

Antiproliferative action of dopamine and norepinephrine in neuroblastoma cells expressing the human dopamine transporter¹

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SPECIFIC AIMS

The aim of our study was to determine the mechanism and site of action of the cytotoxic potential of the catecholamine transmitters dopamine (DA) and norepinephrine (NE). We established SK-N-MC neuroblastoma cell lines stably transfected with the cDNAs of the human DA transporter (DAT) or NE transporter and studied the effects of DA and NE in cells with and without catecholamine uptake.

PRINCIPAL FINDINGS

1. Low micromolar concentrations of DA or NE (1–10 μ M) inhibit cell growth

Exposure of cells expressing DA uptake at a maximal initial rate (V_{\max}) of 18 to 24 pmol/min \cdot 10⁵ cells to 10 μ M DA in the culture medium had a profound effect on cell growth: instead of the normal time-dependent increase, there was a gradual loss of viable cells (**Fig. 1A**). The effect of DA was related to the level of DAT expression (**Fig. 1B**) and could be prevented by the transport blocker mazindol; in this low micromolar range, DA had no effect in parental cells lacking the DAT (**Fig. 1C**). NE, which is a substrate of the DAT, had an effect slightly stronger than DA on growth of our DAT-expressing cells (**Fig. 1D, E**). The noncatecholamine substrate of DAT 1-methyl-4-phenylpyridinium (MPP⁺) was, in contrast, less effective than the two catecholamines (**Fig. 1D, E**).

We could rule out the possibility that transporter activation and the necessary higher energy demand per se elicited cell growth inhibition. Neither DA or NE receptor blockers nor cell-permeable blockers of intracellular signaling pathways modified the catecholamine effect.

2. Oxidative stress is not involved in catecholamine inhibition of cell growth

None of the known experimentally effective antioxidants prevented the cell loss induced by DA in the

DAT-expressing cells. Nor did we find any accumulation of intracellular oxidants. We used the inhibition of aconitase activity and the fluorescence of hydroethidine as a measure of intracellular superoxide radicals and the fluorescence of dichlorofluorescein as an indicator of hydrogen peroxide. Whereas the toxic transporter substrate MPP⁺ produced effects indicative of oxidative stress in our cells, intracellular accumulation of DA or NE did not. Thus, a major contribution of oxidative stress to the catecholamine effects studied can be excluded.

3. Catecholamines arrest cell cycle progression and induce apoptosis

Since cell growth and cell death are intimately connected to cell cycle events, we determined the effect of DA and NE on cell cycle by flow cytometry based on DNA content. In cells without DA uptake, neither 10 μ M DA nor 10 μ M NE added for 24 h to the culture medium altered the cell cycle progression. However, in cells with DA uptake (**Fig. 2**), the share of the G₀/G₁ population of cells was significantly increased (from 50% to ~80%; after 6 h of exposure, to nearly 60%). The profound effects of the catecholamines on cell cycle distribution was not observed after 10 μ M MPP⁺. Deferoxamine, known to block cellular proliferation before the G₁/S, arrested the progression in the G₀/G₁ phase of the cell cycle, as expected, in cells with and without DAT.

In DAT-expressing cells, low micromolar levels of DA or NA and 100 μ M deferoxamine in the medium for 24 h elicited the pattern of DNA laddering characteristic of apoptosis. Flow cytometric measurements using the fluorescent dye YO-PRO-1 showed the share of apoptotic cells to be doubled relative to controls.

