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Tianeptine interferes with microtubule organization and hormone secretion of pheochromocytoma cells

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Abstract

Pheochromocytoma originates from chromaffin cells in the adrenal medulla and sympathetic paraganglia. 36–53% of pheochromocytoma becomes malignant and, thereafter, resistant to conventional treatments. Pheochromocytoma also causes hyper-secretion of catecholamines that cause severe hypertension. We found that an antidepressant, tianeptine, interfered with normal life cycle of pheochromocytoma cells at its clinical doses. Treatment with tianeptine caused microtubule bundling and specific degradation of cytoplasmic dynein, a retrograde microtubule motor that mediates various microtubule-dependent processes during interphase and mitosis, in the rat pheochromocytoma PC12 cells. Tianeptine also increased the levels of pro-apoptotic proteins, slowed cell cycle progression, and increased apoptosis in PC12 cells. Importantly, tianeptine treatment decreased high K⁺-stimulated secretion of norepinephrine and chromogranin A in PC12 cells and of epinephrine in the mouse pheochromocytoma MPC cells. Our study demonstrates, for the first time, that tianeptine interferes with normal life cycle of pheochromocytoma cells.

Keywords

pheochromocytoma; tianeptine; microtubule bundling; cytoplasmic dynein

1. Introduction

Pheochromocytoma is an endocrine tumor arising in the adrenal medulla and paraganglia. 36–53% of pheochromocytomas become malignant with high mortality rates (Amar et al., 2007; Brouwers et al., 2005; King et al., 2011; Timmers et al., 2009). The 5-year survival rate for metastatic pheochromocytomas has been <50% (Ahlman, 2006). Surgery in combination with chemotherapy using cyclophosphamide, vincristine, dacarbazine, anthracycline, etoposide, cisplatin, and sunitinib are used for the treatment of metastatic pheochromocytoma (Adjalle et al., 2009; Huang et al., 2008; Park et al., 2009). If patients have unresectable metastatic pheochromocytoma, ¹³¹I-metaiodobenzylguanidine (MIBG) and somatostatin receptor radionuclide are used as an alternative treatment (Shah et al.,

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2012). However, the prognosis of the above treatments for malignant pheochromocytoma is still poor (Fassnacht et al., 2009; Shah et al., 2012). Thus, it is in great demand to find a better strategy for the treatment of metastatic pheochromocytoma.

Pheochromocytoma also causes hyper-secretion of catecholamines, such as norepinephrine and epinephrine, resulting in development of hypertension (Guerin et al., 2010; Shah et al., 2012), headache, palpitation, anxiety, and sweating, which makes a poor quality of life (Adler et al., 2008). Surgical debulking of pheochromocytoma mass is used to cut down catecholamine hypersecretion (Adler et al., 2008; Shah et al., 2012). Pre-operative and intra-operative pharmacological treatments are also used to prevent a sudden surge of blood pressure caused by excessive catecholamine secretion during surgery (Adler et al., 2008; Shah et al., 2012). Hence, it is desired to suppress or not to invoke hyper-secretion of catecholamines during pheochromocytoma treatment.

During our examination of the neuritogenic effect of an atypical antidepressant, tianeptine, on the rat pheochromocytoma PC12 cells, we have found that tianeptine reduces the survival and norepinephrine secretion of PC12 cells. Tianeptine is already used in clinics as an antidepressant to alleviate the symptoms of mild-to-severe major depression (Lepine et al., 2001; Szadoczky and Furedi, 2002). It has few side effects on autonomic, cardiovascular, attention, sexual, learning and memory functions (Kasper and McEwen, 2008; Wagstaff et al., 2001). Thus, tianeptine should be a safe supplement if it is used for the treatment of pheochromocytoma.

We also found that tianeptine caused specific and rapid reduction of cytoplasmic dynein in pheochromocytoma cells. Cytoplasmic dynein is a retrograde microtubule motor that mediates anchoring of microtubule minus ends to the centrosomes, thereby establishing a radial array of microtubules from the centrosomes to the periphery of cells in interphase (Burakov et al., 2008). Cytoplasmic dynein is also involved in the maintenance of the hierarchy of intracellular structures and the inter-compartmental trafficking of proteins and vesicles (Hirokawa and Takemura, 2004; Schroer, 2004). During mitosis, cytoplasmic dynein mediates attachment of mitotic spindles to the kinetochores on condensed chromatin (Pfarr et al., 1990; Steuer et al., 1990) and to the cell cortex (Busson et al., 1998; O'Connell and Wang, 2000). The dynein-based spindle attachment facilitates movement of chromatin to the mid-plane during prometaphase (Sharp et al., 2000). Therefore, reduction of cytoplasmic dynein in tianeptine-treated pheochromocytoma cells should interfere with the dynein-dependent processes during interphase and mitosis, which may lead to prolonged cell cycle progression and cell death.

Here, we demonstrate how tianeptine, an antidepressant with few side effects, perturbs microtubule organization, increases apoptosis, and decreases norepinephrine secretion in rat pheochromocytoma cells. Tianeptine also has a similar effect on mouse pheochromocytoma cells. Our study shows, for the first time, the anti-tumor properties of tianeptine against pheochromocytoma cells.

2. Materials and methods

2.1. Materials

The antidepressant, (+/-)-tianeptine (>99% pure), was purchased from Tocris Bioscience (Minneapolis, MN) and dissolved at 100 mM in de-ionized H₂O. Nocodazole (>99% pure) was purchased from Sigma (St. Louis, MO) and dissolved at 33.3 mM in DMSO. Paclitaxel (>99.5% pure) was from Cytoskeleton Inc. (Denver, CO) and dissolved at 4 mM in DMSO.

2.2. Cell culture and drug treatment

The rat pheochromocytoma cell line, PC12, was a kind gift from Dr. Y. Peng Loh (NIH). The mouse pheochromocytoma cell line, MPC (Korpershoek et al., 2012), was a kind gift from Dr. Pacak (NIH). Both PC12 (passage 8–10) and MPC (passage 39–40) cells were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS), 5% horse serum (HS), 4.5 g/L D-Glucose, 110 mg/L sodium pyruvate, and 1 x Pen Strep (Life Technologies, Carlsbad, CA). All the cell cultures were incubated at 37°C in a humidified 5% CO₂ incubator. PC12 and MPC cells were treated with tianeptine (1, 10, or 100 µM) or mock (de-ionized water) once for 18 h or twice for 24 h at a 12-h interval. Then, cells were processed for immunoblotting and immunocytochemistry.

