Inhibition of neuronal nitric oxide synthase by 6-nitrocatecholamines, putative reaction products of nitric oxide with catecholamines under oxidative stress conditions

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6-Nitrodopamine and 6-nitronoradrenaline (6-nitronorepinephrine), putative products of the nitric oxide (NO)-dependent nitration of dopamine and noradrenaline, are reported to be reversible, competitive inhibitors of neuronal nitric oxide synthase (nNOS) with K_i values of 45 and 52 μM respectively. The nitrocatecholamines inhibited H_2O_2 production in the absence of L-arginine and tetrahydrobiopterin (BH_4) (the IC_{50} values for 6-nitrodopamine and 6-nitronoradrenaline were 85 and 55 μM respectively) but without affecting cytochrome *c* reduction. The apparent K_i values for nitrocatecholamine inhibition of enzyme activation by BH_4 were 18 μM for 6-nitrodopamine and 40 μM for 6-nitronoradrenaline. Both nitrocatecholamines antagonized the dimerization of nNOS induced by BH_4 and by L-arginine, the effect being reversed by BH_4 (more than 10 μM) and L-arginine (e.g. 100 μM). Overall, these results suggest that nitrocatecholamines interfere with nNOS activity by binding to the enzyme in the proximity of the substrate and BH_4-binding sites near the haem group.

Key words: dimerization, haem, tetrahydrobiopterin.

INTRODUCTION

The origin and possible functional significance of 6-nitrocatecholamines, including 6-nitrodopamine and its congener from noradrenaline (norepinephrine), 6-nitronoradrenaline (Figure 1), have attracted increasing interest since the initial identification of 6-nitronoradrenaline in mammalian brain [1] in levels proportional to the activity of nitric oxide synthase (NOS), the enzyme assigned the synthesis of the physiological mediator nitric oxide (NO) [2]. The same metabolite has recently also been detected in the rat spinal cord, suggesting a wide distribution throughout the nervous system [3].

Chemical studies under biomimetic conditions have indicated that 6-nitrocatecholamines can arise by different interrelated NO-dependent processes that share an absolute requirement for an oxidative step [4,5]. Formation of these metabolites would thus entail the existence of hitherto unrecognized interactions between the catecholaminergic and nitrergic signalling pathways that are sustained by an acute or chronic oxidative stress state.

A number of possible biological properties have been ascribed to 6-nitrocatecholamines, including modulatory effects on catecholaminergic neurotransmission derived from a lack of activity on catecholamine receptors [6], the inhibition of catechol-*O*-methyl transferase activity, the transport of noradrenaline into rat synaptosomes and catecholamine reuptake [1], a moderate neurotoxicity after stereotaxic injection into the substantia nigra of laboratory rats [5] and an anti-nociceptive activity [3].

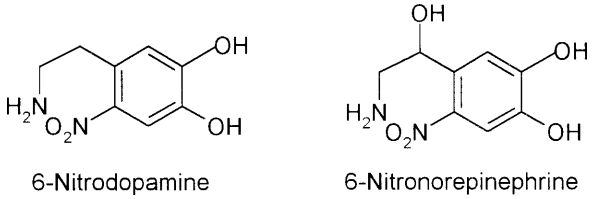

Figure 1 Structures of 6-nitrodopamine and 6-nitronoradrenaline

Little attention has so far been paid to the possible involvement of 6-nitrocatecholamines in NO metabolism and signalling pathways under oxidative stress conditions. As an initial approach to this issue we have now investigated the effects of 6-nitrodopamine and 6-nitronoradrenaline on the activity of the endothelial (eNOS), inducible (iNOS) and neuronal (nNOS) isoforms of NOS. The NOS isoforms use L-arginine as their substrate to form L-citrulline and NO. Whereas nNOS and eNOS are Ca^{2+}/calmodulin-dependent, iNOS is apparently independent of Ca^{2+} [7]. For all isoforms a homodimeric quaternary structure is required for catalytic activity and each subunit displays both a reductase domain and an oxygenase domain. The reductase domain contains binding sites for the cofactors NADPH, FAD and FMN, whereas in the oxygenase domain a protoporphyrin IX haem group forms the catalytic centre together with the binding sites for L-arginine and tetrahydrobiopterin (BH_4), as an additional cofactor. The latter is crucial for enzyme functioning because it exerts multiple allosteric effects, facilitates coupled electron transfer between the reductase and oxygenase domains, shifts the haem iron to its high-spin state, induces enzyme dimerization and stabilizes the enzyme during catalysis [8]. Here we report that 6-nitrodopamine and 6-nitronoradrenaline are novel inhibitors of nNOS, antagonizing BH_4-dependent enzyme activation and dimerization.