¹ To read the full text of this article, go to <http://www.fasebj.org/cgi/doi/10.1096/fj.00-0738fje>; to cite this article, use *FASEB J.* (May 18, 2001) 10.1096/fj.00-0738fje

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4. The catecholamine-induced inhibition of cell growth is antagonized by iron (III)

As shown above, in DAT-expressing cells the effect of DA and NE had striking parallels to the action of deferoxamine: G₁ arrest with a similar time course and induction of apoptosis. In addition, deferoxamine showed an antiproliferative effect, with the growth inhibition being additive to that of DA. As deferoxamine is an effective iron chelator, interaction with cellular iron was investigated. 90 μM FeCl₃ antagonized not only the effect of 100 μM deferoxamine, but also the effect of 3 μM DA on cell growth [% of control cells ± SE (n): 55 ± 6 (4), vehicle; 79 ± 1 (4), FeCl₃; P < 0,05]; the effect of 3 μM MPP⁺ on cell numbers, however, was not influenced. Furthermore, 90 μM FeCl₃ antagonized the arrest of the cell cycle elicited by 1 μM catecholamines. This iron concentration reduced the intracellular DA levels by 18 ± 3% (after 6 h of incubation with 3 μM DA; n=3); the initial DA uptake rate was reduced by 10 ± 2%.

CONCLUSIONS AND SIGNIFICANCE

The effect of catecholamines in the low micromolar range on cell growth and viability clearly had an intracellular site of action: 1) It was absent in parental cells; 2) the susceptibility of cells transfected with the transporter was related to the initial rate of uptake and prevented by uptake blockade (mazindol); and 3) continuous transporter activation (entailing increased energy demand) was not involved. Oxidative stress is commonly considered to be crucial for DA cytotoxicity. This notion is based on the protective action of antioxidants. In fact, in our neuroblastoma cells without DA uptake, cytotoxicity of high micromolar catecholamines was blocked by antioxidants. However, for low micromolar concentrations of DA or NE acting in DAT-expressing neuroblastoma cells, we could rule out oxidative stress as a key mechanism.

Our study was performed in dividing cells with an active cell cycle. The effect of DA was primarily antiproliferative in nature, with the sum of necrotic and apoptotic cells representing only a minor portion of the DA-induced cell loss. DA and NE induced an apparent arrest in the G₁ phase, whereas MPP⁺ (a structurally unrelated cytotoxic DAT substrate) was without effect. Thus, the effect on the distribution of cells within the cycle phases was specific for catecholamines and not shared by every substrate of the DAT.

In view of the known property of catecholamines to bind iron and other metals, iron chelation as a likely mechanism suggested itself. Intracellular iron is essential for cell growth, and iron-chelating compounds have been shown to interfere with the cell cycle by inhibition of ribonucleotide reductase. In our cells expressing the DAT, the antiproliferative action of DA was prevented by addition of FeCl₃ to the medium; the effect was

specific in that the action of MPP⁺ on cell numbers was not affected. Furthermore, addition of FeCl₃ antagonized the arrest of the cell cycle elicited by the catecholamines. This implicates iron chelation as the mechanism of catecholamine-induced antiprolifera-

