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Corticotropin-Releasing Factor-2 Activation Prevents Gentamicin-Induced Oxidative Stress in Cells Derived From the Inner Ear

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Abstract

Generation of reactive oxygen species (ROS) is a common denominator in many conditions leading to cell death in the cochlea, yet little is known of the cochlea's endogenous mechanisms involved in preventing oxidative stress and its consequences in the cochlea. We have recently described a corticotropin-releasing factor (CRF) signaling system in the inner ear involved in susceptibility to noise-induced hearing loss. We use biochemical and proteomics assays to define further the role of CRF signaling in the response of cochlear cells to aminoglycoside exposure. We demonstrate that activity via the CRF₂ class of receptors protects against aminoglycoside-induced ROS production and activation of cell death pathways. This study suggests for the first time a role for CRF signaling in protecting the cochlea against oxidative stress, and our proteomics data suggest novel mechanisms beyond induction of free radical scavengers that are involved in its protective mechanisms.

Keywords

corticotropin-releasing factor receptors; oxidative stress; mass spectrometry; iTRAQ; cochlea; OC-K3 cells

Numerous external agents, including high-intensity noise and various drugs, damage hair cells and/or support cells of the inner ear (Santi and Duvall, 1978; Rybak, 1986; Huizing and de Groot, 1987; Fredelius et al., 1990). Because mammalian hair cells do not regenerate, cytotoxic stress-induced damage can lead to significant hearing loss. Despite differences in the initial targeted compartments of the inner ear by the interactions of ototoxic drugs and damaging noise, these agents ultimately seem to produce similar cellular pathologies and likely have overlapping mechanisms of action, perhaps including activation of the same cellular signaling pathways, that lead to cell death (Kopke et al., 1999).

Metabolically induced and noise-activated oxidative stress and production of reactive oxygen species (ROS) play a significant role in cochlear injury (Yamane et al., 1995). Given the natural activity state of the inner ear, highly active endogenous defenses against the initiation of oxidative stress/damage must exist, yet little is known of these mechanisms. Indeed, most

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research to date has focused on mitigating ROS-associated damage, despite the fact that the presence of elevated ROS levels indicates that damage has already taken place. That endogenous mechanisms exist to protect against the earliest phases of cochlear damage is exemplified by the efficacy of sound-conditioning paradigms in attenuating both noise-induced and drug-induced hearing loss (Canlon et al., 1988; Campo et al., 1991; Subramaniam et al., 1992; Suryadevara et al., 2009). Two effects induced by sound conditioning suggested as underlying mechanisms by which conditioning protects against cochlear injury are an increase in cochlear antioxidant enzymes (Jacono et al., 1998; Harris et al., 2006; Henderson et al., 2006) and inhibition of apoptosis (Niu et al., 2003). Protective effects of sound conditioning have been reported to last for as long as 60 days (McFadden et al., 1997), suggesting involvement of intracellular signaling pathways. Because exposure to initially nondamaging noise has been shown to result nonetheless in significant functional defects with aging (Kujawa and Liberman, 2006) and because sound conditioning has also been shown to be effective against temporary hearing threshold shifts in humans (Miyakita et al., 1992), a clearer and more detailed definition of the endogenous mechanisms involved in cochlear protection is fundamental not only to our understanding of basic cochlear function, but also for designing future interventional strategies designed to lessen the deleterious effects following exposure to intense stimuli.

We recognized that the inner ear is constantly exposed to stress-inducing stimuli, similar to the extent that skin also must cope with such impacts. Therefore, we have modeled our hypotheses concerning the identity of components making up the endogenous stress response system of the inner ear on that of skin. One signaling system expressed in skin that plays a major role in stress-response involves corticotropin-releasing factor (CRF) and its receptors (Slominski et al., 1999, 2000, 2001). We have previously shown that cells of the inner ear also express CRF, urocortin (a CRF-related peptide), and CRF receptors (Vetter et al., 2002). We have also demonstrated a role for one of the CRF receptors, CRF₂, in controlling auditory brainstem response (ABR) thresholds and establishing susceptibility to noise-induced permanent threshold shifts of the ABR (Graham et al., 2010). Here we define a role for CRF₂ in protection against aminoglycoside-induced ROS generation and its associated activation of cell death pathways. We go on to use a global proteomics approach to begin defining mechanisms that may explain the ability of CRF₂ to provide such protection. These preliminary protein data may hold information on potential novel therapeutic targets for future rational drug design useful in combating damage induced by exposure to ototoxic compounds.