EXPERIMENTAL

Materials

Dopamine and noradrenaline were from Sigma (Italy). 6-Nitrodopamine and 6-nitronoradrenaline were prepared as reported

Abbreviations used: BH_4, tetrahydrobiopterin; eNOS, endothelial NOS; iNOS, inducible NOS; nNOS, neuronal NOS; NOS, nitric oxide synthase.

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previously [4]. Radiolabelled L-[U-^{14}C]arginine monohydrochloride (317 mCi/mmol) was obtained from Amersham Italia (Milan, Italy). Recombinant rat nNOS (purity at least 98 % by SDS/PAGE, 0.7 μmol of L-citrulline/min per mg) from baculovirus-infected Sf9 cells [9] was from Alexis Italia (Vinci, Italy). Recombinant mouse iNOS (100000 ***g*** supernatant, 22.5 units/mg) expressed in *Escherichia coli* was from Alexis Italia. Recombinant bovine eNOS (100000 ***g*** supernatant, 0.8 unit/mg) isolated from a baculovirus overexpression system in Sf9 cells [10] was from Cayman (Ann Arbor, MI, U.S.A.).

Determination of NOS activity

NOS activity was determined by monitoring the conversion of L-[U-^{14}C]arginine into L-[U-^{14}C]citrulline. For nNOS, aliquots (2 μl, 0.18 μg of protein) of commercial recombinant enzyme were incubated for 15 min at 37 °C in 0.1 ml of 20 mM Tris/HCl, pH 7, containing 0.5 mM NADPH, 250 μM $CaCl_2$, 1 μM BH_4, 10 μg/ml calmodulin, 5 μM FAD, 5 μM FMN and 1.57 μM L-[U-^{14}C]arginine. In all cases, each reaction was stopped by the addition of 0.9 ml of ice-cold 100 mM Hepes buffer, pH 5.5, containing 4 mM EDTA and passed through a 1 ml Dowex 50 (Na^+ form) column, which retains L-arginine. The L-[^{14}C]citrulline generated was eluted with water and quantified by liquid-scintillation counting.

For isoform selectivity experiments, comparable activities of NOS were used and the enzymes were assayed under the same experimental conditions.

Reversibility of NOS inhibition was tested as described by Garvey et al. [11]. In brief, nNOS was preincubated at 4 °C for 10 min in the presence of cofactors and with or without 500 μM inhibitor. The reaction was then started by the addition of L-arginine; portions of the mixture were removed at intervals and assayed for NOS activity.

The formation of H_2O_2 by nNOS was determined by the ferrous thiocyanate assay [12]. In brief, commercial recombinant enzyme (1.35 μg) was incubated at 37 °C for 10 min in 0.45 ml of 50 mM triethanolamine/HCl buffer, pH 7, containing 0.5 mM NADPH, 3 μM $CaCl_2$ and 3 μg of calmodulin. Reactions were terminated by the addition of 225 μl of conc. HCl; aqueous solutions of ferrous ammonium sulphate (30 μl) and potassium thiocyanate (45 μl) were added to give final concentrations of 3.2 and 180 mM respectively. Samples were incubated for 10 min at room temperature, followed by the immediate determination of A_{492}. Blank values were determined in the absence of the enzyme but with all cofactors present. Observed changes in A_{492} in the blank during readings did not exceed 15 % of the initial value and were duly considered in H_2O_2 determinations.

The NADPH-dependent reduction of cytochrome *c* in the absence of Ca^{2+} and calmodulin was determined spectrophotometrically as described previously [13].

HPLC analysis

6-Nitrocatecholamines were analysed by HPLC on a Gilson model 302 instrument, with a Sphereclone ODS2 column (5 μm pore size; 250 mm × 4.60 mm) (Phenomenex Chemtek Analytica, Bologna, Italy). The mobile phase was 50 mM sodium phosphate buffer, pH 3, containing 10 mM octanesulphonic acid/acetonitrile (82:18, v/v). The flow rate was maintained at 1 ml/min. Detection was conducted at 280 nm with a Gilson model 116 UV detector.