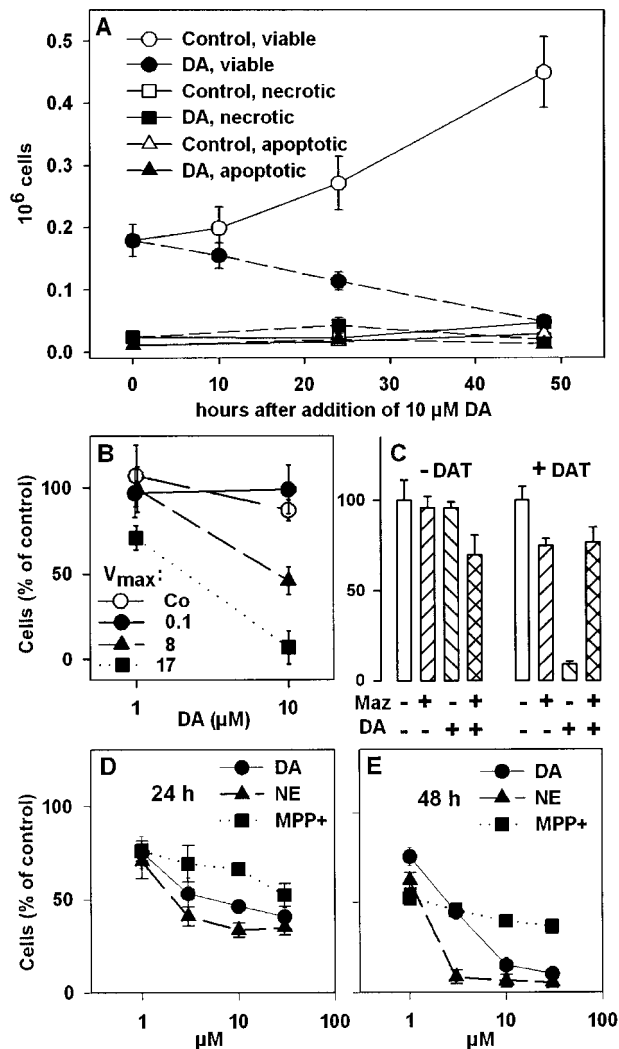


Figure 1. Effect of catecholamines on the viability of neuroblastoma cells expressing the human DAT. *A*) DA inhibited cell proliferation; cells were exposed to 10 μM DA (filled symbols) or vehicle (open symbols) and the number of viable (circles), necrotic (squares), and apoptotic (triangles) cells was determined at the time points indicated. *B*) The effect of DA depended on the expression level of DA uptake: cells with different maximum uptake initial rates (V_{max} in pmol/min · 10⁵ cells: zero uptake, open circles; 0.09, closed circles, 8, triangles; 17, squares) were exposed to DA at the concentrations indicated and the number of viable cells was determined 72 h later. *C*) The effect of DA was inhibited by uptake blockade: cells without (left panel) and with DA uptake (V_{max} 24 pmol/min · 10⁵ cells, right panel) were exposed to 10 μM DA in the absence and presence of 10 μM mazindol in the medium and the number of viable cells was determined 72 h later. *D, E*) The effect of NE was stronger than that of DA and MPP⁺: cells were exposed to different concentrations of DA (circles), NE (triangles) or MPP⁺ (squares) in the medium and the number of viable cells was determined 24 h (*D*) or 48 h (*E*) later. The results are means ± SE calculated from 3–4 independent experiments, each carried out in triplicate.

tion. Another explanation might be that iron (III) destabilized DA in the medium by accelerating its autoxidation. However, intracellular levels of the catecholamines determined in the absence or presence of 90 μM FeCl_3 make it unlikely that accelerated decomposition of extracellular DA is a major factor in the protective action of iron(III).

Binding of intracellular iron as a mechanism of catecholamine-induced growth inhibition is greatly supported by the similar time course of cell cycle arrest at G_1 and growth inhibition induced by the known iron chelator deferoxamine. The finding that at submaximal concentrations the effect of deferoxamine was only additive to that of DA, being antagonized by addition of FeCl_3 , favors an identical site of action of these two otherwise quite different compounds.

The cell cycle effect of DA, NE, and deferoxamine was accompanied by apoptosis as revealed by DNA laddering and flow cytometry. Interfering with the cell cycle has been demonstrated to be a potent trigger of apoptosis. G_1 arrest and apoptosis are often interre-

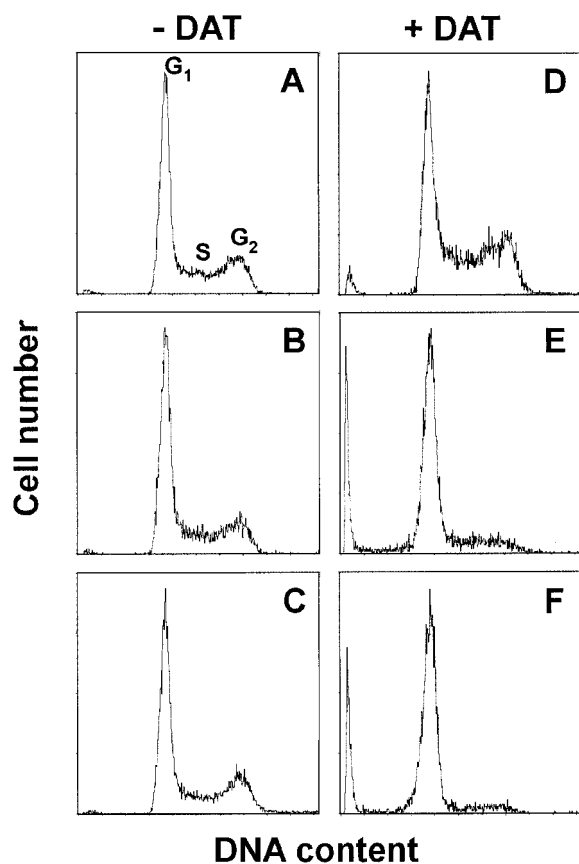
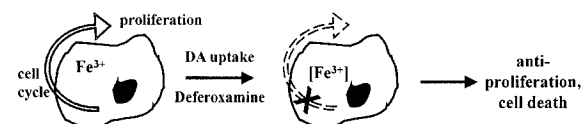