MATERIALS AND METHODS

Reagents

All laboratory-grade chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Reactive oxygen species-detecting dye 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA; DCF) was obtained from Invitrogen (Carlsbad, CA), the micro-BCA protein estimation assay kit from Pierce Inc. (Rockford, IL), fetal bovine serum (FBS) from Hyclone-Thermo-Scientific (Logan UT), and Dulbecco's modified Eagle's medium (DMEM) cell culture medium from Gibco/Invitrogen. The caspase-3 assay kit and SOD assay kit were obtained from BioVision.

Cell Culture

The immortalized organ of Corti cell line (OC-K3) used throughout the experiments was a kind gift from Dr. F. Kalinec (Kalinec et al., 1999, 2003). The cells were initially grown in 250-ml T-neck Falcon flasks. Media consisted of 10 ml DMEM supplemented with 10% fetal bovine serum (FBS) and 50 U/ml recombinant interferon- γ (Sigma) per flask. Cultures were maintained at 33°C, 10% CO₂ for proliferative (permissive) conditions. When 80–90%

confluent, cells were transferred to differentiation conditions. The proliferative culture medium was replaced with DMEM supplemented with 10% FBS without interferon- γ and moved to 39°C, 5% CO₂. Cells were maintained under differentiation conditions for 10 days prior to performing all experiments. No antibiotics were used during cell culturing to avoid potential elevation of antibiotic signaling prior to the evaluation stages of the experiments.

Cellular Signaling Assays

Measurement of ROS levels—To measure ROS levels, OC-K3 cells were grown in six-well plates and allowed to differentiate for 10 days. To perform the experiment, differentiation medium was replaced with serum-free DMEM and stimulated with H₂O₂ (100 μ M), urocortin II (UcnII) peptide (100 nM), gentamicin (200 μ M), or vehicle control for 1 hr. To determine whether UcnII could protect against ROS-activating insults, the cells were first preincubated with UcnII (100 nM) for 1 hr, followed by either H₂O₂ (100 μ M) or gentamicin (200 μ M) for 1 hr. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and incubated at 39°C for 30 min in the presence of 10 μ M DCF, prepared in PBS. Cells were then washed twice with PBS and scraped into 300 μ l of 1% Triton X-100 prepared in PBS. The cells were kept on ice for 30 min and homogenized by trituration using a 27-gauge needle coupled to a 1-ml syringe. Homogenates were centrifuged at 12,000g for 15 min at 4°C, the supernatant collected, and total protein estimation was performed by using a micro-BCA kit (Pierce Inc.). To measure ROS accumulation, 50 μ l of cell suspension was diluted to 500 μ l with PBS, and any resultant fluorescence level was measured in triplicate for each experiment using a Perkin Elmer luminescence spectrometer LS50B (Perkin Elmer, Waltham, MA) at an excitation wavelength of 485 nm and an emission wavelength of 522 nm. As a blank control, 50 μ l of 1% Triton X-100 in PBS was used in place of cell extract. DCF fluorescence intensity was obtained and expressed as percentage of control (blank) fluorescence per milligram protein. As a negative control, cells were incubated with PBS or DMSO, and we followed the same procedure to measure the ROS level. The experiments were repeated three times, and the average and SEM were calculated and plotted in Prism 4.0 (GraphPad Software, San Diego, CA).