BH_4 was determined by an HPLC method as described by Klatt et al. [14].

PAGE

The formation of stable nNOS dimers was analysed by low-temperature SDS/PAGE as described previously [15]. Recombinant nNOS was incubated for 10 min at 37 °C in 20 μl of 50 mM triethanolamine/HCl buffer, pH 7, in the absence and presence of additives as indicated. Incubations were terminated by the addition of chilled Laemmli buffer [16] containing 0.125 M Tris/HCl (pH 6.8), 4 % (w/v) SDS, 10 % (v/v) 2-mercaptoethanol, 20 % (w/v) glycerol and 0.02 % Bromophenol Blue. Samples containing 2.25 μg of proteins were subjected to SDS/PAGE for 110 min at 150 V on discontinuous SDS/6 % polyacrylamide slab gels. Gels and buffers, prepared as described by Laemmli [16], were equilibrated at 4 °C and the buffer tank was cooled in an ice bath during electrophoresis. Gels were stained for protein detection with silver by using the silver stain plus kit from Bio-Rad. Densitometric analysis of the gels was performed by Bio-Rad FLUOR-S™ Multi Imager apparatus with the software Quantity One, version 1.11.

Data analysis

All values were determined in triplicate and means ± S.E.M. for four separate experiments were calculated. Statistical analysis of data was performed with the software GraphPad Prism, version 2.00.

RESULTS

Table 1 shows the effects of 6-nitrodopamine and 6-nitronoradrenaline on the activity of recombinant nNOS in comparison with dopamine and noradrenaline, as determined by the L-[^{14}C]arginine conversion radioassay. The results indicate that

Table 1 Effect of the nitrocatecholamines 6-nitrodopamine and 6-nitronoradrenaline, and their parent catecholamines, on the activity of recombinant nNOS

NOS activity was measured as the production of L-[^{14}C]citrulline from L-[^{14}C]arginine as described in the Experimental section. All substances tested were used at 100 μM. Results are means ± S.E.M. for four separate experiments. Statistical significance: $^{**}P < 0.01$.

Compound	Inhibition of nNOS (%)
6-Nitrodopamine	73 ± 4
Dopamine	5 ± 3
Noradrenaline	45 ± 5**
6-Nitronoradrenaline	71 ± 3**

Table 2 Inhibition of NOS isoforms by 6-nitrodopamine and 6-nitronoradrenaline

NOS activity of the different isoforms was measured as the production of L-[^{14}C]citrulline from L-[^{14}C]arginine as described in the Experimental section. 6-Nitrodopamine and 6-nitronoradrenaline were tested at 500 μM. Results are means ± S.E.M. for four separate experiments. Statistical significance of inhibition by 6-nitrodopamine: $^{***}P < 0.001$ compared with eNOS; $^{**}P < 0.01$ compared with nNOS; $^{*}P < 0.05$ compared with iNOS [analysis of variance (ANOVA), Bonferroni's post-hoc test]. Statistical significance of inhibition by 6-nitronoradrenaline: $^{***}P < 0.001$ compared with eNOS; †$P < 0.001$ compared with iNOS; $^{**}P < 0.01$ compared with iNOS (ANOVA, Bonferroni's post-hoc test).

	Inhibition (%)		
Compound	nNOS	iNOS	eNOS
6-Nitrodopamine	92 ± 5**	60 ± 3**	40 ± 3*
6-Nitronoradrenaline	90 ± 3†	52 ± 4***	24 ± 2**

