Pergamon

Bioorganic & Medicinal Chemistry, Vol. 3, No. 7, pp. 923–927, 1995

0968-0896/95 $9.50 + .00

0968-0896(95)00083-6

Nitric Oxide-Induced Nitration of Catecholamine Neurotransmitters: a Key to Neuronal Degeneration?

Marco d'Ischia* and Claudio Costantini

Department of Organic and Biological Chemistry, University of Naples "Federico II", Via Mezzocannone 16, I-80134 Naples, Italy

Abstract—Exposure of the neurotransmitters dopamine (**1a**) and norepinephrine (**1b**), as well as of other catechol compounds (**1c–e**), to nitric oxide (NO) in aerated phosphate buffer at room temperature leads to the corresponding 6-nitroderivatives (**2a–e**) in yields higher than 80%. Formation of nitration products depends on the presence of oxygen and is inhibited by excess ascorbic acid, whereas sulfhydryl compounds, e.g. cysteine, and scavengers of reactive oxygen species, such as catalase and superoxide dismutase, exert no significant inhibitory effect. O-Methylated catechols are poorly or not reactive toward NO. These and other observations are consistent with a mechanism involving coupling of a semiquinone radical with NO or a higher oxide, e.g. nitrogen dioxide (NO_2). The observed formation of potentially toxic 6-nitrocatecholamines under physiologically relevant conditions may open new perspectives to an understanding of the biochemical processes underlying NO-induced toxicity and neuronal degeneration.

Introduction

Nitric oxide (NO) has emerged over the past few years as one of the most pervasive regulators of human physiology yet described.[1–3] Besides acting as a central mediator in relaxation of vascular smooth muscle, inhibition of platelet aggregation and immune regulation, NO appears to exert a number of diverse activities in the central nervous system (CNS).[4,5] Within specific populations of glutamate neurons, NO is produced by the action of a Ca^{2+}-dependent NO synthase (NOS) on arginine, and diffuses out to the closest anatomical structures, i.e. the presynaptic terminal and astrocytic processes, to act as a non-receptor-dependent intercellular messenger. Signal transduction processes are usually brought about at very low concentrations of NO, and involve mainly interaction with the heme iron of guanylate cyclase.

In addition to neurons, other cells in the CNS, i.e. astrocytes, microglia and macrophages, share the ability to produce NO by Ca^{2+}-independent NOS.[6] When generated in abnormally high fluxes, e.g. following immunostimulation of glial cells or excessive activation of *N*-methyl-D-aspartate (NMDA)-dependent excitatory pathways, NO gains unhindered access to aqueous and lipid environments where it may react with oxygen or oxygen radicals, e.g. superoxide ions, to form highly reactive species which may elicit a neurotoxic response.

Similar mechanisms have been suggested to intervene in a number of pathological conditions of the CNS, including the degeneration of nigrostriatal dopaminergic neurons in Parkinson's disease.[7,8] This view is supported by the observation that in parkinsonian substantia nigra there is a marked proliferation of reactive macrophages–microglia, and that macrophages can cause the death of mesencephalic dopaminergic neurons in culture via an NO-dependent process. The detailed molecular events underlying NO-induced neurotoxicity have, however, remained largely unknown.

Recently, in the course of a research programme[9] on the biochemical mechanisms of neuronal degeneration, we found that at physiological pH, NO reacts efficiently with catecholamine neurotransmitters, e.g. dopamine (**1a**) and norepinephrine (**1b**), to give the corresponding 6-nitroderivatives[10] (**2a** and **b**) as the major isolable products. This finding prompted us to examine in some detail the general features of the reaction, and to extend the study to those factors that may affect its course under biologically relevant conditions.

Results and Discussion

In a typical experiment, purified NO gas was slowly bubbled through a stirred solution of the catecholamine (1×10^{-3} M) in 0.5 M phosphate buffer, pH 7.0, in air at 25 °C or at room temperature. Under these conditions, the 6-nitrocatecholamine was formed in more than 80% yield after a few minutes. The kinetic profiles of dopamine decay and 6-nitrodopamine formation are depicted in Figure 1. Prolonged exposure of the mixture to NO resulted in a significant degradation of the nitrocatecholamine formed, with consequent decrease in the yield.