Superoxide dismutase (SOD) assay—SOD activity was measured by using an SOD Assay Kit according to the manufacturer's protocol (BioVision). Briefly, OC-K3 cells were grown in six-well plates and differentiated for 10 days. Medium was replaced with serum-free DMEM and stimulated with or without UcnII peptide (100 nM) and gentamicin (200 μ M) for 1 hr. After drug treatment, cells were washed twice with ice-cold PBS and scraped into ice-cold 0.5% Triton X-100 prepared in PBS. The cells were kept on ice for 30 min and homogenized by trituration with a 27-gauge needle coupled to a 1-ml syringe. Homogenates were centrifuged at 12,000g for 15 min at 4°C, the supernatant was collected, and total protein estimation was performed by using a micro-BCA kit. (Pierce Inc.). Next, 40 μ g protein lysate was loaded into a 96-well microplate and appropriate buffer and enzyme solution added, followed by incubation at 37°C for 30 min. Absorbance was read at 450 nm using a Bio-Rad microplate reader. The experiments were repeated three times, and the average and SEM were calculated. The inhibition rate of SOD on WST-1 dye formation was expressed as SOD activity per milligram protein.

Caspase-3 activity measurement—Activation of caspase-3 was evaluated in OC-K3 cell lysates using a Caspase-3 Fluorometric Assay Kit, following the manufacturer's recommendations (BioVision). Briefly, OC-K3 cells were maintained under differentiation conditions for 10 days. Prior to the experiment, cultures were serum starved for 2 hr, prestimulated with UcnII for 5 min, and then challenged with 200 μ M gentamicin for 1 hr. The cells from each condition were washed twice with ice-cold PBS and scraped into 200 μ l ice-cold lysis buffer. The cells were homogenized by trituration through a 27-gauge needle and 1-

ml syringe. The homogenates were centrifuged at 12,000g for 15 min at 4°C, the supernatant was collected, and total protein estimation was performed by using a micro-BCA kit. (Pierce Inc.). Caspase-3 activity was performed with 100 µg protein per assay. Fold increase in caspase-3 activity was determined by comparing fluorescence of 7-amino-4-trifluoromethyl coumarin in control and drug-treated cell lysates using a Perkin Elmer luminescence spectrometer LS50B at an excitation wavelength of 400 nm and an emission wavelength of 505 nm. The caspase-3 activity was expressed per milligram protein, and relative activity for treated samples was expressed as percentage relative to blank control. The experiments were repeated three times, and average and SEM of the three experiments were calculated.

Peptide Labeling Using Isobaric Tagging for Relative and Absolute Quantification Techniques and LC-MS/MS Analysis

Cell culture—OC-K3 cells were initially grown in 250-ml T-neck flasks (Falcon) consisting of 10 ml DMEM supplemented with 10% FBS and 50 U/ml recombinant interferon-γ (Sigma). Cultures were maintained at 33°C, 10% CO₂ for proliferative (permissive) conditions. When 80–90% confluent, the cells were differentiated. The culture medium was replaced with DMEM supplemented with 10% FBS without interferon-γ and moved to 39°C, 5% CO₂. After 10 days, plates were assigned to one of four conditions: baseline, no stimulation; gentamicin (200 µM) stimulation for 1 hr; UcnII (100 nM) stimulation for 1 hr; or prestimulation for 1 hr with 100 nM UcnII, followed by a wash and a sequential challenge with 200 µM gentamicin for 1 hr. Cells were washed twice with ice-cold PBS and scraped into mammalian cell lysis buffer (T-Per; Pierce), without adding any protease inhibitors. The cells were homogenized by triturating through a 27-gauge needle and 1-ml syringe. The homogenates were centrifuged at 12,000g for 15 min at 4°C, supernatant collected, and the protein estimation performed by using a micro-BCA kit (Pierce Inc.).

