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Research paper

Proteomic analysis of cisplatin-induced cochlear damage: Methods and early changes in protein expression

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Abstract

To identify early changes in protein expression associated with cisplatin ototoxicity, we used two dimensional-difference gel electrophoresis (2D-DIGE) and matrix-assisted laser desorption-time-of-flight (MALDI-TOF) mass spectrometry to analyze proteins from P3 rat cochleae that were cultured for 3 h with or without 1 mM cisplatin. Replicate analysis of fluorescent images from six gels revealed significant ($p < 0.01$) cisplatin-induced changes (greater than 1.5-fold) in expression of 22 cochlear proteins. These include increases in the expression of five proteins, four of which were identified as nucleobindin 1, a nuclear calcium signaling and homeostasis protein (2.1-fold), heterogeneous nuclear ribonucleoprotein C, an RNA processing protein (1.8-fold), a 55 kDa protein that is either endothelial differentiation-related factor 1 or alpha-6 tubulin (1.7-fold), and calreticulin, a calcium binding chaperone of the endoplasmic reticulum (ER, 1.6-fold). The expression of 17 proteins was significantly ($p < 0.01$) decreased by greater than 1.5-fold. These include ribonuclease/angiogenin inhibitor 1 (1.6-fold), RAS-like, family 12 (predicted), ras association (RalGDS/AF-6) domain family 5 (4.5-fold), homologous the RAS family of GTPase signaling proteins (2.4-fold), and Protein tyrosine phosphatase domain containing 1 (predicted, 6.1-fold). We identified seven cochlear proteins with either smaller (1.2–1.5-fold) or less significant ($p < 0.05$) cisplatin-induced changes in expression. Notably, heat shock 70 kDa protein 5 (Hspa5, Grp78, and BiP), an ER chaperone protein involved in stress response, decreased 1.7-fold. We observed changes consistent with phosphorylation in the level of isoforms of another ER stress-induced protein, glucose-regulated protein Grp58. Changes in cisplatin-induced protein expression are discussed with respect to known or hypothesized functions of the identified proteins.
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1. Introduction

Cisplatin is one of the most effective drugs for treatment of solid tumors (Siddik, 2003). Because its use is limited by ototoxic and nephrotoxic side effects (Rybak et al., 2005; Taguchi et al., 2005), there has been a large effort to develop safer analogs (Abu-Surrah et al., 2006). The first of these to gain clinical usage, carboplatin, is generally less toxic and can be used to replace cisplatin in the treatment of some, but not all cancers. Even since the introduction of a third derivative, oxaliplatin, cisplatin remains a first choice for treating a wide variety of tumors in spite of its toxic side effects (Lokich, 2001). Thus, it is important to understand the toxic effects on a molecular level in order to improve the preservation of hearing and kidney function during treatment.

DNA is considered the primary target of platinum-based antineoplastic drugs. DNA damage results from

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the formation of adducts consisting mostly of intra-strand crosslinks. DNA adducts, in turn, lead to an arrest of the cell cycle by blocking DNA synthesis, inhibiting of RNA transcription at the site of the adduct, and inducing pro-apoptotic genes. Pro-survival genes may also be upregulated. These include genes encoding chaperone proteins to restore folding of damaged proteins, DNA damage repair enzymes to repair sites of lesion and antioxidant enzymes to inactivate ensuing reactive oxygen species (ROS). The state of the cell is then in a delicate balance. If the cisplatin-induced damage is low, the cell may repair the damage and acquire a cisplatin resistance. If the damage is great enough, the apoptotic machinery may overcome the survival response (Siddik, 2003). Interestingly, some cancers, like colon carcinomas, are resistant to cisplatin and carboplatin, yet may be treated with oxaliplatin. The differential molecular details are not yet understood.

