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Mitochondrial damage by nitric oxide is potentiated by dopamine in PC12 cells

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Abstract

Mitochondrial damage in PC12 cells, a model for dopaminergic cells, was examined in terms of the contribution of oxidative stress, nitric oxide (·NO), and dopamine to impairment of mitochondrial respiratory control (RC). A kinetic analysis suggested that the oxidative deamination of dopamine catalyzed by monoamine oxidase (MAO) was not a significant source of hydrogen peroxide, because of constrains imposed by the low cytosolic level of dopamine. ·NO induced irreversible damage of mitochondrial complex I in PC12 cells: this damage followed a sigmoid response on ·NO concentration with a well-defined threshold level. Dopamine did not elicit damage of mitochondria in PC12 cells; however, the amine potentiated the effects of ·NO at or near the threshold level, thus leading to irreversible impairment of mitochondrial respiration. This synergism between ·NO and dopamine was not observed at ·NO concentrations below the threshold level. Depletion of dopamine from the storage vesicles by reserpine protected mitochondria from ·NO damage. Dopamine oxidation by ·NO increased with pH, and occurred at modest levels at pH 5.5. In spite of this, calculations showed that the oxidation of dopamine in the storage vesicles (pH 5.5) was higher than that in the cytosol (pH 7.4), due to the higher dopamine concentration in the storage vesicles (millimolar range) compared to that in the cytosol (micromolar range). It is suggested that storage vesicles may be the cellular sites where the potential for dopamine oxidation by ·NO is higher.

These data provide further support to the hypothesis that dopamine renders dopaminergic cells more susceptible to the mitochondrial damaging effects of ·NO. In the early stages of Parkinson's disease, ·NO production increases until reaching a point near the threshold level that induces neuronal damage. Dopamine stored in dopaminergic cells may cause these cells to be more susceptible to the deleterious effects of ·NO, which involve irreversible impairment of mitochondrial respiration.

Keywords: Nitric oxide; Mitochondrion; Storage vesicle; Complex I; Reserpine; Synergism

1. Introduction

Parkinson's disease is characterized by a progressive loss of muscular coordination caused by a lower rate of production of dopamine as a result of the damage of dopaminergic nigrostriatal neurons. Although the mechanism underlying the selective damage to dopaminergic neurons remains to be elucidated, the specific mitochondrial dysfunction in dopaminergic neurons inherent in Parkinson's disease is widely recognized [1]. A current hypothesis purports that dopamine itself renders dopaminergic neurons more susceptible to damage, a view supported by, on the one hand, the oxidative stress produced by H_2O_2 generated during the oxidative deamination of dopamine catalyzed by monoamine oxidase (MAO), located on the mitochondrial outer membrane [2,3] and, on the other hand, the electrophilic character of quinones derived from dopamine oxidation and involved in damage of cellular components [4], among them mitochondria [5,6].

An important recent discovery is that nitric oxide (·NO) production increases during the progress of Parkinson's disease as a result of inflammation-like processes and that this species plays a key role in the damage of dopaminergic

Abbreviations: MAO, monoamine oxidase; iNOS, inducible nitric oxide synthase; RC, respiratory control

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neurons: for example, mutant mice lacking inducible nitric oxide synthase (iNOS) were more resistant to MPTP-induced dopaminergic neurodegeneration [7,8].

Taking together these observations, the aforementioned hypothesis may be broadened by considering that dopamine renders dopaminergic cells more susceptible to the mitochondrial damaging effects of ·NO; this hypothesis is supported by two features of ·NO biological reactivity: first, it oxidizes dopamine in aerobic conditions [9] and, second, it regulates mitochondrial functions in a gradient-dependent manner: at low concentrations ·NO binds to cytochrome oxidase [10], whereas at higher concentrations it inhibits electron transfer at the bc_1 segment [10,11] and oxidizes ubiquinol [12,13], two effects associated with H_2O_2 production.

