate, 2:8). λ_{max} (nm) (log ε) 551.0 (2.78), 370.0 (3.02), 310.00 (3.25), 234.50 (3.60), 206.0 (3.57). ν_{max} (KBr) (cm^{-1}) 1635(C=O). ^{1}H-NMR 400 MHz, δ 9.42 (dd, $J = 8.8$, 1H), 7.93 (d, $J = 7.6$, 1H), 7.51 (dd, $J_1 = 8.8$, $J_2 = 6.8$, 1H), 7.30 (d, $J = 7.6$, 1H), 7.08 (d, $J = 6.8$, 1H), 7.02 (d, $J = 7.6$, 2H), 6.88 (dd, $J_1 = J_2 = 7.6$, 1H), 6.39 (s, 1H), 3.46–3.32 (m, 3H), 3.28–3.00 (m, 7H), 2.66–2.58 (m, 1H), 2.60 (s, 3H), 2.47 (s, 3H). ^{13}C-NMR δ 188.06 (C), 180.90 (C), 150.54 (C), 145.55 (C), 142.59 (C), 137.16 (C), 135.22 (C), 134.99 (C), 133.99 (C), 133.58 (C), 133.44 (C), 132.22 (C), 131.09 (C), 130.62 (CH), 129.60 (CH), 129.03 (CH), 128.59 (CH), 127.61 (CH), 125.99 (CH), 125.69 (CH), 124.18 (CH), 120.65 (C), 113.71 (C), 108.88 (CH), 61.71 (CH), 53.23 (CH_2), 50.55 (CH_2), 44.42 (CH_3), 39.32 (CH_3), 36.18 (CH_2), 29.44 (CH_2), 29.26 (CH_2). MS (%), 526 (2), 470 (20), 454 (100), 453 (85), 440 (40).

4.1.4. 6-Methyl-5,6-dihydro-4H-benzo[de]quinolin-8,9-diona 4 from 6-methyl-5,6-dihydro-4H-dibenzo[de,g]quinolin-10,11-diona 2

A solution of compound **2** (5 mg) in dioxane (10 mL)/water (10 mL) and glacial acetic acid (3 mL) was cooled in an ice bath and was stirred for 2 h. Then an aqueous solution of potassium dichlorocromate 0.4 N (30 mL) was added with stirring, was left to rise to room temperature overnight. Neutralised with sodium bicarbonate, extracted with chloroform, dried, evaporated,

and purified by silica chromatography, giving compound **4** almost in quantitative yield.

4.2. Pharmacology

4.2.1. Preparation of rat cortical membranes

Membranes were prepared from cerebral cortex of female Wistar rats (200–220 g). The tissue was homogenised in 10 vol.(w/v) of ice-cold buffer (Tris HCl 5 mM, sucrose 250 mM and EDTA 1 mM, pH 7.5 at 25 °C) using an Ultra-Turrax (Janke & Kunkel, IKA, Germany; twice, 15s). The homogenate was centrifuged for 10 min at $1000 \times g$. The pellet was discarded and the supernatant was centrifuged at $50\,000 \times g$ for 15 min at 4 °C. The pellet was resuspended in the same volume of assay buffer (Tris HCl 50 mM, pH 7.5) and centrifuged at $50\,000 \times g$ for 15 min at 4 °C. The final pellet was resuspended in assay buffer and stored at −70 °C for later use. All membrane-preparation procedures were carried out at 4 °C.

4.2.2. Radioligand binding to native receptors from rat cerebral cortex

Binding of [^{3}H]-prazosin, [^{3}H]-nitrendipine or [^{3}H]-(+)-*cis*-diltiazem was measured using a total volume of 1 mL including diluted membranes (250 μg protein/tube) in 50 mM Tris HCl (pH 7.5). Competition curves were determined in triplicate, with a final [^{3}H]-prazosin, [^{3}H]-nitrendipine or [^{3}H]-(+)-*cis*-diltiazem concentration of 0.2, 0.3 or 3 nM, respectively and a range of concentrations for each compound from 1 nM to 100 μM. Incubations were carried out at 25 °C for 30 min ([^{3}H]-prazosin), 90 min ([^{3}H]-nitrendipine) or 120 min ([^{3}H]-(+)-*cis*-diltiazem), after which the entire reaction mixture was filtered rapidly over fiberglass filters (Schleicher and Schuell, No. 30) by using a Brandel harvester. Filters were pre-treated with polyethyleneimine 0.3%. Non-specific binding was determined in the presence of phentolamine (1 μM), nifedipine (1 μM) or diltiazem (10 μM), respectively. Curves were fit and pK_i calculated by using non iterative regression analysis (GraphPad, San Diego, CA).