Protein precipitation for the isobaric tagging for relative and absolute quantification protocol—Isobaric tagging for relative and absolute quantification (iTRAQ) protein labeling and mass spectrometry were performed at the University of Victoria Proteomics Centre (Victoria, British Columbia, Canada). One hundred micrograms of protein was precipitated by adding 9 volumes of ice-cold acetone and incubating overnight at 4°C. After removal of the acetone, the protein pellet was resuspended in 30 µl of 0.5 M triethyl ammonium bicarbonate.

iTRAQ labeling—Numerous methods have evolved to label proteins for quantitative proteomics analysis. One of the methods, iTRAQ, is well suited for analysis of protein expression changes over discrete time intervals. Briefly, peptides are labeled with isobaric reagents carrying reporters of differing molecular weights. In the system used here, four reagents are used that show up in the mass spectrometer as peaks at 114, 115, 116, or 117 Da and hence are simply termed reagents 114, 115, 116, and 117 (for further description see Vetter et al., 2009). Samples undergoing the different treatments (no treatment control, gentamicin treatment, urocortin II treatment, and urocortin pretreatment followed by gentamicin challenge) can then be labeled with one of the reporter tags to differentiate protein expression levels for each condition (see below for assignments). Labeling of protein lysates for all conditions was performed using the iTRAQ labeling kit (Applied Biosystems) and following the manufacturer's recommendations (see also Vetter et al., 2009). Briefly, the samples were each denatured using a final concentration of 0.2% SDS and cysteines were reduced with 2 µl 50 mM TCEP. Tubes were held at 60°C for 1 hr and spun briefly, and 1 µl iodoacetamide was added to block cysteines. Finally, samples were trypsinized at 37°C overnight.

On the following day, the digested samples were labeled with the iTRAQ reagents, following the manufacturer's protocol such that the control, unstimulated sample was combined with

reagent 114, gentamicin-stimulated sample was combined with reagent 115, UcnII-stimulated sample was combined with reagent 116, and the UcnII-pretreated gentamicin-challenged sample was combined with reagent 117. Labeling proceeded at room temperature for 1 hr. Labeled samples were then combined in a 1:1:1:1 ratio in a fresh Eppendorf tube.

Strong cation exchange (SCX) HPLC—A Vision Workstation (Applied Biosystems, Foster City, CA) was equipped with a polysulfoethyl A (poly-LC) 100 mm × 4.6 mm, 5- μ m, 300-Å SCX column. Buffer A was 10 mM KPO₄ (pH 2.7), 25% ACN. Buffer B was 10 mM KH₂PO₄, 25% ACN, 0.5 M KCl. The flow rate was set to 0.5 ml/min.

Samples were brought up to 2 ml with buffer A and injected onto the column. The column was allowed to equilibrate for 20 min in buffer A before a gradient was applied, 0–35% B in 30 min. Fractions were collected every minute after injection. The collected fractions were then reduced in volume in a Speed-Vac (Savant Instruments) and transferred to autosampler vials (LC Packings)

LC-MS/MS materials and methods—LC-MS/MS analysis was performed with an integrated Famos autosampler, Switchos-II switching pump, and UltiMate micropump (LC Packings) system with a hybrid quadrupole-time of flight (TOF) LC/MS/MS mass spectrometer (QStar Pulsar i) equipped with a nanoelectrospray ionization source (Proxeon) and fitted with a 10-lm fused-silica emitter tip (New Objective). Chromatographic separation was achieved on a 75 μ m × 15 cm C18 PepMap Nano LC column (3 μ m, 100 Å; LC Packings), and a 300 μ m × 5 mm C18 PepMap guard column (5 μ m, 100 Å; LC Packings) was in place before switching inline with the analytical column and the mass spectrometer (MS). The mobile phase (solvent A) consisted of water/acetonitrile (98:2 v/v) with 0.05% formic acid (FA) for sample injection and equilibration on the guard column at a flow rate of 100 μ l/min. A linear gradient was created upon switching the trapping column inline by mixing with solvent B, which consisted of acetonitrile/water (98:2 v/v) with 0.05% formic acid, and the flow rate was reduced to 200 nl/min for high-resolution chromatography and introduction into the mass spectrometer.