Figure 2. Cell cycle analysis of neuroblastoma cells exposed to catecholamines. Cells without catecholamine uptake (A–C) or cells expressing the human DAT (D–F) were exposed for 24 h to vehicle (A, D), 10 μM DA (B, E), or 10 μM NE (C, F) in the medium, harvested, fixed with ethanol, stained with propidium iodide, and analyzed using flow cytometry. Shown are DNA histograms from representative experiments.

Dividing Neuroblastoma Cells with DAT



Postmitotic DA Neurons

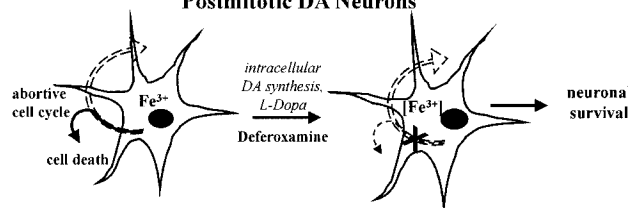


Figure 3. Schematic diagram. Different effect of cell cycle arrest (X) in dividing cells and differentiated neurons. Upper panel: G_1 arrest by DA uptake or deferoxamine due to intracellular Fe^{3+} chelation ($[\text{Fe}^{3+}]$) results in antiproliferation and cell death in neuroblastoma cells expressing the DAT. Lower panel: Hypothesis suggesting how increased intracellular DA (via increased DA synthesis or L-dopa) might promote survival of postmitotic DA neurons. When compromised by, for example, withdrawal of neurotrophic support, differentiated neurons may enter an abortive cell cycle, resulting in cell death. DA might be neuroprotective (as shown recently for deferoxamine in differentiated PC12 cells and sympathetic neurons) by blocking the abortive cell cycle (X).

lated. Thus, in agreement with our observations, data in the literature show that apoptosis of proliferating neuronal cells after growth factor withdrawal correlated with arrest in G_1 .

The most obvious relevance of our observations lies in aspects of neurodevelopment and neuroprotection. Regulation of the cell division cycle apparently has an effect on neuronal differentiation. It is believed that the final division of neuronal progenitor, especially events during the G_1 phase of the cycle, influences to some degree its final differentiation.

We made our observations in actively dividing neuroblastoma cells. Could our findings also be relevant for adult neurons? Agents that block the G_1/S transition, including the iron chelator deferoxamine, have been shown to suppress the apoptosis of differentiated PC12 cells and sympathetic neurons. These findings are in line with the suggestion that, although antiproliferative agents will cause apoptosis in cycling cells, by preventing re-entry into an abortive cycle they will promote survival in terminally differentiated cells, such as neurons. The parallels between deferoxamine and catecholamines in our proliferating neuroblastoma cells in terms of growth inhibition, G_1 arrest, and induction of apoptosis suggest that, similar to deferoxamine, catecholamines might be neuroprotective in mature NE and DA neurons (Fig. 3). The possibility of neuroprotection stands in contrast to the current hypotheses implicating DA in the etiology of nigral cell death in Parkinson's disease. [F]