The reaction seems quite general in scope, since other catechols, including dopa (**1c**), catechol (**1d**) and 4-

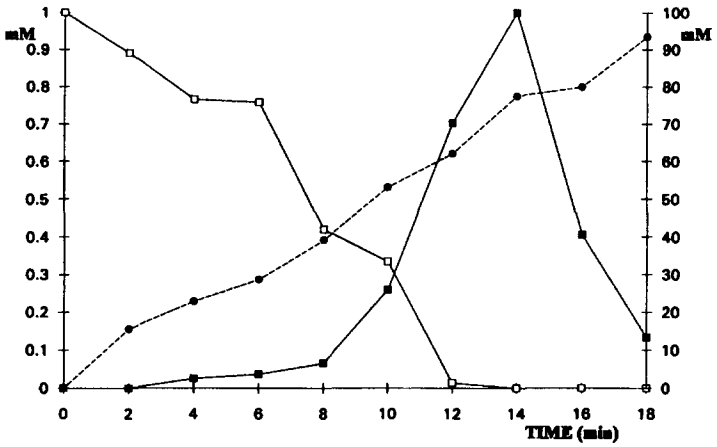
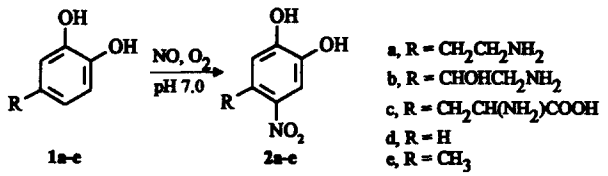


Figure 1. Time course of dopamine consumption, 6-nitrodopamine formation and nitrite accumulation by reaction of 1×10^{-3} M dopamine with NO in air in 0.5 M phosphate buffer, pH 7.0 at 25 °C. Dopamine (□) and 6-nitrodopamine (■) concentrations are reported on the left axis; nitrite (●) concentration is on the right axis. Each point represents the mean of at least three experiments. S.D. did not exceed 10%.

methylcatechol (**1e**), were converted to the corresponding nitroderivatives **2c–e** in comparable yields. Products could be recovered by conventional chromatographic techniques, after removal of excess NO gas by flushing with N_2 or Ar. Attempts to isolate and characterize other reaction products or intermediates were unsuccessful. Usually only minor peaks corresponding to unstable compounds could be detected by HPLC.



Under oxygen depleted atmosphere, formation of nitrocatechols was virtually negligible over a period of 1 h or more. At slightly acidic pH values, e.g. 6.0, 6-nitrodopamine formation was somewhat faster, whereas at pH higher than 7.0, e.g. 8.0, product yield was reduced, due to the apparent oxidation of the product.

Addition of 5.0×10^{-4} M $NaNO_2$ did not affect the kinetic and chemical course of the reaction of 5.0×10^{-4} M dopamine with NO in 0.5 M phosphate buffer, pH 7.0. When oxygen was rigorously excluded from the mixture, dopamine remained virtually unchanged over 1 h or more in the presence of equimolar amounts of $NaNO_2$ in aqueous buffer at pH 7.0, whereas small amounts of 6-nitrodopamine (1–2%) could be detected in aerated medium over the same period of time. Under these latter conditions, product formation resulted probably from nucleophilic attack of NO_2^- to the small dopamine quinone generated by autoxidation. It is noteworthy, in this connection, that when 4-methyl-*o*-benzoquinone (1×10^{-3} M) was allowed to react with an excess of $NaNO_2$ (2.0×10^{-2} M) in 0.5 M phosphate buffer pH 7.0, only 5% of the quinone was consumed after 10 min, without detectable formation of 4-methyl-5-nitrocatechol (**2e**), whereas 4-methylcatechol was almost quantitatively nitrated with NO in air.

Scavengers of reactive oxygen species, such as superoxide dismutase (SOD) (150 U mL^{-1}), catalase (150 U mL^{-1}) and sulfhydryl compounds, e.g. cysteine (up to 1×10^{-2} M) did not affect the rates of dopamine consumption and 6-nitrodopamine formation in the early stages of reaction of 1×10^{-3} M dopamine with NO. Notably, no detectable generation of thiol–quinone adducts, i.e. 5-S-cysteinyldopamine,[11] was observed in the presence of cysteine, the thiol being rapidly oxidized to cystine.[12] Ascorbic acid, by contrast, completely suppressed nitrocatecholamine formation, provided that it was present in sufficiently high concentration (more than 5×10^{-3} M) to prevent catechol oxidation.

When the experiments described in the present study were completed, a paper appeared in the literature[13] describing the nitration of catecholamines with NO and proposing an interesting hypothesis on the biological significance of the reaction. In that paper, the authors postulated a mechanism of catecholamine nitration involving the generation of an N_2O_4 species by autoxidation of NO, followed by electrophilic attack to the nucleophilic C-6 position of the catechol ring. Such a mechanism, in our opinion, does not withstand critical analysis.