Transfection of DNA constructs (pEGFP and pEGFP-dynamin [p50]) was performed using Lipofectamine 2000 according to the manufacturer's protocol (Life Technologies). Briefly, cells were grown in 35-mm dishes to 50% confluency, washed with 1 x PBS, and then DNA (4 µg) + lipofectamine (10 µL) in OPTI-MEM I (Life Technologies). Transfected cells were incubated in DMEM + 10% FBS + 5% HS for 16–18 h prior to immunocytochemistry. Transfection efficiencies of 40–50% were routinely obtained for PC12 cells.

2.3. Immunoblotting and densitometry

PC12 and MPC cells treated with mock (H₂O) or tianeptine were harvested in PMEE buffer (pH 7.0; 35 mM KOH, 35 mM PIPES, 5 mM MgSO₄, 1 mM EGTA, and 0.5 mM EDTA) containing 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (Sigma) and 1 x HaltTM protease inhibitor cocktail (Thermo Scientific, West Palm Beach, FL, 1 mM AEBSF, 0.8 mM aprotinin, 50 µM bestatin, 50 µM E64, 20 µM leupeptin, 10 µM pepstatin A, 5 mM EDTA). Cells were then lysed in PMEE buffer plus 0.5% Igepal CA-630 (Sigma) on ice for 20 min. Cell debris and nucleus were removed by centrifugation for 5 min at 14,500 × g. Proteins (50 µg per well) in the post-nuclear supernatant were separated in NuPAGE 4–11% Bis-Tris protein gels (Life Technologies). Immunoblotting was performed using antibodies to α -tubulin, α -actin (Sigma), p150 (dynactin side arm), dynein intermediate chain (DIC), p53, and p27 (Santa Cruz Biotech, Santa Cruz, CA) as described in (Park et al., 2008). The densitometry of the protein bands on immunoblots was performed using 'Image J' software. Briefly, protein bands on films were selected with boxes to measure integrated band densities using 'Analyze/Gels' menu of Image J. The integrated band densities of loading controls were measured similarly and used to normalize the band densities of different proteins.

2.4. Immunocytochemistry

For microscopic purposes, PC12 cells were cultured on glass coverslips coated with 0.001% poly-L-lysine (Sigma) in PBS. Cells were fixed in 100% methanol for 15 min at –20°C for staining microtubules. Cells were fixed in 3.7% paraformaldehyde in PBS at 37°C and permeabilized in 0.1% Tx-100 in PBS for staining microtubules and large dense core vesicles. For microtubule depolymerization and regrowth assays, Cells were fixed and permeabilized in –20°C methanol prior to immunocytochemistry. After blocking with 2% BSA in T-TBS (TBS + 0.1% Tween 20), cells were immunostained with primary antibodies (α -tubulin [Sigma], lamin-B1 [Santa Cruz], and chromogranin A (CgA) [Dr. Loh, NIH]). MPC cells were also fixed in 100% methanol for 15 min at –20°C for staining microtubules. Bound primary antibodies were detected using their corresponding secondary antibodies conjugated with either Alexa_{488nm} or Alexa_{594nm} (Life Technologies). Non-specific binding of primary and secondary antibodies was reduced by extensive washing. Coverslips were then mounted on glass slides using Fluoromount G (Fisher Scientific, Pittsburgh, PA).

2.5. Confocal microscopy and image analysis

The images of immunostained PC12 and MPC cells were taken using a TCS SP5 multi-photon laser scanning confocal microscope (Leica Microsystems, Bannockburn, IL). The confocal microscope is equipped with conventional solid state, a ti-sapphire tunable multi-photon laser (Coherent, Santa Clara, CA), and acousto optical beam splitter AOBs. Images were taken with 63 x Zeiss alpha plan fluor oil objective (1.4 NA). Metamorph software (Life Technologies) was used to count the number of PC12 cells showing bundled microtubules and aggregated lamin-B1. Metamorph was used to quantify the average intensity of immunostained CgA at the cell periphery within ~300 nm from the plasma membrane of PC12 cells. In order to obtain data objectively, three different persons counted blindly the numbers of cells per condition in three different experiments. In addition, all of the image analyses using Metamorph were performed blindly.

2.6. Microtubule polymerization assay

Microtubule polymerization assay was performed to determine the effect of tianeptine on the *in vitro* microtubule polymerization in the cytosols of PC12 cells. High-speed cell cytosol was prepared using 1 X PMEE buffer plus protease inhibitor cocktail (Park et al., 2008). Endogenous microtubules were polymerized with 1 mM GTP/MgSO₄ plus none, 20 μ M paclitaxel, or 50 μ M tianeptine. The mixture was then incubated for 20 min at 27°C and centrifuged at 161,000 x *g* for 45 min through a double sucrose cushion (12.5%/25%) that prevents non-specific pelleting. This process separates free tubulins in the supernatant from polymerized microtubules at the pellet. α -tubulins in the supernatant and pellet were detected by immunoblotting.

2.7. Fluorescence-activated cell sorting (FACS) analyses

For FACS analyses of cell cycle in tianeptine-treated cells, PC12 cells were starved in serum-free medium plus mock or 1 μ M tianeptine (added every 12 h) for 24 h in order to synchronize the cell cycle of a whole population in G0/G1 phase. Then, the cells were incubated in serum-containing medium plus mock or 1 μ M tianeptine (added every 12 h) for release from G0/G1 phase. At 0, 24, and 48 h after the release, 1×10^6 cells per condition were resuspended in 0.5 mL PBS, fixed in 0.5 mL of 100% ethanol for 1 h at 4°C, and stained with propidium iodide (FxCycle PI/RNase staining solution, Molecular Probes, Eugene, OR) for 30 min in the dark. DNA content was analyzed immediately by a FACSCalibur flow cytometer equipped with CellQuest software (BD Biosciences, San Jose, CA). Cell doublets were separated from single cells in G2/M phase using pulse-width/pulse-area signal. At least 5,000 cells were acquired in a histogram and cell cycle data were analyzed using Modfit LT software (BD Biosciences). Results were expressed as mean $\pm$ standard error of mean (SEM) of three independent experiments.

For FACS analyses of cell death, PC12 cells were treated with mock or 1 μ M tianeptine (added every 12 h) for 48 h. Cells were harvested in 0.5 mL PBS and spun at 1,000 rpm for 3 min. Cell pellet was then resuspended in 250 μ L of PI/RNase staining solution (5 μ g/mL propidium iodide). After 5-min incubation, cells were analyzed by flow cytometry. At least 10,000 cells were acquired in a histogram using CellQuest software. Cells stained by propidium iodide were counted as dead cells. The data were shown as mean percent of dead cells $\pm$ SEM of at least three independent experiments.

