Effects of testosterone on differentiation and oxidative stress resistance in C1300 neuroblastoma cells

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Submitted: November 23, 2006 *Accepted: November 26, 2006*

Key words: **testosterone; neural differentiation; neuroprotection; oxidative stress; tubulin; 3-nitro-L-tyrosine; N^6,2'-O-dibutyryl-adenosine-3',5'-cyclic-mono-phosphate; C1300 neuroblastoma cells**

Neuroendocrinol Lett 2006; **27**(6):807–812 **PMID:** 17187012 NEL270606A15 © Neuroendocrinology Letters **www.nel.edu**

Abstract

OBJECTIVES: Using undifferentiated mouse neuroblastoma cells (C1300), we have previously observed that testosterone (T) exerts a neuroprotective action against oxidative stress. Nitrogen intermediates induce the production of 3-nitro-L-tyrosine (3NT), an amino acid analogue involved in many neurodegenerative disorders. The aim of our work is to investigate T capability on C1300 cell differentiation. It is also evaluated whether differentiation could mitigate the nitrosative effects of 3NT.

METHODS: The effects of both T and 3NT were studied on an undifferentiated cell line of neural origin (C1300). For this purpose, cell cultures underwent morphometric investigation, blot analyses and catalase activity assay. All data obtained were expressed as mean ±SD and tested by one-way ANOVA or Student's *t* test.

RESULTS: The results were compared with those gathered by means of N^6,2'-O-dibutyryl-adenosine-3',5'-cyclic-mono-phosphate (db-cAMP), a well-known differentiating agent. T-exposed cells showed an irregular shape and exhibited long branching cytoplasmic extensions, which were longer than in db-cAMP cells. Moreover, T-exposure induced an increase in the expression of tyrosinated and acetylated α-tubulin while 3NT-incorporation into tubulin was markedly reduced. The action of antioxidant defence systems, namely catalase activity, was enhanced in cells exposed to T.

CONCLUSION: This work highlighted the effects of db-cAMP on differentiation and neuroprotection, but even indicated that T exposure induced differentiation in C1300 cells and this process matches a significant neuroprotective effect. This action seemed to be more effective than in db-cAMP-treated cells. T is suggested, like other substances having antioxidant properties, to be of potential interest in the experimental therapy of neuropathological conditions.

ORIGINAL ARTICLE

Introduction

It has been reported that neural cells are differentiated when they show at least one cytoplasmic process longer than the cell body [7]. Inhibition of cell proliferation, hypertrophy of cell bodies and nuclei, elongation of cytoplasmic processes, as well as the tendency to flatness and multipolarity of cells, are typical of differentiated neurons [13]. Moreover, axonal extension is accompanied by an increase in the number and length of microtubules, which in the axon are typically arranged parallel to the axis. Both intrinsic capability to elongate axons, and extrinsic signals, such as serum deprivation, dimethyl sulfoxide, retinoic acid, N^6,2'-O-dibutyryl-adenosine-3',5'-cyclic-mono-phosphate (db-cAMP) regulating the outgrowth at the axonal tip, play a role in cellular differentiation [7,19].

In Sertoli cells, testosterone (T) halts the proliferative phase and upregulates the expression of markers associated with cell maturation [6]. The role of androgens and their derivatives in neural differentiation is still controversial. T and 17-β-estradiol inhibit cell growth and cause cell death at high concentrations with no real differentiating effect in a murine neuroblastoma clone [17]. Moreover, a T analogue, 19-nortestosterone (nandrolone), can have important effects on the proliferation and differentiation as it influences neurogenesis of embryonic neural stem cells [4]. A recent study carried out at our laboratories has revealed that undifferentiated C1300 neuroblastoma cells exposed to T for 96 hrs undergo a weak decrease in proliferation [8]. The first aim of this work is to verify whether a longer exposure to T may induce differentiation in C1300 cells.