A major point is that autoxidation of NO in neutral aqueous buffer does not lead to the formation of sufficient amounts of N_2O_4 to account for the fast and efficient nitration observed. Reaction of NO with oxygen-containing aqueous solutions leads only to the formation of NO_2^- but not of NO_3^-.[14–17] Since NO_3^- arises by hydrolysis of N_2O_4 formed by dimerization of NO_2, it follows that during the autoxidation of NO, NO_2 does not dimerize, but couples with excess NO present in the medium to form NO_2^- via N_2O_3:

$$2\,NO + O_2 = 2\,NO_2 \quad (1)$$
$$NO_2 + NO = N_2O_3 \quad (2)$$
$$2\,NO_2 = N_2O_4 \quad (3)$$
$$N_2O_3 + H_2O = 2\,NO_2^- + 2\,H^+ \quad (4)$$
$$N_2O_4 + H_2O = NO_2^- + NO_3^- + 2\,H^+ \quad (5)$$

The trapping of NO_2 by NO is favored also on a kinetic basis, since it is characterized by a rate constant of 1.1 $\times$ 10^9 M^{-1} s^{-1} which is significantly higher than that reported for the dimerization of NO_2 (4.5 $\times$ 10^8 M^{-1} s^{-1}).[18] Further oxidation of NO and NO_2^- to NO_3^- may occur *in vivo* or *in vitro* in the presence of oxyhemoglobin or related oxyhemoproteins.

Another critical point in the N_2O_4-based mechanism is that N_2O_4, like NO_2, has a sufficiently high reduction potential (E_0 for the couple NO_2/NO_2^- = 0.9 V)[19] to induce one-electron oxidation of the catecholamine in competition with the postulated electrophilic attack. With a view to addressing this point, we compared the reactivity toward NO of catechol (**1d**) versus veratrole, which would expectedly exhibit similar nucleophilicity but a markedly different behavior to oxidation. Upon exposure to NO in 0.5 M phosphate buffer at pH 7.0, catechol was readily consumed to give **2d**, whereas veratrole remained virtually unchanged over relatively prolonged periods of time. This would indicate that an oxidation step is essential for nitration of the catechol ring to occur.

A mechanism that could in principle be considered involves nucleophilic attack of NO_2^- to the catecholamine quinone.[20] This is however ruled out by: (a) the low reactivity of 4-methylbenzoquinone toward NO_2^-; (b) the failure of added NO_2^- to accelerate 6-nitrocatecholamine formation; (c) the negligible inhibitory effect of sulfhydryl compounds, among the best quinone trapping reagents.

From the balance of available evidence, it seems reasonable to conclude that formation of 6-nitrocatecholamines proceeds via the initial one-electron oxidation of the catecholamine to yield a semiquinone, which would be trapped by either NO or an oxidized species, most conceivably NO_2 (Fig. 2).

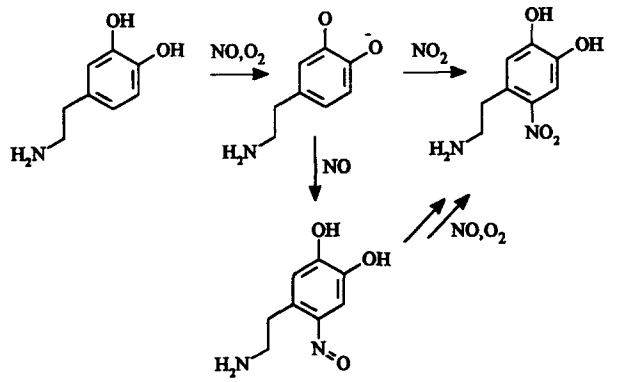


Figure 2. Suggested mechanisms of nitration of catecholamines with NO in air.

The former route would be supported by the known tendency of NO to couple with phenoxyl radicals[21,22] and implies formation of a nitrosocatechol intermediate (or the quinone oxime tautomer). As an attempt to verify the possible involvement of nitroso compounds in the nitration process, we reacted the model 4-nitrosophenol[23] with NO in phosphate buffer at pH 7.0 in air. Under such conditions, the starting material was rapidly consumed, but no significant amount of 4-nitrophenol could be detected. This observation, however, is far from being conclusive, given the inherent limitations of the model chosen.

The latter path would be akin to that suggested by Prutz and coworkers[19] for the nitration of tyrosine with NO_2 in aqueous neutral buffer, but requires generation of substantially high fluxes of NO_2 to ensure efficient capture of the transient semiquinone. The observed regioselectivity of the reaction would be consistent in any case with estimates of spin density distribution in *o*-benzosemiquinone,[24] suggesting a more pronounced radical reactivity at the 4(5)-positions as compared to the 3(6)-positions adjacent to the oxygen-bearing carbons. Unfortunately, all efforts aimed at providing experimental support to the proposed nitration paths were thwarted by the marked complexity of the chemistry involved, so alternative reaction mechanisms cannot be definitively ruled out at present.