2.8. Secretion assay

PC12 cells in six-well plates were grown to about 60% confluency in DMEM + 10% FBS + 5% HS in a 37°C incubator maintained at 5% CO₂. Each well was rinsed twice and then incubated with 0.5 ml DMEM for two 30-min periods for basal secretion. Basal secretion

was collected from individual wells and centrifuged at $1,000 \times g$ for 3 min to remove cell debris, after which 0.5 ml of the supernatant was transferred to a 1.5-ml microtube. The cells were then incubated with 0.5 ml DMEM containing 50 mM KCl/2 mM BaCl₂ for 30 min. Each medium (stimulated) was collected from individual wells. 30 μ L aliquots of basal and stimulated media were loaded into NuPAGE 4–11% Bis-Tris protein gels. Basal secretion of CgA was analyzed by comparing CgA in medium during the first 30-min period *vs.* the second 30-min period in the basal condition. Stimulated secretion of CgA was analyzed by comparing CgA in medium during the second 30-min period in the basal condition *vs.* stimulated condition. The band densities of protein bands on immunoblot were quantified using 'Image J' program as described above. The mean band density $\pm$ SEM was calculated from three independent secretion assays.

2.9. Enzyme-linked immunosorbent assay (ELISA)

In order to examine the effect of tianeptine on the stimulated secretion of norepinephrine and epinephrine, PC12 and MPC cells were treated with mock, 1 or 10 μ M tianeptine for 24 h prior to 30-min stimulation with 50 mM KCl and 2 mM BaCl₂ after basal secretions. The media over stimulated cells were collected for quantification of norepinephrine and epinephrine using an ELISA kit (Abnova, Walnut, CA). Briefly, norepinephrine and epinephrine were extracted from 10 μ L of norepinephrine standards (0, 5, 20, 75, 250, and 1000 ng/mL), epinephrine standards (0, 1, 4, 15, 50, 200 ng/mL), positive controls, and media using a cis-diol-specific affinity gel. Then, 25 μ L of enzyme solution containing catechol-*o*-methyl transferase was mixed with the extractions containing norepinephrine and epinephrine in microtiter plates. After 30-min incubation at room temperature on a shaker, 50 μ L of rabbit antiserum that detects either norepinephrine or epinephrine was added to their corresponding microtiter plates. After 2-h incubation at room temperature on a shaker, the wells of each plate were washed with wash buffer three times. 100 μ L of anti-rabbit IgG tagged with peroxidase was added to each well and the plates were incubated on a shaker for 30 min at room temperature. After the plates were washed with wash buffer three times, 100 μ L of substrate solution containing tetramethylbenzidine was added to each well and the plates were incubated for 30 min at room temperature on a shaker. Finally, 100 μ L of 0.25 M sulfuric acid solution was added to each well to stop the enzymatic reaction of peroxidase. The absorbance of the reactions in the wells was measured at 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). The absorbance at 650 nm was used as a reference wavelength. The standard curves for norepinephrine and epinephrine were generated by plotting the absorbances versus the concentrations of standards. The standard curves were used to calculate the concentrations of norepinephrine and epinephrine in the media over stimulated PC12 and MPC cells. The amounts of norepinephrine and epinephrine were expressed as 'ng/mL.' The results were expressed as mean $\pm$ SEM of at least 3 independent experiments.

2.10. Statistical Analysis

All experiments were replicated at least three times with different batches of cell cultures. Microscopic analysis was performed by students blinded to the experimental setup. Statistical significance between two groups was calculated using unpaired student's *t*-test, with a value of $p < 0.05$ considered statistically significant. Multiple comparisons were done using one-way ANOVA followed by Dunnett's multiple comparisons test (GraphPad Prism software, La Jolla, CA).

3. Results

3.1. Tianeptine causes microtubule bundling in the rat pheochromocytoma PC12 cells

We had examined whether tianeptine had a neuritogenic effect on the rat pheochromocytoma PC12 cells. Cells grown in DMEM containing 10% FBS and 5% HS were treated with mock (H₂O) or different concentrations of tianeptine (1, 10, or 100 μ M) for 18 h. After the treatment, cells were fixed in -20°C methanol for 15 min and processed for immunocytochemistry using an antibody to α -tubulin. In mock-treated cells, $96.3 \pm 1.4\%$ (mean $\pm$ SEM) showed well-spread interphase microtubules (Fig. 1A and I). In contrary to our expectation, cells treated with tianeptine did not form neurites and, instead, became shrunken and rounded. $32.8 \pm 2.3\%$ and $35.7 \pm 1.3\%$ of the cells treated with 1 and 10 μ M tianeptine, respectively, lost normal interphase microtubule networks and showed bundled microtubules at the cell periphery (Fig. 1B, C, and I; $n=3$, $p<0.001$). At higher tianeptine concentration (100 μ M), $88.2 \pm 1.1\%$ of cells showed bundled microtubules and $11.8 \pm 1.1\%$ formed aberrant mitotic spindles, such as single or multiple microtubule aster(s), sparse metaphase spindles and incomplete abscission (Fig. 1D and I). We also treated cells twice with mock, 1, 10, or 100 μ M tianeptine at a 12-h interval to affect the cells that escaped the initial impact of tianeptine because they were at cell-cycle stages insensitive to tianeptine. This double treatment with 1 and 10 μ M tianeptine increased microtubule bundling to $47.2 \pm 2.9\%$ and $58.7 \pm 1.5\%$, respectively (Fig. 1F, G, and J; $n=3$, $p<0.001$). Double treatment with 100 μ M tianeptine caused more massive microtubule bundling with severe abnormal mitotic spindles in $85.9 \pm 6.5\%$ cells (Fig. 1H and J). These results indicate that tianeptine treatment perturbs normal microtubule organization in the pheochromocytoma PC12 cells.