Although differences in the response to oxidative agents between undifferentiated and differentiated neural cells have been ascertained, some aspects are still matter of debate. In the N1E neuronal cell line, clonally derived from neuroblastoma cells, treatment with glutamate resulted in decreased viability of differentiated cells [18]. By contrast, an increased resistance to oxidative stress has been associated with db-cAMP-induced differentiation in neuroblastoma-glioma hybrid cells [12].The stimulation of N-methyl-D-aspartate (NMDA) glutamate receptors involved in the production of nitric oxide leads to the formation of the amino acid analogue 3-nitro-L-tyrosine (3NT), which is considered a marker of oxidative processes. Frequent reports in the literature document the incorporation of this oxidant into many cellular and extracellular target proteins such as $p130^{cas}$, α-tubulin and albumin, among others [9,11,16]. In the present study, the possible differences have been evaluated in the vulnerability to the nitrosative action of 3NT between undifferentiated and differentiated neuroblastoma cells. For our purposes, a mouse neuroblastoma cell line (C1300) was employed. Indeed, its capability to differentiate in response to biological agents is well-known, since it has been widely recognized as a good model for the study of the specific changes which occur during cell differentiation [20].

Material and methods

Undifferentiated C1300 cells (*American Type Culture Collection,* Rockville, MD, USA), a mouse neuroblastoma cell line at passage 60, were seeded at a concentration of 5.6×10^5 per ml and grown in phenol-red-free RPMI-1640 medium supplemented with 10% heat inactivated dextran-coated charcoal-stripped newborn calf serum, 2 mM L-glutamine, 100 units/ml penicillin G and 100 μg/ml streptomycin sulphate. Cells were incubated for 6 days at 37 °C in a 5% CO_2 humidified atmosphere according to the following protocols:

i) 1mM db-cAMP (db-cAMP cells);
ii) 280 μM 3NT (3NT cells);
iii) 1 mM db-cAMP + 280 μM 3NT (db-cAMP&3NT cells);
iv) 50 nM T (T cells);
v) 50 nM T + 280 μM 3NT(T &3NT cells).

T was dissolved in absolute ethanol and then diluted in basal medium to the final concentration. To check the possible toxic effects of ethanol, a further control was performed incubating cells for 6 days in medium containing absolute ethanol at the same concentration used to dissolve T (0.0001% v/v).

In order to estimate morphological features, monolayers were observed in the living state under phase-contrast optics and photographed. Pictures were captured in bitmap format and the length of cytoplasmic processes was analyzed using a validated software (*Scion*, Frederick, MD, USA). In details, each sample underwent measurement of the longest cytoplasmic process in 30 cells randomly chosen. The average was calculated from the values obtained.

Cells were suspended in microtubule-stabilizing solution (5 mM TRIS HCl pH 8.0, 2 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride), supplemented with protease inhibitors (Complete-mini, *Roche*, Basel, Switzerland). Cells were homogenized mechanically and then sonicated. Protein content was determined (DC Protein Assay, *BioRad*, Hercules, CA, USA) and aliquots of 15 μg proteins each were fractionated in SDS-PAGE 12% gel and then transferred onto nitrocellulose membranes. Non-specific protein binding was blocked incubating membranes with 0.1% Tween 20, 5% skim milk in PBS. Nitrocellulose membranes were incubated with primary monoclonal antibodies against tyrosinated α-tubulin (TUB-1A2, *Sigma*, St. Louis, MO, USA), acetylated-α-tubulin (611B1, *Sigma*), 3NT (1A6, *Upstate*, Lake Placid, NY, USA), overnight at 4 °C at 1:1 000, 1:500, 1:250, dilution respectively. An anti-mouse IgG-alkaline phosphatase-conjugated antibody (*Sigma*) was then used at 1:1 000 dilution for 1h at room temperature. The immunoreactive bands were visualized by incubating the membrane with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) and heat fixed. Signals were quantified using Scion Image software.

Catalase activity was determined using a method based on the decomposition of hydrogen peroxide.