LC-MS/MS procedure—Samples were brought up to 20 μ l with 5% ACN and 3% FA and transferred to autosampler vials (LC Packings). Ten microliters of sample was injected in 95% solvent A and allowed to equilibrate on the trapping column for 10 min to wash away any contaminants. Upon switching inline with the MS, a linear gradient from 95% to 40% solvent A was developed for 40 min, and in the following 5 min the composition of the mobile phase was increased to 20% A, before decreasing to 95% A for a 15 min equilibration before the next sample injection. MS data were acquired automatically in Analyst QS 1.0 software (Service Pack 8; ABI MDS SCIEX). An information-dependent acquisition method consisted of a 1-sec TOF-MS survey scan of mass range 400–1,200 amu and two 2.5-sec product ion scans of mass range 100–1,500 amu. The two most intense peaks over 20 counts with charge states 2–5 were selected for fragmentation, and a 6-amu window was used to prevent the peaks from the same isotopic cluster from being fragmented again. Once an ion was selected for MS/MS fragmentation, it was put on an exclude list for 180 sec. Curtain gas was set at 23; nitrogen was used as the collision gas, and the ionization tip voltage used was 2,700 V. If the observed A₂₁₅ was greater than 0.1 for any fraction collected during the SCX, a 2.5-hr gradient (95–50% solvent A) was used to compensate for the higher peptide concentration in that fraction.

Data Analysis

Data files were processed offline at Tufts University School of Medicine in ProteinPilot software (v 2.0.1, revision 67476; Applied Biosystems MSD SciEx). Identification of significant expression changes was accomplished by first establishing an Unused ProtScore

(confidence level) cutoff of 1.3 (this metric establishes the number of peptides of any spectrum that have not already been used by a higher scoring, “winning” protein; the greater number of unused peptides, the greater the confidence in the protein identification), thereby filtering the data set for detected proteins with a 95% or greater confidence ID call. This first filter ensured that all proteins examined further were identified with a high level of confidence. Next, this filtered data set was again filtered by ratio change of any reaction ratio, with proteins that attained a 1.2 ratio or higher or a 0.8 ratio or lower arbitrarily considered to be proteins of potential interest. Finally, the data set was again filtered by *P* value of the reaction ratios (relative to the mean ratio change of the entire data set), with a cutoff first of 0.05 to establish the protein ID list and next with a cutoff of 0.01 to establish proteins that were examined further for functional classification. After these steps, only the most stringently identified proteins of highest statistical significance in terms of expression level changes were considered in this analysis. A Venn diagram was drawn using the Venny software (Oliveros, 2007) with the statistically significant proteins as input for each condition. R statistical framework was used to cluster differentially expressed proteins that met the highest stringency conditions to generate a heat map of functional clusters, color coded according to their row z-scores. Heat maps were created using the heatmap.2 function in the gplots package.

RESULTS

CRF₂ Activation Protects Against Cytotoxic Effects of Aminoglycoside Exposure

ROS formation is decreased by activation of CRF₂—ROS formation was initiated in OC-K3 cells by a 1-hr exposure to either 100 μ M H₂O₂ (Fig. 1) or 200 μ M of the aminoglycoside gentamicin (Fig. 2). ROS detection and quantification were performed using H₂DCFDA dye (DCF) by monitoring fluorescence intensity. Baseline measures of unstimulated samples served as control. Both H₂O₂ (Fig. 1) and gentamicin (Fig. 2) increased DCF intensity approximately two-fold over baseline measures. Ucn (100 nM), which is maximally active at CRF₂ but has some efficacy also at CRF₁, and UcnII (100 nM), which is active only at CRF₂, significantly decreased basal DCF signal ($P = 0.037$, $P = 0.0027$, respectively; Fig. 1). Pretreatment for 1 hr with either Ucn or UcnII prior to challenge with H₂O₂ (Fig. 1) produced a significant block of H₂O₂-induced DCF signal (Ucn, $P = 0.0035$; UcnII, $P = 0.0013$). Simultaneous application of the CRF₁ and CRF₂ antagonists antalarmin and astressin2B, respectively, prior to H₂O₂ application blocked the protective effect of Ucn, whereas application of astressin2B alone was sufficient to block the protective effects of UcnII application on H₂O₂-induced DCF fluorescence increase. Antalarmin did not block the protection offered by UcnII against H₂O₂-induced ROS formation (DCF fluorescence; Fig. 1). In a manner similar to protection against H₂O₂, UcnII preapplication 1 hr prior to gentamicin challenge protected the cells from gentamicin-induced DCF increase ($P = 0.0032$; Fig. 2).

