NeuroToxicology 37 (2013) 51–62

Contents lists available at SciVerse ScienceDirect

NeuroToxicology

Trichostatin A protects against cisplatin-induced ototoxicity by regulating expression of genes related to apoptosis and synaptic function

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ARTICLE INFO

Article history:
Received 4 October 2012
Accepted 18 March 2013
Available online 1 April 2013

Keywords:
Ototoxicity
Trichostatin A
Cochlea
Cisplatin
Global expression profile microarray analysis

ABSTRACT

Objective: Although inhibition of histone deacetylases (HDACs) has been shown to protect against cisplatin-induced hearing loss, the underlying mechanism is still poorly understood. In the present study, we aim to investigate the protective effect of trichostatin A (TSA), a specific inhibitor of HDACs, on cisplatin-induced ototoxicity and to determine the differentially expressed genes involved in this process.

Methods: The basilar membrane of the cochlea was isolated from 3-day newborn Wistar rats. Organotypic cultures were treated with 150 μM cisplatin or 200 nM TSA. For combination treatment, cells were pre-incubated with TSA for 1 h, followed by TSA plus cisplatin treatment. Rhodamine-phalloidin staining was used to label hair cells, and immunocytochemistry with an anti-neurofilament-200 antibody was applied to label spiral ganglion neurons (SGNs). Global expression profile microarray analysis was used to identify differentially expressed genes. Molecular function and signal pathway analysis were performed using a protein analysis through evolutionary relationships (PANTHER) classification system. Real-time quantitative PCR (qPCR) was carried out for data validation.

Results: Severe loss of hair cells and SGNs occurred after 48 h of cisplatin incubation, while TSA significantly increased the number of hair cells and SGNs in the combination treatment group ($P < 0.05$). Compared with control, expression of 71 genes were up-regulated and 383 genes were down-regulated upon cisplatin treatment. Addition of TSA induced the up-regulation of 1387 genes and down-regulation of 1226 genes as compared with cisplatin administration alone. After cisplatin treatment, we observed significant down-regulation of mRNA for several genes related to synaptic function genes, including Camk2a, Camk2b, Vglut1, Snap25 and Rab3b, whereas pretreatment with TSA elevated mRNA levels of these genes. TSA greatly decreased expression of genes related to the calcium signaling pathway (Capn1 and Capn2) and apoptosis signaling pathway (Tnfrsf1a and Tp53), while addition of TSA significantly reduced levels of Tnfrsf1a and Tp53 compared with cisplatin alone ($P < 0.01$).

Conclusions: Our results suggested that TSA might protect against cisplatin-induced ototoxicity via mediating expression of genes responsible for regulating apoptosis, intracellular calcium homeostasis, neurotransmitter synthesis and release, and synaptic plasticity.

1. Introduction

Although cisplatin has been demonstrated to be an effective and widely used chemotherapeutic agent for patients with malignant disease, severe toxic side-effects such as ototoxicity occur frequently after cisplatin therapy (Rybak et al., 2007). Audiometric studies have shown that elevated hearing thresholds, which may be associated with tinnitus and hearing loss, were detected in 75–100% of patients treated with cisplatin (McKeage, 1995). Combinations of neuroprotective drugs, anti-oxidants or anti-apoptosis agents have been used for ameliorating ototoxicity, but the clinical outcomes are still disappointing (Rybak et al., 2007). Strategies to eliminate ototoxicity without interfering with the desired therapeutic effects of cisplatin are urgently needed.

Cisplatin-induced ototoxicity occurs mainly in the cochlea, especially in the outer hair cells (OHCs) of the organ of Corti (Ramirez-Camacho et al., 2004). The stria vascularis and the spiral ganglion are also major targets of cisplatin-induced toxicity in the

Abbreviations: CaMK, calcium/calmodulin-dependent protein kinase; HDAC, histone deacetylase; PBS, phosphate-buffered saline; PANTHER, Protein ANalysis THrough Evolutionary Relationships; qPCR, quantitative PCR; SGNs, spiral ganglion neurons; SNAP, synaptosomal-associated protein; TGF-β, transforming growth factor beta; TSA, trichostatin A; VGLUT1, vesicular glutamate transporter 1.

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http://dx.doi.org/10.1016/j.neuro.2013.03.007

cochlea (Hamers et al., 2003). Apoptosis contributes to cochlear cell loss following exposure to an ototoxic level of cisplatin. Additionally, cisplatin activates apoptotic cell death by inducing the activation of caspase-3, caspase-8 and caspase-9, triggering the release of cytochrome *c*, reducing the expression of Bcl-2, and increasing the expression of Bax (Jeong et al., 2007; Garcia-Berrocal et al., 2007). Moreover, cisplatin generates reactive oxygen species (ROS) and induces calcium influx, which contributes to ototoxicity (Rybak et al., 1999). However, all the mechanisms underlying cisplatin-induced ototoxicity have not been fully elucidated.