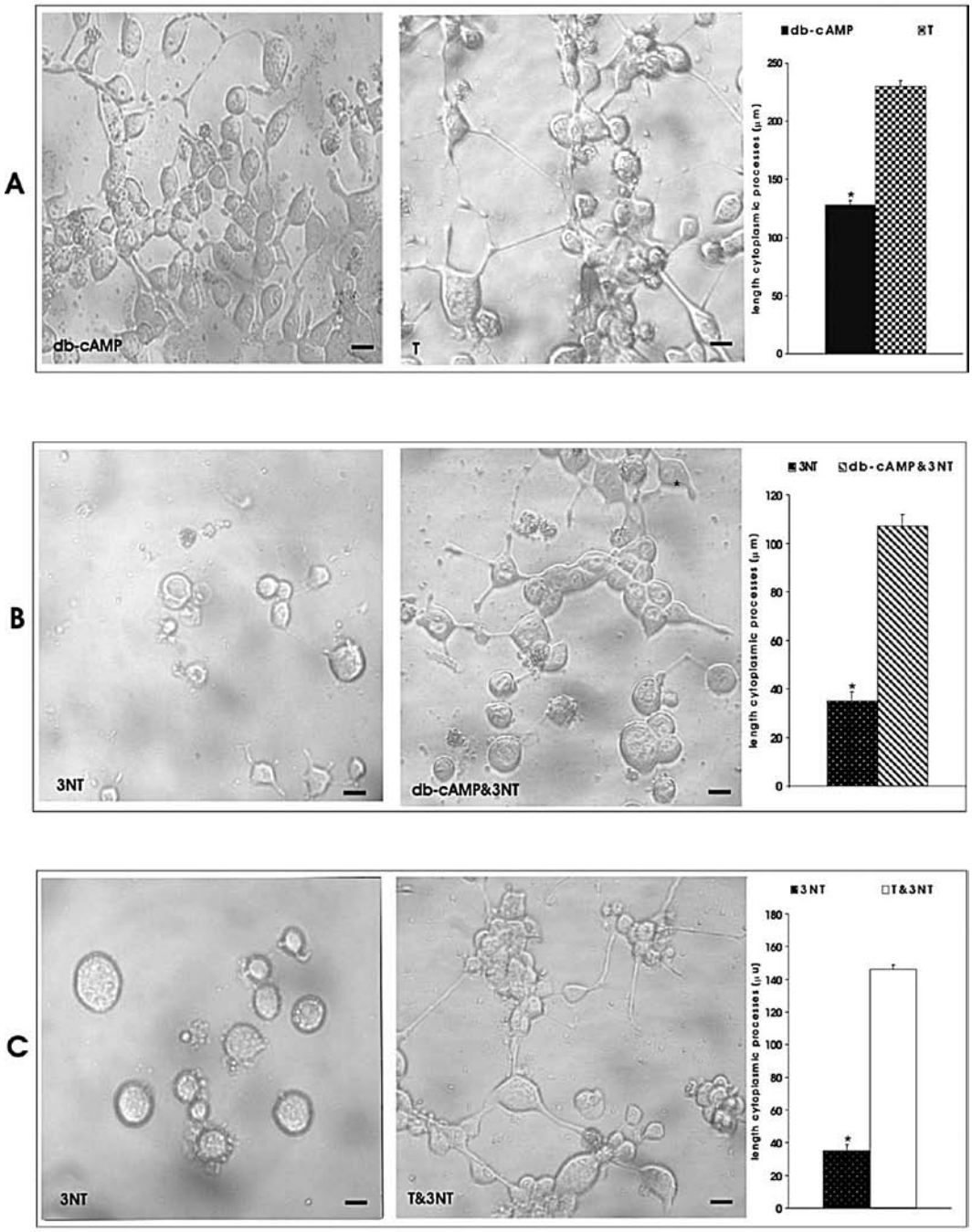

Figure 1. Differentiating effects of T in C1300 cells. T cells exhibit cytoplasmic processes (average length 230 μm) longer than those in db-cAMP cells (128 μm) (**A**). Db-cAMP&3NT cells recover a neuron-like shape with average length of 107 μm vs 35 μm in 3NT cells (**B**). Number and length of cytoplasmic processes (146 μm) are higher in T&3NT cells than in 3NT cells (**C**). Phase contrast optics. Bar = 25 μm. *Significant difference from T (**A**), db-cAMP&3NT (**B**) and T&3NT cells (**C**) respectively. P<0.05.