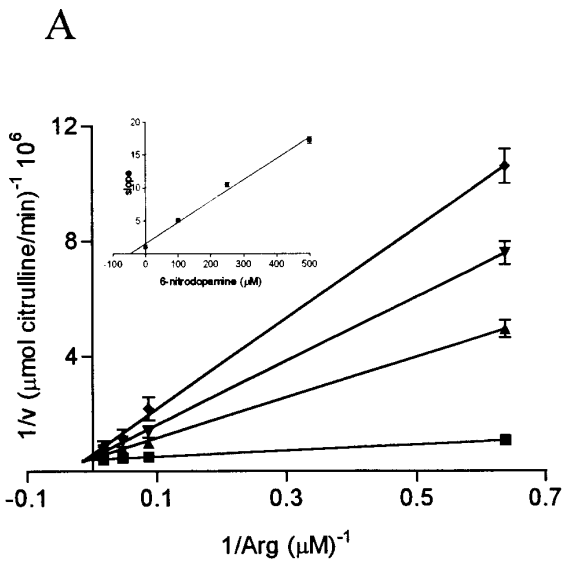

Figure 2 Kinetic analysis of nNOS inhibition by 6-nitrodopamine and 6-nitronoradrenaline

Reaction mixtures were as described in the Experimental section. The concentration of L-[^{14}C]arginine was varied over the range shown (1.57–61.57 μM). 6-Nitrodopamine (**A**) and 6-nitronoradrenaline (**B**) concentrations were 0 μM (■), 100 μM (▲), 250 μM (▼) and 500 μM (◆). The plots shown are representative of four separate experiments. Results are means ± S.E.M. for four different observations (some S.E.M. lie within the dimensions of the symbols).

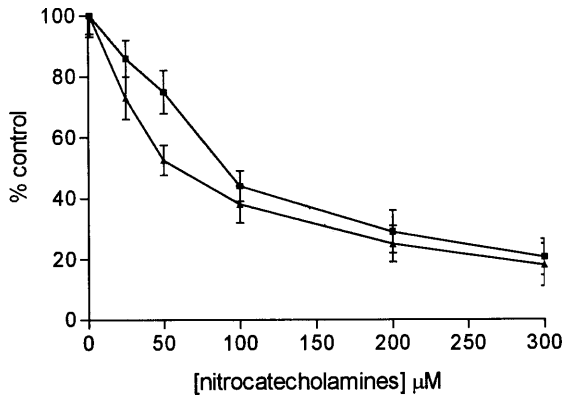

Figure 3 Effect of 6-nitrodopamine and 6-nitronoradrenaline on H_2O_2 formation by nNOS

The enzyme was incubated with increasing concentrations of 6-nitrodopamine (■) and 6-nitronoradrenaline (▲); H_2O_2 formation was measured as described in the Experimental section. Values are expressed as percentages of control values of incubations containing no added nitrocatecholamines. Results are means ± S.E.M. for four separate experiments.

6-nitrocatecholamines exert a markedly inhibitory effect on the enzyme activity, whereas, at the same concentration, noradrenaline was only moderately active and dopamine was virtually ineffective.

Both 6-nitrodopamine and 6-nitronoradrenaline exhibited a moderate selectivity for nNOS (Table 2).

6-Nitrodopamine and 6-nitronoradrenaline inhibited nNOS activity in a concentration-dependent manner, with IC_{50} values of 71 ± 1 and 78 ± 1 μM, when assayed in the presence of 1.57 μM L-arginine. More than 90% inhibition of nNOS activity was observed with 6-nitrodopamine and 6-nitronoradrenaline at 1 mM (results not shown).

The progress curve for inhibited nNOS was almost linear, ruling out significant irreversible inhibition during the 15 min time course examined, and the onset of inhibition was rapid (results not shown). In accordance with these results, preincubation of nNOS with 6-nitrodopamine or 6-nitronoradrenaline in the absence of L-arginine for 10 min before initiation of the reaction with L-arginine did not cause additional inhibition, confirming that the inhibition was in rapid equilibrium and reversible.

Initial-rate kinetic studies were performed over a range of concentrations of L-[^{14}C]arginine, 6-nitrodopamine and 6-nitronoradrenaline, to determine the nature of nNOS inhibition. The inhibition proved to be competitive with respect to L-arginine for both compounds (Figure 2). In the same studies, V_{max} and K_m values for L-arginine were determined as 0.61 ± 0.06 μmol/min per mg and 5.6 ± 0.8 μM respectively. In the presence of 100 μM 6-nitrodopamine and 6-nitronoradrenaline, the K_m values increased to 15.7 ± 2.4 and 16.3 ± 3.2 μM respectively (means ± S.E.M., $n = 4$; both increases were statistically significant, $P < 0.01$). V_{max} decreased to 0.48 ± 0.05 and 0.52 ± 0.09 μmol/min per mg for 6-nitrodopamine and 6-nitronoradrenaline respectively (means ± S.E.M., $n = 4$), only the former difference being statistically significant ($P < 0.05$).