SOD activity is increased by stimulation of CRF₂—The SOD activity assay performed measures SOD-induced inhibition of soluble dye formation as a result of WST-1 reduction following superoxide anion formation. Dye formation is linearly related to the ability of SOD to catalyze dismutation of superoxide anions to O₂ and H₂O₂ and therefore is indicative of the amount of SOD present. Thus, rising levels of SOD will inhibit dye formation, whereas lower SOD levels result in enhanced dye formation. Exposure to 200 μ M gentamicin inhibited SOD activity approximately three times over basal activity ($P = 0.0004$; Fig. 3). Similar to the effects observed for H₂O₂-induced ROS formation, UcnII pretreatment significantly blocked the gentamicin inhibition of SOD activity ($P = 0.0015$), although it was not able to return the measure completely to baseline (difference between UcnII + gentamicin and baseline, $P = 0.0020$). However, the protective effect of pre-exposure to UcnII decreased the SOD inhibition by approximately half (Fig. 3).

Caspase-3 activity is inhibited by stimulation of CRF₂—Caspases are intimately involved in apoptotic responses to both extrinsic and intrinsic cell death signals. In the inner ear, caspase-3 has been shown to play a major role in hair cell apoptosis following numerous insults (Van De Water et al., 2004). We therefore examined the ability of CRF₂ activity to inhibit caspase-3 activity in a caspase-3 assay. Application of gentamicin increased caspase-3 activity by approximately 53% ($P = 0.0132$; Fig. 4). However, application of UcnII alone was able to decrease the baseline caspase-3 activity by 63% ($P = 0.0028$) over untreated cells. UcnII pretreatment similarly protected the cells from a gentamicin-mediated increase in caspase-3 activity (67% decrease; $P = 0.0008$ vs. gentamicin treatment alone). Thus, UcnII treatment was able to control one of the most important effectors of apoptosis induced in the inner ear significantly.

Differential Protein Expression Assayed by iTRAQ Labeling and LC-MS/MS

To ascertain more precisely the potential mechanisms underlying the ability of UcnII, and therefore of CRF₂ activation, to protect against oxidative stress and its accompanying signaling cascades, we used a shotgun proteomics procedure to perform a differential proteomic expression analysis on the OC-K3 cells under various conditions. The iTRAQ technique uses a tagging procedure that allows multiplexed samples to be compared quantitatively (Ross et al., 2004; Choe et al., 2007; Vetter et al., 2009) against each other or against a baseline condition. OC-K3 cells were grown under differentiation conditions for 10 days, and separate plates were then treated with 200 μ M gentamicin for 1 hr; 100 nM UcnII for 1 hr; pretreated first for 1 hr with UcnII, followed by gentamicin exposure for 1 hr; or left untreated. Lysates were generated and samples were labeled with iTRAQ reagents, following the manufacturer's instructions. Control, untreated samples were labeled with reagent 114, gentamicin treated samples with reagent 115, UcnII treated samples with reagent 116, and the double treatment, UcnII + gentamicin samples with reagent 117. Samples were mixed and subjected to LC-MS/MS analysis.