3.2. Tianeptine interferes with the formation of interphase microtubule network in PC12 cells

To examine the effect of tianeptine on the organization process of interphase microtubules, we conducted microtubule re-growth assays using nocodazole (Quintyne et al., 1999). PC12 cells were treated with mock, 1, 10, or 100 μ M tianeptine for 18 h prior to nocodazole treatment (33 μ M) for 2 h at 37°C that induces microtubule depolymerization. Then, nocodazole was washed out from the cells to allow microtubule re-growth. After nocodazole washout, cells were incubated at 37°C for 0, 15, 30, and 60 min, fixed in methanol, and processed for immunocytochemistry using anti- α -tubulin antibody. At 0 min after nocodazole washout, microtubules were completely depolymerized in the cells treated with mock, 1, or 10 μ M tianeptine while those in the cells treated with 100 μ M tianeptine remained in a bundled form (Fig. 2A). At 15 min after nocodazole washout, the cells treated with mock, 1, or 10 μ M tianeptine showed small microtubule asters at the peri-nuclear region while those treated with 100 μ M tianeptine still contained bundled microtubules. At 30 min after nocodazole washout, mock-treated cells recovered normal interphase microtubules while those treated with either 1 or 10 μ M tianeptine formed bundled microtubules. Conversely, the cells treated with 100 μ M tianeptine showed small microtubule asters at the peri-nuclear region without bundled microtubules. At 60 min after nocodazole washout, mock-treated cells restored normal interphase microtubule network while those treated with 1–100 μ M tianeptine did not. The cells treated with 1–100 μ M tianeptine were shrunken with bundled microtubules (Fig. 2A). The number of the cells showing abnormal microtubules after 60-min microtubule regrowth were counted using Metamorph software. $43.3 \pm 3.1\%$ and $61.2 \pm 5.8\%$ (mean $\pm$ SEM) of the cells treated with 1 and 10 μ M tianeptine, respectively, could not restore normal interphase microtubules compared to control ($2.0 \pm 1.2\%$) (Fig. 2B, $n=3$, $p<0.001$). $70.4 \pm 2.7\%$ of the cells treated with 100 μ M tianeptine showed bundled microtubules and the rest of the cells showed aberrant microtubule network (Fig. 2B). These results suggest that tianeptine interferes with the formation of normal interphase microtubule network.

3.3. Bundled microtubules in tianeptine-treated PC12 cells are resistant to cold-induced depolymerization

It is possible that bundled microtubules in tianeptine-treated cells are not as dynamic as those in mock-treated cells. Therefore, we examined whether bundled microtubules in tianeptine-treated cells were easily depolymerized upon incubation on ice (Quintyne et al., 1999). After 18-h treatment with either mock or 100 μ M tianeptine, PC12 cells were incubated on ice for 0, 10, 20, and 30 min prior to fixation in -20°C methanol. Depolymerized microtubules in the fixed cells were visualized using anti- α -tubulin antibody. Free cytosolic tubulins generated by microtubule depolymerization are usually not detected by immunocytochemistry because they are diffused out of cells that are permeabilized by methanol. Mock-treated cells showed a large amount of intact microtubule fibrils in the cytoplasm prior to incubation on ice (Fig. 3A). At 10 min after incubation on ice, there were no microtubule fibrils remaining in the cytoplasm of mock-treated cells while some tubulins were detected at the cell periphery. The overall levels of immunostained tubulins in the cytoplasm were decreased. This should be attributed to loss of free tubulins generated by microtubule depolymerization to outside of cells. At 20 min after incubation on ice, very few microtubules remained in the cytoplasm, suggesting that microtubules in mock-treated cells are completely depolymerized between 10 and 20 min on ice (Fig. 3A). On the other hand, bundled microtubules in tianeptine-treated cells were not depolymerized as quickly as those in mock-treated cells (Fig. 3B). At 0 min on ice, tianeptine-treated cells showed bundled microtubules at the cell periphery while little microtubules were present in the cell center. Bundled microtubules at the cell periphery were not depolymerized after 10-min incubation on ice. Even at 20 min after incubation on ice, large amounts of bundled microtubules still remained at the cell periphery while some were depolymerized. However, 30-min incubation on ice caused almost complete microtubule depolymerization (Fig. 3B). We also quantified the average intensities of microtubules at the periphery of cells treated with mock or 100 μ M tianeptine and then incubated on ice. The average intensities of immunostained microtubules at the periphery of mock-treated cells were decreased from 93.33 ± 3.8 at 0 min to 31.74 ± 6.3 at 10 min after incubation on ice and not restored to normal levels (31.74 ± 6.3 [20 min], 47.46 [30 min], Fig. 3C). On the other hand, the average intensities of bundled microtubules at the cell periphery of tianeptine-treated cells were not decreased during 20-min incubation on ice (94.06 ± 5.4 [0 min], 116 ± 122 [20–30 min]) and later reduced to 73.02 ± 3.6 after 30-min incubation on ice. Bundled microtubules in the cells treated with either 1 or 10 μ M tianeptine were also resistant to cold-induced microtubule depolymerization but to less extents than those with 100 μ M tianeptine (data not shown). These results suggest that bundled microtubules in tianeptine-treated cells are less dynamic than those in mock-treated cells.

3.4. Tianeptine does not stabilize microtubules directly

Formation of cold-resistant microtubules in tianeptine-treated PC12 cells raises a possibility that tianeptine stabilizes polymerized microtubules directly as paclitaxel does. Therefore, we examined the possibility by *in vitro* microtubule polymerization assay (Park et al., 2008). Tubulins in the cytosol (3 mg/ml) of mock-treated cells were polymerized with 1 mM GTP/ MgSO_4 plus none, 20 μ M paclitaxel, or 50 μ M tianeptine at 37°C for 20 min and spun through 12.5%/25% sucrose layers at $161,000 \times g$ at 27°C for 45 min. The supernatant (S) and pellet (P) were processed for immunoblotting using anti- α -tubulin antibody. All of the paclitaxel-treated tubulins were polymerized and appeared in the pellet. On the other hand, addition of tianeptine did not increase the amount of polymerized microtubules in the pellet compared to no treatment ($n = 2$, Fig. 3D), suggesting that tianeptine does not directly stabilize polymerized microtubules.

3.5. Tianeptine causes rapid and specific reduction of cytoplasmic dynein

Given that the formation of aberrant microtubule network was not due to the direct effect of tianeptine on microtubules, we speculated that accumulation of bundled microtubules at the cell periphery in tianeptine-treated cells might be caused by loss of anchoring of microtubule minus ends to the centrosomes. Therefore, we examined the effect of tianeptine on the protein levels of cytoplasmic dynein and its activator, dynactin, that mediate microtubule anchoring to the centrosomes (Quintyne et al., 1999). PC12 cells were treated with mock, 1, 10, or 100 μM tianeptine for 18 h and processed for immunoblotting using antibodies to cytoplasmic dynein (DIC: dynein intermediate chain), dynactin (p150), and α -tubulin. The average band densities of p150 and α -tubulin were not significantly changed in the cells treated with 1–100 μM tianeptine while those of DIC were rapidly decreased by treatment with tianeptine (Fig. 4A–C). The relative densities of the protein bands of DIC to those of α -tubulins were measured using 'Image J' software. Treatment with 1, 10, and 100 μM tianeptine decreased the protein levels of DIC to $58\pm 10\%$, $35\pm 9\%$, and $16\pm 10\%$, respectively, of those in mock-treated cells (Fig. 4B). Thus, tianeptine causes specific and rapid reduction of cytoplasmic dynein, which appears to contribute to dispersion of unanchored microtubules, resulting in accumulation of bundled microtubules at the cell periphery.