On the assumption of a purely competitive inhibition, replots of the slopes of primary plots against 6-nitrodopamine and 6-nitronoradrenaline concentrations (Figure 2, insets) yielded K_i values for 6-nitrodopamine and 6-nitronoradrenaline of 45 and 52 μM respectively.

At 0.1 mM, 6-nitrodopamine and 6-nitronoradrenaline did not affect cytochrome *c* reduction in the absence of Ca^{2+}/calmodulin (results not shown), although they induced a significant inhibition of H_2O_2 production in the absence of L-arginine and BH_4 (Figure 3).

From these data, IC_{50} values of 85 ± 4 and 55 ± 5 μM were calculated for 6-nitrodopamine and 6-nitronoradrenaline respectively. With both nitrocatecholamines the inhibition of H_2O_2 production did not exceed 80%. Control experiments showed

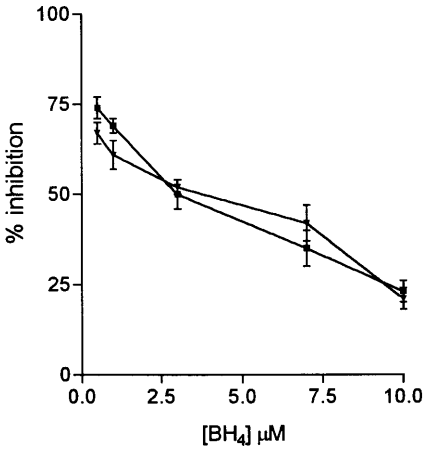

Figure 4 Effect of BH_4 concentration on nNOS inhibition by 6-nitrodopamine and 6-nitronoradrenaline

The enzyme was incubated with increasing concentrations of BH_4 in the presence of 500 μM 6-nitrodopamine (▼) and 6-nitronoradrenaline (■); NOS activity was measured as described in the Experimental section. Results are means ± S.E.M. for four separate experiments.

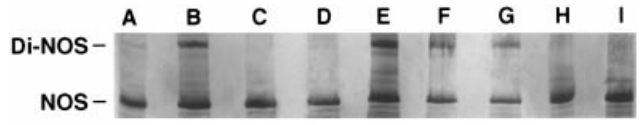

Figure 5 Effect of 6-nitrodopamine and 6-nitronoradrenaline on BH_4-induced nNOS dimerization

nNOS was incubated as described in the Experimental section with or without BH_4 in the absence or presence of 6-nitrodopamine and 6-nitronoradrenaline (500 μM), followed by low-temperature SDS/PAGE analysis. Lane A, no BH_4; lane B, 1 μM BH_4; lane C, 1 μM BH_4 plus 6-nitrodopamine; lane D, 1 μM BH_4 plus 6-nitronoradrenaline; lane E, 10 μM BH_4; lane F, 10 μM BH_4 plus 6-nitrodopamine; lane G, 10 μM BH_4 plus 6-nitronoradrenaline; lane H, 6-nitrodopamine; lane I, 6-nitronoradrenaline. NOS and Di-NOS refer to nNOS monomer and dimer, with apparent molecular masses of approx. 160 and 320 kDa respectively. The amounts of monomer and dimer were quantified by densitometry and the percentages of dimer are as follows: lane A, 5%; lane B, 30%; lane C, 0%; lane D, 0%, lane E, 47%; lane F, 27%, lane G, 26%, lane H, 1%; lane I, 2%. The gel shown is representative of four separate experiments.

that 6-nitrocatecholamines did not react with H_2O_2 under the specific conditions of the assay, as revealed by the ferrous thiocyanate assay.

The inhibitory effects of 6-nitrocatecholamines on L-citrulline formation were not affected by varying the FAD concentration. Notably, however, reversal of inhibition was observed in the presence of BH_4 in a concentration-dependent manner (Figure 4). The apparent K_i for the nitrocatecholamine inhibition of enzyme activation by BH_4 were 18 μM for 6-nitrodopamine and 40 μM for 6-nitronoradrenaline. This was obtained by initial rate kinetic studies performed over a range of concentrations of BH_4, 6-nitrodopamine and 6-nitronoradrenaline and in the presence of 1.57 μM L-arginine. Double-reciprocal plots of the nNOS activity as a function of BH_4 concentration showed a competitive pattern of inhibition (results not shown).