Global analysis of alterations to baseline protein expression induced by gentamicin, UcnII, or combined exposure—iTRAQ results generate sequence and quantitative expression information for each peptide detected. Criteria used for acceptable protein identification and differential expression assessment are described in Materials and Methods. Seventeen thousand one hundred seventy-seven spectra were obtained, among which 81% (13,956) met the 95% confidence level cutoff criterion for protein identification [Unused ProtScore cutoff 1.3 or higher; represented by a $-\log_{10}(P \text{ value}) \leq 0.05$ established under the Paragon algorithm (Shilov et al., 2007) run within ProteinPilot for confidence in protein identification], resulting in identification of 5,667 peptides. These peptides were mapped to 942 total proteins identified by the mass spectrometer (see Supp. Info. Table I). Further refinement of the initial protein list was performed so that proteins from the initial list were then filtered to meet requirements for a ratio change relative to baseline (i.e., ratio with reaction 114) of 1.2 or greater or 0.8 or less (i.e., over- or under-expressed, respectively) and at least one ratio associated with the different conditions for each protein demonstrating a P value ≤ 0.05 . This second filter produced Table I. A further refinement of the Table I data set was performed to select only for those proteins having an Unused ProtScore of 2.0 or greater [i.e., a $\log_{10}(P \text{ value}) = 0.01$], representing a protein identification confidence score of 99% or greater and $P \leq 0.05$. This list was generated to define those proteins that are likely to define future validation targets for therapeutic intervention, because confidence in identity is highest. This filter defined the 173 proteins of Table I that met such a criterion, and these proteins were used to investigate further the occurrence of contraregulated proteins between the gentamicin exposure and UcnII exposure conditions. Interestingly, a four-way Venn diagram (Fig. 5) demonstrated that only five proteins were contraregulated between these conditions, four that were down-regulated under gentamicin exposure while being up-regulated upon UcnII

exposure and one that was up-regulated under gentamicin exposure while being down regulated with UcnII exposure (listed in Table II). These data seem to indicate that the mechanism by which UcnII exerts its protective effects against gentamicin-induced damage does not include a simple rescue of protein expression levels altered by the actions of gentamicin.

Functional clustering—Results from the iTRAQ-based analysis were examined to determine whether functional clusters could be revealed that would be useful in better understanding potential mechanisms whereby UcnII exerts its protective effects over gentamicin-induced damage. Data from Table I were further refined and selected for functional clustering if the absolute $\log_2$ (-fold change) $P \leq 0.01$. Although these criteria were highly stringent along both the expression and identification dimensions, they nonetheless returned 37 proteins that met or exceeded this overall metric. Expression level ratios relative to baseline were assessed for each protein, and a Z-score was generated for each row and used to map the maximal expression ratio for each protein between gentamicin only (115:114) and UcnII pretreatment followed by gentamicin (117:114) conditions. Proteins were manually curated into biologically relevant functional groups, clustered by this annotation, and visualized using a heat map (Fig. 6). The 37 proteins separated into five mutually exclusive functional clusters that represented distinct biological processes. These involved protein degradation and signal transduction, which were decreased in the gentamicin condition relative to baseline, while being significantly up-regulated following UcnII pretreatment followed by gentamicin challenge. Proteins related to cytoskeletal processes also clustered, and, although the data indicated contraregulated expression between the two main conditions, as a cluster the proteins did not behave similarly within each condition (i.e., some were up- and some were down-regulated, but always opposite the other treatment paradigm). Apoptosis-related proteins also formed a cluster, and most of these proteins were overexpressed under gentamicin conditions and suppressed with UcnII pretreatment. Finally, the largest cluster generated was composed of proteins involved in nucleotide binding and transcriptional regulation, most of which were up-regulated following gentamicin exposure and suppressed following UcnII pretreatment, indicating the normalizing effects of UcnII pretreatment.