In order to confirm that microtubule bundling is caused by inhibition of the function of cytoplasmic dynein in anchoring microtubules to the centrosomes, we performed overexpression study using dynamitin (p50), a subunit of dynactin complex. Overexpression of dynamitin disassembles dynactin complex, thereby inhibiting cytoplasmic dynein (Quintyne et al., 1999). PC12 cells were transfected with GFP tag alone or GFP-tagged dynamitin and stained with anti- α -tubulin antibody. All of the cells over-expressing GFP-dynamitin (p50) showed bundled microtubules (Fig. 4E) while GFP-expressing cells did not (Fig. 4D). Thus, it is clear that inhibition of cytoplasmic dynein in tianeptine-treated cells causes microtubule bundling.

3.6. Tianeptine has a pro-apoptotic effect on PC12 cells

We also found that the nuclear membrane protein, lamin-B1, was aggregated in tianeptine-treated PC12 cells while not in mock-treated cells (Fig. 5A–C), suggesting that tianeptine has a pro-apoptotic effect. Thus, we examined whether tianeptine increased the levels of the pro-apoptotic proteins, p53 and p27. PC12 cells were treated with mock, 1, or 10 μM tianeptine for 18 h and processed to obtain the cytosols for immunoblotting using antibodies to p53 and p27. Treatment with 1 and 10 μM tianeptine increased the protein levels of p53 (1 μM : 1.43 ± 0.13 , 10 μM : 2.5 ± 0.17 [values normalized to control]) (Fig. 5D and E). The protein levels of p27 were increased after treatment with tianeptine (1 μM : 1.89 ± 0.33 , 10 μM : 4.7 ± 2.01 [values normalized to control]) (Fig. 5D and F). Since p53 and p27 are tumor suppressor proteins that slow cell cycle progression (North and Hainaut, 2000; Sgambato et al., 2000), we speculate that the increase of both proteins in tianeptine-treated cells might interfere with cell cycle progression. Thus, we performed FACS analyses to examine the effect of tianeptine on cell cycle progression in PC12 cells. Cells were starved in serum-free medium containing mock or 1 μM tianeptine (added every 12 h) for 24 h to synchronize the cell cycle of a whole population in G0/G1 phase. Then, cells were incubated in serum-containing medium plus mock or 1 μM tianeptine (added every 12 h) for release from G0/G1 phase. Cells were harvested at 0, 24, and 48 h after release from G0/G1 phase prior to FACS analysis. The majority (>60%) of the cells treated with mock or tianeptine were accumulated in G1 phase at 0 h after the release. After 24-h post-release, the percent of cells accumulated in S phase was increased in tianeptine-treated cells compared to mock-treated cells (mock: $39.52\pm 0.02\%$, 1 μM tianeptine: 48.66 ± 0.03 , $p<0.05$ [$n=3$]). After 48-h post-release, there was a significant increase in the percent of tianeptine-treated cells that had

accumulated in G2/M phase compared to those of mock-treated cells (Fig. 5G, mock: $18.3 \pm 3.0\%$, tianeptine: $29.0 \pm 2.0\%$, $p < 0.05$ [$n=3$]). These results suggest that treatment with $1 \mu\text{M}$ tianeptine slows cell cycle progression through S and G2/M phases in PC12 cells. In addition, we examined whether tianeptine caused cell death in PC12 cells because prolonged cell cycle arrest (Mollinedo and Gajate, 2003) and microtubule bundling (Aonuma et al., 2005; Iliev et al., 2009) are reported to cause apoptosis. After 48-h treatment with mock, 1, or $10 \mu\text{M}$ tianeptine, dead cells were stained with propidium iodide (PI)/RNase buffer and identified by FACS. Some mock-treated cells ($23 \pm 2.2\%$) died during the procedures for FACS (Fig. 5H). Compared to mock-treated cells, 48-h treatment with 1 and $10 \mu\text{M}$ tianeptine significantly increased the percentage of dead cells to $38 \pm 5.9\%$ (Fig. 5I) and $43 \pm 1.7\%$, respectively. These results suggest that tianeptine not only slows cell cycle progression but also causes cell death in PC12 cells.

3.7. Tianeptine treatment decreases stimulated secretion of chromogranin A and norepinephrine in PC12 cells

Since bundled microtubules are accumulated beneath the plasma membrane of tianeptine-treated cells, it is possible that the bundled microtubules interfere with access of large dense core vesicles (LDCVs) containing catecholamines and CgA to the plasma membrane during the regulated exocytosis. We examined the possibility by secretion assay (Park et al., 2008). PC12 cells were treated with mock, 1, or $10 \mu\text{M}$ tianeptine for 18 h and incubated for 30 min twice in DMEM to determine basal releases (B1 and B2; B2 is shown as 'B' in Fig. 6A). The basal releases were followed by 30-min stimulation with 50 mM KCl and 2 mM BaCl_2 in DMEM (S in Fig. 6A). The 62-, 68-, and 80-kD forms of CgA were released in a stimulated manner (3.0-fold: compare the lanes B2 and S) in mock-treated cells (Fig. 6A). Although cells treated with $1 \mu\text{M}$ tianeptine showed stimulated secretion (3.1-fold) of three CgA forms, the stimulated secretion of those CgA forms was blocked in cells treated with $10 \mu\text{M}$ tianeptine (Fig. 6A and B).

We also examined the effect of tianeptine on high K^+ -stimulated secretion of norepinephrine and epinephrine by ELISA. PC12 cells were treated with mock, 1 or $10 \mu\text{M}$ tianeptine for 24 h, followed by 30-min stimulation with 50 mM KCl and 2 mM BaCl_2 . Stimulated secretion of norepinephrine was significantly decreased in cells treated with $1 \mu\text{M}$ tianeptine compared to that in mock-treated cells (Fig. 6C: $2.0 \pm 0.36 \text{ ng/mL}$ [mock] vs. $1.08 \pm 0.19 \text{ ng/mL}$ [tianeptine], $p < 0.05$). PC12 cells treated with $10 \mu\text{M}$ tianeptine also showed a slight decrease in norepinephrine secretion (Fig. 6C: $2.0 \pm 0.36 \text{ ng/mL}$ [control] vs. $1.3 \pm 0.23 \text{ ng/mL}$ [$10 \mu\text{M}$ tianeptine], $p = 0.08$). Although PC12 cells secrete lower levels of epinephrine compared to norepinephrine (Byrd et al., 1986), we examined whether tianeptine affected stimulated epinephrine secretion. We could not detect a significant difference in stimulated epinephrine secretion between cells treated with tianeptine and those with mock (Fig. 6D). These results indicate that treatment with 1– $10 \mu\text{M}$ tianeptine does not invoke but decreases stimulated secretion of CgA and norepinephrine in the rat pheochromocytoma PC12 cells.

