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Taxol induces oxidative neuronal cell death by enhancing the activity of NADPH oxidase in mouse cortical cultures

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ABSTRACT

We examined the involvement of oxidative stress in neuronal cell death induced by taxol, a microtubule-stabilizing anti-cancer drug and investigated whether NADPH oxidase plays a role in taxol-induced neuronal cell death in mouse cortical cultures. Cell death was assessed by measuring lactate dehydrogenase in the bathing media after 24-h exposure to taxol. Taxol (30–1000 nM) induced the concentration-dependent neuronal death with apoptotic features. The neuronal death induced by taxol was significantly attenuated not only by anti-apoptotic drugs such as z-VAD-fmk and cycloheximide but also by antioxidants such as trolox, ascorbic acid and tempol. Vinblastine, a microtubule-depolymerizing anti-cancer drug, also induced neuronal death. The neuronal cell death induced by vinblastine was also attenuated by z-VAD-fmk, but not by antioxidants and NADPH oxidase inhibitors. Exposure the cortical cultures to taxol for 80 min formed neurite beadings visualized by fluorescence immunocytochemistry for tubulin. Treatment with either trolox or apocynin, an NADPH oxidase inhibitor, did not affect formation of the neurite beadings. RT-PCR and Western blot analysis revealed that exposure to taxol increased the expression of $p47^{phox}$ and $gp91^{phox}$ and induced translocation of the $p47^{phox}$ to the membrane in cortical cultures. Exposure to taxol markedly increased cellular 2,7-dichlorofluorescin diacetate fluorescence, an indicator for reactive oxygen species. Apocynin and trolox markedly inhibited the taxol-induced increase of the fluorescence. Moreover, treatment with NADPH oxidase inhibitors or suppression of $gp91^{phox}$ by siRNA significantly attenuated the taxol-induced neuronal death. These results indicate that taxol induces oxidative neuronal apoptosis by enhancing the activity of NADPH oxidase.

Taxol has been widely used as an anti-cancer drug for ovarian, breast, lung and prostate cancer [4]. It inhibits the normal function of microtubules, which causes the failure of mitosis and the death of proliferating cells, and also affects other cellular functions such as intracellular signaling, organelle transport and cellular locomotion [8].

Taxol induces apoptosis in cortical neurons by a mechanism distinct from that in non-neuronal cells. In contrast to non-neuronal cells, expression of wild type Bcl-2 in cortical neurons protects against taxol-induced apoptosis [7]. Moreover, taxol induces activation of N-terminal c-Jun protein kinase (JNK) and phosphorylation of Bcl-2 in cancer cells [4], whereas it induces neither JNK activation nor phosphorylation of Bcl-2 in cortical neurons [7].

NADPH oxidase is a superoxide-producing enzyme that consists of membrane ($gp91^{PHOX}$ and $p22^{PHOX}$) and cytosolic ($p47^{PHOX}$, $p67^{PHOX}$, and $p40^{PHOX}$) components [3,5]. Although NADPH oxidase was originally characterized as part of the host defense machinery in phagocytes, all of the subunits of NADPH oxidase are also expressed in cortical neurons [10,15] and are involved in ROS-induced oxidative cell death in some neurodegenerative diseases [16,18].

In the present study, we examined the effects of some antioxidants on neuronal cell death induced by taxol and investigated whether NADPH oxidase plays a role in taxol-induced neuronal death.

4-(2-Aminoethyl)benzenesulfonylfluoride (AEBSF), apocynin, tempol and N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) were obtained from Calbiochem Corporation (San Diego, CA, USA). Fetal bovine serum and horse serum were from Hyclone (Logan, UT, USA). Media for cell culture were purchased from Gibco BRL (Rockville, MD, USA) and other reagents were from Sigma (St Louis, MO, USA).

Mixed cortical cell cultures, containing both neurons and astroglia, were prepared from fetal ICR mice at 14–15 days of

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gestation as described previously [14]. Briefly, dissociated cortical cells were plated onto a previously established astroglial cell monolayer at 3 hemispheres per 24-well plate (Primaria, Falcon, USA) in plating medium (Modified Eagle medium supplemented with 21 mM glucose, 26.5 mM sodium bicarbonate, 2 mM glutamine, 5% fetal bovine serum, and 5% horse serum). Cytosine arabinoside (10 μM) was added 5–6 days after plating to halt the growth of non-neuronal cells.

Treatments with taxol and other drugs were performed at DIV 12–14 in serum-free MEM (minimal essential medium with Earle's salts). The stock solutions of drugs were made at 100× concentration of working solution in appropriate solvent and diluted in MEM immediately before experiments. In addition to morphological assessment under a phase-contrast microscopy, overall neuronal cell injury in mixed cortical cultures was quantitatively assessed by measuring lactate dehydrogenase (LDH) activity released from damaged cells into the bathing medium [11]. Each LDH value, after subtracting the mean background value in sham-washed controls (=0), was scaled to the mean value in positive control cultures treated with 500 μM NMDA for 24 h (=100), which induced near complete neuronal death in the absence of glial damage.

SYTOX Green (Invitrogen, Carlsbad, CA, USA) staining was used for morphological evaluation of nuclei. After fixing the cells with 4% paraformaldehyde for 50–60 min at room temperature, the cells were permeabilized with 0.5% Triton X-100 for 10 min and treated with 1 μM SYTOX Green stock for 15 min.