Ice cold cells were collected, washed and sonicated to rupture cell membranes. The reaction started by adding the sample to 10mM H_2O_2 in PBS. Catalase activity was measured by monitoring the absorbance setting the spectrophotometer at 240nm [1].

All data are expressed as mean ±SD of 5 single experiments. Data were tested by one-way ANOVA or Student's *t* test. A probability of 0.05 or less was considered significant.

Results

Cell morphology

Pictures from both db-cAMP and T cells were similar. Most cells exhibited an irregular shape and extended long branching cytoplasmic processes. However, cytoplasmic processes were longer in T cells (average length 230 μm) than in db-cAMP cells (average length 128 μm), as shown in the diagram of Figure 1A. 3NT cells were scattered displaying a spherical form. Cytoplasmic vacuoles

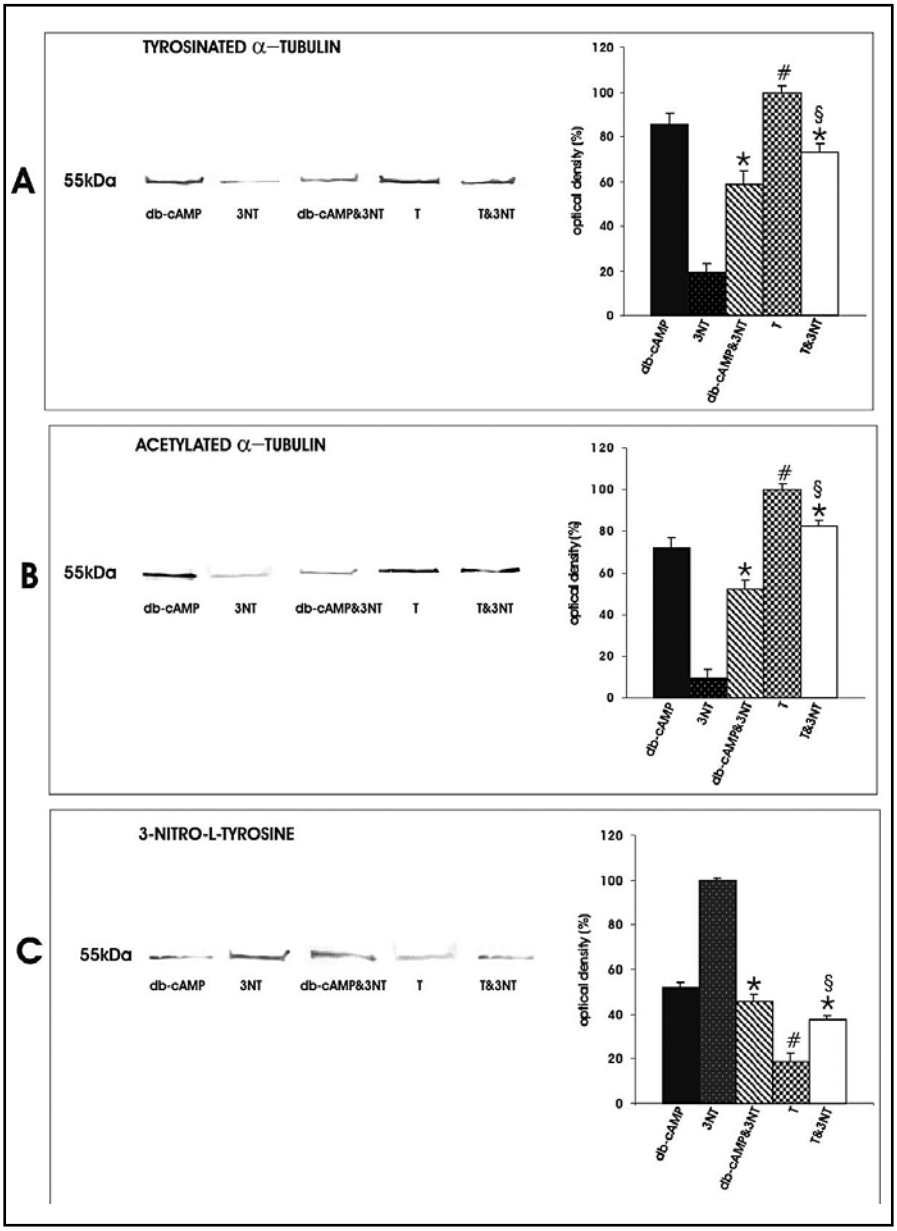

Figure 2. Changes in tubulin expression induced by T. T upregulates tubulin isoforms. In db-cAMP&3NT and mostly in T&3NT cells the expression is markedly higher than 3NT cells (**A, B**). 3NT is incorporated into tubulin. The highest amount is in 3NT cells. Anti-oxidative effects of T (T&3NT cells) are stronger than those of db-cAMP (db-cAMP&3NT cells) (**C**). *Significant difference from 3NT cells. #Significant difference from db-cAMP cells. §Significant difference from db-cAMP&3NT cells. P<0.05.