3.8. Tianeptine treatment decreases CgA-containing LDCVs at the proximity of the plasma membrane

The reduction in hormone secretion in tianeptine-treated cells may be due to bundled microtubules that decrease the localization of hormone-containing LDCVs to the plasma membrane. Therefore, we quantified the average intensity of LDCVs at the cell periphery within $\sim 300 \text{ nm}$ below the plasma membrane (Fig. 7B) after immunostaining PC12 cells with antibodies to α -tubulin and CgA in PC12 cells treated with mock or tianeptine (1 and $10 \mu\text{M}$) (Fig. 7A). Compared to mock-treated cells (17.40 ± 1.69), the average intensity of CgA at the cell periphery of tianeptine-treated cells was reduced to 9.82 ± 0.95 ($1 \mu\text{M}$) and 10.47 ± 1.47 ($10 \mu\text{M}$) (Fig. 7C). Thus, bundled microtubules in tianeptine-treated cells appear

to decrease the localization of CgA-containing LDCVs at the proximity of the plasma membrane.

3.8. Mouse pheochromocytoma MPC cells are affected by tianeptine similarly to PC12 cells

We examined whether tianeptine affected the mouse pheochromocytoma MPC cells similarly to the rat pheochromocytoma PC12 cells. First, we examined the effect of tianeptine on high K^+ -stimulated secretion of norepinephrine and epinephrine in MPC cells. Treatment with 10 μM tianeptine slightly, but not significantly, decreased stimulated secretion of norepinephrine (Fig. 8A: 3.50 ± 0.58 ng/mL [mock], 2.90 ± 0.04 ng/mL [1 μM], and 2.20 ± 0.19 ng/mL [10 μM], $p=0.06$ vs. control). Although the amounts of epinephrine secreted from stimulated MPC cells were lower than those of norepinephrine, 1 and 10 μM tianeptine decreased stimulated epinephrine secretion in MPC cells compared to control (Fig. 8A: 0.59 ± 0.15 ng/mL [mock], 0.26 ± 0.04 ng/mL [1 μM], and 0.18 ± 0.04 ng/mL [10 μM], $p<0.05$ vs. control). These results suggest that tianeptine decreases stimulated secretion of epinephrine in the mouse pheochromocytoma cells.

Then, we examined the protein levels of cytoplasmic dynein (DIC) and p53 in tianeptine-treated MPC cells. Those of p150 (dynactin), α -tubulin, and α -actin were examined as controls. The protein levels of DIC in MPC cells treated with 10 μM tianeptine (0.79 ± 0.13) were lower than those in mock-treated cells (1.31 ± 0.06) (Fig. 8B and C, $p<0.05$, $n=3$). Conversely, the protein levels of p53 in MPC cells treated with 10 μM tianeptine (0.87 ± 0.29) were much higher than mock-treated cells (0.17 ± 0.11) (Fig. 8B and D, $p<0.05$, $n=3$). 1 μM tianeptine decreased and increased DIC and p53, respectively, but to a lesser extent than 10 μM tianeptine (data not shown). There was no significant change in the protein levels of p150 between mock- and tianeptine-treated cells (mock: 0.54 ± 0.04 , tianeptine: 0.39 ± 0.05 , $n=3$) (Fig. 8B and E). Thus, tianeptine has the same effect on cytoplasmic dynein and p53 in the mouse pheochromocytoma MPC cells as in the rat pheochromocytoma PC12 cells. Additionally, we tried to stain microtubules in MPC cells to examine the effect of tianeptine on microtubules in MPC cells, but could not because of an invisible sac around clustered MPC cells. The invisible sac appeared to interfere with the access of antibody to microtubules in the cells within the sac (Fig. 8F).

4. Discussion

Pheochromocytoma is a neuroendocrine tumor that arises from the adrenal medulla and paraganglia. 36–53% of pheochromocytoma becomes malignant. There is currently no cure for malignant pheochromocytoma. Hence, it is of the utmost urgency to develop a better strategy for the treatment of pheochromocytoma. We have discovered that an antidepressant, tianeptine, interferes with the normal life cycle of the rat pheochromocytoma PC12 cells by bundling microtubules, reducing cytoplasmic dynein, slowing cell cycle progression, and increasing apoptosis. In addition, tianeptine decreases stimulated secretion of norepinephrine in PC12 cells and of epinephrine in the mouse pheochromocytoma MPC cells.

The plasma concentrations of tianeptine reach ~ 332 ng/mL (~ 1 μM) with a single 12.5-mg oral dose per day (Edwards et al., 1995). 1–5 μM tianeptine is expected to be reached in the plasma with a single 75-mg oral dose per day on the basis of bioavailability, distribution volume, etc. (Ginestet, 1997; Wagstaff et al., 2001). One case report about tianeptine abuse showed that a woman who took 150 tablets of 12.5 mg per day for 1–2 months did not exhibit severe toxic effects and hepatic damage but some minor side-effects such as nausea, vomiting, constipation, etc (Vandel et al., 1999). After four-day treatment for discontinuation of tianeptine, she returned to normal with minor symptoms like myalgia and cold feeling. The case study suggests that treatment with over-doses (>5 μM) of tianeptine may be tolerable for patients.

Currently, the partition coefficient of tianeptine between the plasma and the adrenal gland is unknown while one study showed that orally administered tianeptine was targeted to the adrenal glands (Dziedzicka-Wasylewska et al., 2002). Male Wistar rats that were orally administered with 10 mg/kg tianeptine twice a day for 14 days showed a significant increase in the expression of Met-enkephalin-encoding mRNA in the adrenal gland but not in other tissues and brain. Thus, orally administered tianeptine can reach the adrenal gland and directly affect the cells in the adrenal gland.

In our study, single treatment with 1 μ M tianeptine caused the formation of bundled microtubules at the cell periphery in ~33% of PC12 cells and double treatments with 1 μ M tianeptine caused the formation of bundled microtubules at the cell periphery in ~47%. Conversely, single treatment with 10 μ M tianeptine caused microtubule bundling in ~36% of the cells and double treatments did so in ~59%. Both single and double treatment with 100 μ M tianeptine caused massive microtubule bundling in 85–88%. Tianeptine-induced microtubule bundling appears to slow down cell cycle progression as the percent of cells paused at M phase was increased after 48-h treatment with tianeptine.