Although, the interaction between ·NO—or ·NO-derived species—and dopamine has been investigated in vitro [9,14–16], a possible synergism between dopamine and ·NO that causes cellular damage has not been addressed. In this study, a synergism between ·NO and dopamine leading to mitochondrial damage in PC12 cells is reported. Undifferentiated PC12 cells produce dopamine [17] and are a good model to study dopamine-related metabolism [18]. In addition, simple mathematical calculations were applied to integrate the knowledge available on this issue and analyze the validity of alternative mechanisms by which dopamine could render dopaminergic neurons more susceptible to oxidative and/or nitrosative damage.

2. Materials and methods

2.1. Chemicals and biochemicals

·NO gas was from Praxair (Danbury, CT, USA). Dopamine and digitonin were from Fluka (Buchs, Switzerland). H_2O_2, aurothioglucose, reserpine, diethylamine/·NO complex, Cu,Zn-superoxide dismutase (from bovine red blood cells), malate, glutamate, ADP, horseradish peroxidase type VI, *p*-hydroxyphenylacetic acid, succinate, antimycin A, and tyramine were from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Cell culture

PC12 cells from ATCC were cultured in complete medium (RPMI-1640 medium supplemented with 10% horse serum, 5% fetal calf serum, L-glutamine, and antibiotics).

Cells were incubated at 37 °C in humidified air with 5% CO_2 and kept in logarithmic phase by routine passage.

2.3. Incubation conditions

Cells (1.25 mg of protein) were incubated with the ·NO donor (diethylamine/·NO complex) for 30 min at 37 °C (·NO release rate at t_0 was 3 μM s^{-1}). The half-life of the donor was ~2.1 min; hence, every 2.1 min, ·NO release was decreased by 50%. Incubations were carried out in the presence or absence of exogenous dopamine (1 mM). Cells were spun down, collected, and used for mitochondrial respiration measurements.

2.4. Biochemical measurements

H_2O_2 production by PC12 cells was measured by monitoring fluorescence originating from *p*-hydroxyphenylacetate oxidation by horseradish peroxidase compound I [19]. Fluorescence measurements ($\lambda_{ex}=320$ nm; $\lambda_{em}=400$ nm) were performed in a Perkin-Elmer LS-5 spectrofluorometer equipped with a thermal-controlled and magnetic stirring sample compartment. For all measurements, PC12 cells (2.5 mg of protein) were incubated at 37 °C in respiration buffer (0.07 M sucrose, 0.23 M mannitol, 30 mM Tris HCl, 4 mM $MgCl_2$, 5 mM KH_2PO_4, 1 mM EDTA, and 0.5% bovine serum albumin, pH 7.4) containing 0.01% digitonin, 40 μM aurothioglucose, and 0.015 mg/ml superoxide dismutase.

2.5. Mitochondrial damage

Complex I-driven respiration was measured in respiration buffer in the presence of malate/glutamate (20 mM) (state 4) and malate/glutamate plus ADP (0.125 mM) (state 3) at room temperature in digitonin-permeabilized cells (1.25 mg of protein). Mitochondrial damage was expressed as inhibition of the respiratory control (RC) calculated as $(RC_{control} - RC_{sample})/RC_{control} - 1)$. The $RC_{control}$ obtained was 2.36 ± 0.37 ($n=8$). This RC cannot be compared directly with the classical values obtained with isolated mitochondria because in permeabilized cells there is endogenous ADP present before the addition of exogenous ADP, and so the state 4 measured is not a "true" state 4. Because RC showed some variation from day to day, we reported inhibition of RC as percentage of control.

3. Results and discussion

The potential synergistic effects of dopamine and ·NO on mitochondrial function were examined with a widely used model for dopaminergic cells, PC12 cells [17,18]. The assessment of mitochondrial functions described below was performed on PC12 cells treated with a low dose of digitonin (0.01%) that selectively disrupts plasma membranes without affecting mitochondrial membranes [20–22]. Hence, this experimental approach may be viewed as assessing mitochondrial functions in situ. The model was used to (a) quantify mitochondrial damage by dopamine and/or ·NO, (b) ascertain a synergism arising from exposure of cells to both agents, and (c) establish the implications of this process for dopaminergic neurons in vivo.