Changes of microtubule network after exposure to taxol were visualized by fluorescence immunocytochemistry for tubulin. After fixation with 4% paraformaldehyde for 30 min and blocking, cells were labelled with rabbit anti-tubulin antibody (Sigma) at 1:80 dilution at 4 °C overnight. After washes, signals were visualized by Cy3-conjugated affinity purified anti-rabbit IgG (1:200, Jackson ImmunoResearch, West Grove, PA) using epifluorescence microscopy.

Intracellular free radical generation was measured using 2,7-dichlorofluorescin diacetate (DCF) (Invitrogen, Carlsbad, CA, USA) [15]. Briefly, cells were loaded with 10 μM DCF for 30 min and then treated with taxol alone or in combination with apocynin or trolox. After treatments, ROS generation was monitored at 0.5, 1, 2, 4, 6, and 8 h at 37 °C in a fluorescent multiwell plate reader (Fluoroskan Ascent FL, Labsystems) with excitation at 485 nm and emission at 530 nm.

RNA was prepared with TRIZOL and reverse-transcribed into cDNA with random primer and M-MLV reverse transcriptase (Invitrogen). PCR was performed under the following conditions: denaturing for 1 min at 94 °C; annealing for 1 min at

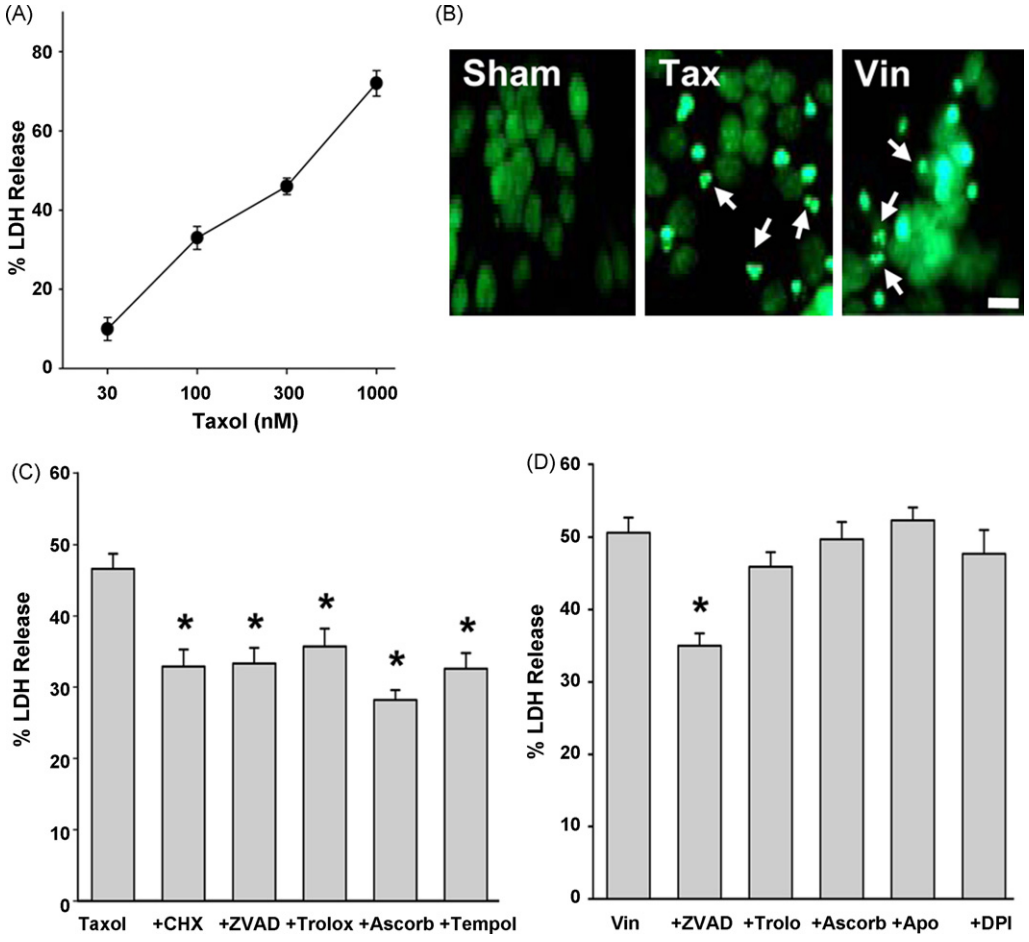

Fig. 1. Taxol-induced concentration-dependent neuronal apoptosis and effect of antioxidants on neuronal cell death induced taxol or vinblastine in mixed cortical cultures. (A) Concentration-dependent neuronal death in mixed cortical cultures. Neuronal death was assessed by LDH release. Each point and bar represents mean ± S.E.M. from 4 to 12 wells. (B) Photomicrographs of Sytox green nuclear staining showing chromatin condensation and nuclear fragmentation (arrows) induced by 8-h exposure to 300 nM taxol (Tax) and 100 nM vinblastine (Vin) in mouse cortical cultures. Sham-wash control (Sham). Calibration bar: 50 μm. (C) Inhibitory effect of 1 μg/mL cycloheximide (+CHX), 100 μM z-VAD-fmk (+ZVAD), 100 μM trolox, 100 μM ascorbic acid, and 5 mM tempol on taxol-induced neuronal death. Each column and bar is the mean ± S.E.M. from 12 to 16 wells. *$P < 0.05$, compared with taxol-treated control group. (D) Effect of 100 μM z-VAD-fmk (+ZVAD), 100 μM trolox, 100 μM ascorbic acid, 500 μM apocynin (+Apo) and 100 nM DPI on the 100 nM vinblastine (Vin)-induced neuronal death in mixed cortical cultures. Each column and bar is the mean ± S.E.M. from 8 to 12 wells. *$P < 0.05$, compared with vinblastine-treated group.