Conclusions

The results of our study have shown that under biologically relevant conditions NO can efficiently convert catecholamine neurotransmitters and other catechol substrates into the corresponding nitro-derivatives by a mechanism probably involving generation of semiquinone intermediates as the crucial step. It is tempting to speculate that liberation of abnormally high fluxes of NO following macrophage activation, increased activity in excitatory pathways or oxidative stress, partially deplete the neuronal catecholamine pool, leading to the concomitant accumulation of nitrocatecholamine derivatives. Given the potential cytotoxicity of nitrocompounds,[25] we might envisage an NO-dependent aberrant path in catecholamine metabolism that could play at least a contributory role in neuronal degeneration. This and other issues relating to NO-induced neurotoxicity are currently under assessment in our laboratory.

Experimental

Materials

Dopamine hydrochloride, (±)-norepinephrine hydrochloride, L-dopa, catechol, 4-methylcatechol, 4-methyl-*o*-benzoquinone, veratrole (1,2-dimethoxy-benzene), 4-nitrophenol, 4-nitrocatechol, ascorbic acid, L-cysteine, sodium nitrite, sulphanilamide and *N*-(1-naphthyl)-ethylenediamine dihydrochloride were from Aldrich. Superoxide dismutase (4,200 U mg^{-1}) and catalase (3,100 U mg^{-1}) were from Sigma. NO gas (electronic grade, 99.99%) was from Air Liquide. It was purified from higher nitrogen oxides by passage through a solution of concentrated NaOH previously purged with Ar for 1 h and then through NaOH pellets. All other

chemicals and solvents were of the highest purity available and were used as received. Reference samples of 6-nitrodopamine, 6-nitronorepinephrine, 6-nitrodopa and 4-methyl-5-nitrocatechol were prepared by a previously described procedure[26] involving reaction of the catechol with sodium nitrite in aqueous sulphuric acid. 4-Nitrosophenol was prepared by a classical procedure.[23] All compounds were identified by analysis of NMR and MS data. Aqueous buffers were prepared in glass distilled, deionized water.

Methods

UV spectra were determined with a Perkin–Elmer Lambda 7 spectrophotometer. EIMS spectra were determined with a Trio 2000 Fisons spectrometer. Samples were ionized with a 70 eV electron beam. ^{1}H NMR (270 MHz) and ^{13}C NMR (67.9 MHz) were carried out on a Bruker AC 270 spectrometer equipped with an Aspect 3000 computer.

Analytical HPLC was performed using a Gilson model 302 pump, a Gilson 316 UV detector and a Spherisorb S50DS2 (4.6 × 250 mm, Phase separation, Ltd) column. Detection was carried out at 280 nm. The flow rate was maintained at 1 mL min^{-1}. Mobile phases for product analysis were as follows (proportions for mixed solvents are by volume): **1a** and **2a**: 0.05 M citrate, pH 3.0:acetonitrile 95:5 (elution times: **1a**, 4.9 min; **2a**, 14.4 min); **1b** and **2b**: 0.05 M citrate, pH 3.0 (elution times: **1b**, 4.9 min; **2b**, 12.3 min); **1c** and **2c**: 0.1 M formic acid:acetonitrile 92.5:7.5 (elution times: **1c**, 4.7 min; **2c**, 11.1 min); **1d** and **2d**: 0.1 M formic acid:acetonitrile 8:2 (elution times: **1d**, 5.2 min; **2d**, 9.11 min); **1e** and **2e**: 0.05 M citrate, pH 3.0:acetonitrile 7:3 (elution times: **1e**, 4.8 min; **2e**, 6.9 min). Quantification was carried out by comparing integrated peak areas with external calibration curves. All analyses were run at least in triplicate.

Nitrite concentration was determined spectrophotometrically using the Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine in 5% phosphoric acid) to form a chromophore absorbing at 543 nm.[27]

Reaction of catechols with NO. General procedure

In a typical experiment, purified NO gas was slowly bubbled through a stirred solution of the catechol in 0.5 M phosphate buffer, pH 7.0 (or as indicated in the text) thermostatted at 25 °C. Aliquots of the reaction mixture (usually 1 mL) were periodically withdrawn, purged with Ar to remove excess NO and immediately analyzed by HPLC, for monitoring substrate decay and product formation, as well as for nitrite levels. No significant variation in the pH of the buffer was observed after 1 h. When necessary, aliquots of freshly prepared solutions of enzymes or other additives were added prior to bubbling with NO. Even slight variations in certain experimental parameters, such as the rate of bubbling of the gas, the volume of catechol solution, or the shape of the flask may result in significant variations in the rates of substrate consumption and product formation. Accordingly, we found it useful for comparative purposes to refer to the levels of nitrite ions, which directly relate to the amount of NO gas absorbed by the mixture.

CAUTION: nitrogen oxides are very toxic. All operations must be carried out under an efficient hood.

Acknowledgments

This work was supported by grants from MURST and CNR (Rome). We thank Professor G. Prota and Professor M. B. H. Youdim for helpful discussions.

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(*Received in U.S.A.* 30 *December* 1994; *accepted* 20 *March* 1995)