3.1. Oxidative stress exerted by dopamine

A possible mechanism by which dopamine damages cells is through its oxidative deamination catalyzed by MAO present in the outer mitochondrial membrane leading to production of H_2O_2. This view was examined in PC12 cells—endowed with MAO-A activity [23]—in which the production of H_2O_2 during dopamine metabolism and by the respiratory chain was measured (Table 1). At variance with measurements in isolated brain mitochondria, the rate of H_2O_2 production ascribed to MAO catalysis was lower than that observed during oxidation of succinate and ascribed to the respiratory chain. In agreement with these results, mitochondria were not damaged when PC12 cells were incubated with dopamine (see below).

Experiments carried out with isolated brain mitochondria [3] yielded H_2O_2 production rates, during oxidative deamination of amines, ~ 50-fold higher than those arising from the electron-transport chain. These findings are at variance with the contribution of MAO to H_2O_2 production in permeabilized PC12 cells (Table 1). Dopamine concentration in storage vesicles of peripheral sympathetic neurons can be as high as 50 mM [2]. However, cytosolic levels of dopamine are expected to be in the low micromolar range. Assuming a cytosolic concentration of dopamine of 1 μM [25] and the kinetic data obtained with isolated brain mitochondria (Fig. 1 legend) [3], it can be estimated that the production of H_2O_2 derived from MAO catalysis is ~ 40-fold lower than that from the respiratory chain (Fig. 1). Moreover, it can be argued that H_2O_2 production by the isolated brain mitochondria respiratory chain is an overestimation over the physiological levels, for these measurements were performed in the presence of antimycin A [3]. Yet, these values may bear physiological significance when considering that ·NO exerts an antimycin A-like effect on the respiratory chain by inhibiting electron transfer at the bc_1 segment and, thus, eliciting H_2O_2 production [10].

It may be concluded from experimental (Table 1) and calculated (Fig. 1) data that the rate of production of H_2O_2 via MAO catalysis is lower than that originating from the electron-transfer chain, a situation that could be also expected to occur in dopaminergic neurons in vivo, taking into consideration the kinetic data available for brain MAO.

Table 1
Monoamine oxidase- and respiratory chain-dependent production of H_2O_2 in permeabilized PC12 cells

	$d[H_2O_2]/dt$ (nmol/min/mg protein)
Succinate plus antimycin A	0.09 ± 0.01 (3)
Tyramine plus dopamine	< 0.01 (not detectable)

Assay conditions as described in Materials and methods. The assay mixture consisted of cells (2.5 mg protein) supplemented with either 10 mM succinate and 1 μg antimycin A/ml or 2 mM tyramine and 1 mM dopamine (which are substrates for both MAO A and B isoforms).

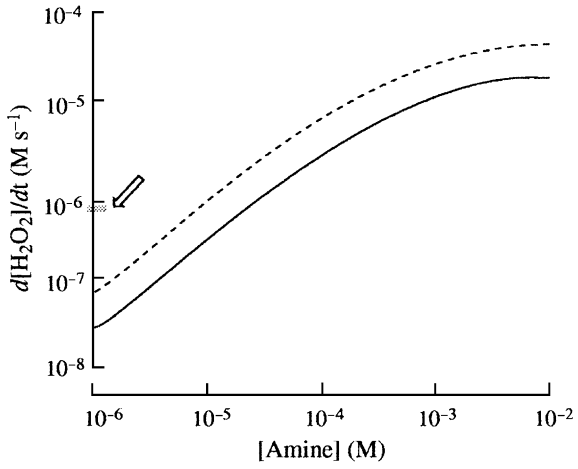

Fig. 1. H_2O_2 production by brain mitochondria. Production of H_2O_2 originating from either MAO-catalyzed oxidation of dopamine and tyramine or the electron-transfer chain was calculated assuming the following data taken from Ref. [3]: (a) oxidation of tyramine by MAO follows Henri–Michaelis–Menten kinetics (K_M of 0.65 mM and V_{max} of 4.5×10^{-5} M s^{-1}); (b) oxidation of dopamine catalyzed by MAO is 2.7-fold lower than that of tyramine; and (c) production of H_2O_2 by the respiratory chain is 9.5×10^{-7} M s^{-1}. Dotted line indicates calculations obtained with tyramine and straight line with dopamine. The arrow indicates the level of H_2O_2 originating from the mitochondrial respiratory chain.