Histone deacetylases (HDACs) play key roles in mediating gene expression by post-translational modification of chromatin (Marks et al., 2003). A group of small-molecule HDAC inhibitors (HDACi) exist (Marks et al., 2003), among which trichostatin A (TSA) has been recognized as an efficient and specific HDACi (Codd et al., 2009). Emerging evidence, including the promotion of neuronal differentiation (Hsieh et al., 2004), reduction of neuron loss (Gardian et al., 2005) and suppression of apoptotic or spontaneous neural cell death (Jeong et al., 2003; Kanai et al., 2004), show the neuroprotective potential of HDACi. Furthermore, Drottar et al. revealed that the HDACi sodium butyrate almost completely protected cisplatin-induced hearing loss in guinea pigs (Drottar et al., 2006). In addition, Chen et al. found that the application of 0.2 mM gentamicin to organotypic cultures of the mouse organ of Corti significantly decreased the acetylation of histone core proteins (H2A Ack5, H2B Ack12, H3 Ack9, and H4 Ack8) (Chen et al., 2009). After 8 h of incubation with gentamicin, the protein expression levels of HDAC1, HDAC3 and HDAC4 were clearly elevated. Notably, administration of 200 nM TSA protected against gentamicin-induced loss of inner and outer hair cells. Although the mechanisms involved remain unclear, these reports suggest a potential clinical benefit for application of HDACi during cisplatin chemotherapy.

In this present study, we investigated the protective effect of TSA on cisplatin-induced ototoxicity and used a global expression profile microarray technique to identify several differentially expressed genes that may be involved. We found that TSA might have prevented cisplatin-induced ototoxicity by regulating the expression of genes responsible for apoptosis, intracellular calcium homeostasis, neurotransmitter synthesis and release, and synaptic plasticity.

2. Materials and methods

2.1. Reagents

TSA, rhodamine-phalloidin, and cisplatin were purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS), DMEM/F12 culture medium, and Alexa Fluor 555-labeled goat anti-mouse IgG were obtained from Invitrogen (Carlsbad, CA). Anti-neurofilament-200 primary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). RNA isolation kits and Real-Time qPCR Universal Kits were purchased from CapitalBio (Beijing, China). NimbleGen Gene Expression 12×135K Arrays were purchased from Roche NimbleGen (Madison, WI)

2.2. Cochlear organotypic explant culture and experimental assignment

A total of 64 three-day-old Wistar rats (male and female) were used in this study. Each independent experiment used 16 rats, and experiments were performed in quadruplicate. Isolation of the basilar membrane of the cochlea in neonatal rats and cochlear hair cell culture was carried out as previously described (Ding et al., 2002). Briefly, after being cleaned with 75% ethanol, rats were decapitated and the cochlea, utricle, and semicircular canals were carefully dissected. Soft tissues and cartilage were removed, and the basilar membrane of the cochlea was isolated by dissecting away other structures. The organotypic cultures were maintained in DMEM/F12 culture medium supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2. After 24 h, culture medium was removed and replaced with media containing the drug treatment. Cochlear explants were randomly divided into four groups: control (without drug treatment), cisplatin (150 μM), TSA (200 nM), or cisplatin plus TSA combination (150 μM cisplatin plus 200 nM TSA). For the combination treatment, cochlear explants were pre-incubated with TSA for 1 h, followed by TSA plus cisplatin treatment. All efforts were made to minimize animal suffering and to reduce the number of animals used. Furthermore, all animal procedures were approved by the Jilin University Animal Case and Use Committee, which is certified by the Chinese Association of Accreditation of Laboratory Animal Care.

2.3. Rhodamine-phalloidin labeling

Forty-eight hours after drug treatment, cochlear explants were fixed with 4% paraformaldehyde (PFA) for 30 min. After rinsing with 0.1 mM phosphate-buffered saline (PBS), cochlear explants were treated with 0.25% Triton X-100 for 5 min. Cochlear explants were treated with 200× rhodamine-phalloidin diluted in 1 mL of PBS for 30 min at room temperature in the dark to label hair cell stereocilia. After double-staining with Hoechst 33342, samples were mounted in glycerol. Fluorescence was detected with a confocal scanning microscope (FluoView FV1000, Olympus, Japan). Ten fields were randomly selected from the apical to the basal turns of the cochlea, and the number of hair cells in each field was counted. Each experiment was carried out in quadruplicate.

2.4. Immunocytochemical analysis

Each independent experiment used eight rats, and experiments were performed in quadruplicate. Three or four cochlear samples were used for each group. For immunostaining of neurofilament-200, 4% PFA-fixed, Triton X-100-treated cells were blocked with 5% goat serum for 1 h. Samples were incubated with a mouse anti-neurofilament-200 primary antibody at a dilution of 1:200 overnight at 4 °C. After three washes in PBS, cells were incubated with an Alexa Fluor 555-labeled goat anti-mouse IgG secondary antibody (1:400 dilution) for 1 h at room temperature in the dark. After PBS washing, cells were mounted with glycerol. Negative controls, in which serum was substituted for the primary antibody, were used to examine antibody specificity. The immunofluorescence was evaluated with a confocal scanning microscope (FluoView FV1000; Olympus, Japan) and the percentage of neurofilament-200-positive SGNs per unit area (0.08 mm^2) was calculated.

2.5. Gene microarray and data analysis

For gene microarray analysis, cochlear explants treated for 6 h with cisplatin or cisplatin plus TSA were used and compared with cochlear explants without drug treatment or treated with TSA alone. Total RNAs were harvested using Trizol (Invitrogen) and the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After confirming RNA concentration and purity with a Nanodrop ND-1000 and denaturing gel electrophoresis, respectively, the samples were amplified and labeled using a NimbleGen one-color DNA labeling kit and hybridized with the NimbleGen Hybridization System. Then, the processed slides were scanned with an Axon GenePix 4000B microarray scanner (Axon Instruments; Union City, CA). Raw data were extracted as pair files by NimbleScan software (version 2.5), which offers quantile