could be frequently found and most cells lost their cell processes. Only few cells gave rise to short cytoplasmic processes no more than 35 µm in average. Db-cAMP& 3NT cells tended to recover a neuron-like shape showing long cytoplasmic processes with an average length of 107µm (Figure 1B). T&3NT cells increased the number and length of their cytoplasmic processes in comparison with 3NT cells. The morphological features of T&3NT cells resembled those from db-cAMP cells. However, in T&3NT cells the average length of cytoplasmic processes was 146 µm, i.e. higher than that in db-cAMP&3NT cells (Figure 1B,C).

Western blot analysis

Western blot results showed that the anti-tyrosinated and acetylated α-tubulin antibodies produced a band located at approx. 55 kDa. The highest expression of both α-tubulin isoforms was detected in T cells. In db-cAMP&3NT and mostly in T&3NT cells, their expression was markedly higher than in 3NT cells (Figure 2A,B). In addition, the antibody against 3NT revealed a single nitrated protein co-migrating with α-tubulin in all the samples. The band was marked in 3NT cells, whereas it gradually dropped in db-cAMP, db-cAMP&3NT, T&3NT and T cells (Figure 2C).

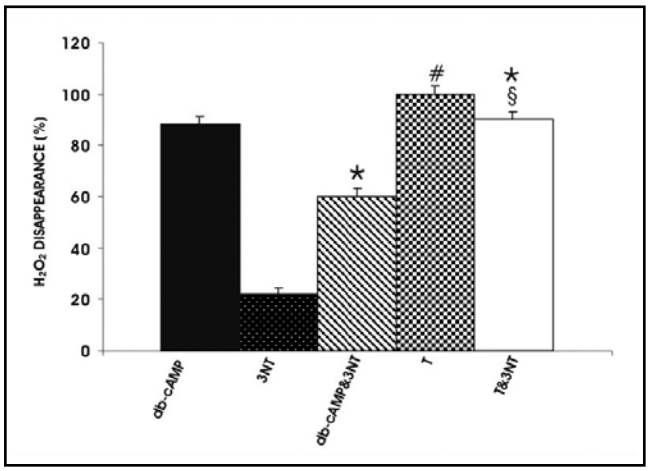

Figure 3. T increases catalase activity in C1300 cells. Catalase activity increases in T cells in comparison with db-cAMP cells. Disappearance of H_2O_2 is more marked in T&3NT cells than in db-cAMP&3NT cells. *Significant difference from 3NT cells. #Significant difference from db-cAMP cells. §Significant difference from db-cAMP&3NT cells. Each column represents the average ±SD of five single experiments. Data are shown as percentage of the maximum value. P<0.05.