3.2. Mitochondrial damage induced by ·NO and its potentiation by dopamine

The irreversible damage to mitochondrial respiration as a function of ·NO levels is shown in Fig. 2. An interesting feature is that within a small range of ·NO concentration, mitochondria are irreversibly damaged. This indicates the occurrence of a threshold level of ·NO below which little

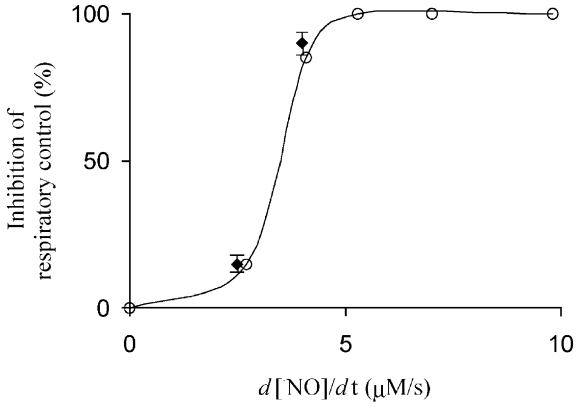

Fig. 2. Effect of ·NO on mitochondrial respiration in PC12 cells. Assay conditions: PC12 cells (1.25 mg of protein) were exposed to a flux of ·NO release from diethylamine/·NO. Mitochondrial respiration was assayed as described in Materials and methods. Values on the *x*-axis indicate initial rates of ·NO release. Open symbols represent a typical experiment carried out with the same cell population, while close symbols were obtained from five and three independent experiments at 2.5 and 4.0 μM/s ·NO production, respectively.

Table 2
Potentiation of ·NO-mediated damage by dopamine

Conditions	Inhibition of respiratory control $(RC_{control} - RC_{sample})/(RC_{control} - 1)$ (%)
+ Dopamine	0.9 ± 0.5 (n = 3)
+ NO (2.5 μM s^{-1})	15.0 ± 5.7 (n = 5)
+ NO (2.5 μM s^{-1}) + dopamine	70.9 ± 8.9 (n = 4)
+ NO (4.0 μM s^{-1})	90.1 ± 7.5 (n = 3)
+ NO (4.0 μM s^{-1}) + reserpine	48.4 ± 8.5 (n = 3)

Assay conditions: PC12 were exposed for 30 min at 37 °C to exogenous dopamine (1 mM) or ·NO (initial flux rate either 2.5 or 4.0 μM s^{-1}) or ·NO plus dopamine. Reserpine concentration was 1 μM. Cells were collected and mitochondrial damage assessed as described in Materials and methods. A flux rate of ·NO of 2.5 μM s^{-1} is at or near the threshold level; that of 4.0 μM s^{-1} is above the threshold level and elicits maximal mitochondrial damage (see Fig. 1). Absolute $RC_{control}$ was 2.36 ± 0.37 (n = 8).

damage is exerted, but once this threshold is reached, mitochondria offer little resistance to further increases in ·NO concentration. Mitochondrial damage by ·NO may involve the reported inactivation of complex I and protein nitrosation [24].