Low-temperature SDS/PAGE analysis showed that the enzyme, when preincubated at 37 °C without added BH_4, migrated mainly as the 160 kDa monomeric form (95%), whereas after incubation with 1 μM BH_4 it gave rise to a clearly detectable 320 kDa band (30%) suggestive of the dimeric form (Figure 5, lanes A and B respectively) [15]. In the presence of 500 μM 6-nitrodopamine or 6-nitronoradrenaline, the 320 kDa band was completely absent at 1 μM BH_4 concentration [Figure 5, lanes C (6-nitrodopamine) and D (6-nitronoradrenaline)]. On increasing the BH_4 concentration to 10 μM, a significant proportion of the dimeric form was observed in the presence of the same concentration of nitrocatecholamines [Figure 5, lanes F (6-nitrodopamine) and G (6-nitronoradrenaline)] amounting to approx. 50% of the control (Figure 5, lane E). In control experiments the nitrocatecholamines failed to induce dimer formation in the absence of added BH_4 [Figure 5, lanes H (6-nitrodopamine) and I (6-nitronoradrenaline)]. In the presence of 2 μM L-arginine, but in the absence of BH_4, dimer formation was approx. 60% of the total enzyme (results not shown). The addition of 500 μM 6-nitrodopamine and 6-nitronoradrenaline completely suppressed L-arginine-induced dimer formation. With higher concentrations of L-arginine, such as 100 μM, 500 μM 6-nitrodopamine and 6-nitronoradrenaline failed to inhibit dimer formation, which is consistent with kinetic data.

HPLC analysis was then used to investigate possible chemical reactions of nitrocatecholamines with BH_4. Incubation of 1 μM BH_4 at 37 °C for 15 min with 500 μM 6-nitrodopamine or 6-nitronoradrenaline and all cofactors present, but without NOS, resulted in 10 ± 2% BH_4 consumption, as determined by pterin formation after alkaline oxidation [8,14]. A similar value was obtained in the absence of added nitrocatecholamines, which argued against a possible chemical reaction. Consistent with this result was the observation that 100 μM 6-nitrodopamine or 6-nitronoradrenaline remained unchanged in the presence of up to 100 μM BH_4 after incubation as above. Attempts to investigate the possible interaction of nitrocatecholamines with the haem iron of nNOS were thwarted by their marked absorption in the 400 nm region, preventing the observation of spectral changes and perturbations of the haem spin equilibrium. However, in separate experiments it was found that, like the parent catecholamines, both 6-nitrodopamine and 6-nitronoradrenaline exhibit a high affinity for Fe^{3+} ions, leading to spectrophotometrically detectable charge transfer complexes (λ_{max} 382 nm) that are titratable with EDTA (results not shown). These complexes proved fairly stable, to an extent comparable to that of the purplish-blue catecholamine–Fe^{3+} complexes, and could not be displaced by various iron ligands at the same concentrations, including L-arginine, 7-nitroindazole [17] and imidazole [18], the latter two being established inhibitors of nNOS with high affinity for the haem centre.

In a final set of experiments it was found that the inhibitory effects of 6-nitrodopamine and 6-nitronoradrenaline varied with pH, being maximal at pH values between 6 and 7 and decreasing to approx. 60% at pH 8 (results not shown).

DISCUSSION

The 6-nitrocatecholamines 6-nitrodopamine and 6-nitronoradrenaline represent a novel class of potential endogenous NOS inhibitors with apparent selectivity for nNOS. Mechanistically, both 6-nitrodopamine and 6-nitronoradrenaline seem to act as antagonists of BH_4-mediated nNOS activation and dimerization. This finding, together with the essentially competitive pattern of inhibition, would be consistent with nitrocatecholamines' interacting with the enzyme in the proximity of the binding sites for BH_4 and L-arginine, most probably near the haem. In agreement with this interpretation, 6-nitrodopamine and 6-nitronoradrenaline inhibited haem-catalysed oxygen activation and H_2O_2 production but not cytochrome *c* reduction, in the absence of Ca^{2+}/calmodulin, indicating that they did not affect the reductase domain.