First, we raised a question of how tianeptine interfered with the formation of normal microtubule network and formed bundled microtubules. Tianeptine may freeze microtubules like paclitaxel, thus preventing the formation of normal microtubule network. However, our *in vitro* microtubule polymerization assay shows that tianeptine does not directly stabilize microtubules as paclitaxel does. Therefore, we examined whether tianeptine interfered with the organization process of microtubule network by microtubule re-growth assay. Microtubule nucleation at the centrosomes occurred normally in the cells treated with 1–100 μ M tianeptine. However, tianeptine-treated cells could not restore the normal microtubule network but, instead, formed bundled microtubules in ~43% (1 μ M), ~61% (10 μ M), and ~70% (100 μ M) of the cells during microtubule re-growth. Accordingly, it is clear that tianeptine interferes with the organization process of microtubule network, thus causing microtubule bundling. Reduction of cytoplasmic dynein in tianeptine-treated cells appears to be the major culprit for the formation of bundled microtubules at the cell periphery. Since cytoplasmic dynein is required for anchoring of microtubules to the centrosomes (Quintyne et al., 1999), reduction of cytoplasmic dynein should cause dispersion of unanchored microtubules to the cell periphery. Overexpression study using dynamitin confirmed that loss of functional cytoplasmic dynein led to microtubule bundling.

In addition, the reduction of cytoplasmic dynein should interfere with the survival of PC12 cells because cytoplasmic dynein is required not only for cell cycle progression (Pfarr et al., 1990; Sharp et al., 2000; Steuer et al., 1990) but also for various intracellular processes during interphase (Hirokawa and Takemura, 2004; Schroer, 2004). Moreover, microtubule bundling itself was shown to kill tumor cells. Treatment with the cytolysin pneumolysin, a major virulence factor of *S. pneumoniae*, causes microtubule bundling and apoptosis in neuroblastoma cells (Iliev et al., 2009). Over-expression of the N-terminal domain of cytoplasmic linker associated protein 1 (CLASP1) causes microtubule bundling and apoptosis in the tumor cell line, HeLa (Aonuma et al., 2005). In accordance with our expectation, treatment with 1–10 μ M tianeptine treatment not only increased the levels of the proapoptotic proteins, p53 and p27, and aggregation of lamin-B1 but also caused the death of ~15% of tianeptine-treated cells. Thus, tianeptine treatment triggers apoptosis in pheochromocytoma cells.

An unsettled question was whether tianeptine treatment caused hypersecretion of catecholamines in the adrenal medulla, resulting in lethal hypertensive crisis, because tianeptine facilitates fast turnover of serotonin at synaptic cleft (Uzbekov, 2009). However, the results of two previous studies are not in compliance with this presumption. In 50

healthy human subjects who took a single oral dose (12.5 mg) of tianeptine, the plasma levels of norepinephrine and serotonin were actually decreased to ~50% of those in placebo-treated subjects after 4-h post-administration (Lechin et al., 2006). In male Wistar rats, the oral administration of tianeptine (5 mg/kg) did not increase the plasma levels of norepinephrine but rather decreased those in stressed animals (Trujillo et al., 2009). Consistent with the previous studies, our study showed that tianeptine treatment reduced stimulated secretion of norepinephrine and CgA in PC12 cells. Stimulated epinephrine secretion in PC12 cells was not affected by tianeptine. Our image analysis showed that the localization of CgA-containing LDCVs at the proximity of the plasma membrane was significantly decreased in tianeptine-treated PC12 cells. Bundled microtubules beneath the plasma membrane appear to decrease the access of the LDCVs to the plasma membrane. Thus, in contrast to the presumption, tianeptine treatment does not increase catecholamine secretion.

In addition, we examined whether tianeptine exerted the same effects on another pheochromocytoma cell line, MPC, a mouse pheochromocytoma cells (Korpershoek et al., 2012). Consistent with the results for the rat pheochromocytoma PC12 cells, tianeptine treatment decreased cytoplasmic dynein and increased p53 in MPC cells. Tianeptine also reduced stimulated secretion of epinephrine without affecting norepinephrine secretion. Thus, tianeptine has similar inhibitory effects on both rat and mouse pheochromocytoma cells.

We also found that the negative effects of tianeptine on microtubule organization and cytoplasmic dynein were specific to pheochromocytoma cells. We treated AtT20 cells (anterior pituitary tumor cells), HeLa cells (cervical cancer cells), and primary mouse neurons with 100 μ M tianeptine but could not detect abnormal microtubule organization and degradation of cytoplasmic dynein in those cells (data not shown). It indicates that tianeptine specifically affects pheochromocytoma cells. We still need to examine whether other types of tumor cells are affected by tianeptine or not to confirm the specificity of tianeptine to pheochromocytoma cells.

In closing, we discovered, for the first time, anti-tumor properties of tianeptine against pheochromocytoma cells. Tianeptine causes microtubule bundling, reduces cytoplasmic dynein and increases p53, which slows cell cycle progression and causes apoptosis. Moreover, tianeptine does not increase catecholamine secretion, which quells the worry about hypertension crisis that might occur during tianeptine treatment. Given that tianeptine is well tolerated and has few side effects on autonomic, cardiovascular, attention, sexual, learning and memory functions (Kasper and McEwen, 2008; Wagstaff et al., 2001), it may be a good supplement to chemotherapy of pheochromocytoma for which there is currently no cure.

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Highlights

- Tianeptine causes microtubule bundling in pheochromocytoma cells.
- Tianeptine causes rapid and specific degradation of cytoplasmic dynein.
- Tianeptine causes apoptosis in pheochromocytoma cells.
- Tianeptine decreases norepinephrine secretion in rat pheochromocytoma cells.
- Tianeptine decreases epinephrine secretion in mouse pheochromocytoma cells.

Fig. 1.

Tianeptine causes microtubule bundling in PC12 cells. (A–D) PC12 cells were treated with mock (A), 1 μM (B), 10 μM (C), or 100 μM (D) of tianeptine for 18 h and immunostained using anti- α -tubulin antibody. Scale bars = 10 μm. (E–H) PC12 cells were treated with mock (E), 1 μM (F), 10 μM (G), or 100 μM (H) of tianeptine twice at a 12-h interval for 24 h and immunostained using anti- α -tubulin antibody. Scale bars = 10 μm. (I & J) Bar graphs showing the average percents of cells showing bundled microtubules after 18-h × 1 (I) and 12-h × 2 (J) tianeptine treatment. Three independent experiments (80 cells per experiment) were performed to obtain the mean percent of cells and standard error of mean (SEM). * $p < 0.001$ compared to control.