Approximately 15% inhibition of mitochondrial RC was observed with ·NO delivery rates below or at the threshold level (2.5 μM × s^{-1}; see Fig. 2) (Table 2). This effect was dramatically amplified by dopamine (~ 70% inhibition) (Table 2). The amine by itself elicited negligible respiratory damage. The synergistic effect observed with dopamine and ·NO was slightly affected by Cu,Zn-superoxide dismutase (not shown), thus suggesting that the contribution of peroxynitrite (arising from the reaction of superoxide anion ($O_2^{\cdot -}$), formed during dopamine oxidation, and ·NO) to mitochondrial damage was not significant. The prevalent mechanism underlying the interaction of dopamine with ·NO was described [9] as depending largely on the concentration of the latter: at high ·NO concentrations, dopamine undergoes nitrosation with subsequent nitration, whereas at low ·NO concentrations, dopaminochrome is formed *via* two *o*-semiquinone intermediates and with ensuing formation of hydroxyl radical.

No synergistic effect was observed when PC12 cells were preincubated with dopamine for 30 min, followed by removal of extracellular dopamine and incubation with ·NO (not shown). Likewise, no damage was observed when a mixture of dopamine and ·NO (incubated for 30 min) was added to PC12 cells. These observations suggest that (a) dopamine does not induce cell changes that render them more susceptible to ·NO damage and (b) a stable, long-lived product from the reaction between ·NO and dopamine was not involved in mitochondrial damage.

It may be surmised that if dopamine renders mitochondria more susceptible to ·NO damage, cells depleted of dopamine should be more resistant to the deleterious effects of ·NO. Accordingly, treatment of PC12 cells with reserpine, a compound that depletes the cellular dopamine storage vesicles (including PC12 cells [17]), rendered cells more resistant to damage elicited by ·NO (Table 2). Although long-term (18 h) treatment of cell with reserpine may elicit cellular changes besides dopamine depletion, these results concur with the view that dopamine causes mitochondria in PC12 cells to be more susceptible to damage by ·NO.

3.3. ·NO-mediated oxidation of dopamine in cytosol and storage vesicles

As mentioned above, dopamine, synthesized in the cytosol, is present at the micromolar range in this compartment [25] and is rapidly stored in vesicles at very high concentrations (millimolar range) [26]. The low pH of the vesicles (5.5) is considered to protect dopamine against autoxidation and, as a corollary, the oxidative damage mediated by dopamine is assumed to involve cytosolic or extracellular dopamine. ·NO oxidizes dopamine at pH 5.5, albeit at a rate substantially lower than at pH 7.4 (Fig. 3). The dependence of dopamine oxidation on pH follows the general tenet that deprotonation is a requisite for electron transfer: for each unit of pH increased, the rate of oxidation of dopamine increases 10-fold. However, the calculated data in Table 3 indicate that the rate of dopamine oxidation in the storage vesicles is ~ 17-fold higher than that in the cytosol because the concentration factor overcomes the pH constraints.

These calculations ought to be interpreted as semiquantitative estimations at the order of magnitude level; nevertheless, they suggest that storage vesicles may be the cellular sites where the potential for dopamine oxidation is higher. Biomembranes do not constitute a barrier for ·NO diffusion and, actually, they promote the diffusion of this species [27]; therefore, ·NO has free access to the interior of the storage vesicles. The results in Table 2 also support the interaction between ·NO and dopamine in storage vesicles: reserpine, a compound that releases dopamine from storage vesicles, followed by its secretion in the extracellular milieu

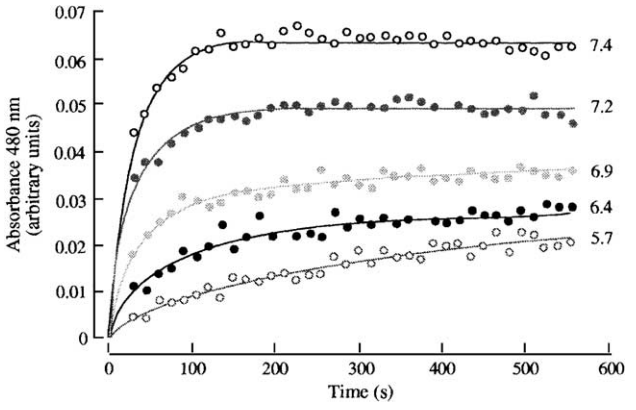

Fig. 3. pH dependence of dopamine oxidation by ·NO. The assay mixture consisted of 1 mM dopamine in 0.1 M phosphate buffer, pH 5.7–7.4. The reaction was initiated upon addition of 70 μM ·NO, delivered as an ·NO-saturated solution kept in anaerobiosis. Dopamine oxidation was followed at 480 nm.