While apoptosis is definitely a beneficial effect for tumor treatment, some otherwise healthy cells, like renal tubule cells and outer hair cells are also particularly sensitive to cisplatin-induced apoptosis. Indeed, hair cells can be protected from cisplatin by inhibitors of proteolytic caspase activity (Liu et al., 1998). In the cochlea, damage may also be incurred by inner hair cells, supporting cells, spiral ganglion neurons and cells of the stria vascularis (Anniko et al., 1986; Comis et al., 1986; Hinojosa et al., 1995; Laurell et al., 1991; Schweitzer, 1993; Stadnicki et al., 1975).

Cisplatin treatment is expected to induce both decreases and increases in the expression of cochlear proteins. Reductions in protein expression are expected to result from inhibition of the synthetic pathway by formation of DNA adducts and from activation of pro-apoptotic protease activity. Selective upregulation of other proteins, including stress response proteins and those which mediate cell death pathways is expected from signaling initiating pro-survival and pro-apoptotic responses. In an effort to define the cochlea's response to cisplatin, here, we report the application of proteomic methodology to identify proteins whose expression is altered by cisplatin in a cochlear organotypic model system. The characterization of these proteins should lead to a better understanding of cellular response to cisplatin and eventually to new therapeutic strategies.

2. Methods

2.1. Animals

Primary cultures were prepared from postnatal day 3 (P3) Fisher-344 rats (Charles River, Wilmington, MA, USA) from untimed pregnant mothers as described in our previous reports (Corbacella et al., 2004; Ding et al., 2002; McFadden et al., 2003; Zhang et al., 2003; Zhu et al., 2003). Experiments were performed according to the rules and regulations of the Institutional Animal Care and Use Committee of the State University of New York at Buffalo and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Culture methods and cisplatin incubation

All cell culture reagents were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO). Cochleae from four litters (an average of 7 pups per litter) were dissected in a sterile hood. The spiral ligament and stria vascularis were removed first. Then, the spiral ganglion and basilar membrane containing spiral limbus, organ of Corti and Hensen's cells were removed together and incubated at 37 °C, 5% CO_2 for 3 h with or without 1 mM cisplatin in 35 mm polystyrene dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ). Cultures were placed in serum-free medium consisting of Basal Medium Eagle, 1% (w/v) bovine serum albumin, 5 mg/ml glucose, 2 mM glutamine, 100 U/ml penicillin G (Sigma P3414) and supplemented with 5 μg/ml bovine pancreas insulin, 5 μg/ml human transferrin, 5 ng/ml sodium selenite (Sigma I-1884).

2.3. Analytical 2 D-DIGE

Two-dimensional difference gel electrophoresis (2D-DIGE) is a relatively new methodology that combines traditional 2D gel electrophoresis with innovative advances using fluorescent protein tags. Like traditional 2D gels, separation in the first dimension is by native charge using isoelectric focusing. Separation in the second dimension is by molecular weight using conventional SDS–PAGE. What distinguishes 2D-DIGE is the use of fluorescent dyes to label protein samples so that multiple samples can be run on the same gel. DIGE fluors take advantage of the sensitivity of Cy dyes to give a linear response over five orders of magnitude. The fluors are specially modified for labeling lysine residues so that the positive charge of the amino acid is not changed. Thus, proteins labeled with DIGE fluors co migrate with unlabeled proteins on isoelectric focusing gels. These features enable "minimal labeling" (on the order of 3% of proteins) so that analytical gels may be used to quantify changes in over 1700 proteins from as little as 10 μg protein (the sensory epithelium from one P3 rat cochlea). The same gels may be loaded with sufficient protein (over 1 mg) for preparative separations for the identification of comigrating unlabeled protein by mass spectrometry. A second advance used by 2D-DIGE is the development of confocal-like gel scanners that permit the analysis of 3 fluorescent protein spot patterns from the same gel. Using combinations of Cy2-, Cy3-, and Cy5-labeled samples, multiple experimental conditions and controls can be run on the same gel and inter-gel controls can be constructed to facilitate the alignment of 2D-gel patterns between gels. The use of DIGE fluors and fluorescent scanning, coupled with contemporary pattern recognition software, have greatly enhanced the quantitative power, ease of use and reliability of 2D gel analysis.

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