Fig. 2.

Tianeptine interferes with the re-formation of interphase microtubule network. (A) PC12 cells were treated with mock, 1, 10, or 100 μ M of tianeptine for 18 h prior to nocodazole (33 μ M) treatment for 2 h at 37°C. After nocodazole was washed out, cells were incubated in nocodazole-free medium at 37°C for 0–60 min to induce microtubule regrowth. Microtubules were stained using anti- α -tubulin antibody. Scale bars = 10 μ m. (B) Bar graphs showing the average percents of cells with bundled microtubules at 60 min after nocodazole washout. Bar graphs represent mean $\pm$ SEM of three independent experiments (* $p < 0.001$ compared to control).

Fig. 3.

Bundled microtubules in tianeptine-treated cells are resistant to cold-induced depolymerization. PC12 cells treated with mock (A) or 100 μ M tianeptine (B) for 18 h were incubated on ice for 0–30 min. Microtubules were stained using anti- α -tubulin antibody. Scale bars = 5 μ m. (C) The average intensities of immunostained microtubules at the cell periphery were quantified using Metamorph software. Results represent mean $\pm$ SEM of three independent experiments (* p <0.001). (D) Microtubules were polymerized in the cell cytosols added with mock, 20 μ M paclitaxel, or 50 μ M tianeptine. Polymerized microtubules were pelleted and free tubulins remained in the supernatant after ultracentrifugation. Tubulins in the pellet (P) and supernatant (S) were detected by immunoblotting.

Fig. 4.

Tianeptine causes rapid and specific reduction of cytoplasmic dynein. PC12 cells were treated with mock, 1, 10, or 100 μM tianeptine for 18 h. Cell cytosols were processed for immunoblotting using antibodies to dynein intermediate chain (DIC), dynactin side arm (p150), and anti- α -tubulin (A). The band densities of cytoplasmic dynein (DIC) and dynactin (p150) on immunoblots were quantified using Image J software. Data represents the relative levels of cytoplasmic dynein (B) and dynactin (C) to α -tubulin. Results represent mean $\pm$ SEM of three independent experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (D–E) PC12 cells transfected with GFP tag alone (D) or GFP-tagged dynamin (p50) (E) were immunostained with anti- α -tubulin antibody. Scale bars = 5 μm .

Fig. 5.

Tianeptide has a pro-apoptotic effect on PC12 cells. PC12 cells treated with none (A) or 100 μ M tianeptide (B) were permeabilized with 0.1% Tx-100 and immunostained with anti-lamin-B1 antibody. Scale bar = 5 μ m. (C) Bar graphs showing the average percent ($\pm$ SEM) of cells with lamin-B1 aggregation. (D–F) PC12 cells were treated with mock, 1, or 10 μ M tianeptide for 18 h and processed for immunoblotting using anti-p53, anti-p27, and anti- α -tubulin antibodies. The protein levels of p53 and p27 were quantified using Image J software. Bar graphs represent the relative levels of p53 (E) and p27 (F) to α -tubulin. (G) Bar graphs show the percents of PC12 cells treated with mock or 1 μ M tianeptide in G2/M phase after 48-h post-release. (H, I) The percentage of dead cells in PC12 cells treated with mock or 1 μ M tianeptide for 48 h was quantified by FACS. Histograms show the numbers of cells positive for propidium iodide (PI) staining. Cells stained with PI were counted as dead cells. All of the above experiments were repeated three times and the results were presented as mean $\pm$ SEM (* $p < 0.05$, ** $p < 0.01$).

Fig. 6.

Tianeptine treatment decreases stimulated secretion of chromogranin A (CgA) and norepinephrine. PC12 cells were treated with mock, 1, or 10 μM tianeptine for 18 h and incubated in serum-free DMEM for 30 min twice for basal releases (B). The basal releases were followed by 30-min stimulation with DMEM containing 50 mM KCl and 2 mM BaCl_2 (S: stimulated secretion). (A) Representative immunoblots show basal and stimulated CgA secretion in cells treated with mock, 1, or 10 μM tianeptine. CgA levels in basal and stimulated secretions were quantified using densitometry (Image J software). (B) Bar graphs show the average fold changes in stimulated CgA secretion in mock, 1, or 10 μM tianeptine-treated cells. (C, D) PC12 cells treated with mock, 1 or 10 μM tianeptine were examined for stimulated secretion of norepinephrine and epinephrine using ELISA assay. Bar graphs represent the amounts of norepinephrine (C) and epinephrine (D) in stimulated secretions. The results are expressed as mean $\pm$ SEM of three independent experiments (* $p < 0.05$).

Fig. 7.

Tianeptine treatment decreases the localization of CgA-containing vesicles to the proximity of the plasma membrane. PC12 cells were treated with mock, 1 or 10 μ M tianeptine for 24 h and immunostained with antibodies to α -tubulin and chromogranin A (CgA). (A) Representative confocal images of α -tubulin (red) and CgA (green) in cells treated with mock, 1, or 10 μ M tianeptine. Scale bar = 10 μ m. The average intensity of CgA staining within ~300 nm from the plasma membrane (PM) was measured using Metamorph software (B). (C) The bar graphs show the average intensities $\pm$ SEM of CgA at the PM (~100 cells per condition in three independent experiments, * $p < 0.05$).

Fig. 8.

Tianeptine decreases epinephrine secretion and cytoplasmic dynein and increases p53 in MPC cells. (A) Mouse pheochromocytoma MPC cells treated with mock, 1 or 10 μ M tianeptine were examined for stimulated secretion of norepinephrine and epinephrine using ELISA assay. Bar graphs show the amounts (mean $\pm$ SEM) of norepinephrine and epinephrine in stimulated secretion in three independent experiments (* $p < 0.05$). (B) MPC cells were treated with mock, 1, or 10 μ M tianeptine for 24 h and processed for immunoblotting using anti-dynein (DIC), anti-p53, anti-dynactin (p150), anti- β -actin, and anti- α -tubulin antibodies. β -actin and α -tubulin were examined as loading controls. The protein levels were quantified using Image J software (C–E). Bar graphs represent the relative protein levels of DIC (C), p53 (D) and p150 (E) to α -tubulin. The data were expressed as mean $\pm$ SEM of the relative protein levels in three different experiments (* $p < 0.05$). (F) MPC cells were fixed in -20°C methanol and immunostained with α -tubulin antibody. A representative image of clustered MPC cells (D.I.C.: differential interference contrast; α -tubulin staining on the right).