Table 3
Relative rates of dopamine oxidation in cytosol and storage vesicles

Cellular site	pH	[Dopamine] (mM)[a]	[Dopamine$^-$] (μM)[b]	Rate of oxidation[c]
Storage vesicles	5.5	1.25	0.50	17
Cytosol	7.4	0.001	0.03	1

[a] Total concentration of dopamine (sum of protonated and anionic forms).
[b] The concentration of the anionic form of dopamine was calculated from [Dopamine]=([Dopamine]$_{total}K_a$/[H$^+$])/(1 + K_a/[H$^+$]) and assuming a pK_a of 8.9.
[c] The relative rate of oxidation is given by the ratio of the concentration of dopamine anion in storage vesicles over that in cytosol.

protected against mitochondrial damage induced by ·NO. Reserpine also causes a temporal increase of the cytosolic levels of dopamine, as observed by the feed-back inhibition of tyrosine hydroxylase [25].

4. Concluding remarks

The low production of H_2O_2 during MAO-catalyzed oxidative deamination of dopamine (with respect to H_2O_2 generated upon oxidation of respiratory chain substrates; Table 1, Fig. 1), the lack of a direct effect of dopamine on mitochondrial respiration (Table 2), and the potentiation of the deleterious effects of ·NO by the amine (Table 2) need be assessed in terms of (a) the MAO content and activity in dopaminergic neurons and the concentration of cytosolic dopamine accessible to this enzyme on the outer mitochondrial membrane, and (b) the cellular site for the interaction between dopamine and ·NO with implications for mitochondrial function.

Although PC12 cells are widely used in neurobiological and neurochemical studies, they do not have a neuronal origin, and extrapolation of the results to dopaminergic neurons deserves caution. Oxidative deamination of dopamine and consequent H_2O_2 production is the domain of outer mitochondrial membrane MAO, the activity levels of which are a matter of controversy in dopaminergic neurons. A recent histochemical study showed only low levels of MAO in these cells [28] and that PC12 cells contain MAO-A activity [23].

In early stages of the onset of MPTP-induced Parkinson's model, preceding dopaminergic neurodegeneration, there is an increase in ·NO production through iNOS in glial cells [7]. According to the data shown here, cells are expected to withstand some increase in ·NO concentration, but once a threshold levels is reached, cells will have a very limited resistance to further ·NO increases that cause irreversible damage to mitochondria. Due to the strong synergism between ·NO and dopamine, the storage of dopamine in dopaminergic neurons causes this threshold to be lower in these cells and, accordingly, they are selectively harmed. The potentiation of ·NO-mediated mitochondrial damage by dopamine was observed in a narrow critical region of ·NO concentration near the threshold level that induces mitochondrial damage: for ·NO concentrations lower than the threshold level, no synergistic effects were observed (not shown), whereas at high ·NO concentrations, the damage observed in the absence of dopamine was already maximal. It may be expected that in dopaminergic neurons in vivo the concentration of ·NO with pathological significance in the development of Parkinson's disease is the one near the threshold level that induces cellular damage. Therefore, the occurrence of a synergism between ·NO and dopamine for the concentration window of ·NO near the threshold level found to induce mitochondrial damage is highly significant. Although data in Table 3 suggest that storage vesicles may be the cellular sites where the potential for dopamine oxidation is higher, alternative pathways cannot be ruled out: the interaction between ·NO and dopamine may occur extracellularly and yield a reactive, long-lived product, which is either endowed with a high permeability constant and able to cross biomembranes and reach mitochondrial targets or capable of interacting with the plasma membrane and thus trigger a cascade that damages mitochondria.

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