Catalase activity assay

As to intracellular catalase activity, the highest levels of H_2O_2 disappearance were ascertained in T cells. Values were lower of about 10% in db-cAMP cells. Moreover, in db-cAMP&3NT and mostly in T&3NT cells catalase activity was enhanced when compared to 3NT cells (Figure 3).

Discussion

It can be assessed that a 6-day exposure to T induces differentiation in C1300 cells. Consistently, neurite outgrowth was observed after a 3-day T treatment in the human neuroblastoma cell line SH-SY5Y [10]. As shown in the diagram of Figure 1A, the length of cytoplasmic processes reaches 128 μm in average after db-cAMP treatment, whereas they hit a 230 μm average length when cells are exposed to T. Therefore, our results suggest, for the first time, that T can play a differentiating effect stronger than db-cAMP, in spite of its widely recognized effectiveness in neuronal differentiation.

In previous studies [8,21], we observed that severe alterations occurred in undifferentiated C1300 cells after a brief exposure (<96 hrs) to 3NT starting from 90 μM concentration. According to the present results, damages induced by nitrosative stress appear to be attenuated after differentiation. Interestingly, cytoplasmic processes of cells differentiated with T (T&3NT cells) are about 40% longer than those extended by db-cAMP&3NT cells. Such results show that not only is T more effective in differentiating cells than db-cAMP but also reduces 3NT effects. On this basis, T could be considered a neuroprotectant against nitrosative stress stronger than a recognized neuroprotective molecule such as db-cAMP [12]. Our results suggest that in C1300 cells, 3NT is incorporated into α-tubulin. As shown in Figure 2C, such attack on this protein is more detectable in undifferentiated than in differentiated neuroblastoma cells. It is noteworthy that when cells are differentiated with T, 3NT incorporation appears to be lower than in db-cAMP cells. Indeed, 3NT immunoreactivity falls of about 10% when the differentiative agent of choise was T rather than db-cAMP. Consistently with what observed in T&3NT cells, the reduced incorporation of 3NT may represent one of the putative mechanisms of T neuroprotective capability. 3NT can substitute the tyrosine residue in the C-terminus of α-tubulin, giving rise to severe alterations in microtubular functions. It is still matter of debate whether such incorporation is irreversible or not [3,9,15].

In addition, T exposure elevates the expression of both the α-tubulin isoforms examined. This upregulation would not be affected by a stimulation of cell proliferation. Indeed, such T-induced tubulin overexpression still occurred when the tubulin content was normalized for histones [5]. The upregulated tubulin expression is consistent with the newly-formed cytoskeletal lattice typical of elongating cell processes, i.e. with concurrent differentiative events.

Catalase is the major enzyme responsible for detoxifying H_2O_2 by converting it to water and represents one of the most effective cellular antioxidant defence systems. Since it has been proposed that 3NT induces the formation of H_2O_2 [14], we postulate that the neuroprotection bestowed by db-cAMP or T on C1300 cells is mediated by the rise in catalase activity. A similar effect of T has been previously observed in cultured cerebellar granule cells exposed to 24hrs H_2O_2, where catalase activity levels were increased 2-fold [2].

In conclusion, not only confirms this work the effects of db-cAMP on differentiation and neuroprotection, but even indicates that T exposure leads to differentiation in C1300 cells and this process matches a significant neuroprotective effect. The present investigation may provide a useful tool in the study of oxidative stress which is known to be involved in the pathogenesis of many neurodisorders. T is suggested, like other substances having antioxidant properties, to be of potential interest in the experimental therapy of neuropathological conditions.

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