4.2.3. Functional study

Wistar rats of both sexes, weighing 200–220 g were decapitated and the thoracic aorta isolated. The connective tissue was removed and the vessels were cut into rings of about 5 mm in length which were suspended in a 10 mL organ bath containing KBS, maintained at 37 °C and gassed with 95% O_2 and 5% CO_2. An initial load of 1 g was applied and maintained throughout a 75–90 min equilibration period. Tension was recorded isometrically on a polygraph (Grass M7) via force-displacement transducers (Grass FT03). KBS had the following composition (mM): NaCl 118, KCl 4.75, $CaCl_2$ 1.8, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11. In Ca^{2+}-free solution $CaCl_2$ was omitted and EDTA (0.1 mM) was added. Depolarising solution, 80 mM, was prepared by equimolar substitution of KCl by NaCl in Krebs solution.

Endothelium-denuded aortic rings were prepared by rubbing the entire intimal surface. To test the presence of vascular endothelium, acetylcholine (100 μM) was added to preparations precontracted with NA (1 μM) [17]. Acetylcholine-induced relaxation was expressed as a percentage of the maximum increment in tension obtained by NA addition. Segments with relaxant responses greater than 80% of previous contraction were considered to have an intact endothelium and those which showed relaxant responses lower than 20% were considered as endothelium-denuded preparations.

Concentration-response curves of contraction or relaxation to oxidised derivatives of apomorphine were obtained by addition of cumulative concentrations of the compounds to vascular rings precontracted or not by NA 1 μM or KCl 80 mM. The results are presented as the mean ± S.E.M. for n determinations obtained from different animals.

4.2.4. Determination of NOS activity

Female Wistar rats (200–220 g) were decapitated. Stomachs were rapidly removed, opened along the greater curvature and placed in a preparative ice-cold buffer (330 mg mL^{-1}; pH 7.4) containing 20 mM HEPES, 320 mM sucrose, 1 mM DL-dithiothreitol, 1 mM EDTA, 10 μg mL^{-1} soybean trypsin inhibitor, 10 μg mL^{-1} leupeptin, 2 μg mL^{-1} aprotinin. Samples were homogenised (Ultra-Turrax), sonicated and centrifuged at $10\,000 \times g$ for 20 min at 4 °C. NO synthetase activity was measured as the rate of conversion of L-[U-^{14}C]-arginine to L-[U-^{14}C]-citrulline [18]. Afterwards centrifugation, 40 μl of supernatant was incubated at 37 °C for 20 min in assay buffer (pH 7.4) containing (mM) K_2HPO_4 50, $CaCl_2$ 0.2, $MgCl_2$ 1, DL-dithiothreitol 1, L-citrulline 1, L-arginine 0.02, L-valine 50 and 100 μM NADPH, 3 μM FAD, 3 μM FMN, 3 μM BH_4, 950 nM L-[U-^{14}C]-arginine (348 mCi mmol^{-1}). The specificity of L-arginine conversion by NOS to L-citrulline was further confirmed using the NO synthesis inhibitor, N^G-nitro-L-arginine methyl esther (300 μM L-NAME). Additionally, 1 mM EDTA, a calcium chelating agent was used to differentiate between Ca^{2+}-dependent and Ca^{2+}-independent isoform of NOS. All activities are expressed as picomol of product generated per minute per gram of tissue. Assays were carried out in triplicate.

4.2.5. Drugs and solutions

The following drugs were used: L-[U-^{14}C]-arginine monohydrochloride (296 mCi mmol^{-1}), [^{3}H]-nitrendipine (70–87 Ci mmol^{-1}), [^{3}H]-prazosin (72–78 Ci mmol^{-1}) (Amersham International, Buckinghamshire, UK); [^{3}H]-HEAT (2200 Ci mmol^{-1}), [^{3}H]-*cis*-diltiazem

(80–85 Ci $mmol^{-1}$), (DuPont-New England Nuclear, Boston, MA); acetylcholine chloride, diltiazem hydrochloride, nifedipine, (−)-noradrenaline bitartrate, superoxide dismutase (SOD) -from bovine liver-, Nω-nitro-L-arginine methyl ester (L-NAME) and all reagents used for determination of NOS activity (Sigma, St. Louis MO); phentolamine mesylate, (R)-apomorphine (RBI Natick MA); dimethyl sulfoxide synthesis grade (Scharlau Chemie S.A., Barcelona, Spain). Other reagents were of analytical grade.

Compound **1** was dissolved in ascorbic acid (0.001%). Compounds **2**, **3**, **4** and **5** were dissolved in ethanol (10^{-2} M) and diluted in deionised water. The other drugs were dissolved in deionised water. All solutions were prepared daily and pH was adjusted to 7.

4.2.6. Data analysis

Where ANOVA showed significant differences ($P < 0.05$) the results were further analysed using the Student–Newman Keuls test and differences were considered significant when $P < 0.05$.

Acknowledgements

Our thanks to Secretaría de Estado de Política Científica y Tecnológica del Ministerio de Ciencia y Tecnología (Proyect PB98-1422) and Generalitat Valenciana (Proyects GV-3215/95 and GV2001-292) for its financial support.

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