The inhibitory properties of nitrocatecholamines seem to reflect the presence of the nitro group on the catechol ring, as indicated by the lower activity, or lack of effect, of the parent catecholamines. The actual factors underlying this specific effect of the nitro group are intriguing and their rationalization could yield new and valuable clues to the elucidation of the delicate cross-talk between L-arginine and BH_4 binding and in mapping the relevant sites. Because of the relative stability to autoxidation imparted by the electron-withdrawing nitro group, 6-nitrodopamine and 6-nitronoradrenaline might interact with nNOS without being engaged in redox processes and/or inducing allosteric enzyme changes and/or dimerization.

Because at physiological pH the catechol rings of 6-nitrocatecholamines are to a substantial extent in the anionic form (pK_a 6.4) [5], they can be engaged in strong ionic-type interactions and/or H-bonding with polar residues near the L-arginine-binding site. Given the marked affinity of catecholamines for ferric ions, it seems conceivable that nitrocatecholamines bind near the ferric centre of the haem group of nNOS. It can be further speculated that the binding of 6-nitrocatecholamines to nNOS would position the nitro group in the proximity of certain critical residues located proximally to the haem. In this regard, incomplete inhibition of H_2O_2 formation would indicate that redox cycling of the haem is only partly blocked by the nitrocatecholamines.

The mechanism proposed above seems especially attractive considering the similarity of the action profile of nitrocatecholamines to that of 7-nitroindazole [17,19,20], an established NOS inhibitor with anti-nociceptive properties.

Like 7-nitroindazole [15,17,19,20], 6-nitrodopamine and 6-nitronoradrenaline bound to the enzyme in a manner competitive with L-arginine, did not affect cytochrome *c* reduction, inhibited H_2O_2 production and affected the pteridine-binding site by antagonizing BH_4-dependent dimerization in a reversible, concentration-dependent manner. By analogy with 7-nitroindazole, at least one of the nitrocatecholamines, 6-nitrodopamine, displayed a non-competitive component of inhibition, as evidenced by a decrease in V_{max}. Moreover, removal of the nitro group rendered the catecholamine system much less active, as occurs for the indazole ring (K_i 2.30 mM) [19].

The observed decrease in the inhibitory effect of nitrocatecholamines at alkaline pH also suggests a prominent role of the protonated aminoethyl side chain. This side chain might establish crucial ionic interactions with the BH_4-binding site, as suggested by the peculiar ability of this site, at least in eNOS, to bind positively charged species [21]. Although the catalytic centre of NOS comprises many conserved residues, it is surrounded by areas with a limited degree of sequence similarity between the various isoforms, which would greatly affect the affinity of nitrocatecholamines for the substrate-binding and pteridine-binding sites, thus accounting for the observed isoform selectivity.

Besides the relevance to the mechanism of action of nNOS and the attractiveness of nitrocatecholamines as novel structural prototypes for inhibitors of the enzyme, most of the interest of this study derives from its possible relevance to several pathophysiological processes implicating an overactivation of nNOS. These include neurodegenerative processes, such as cerebral ischaemia and Parkinson's disease, in which a marked increase in both NO and dopamine levels has been demonstrated [22,23]. Although NO seems to exert a regulatory role on certain catecholamine-dependent synaptic events, including neurotransmitter release from, and uptake into, presynaptic terminals [23], no direct effect of catecholamines on NO metabolism and signalling functions has yet been reported. At this stage, no definitive conclusion can be drawn as to whether the inhibition of nNOS would have any physiological significance in view of the high K_i values for the inhibition of nNOS and the lack of any results on the feasible intracellular concentration of 6-nitrocatecholamines. However, the relevance of these metabolites lies in the fact that their expected levels *in vivo* might fluctuate significantly and might become locally high under specific physiological and/or pathological conditions, for example after ischaemic injury. Whether and by what mechanisms 6-nitrocatecholamines might be available to nNOS is also an open issue and is a focus for further studies in this area. The apparent localization of *N*-methyl-D aspartate ('NMDA') receptors, responsible for nNOS activation, in presynaptic terminals of striatal dopaminergic neurons [23] suggests that, at least in that case, 6-nitrodopamine can be produced and act in the same cells.

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