DISCUSSION

Expression of the corticotropin-releasing hormone family of peptides and their receptors has previously been demonstrated in the inner ear (Vetter et al., 2002; Graham et al., 2010). Although the classically understood role of the CRF system involves activation of the hypothalamic-pituitary-adrenal axis, recent work has demonstrated its involvement more globally in oxidative stress-related biology. In particular, peptides of the CRF family have been shown to be neuro- and cardio-protective. Thus, the family is involved in neurodegenerative disorders such as Alzheimer's disease (Bayatti and Behl, 2005), excitotoxicity (Elliott-Hunt et al., 2002), and ischemia-reperfusion injury, especially of cardiac tissue (Brar et al., 1999, 2000, 2002, 2004; Scarabelli et al., 2002; Burley et al., 2007). The cardiac ischemia-reperfusion injury model demonstrated that the CRF system is involved in cardioprotection via inhibition of free radical activity (Liu et al., 2005).

A myriad of ROS-generating stimuli impinge on the inner ear, including exposure to intense sound and ototoxic drugs. Because mammalian hair cells do not regenerate, significant effort from many groups has gone into understanding the consequences of ROS production in the inner ear in the hopes of neutralizing cellular damage and protecting hearing. However, little is currently known about how the inner ear normally handles the significant everyday stress it is under and what endogenous signaling mechanisms are expressed that allow this very active system to avoid/lessen metabolically related damage over the lifetime of an individual. Hints that an endogenous system not only exists but is capable of significantly altering the response of the inner ear to the effects of oxidative stress has been underscored by a series of findings

reporting the ability of low-level sound exposure (termed *pretreatment*, or *conditioning*) to 1) increase resistance to oxidative stress-related loss of hair cells and hearing function (Harris et al., 2006), 2) increase antioxidant enzyme activity (Jacono et al., 1998), 3) inhibit apoptotic activity (Niu et al., 2003), and finally 4) decrease gentamicin-induced hair cell loss (Suryadevara et al., 2009). Understanding the endogenous defenses mounted against cellular stress and stress-related signaling in the inner ear may reveal novel methods by which one may intervene against environmentally induced damage as well as against age-related hearing loss or that associated with therapeutically necessary exposures to drugs with ototoxic side effects.

The role(s) of SOD and related proteins in protection of the inner ear is well understood. Loss of SOD1 from the cochlea potentiates both noise-induced and age-related hearing loss (McFadden et al., 1999a,b, 2001). Our experiments suggest that mechanisms by which CRF₂-mediated protection against ROS-related damage is established may occur via previously recognized SOD mechanisms as well as by novel, apparently non-SOD-related, mechanisms. With regard to SOD-related protection, our data demonstrate that, in the presence of gentamicin, SOD activity was significantly decreased (almost three times over baseline values), but combining UcnII pretreatment with gentamicin exposure induced a significant up-regulation of SOD activity compared with gentamicin-only treatment. The ability of CRF₂ activity to inhibit numerous other cellular responses linked to oxidative stress, such as caspase-3 activation, suggests that there are clearly other signaling pathways that can be activated to generate cytoprotective responses besides those previously described involving SOD.

In light of the data demonstrating the protective effects of prior activation of CRF₂ on gentamicin-induced ROS generation and activation of cell death pathways, we initiated a top-down proteomics screen. The goal of this procedure was to begin defining novel therapeutic targets useful in combating ROS production and consequent ototoxin-induced damage. We began a directed search using only the most highly reliably identified proteins to define novel cochleoprotective actions mediated by CRF₂ activity. These data represent the first demonstration of novel protective mechanisms invoked by the CRF system of the inner ear. Unexpectedly, very few proteins were contraregulated between UcnII exposure and gentamicin exposure despite the ability of UcnII pretreatment to block cellular response to gentamicin. Only five proteins were contraregulated between UcnII and gentamicin stimulation. Among these, prothymosin α (Ptma) did not change in a statistically relevant manner from baseline under either gentamicin-only or UcnII + gentamicin treatment ($P = 0.102$ and $P = 0.746$, respectively) but underwent a highly significant change in expression from baseline under UcnII exposure ($P = 0.002$). We interpret these findings as suggestive of an early rise in Ptma expression during the UcnII pretreatment phase that is protective against gentamicin exposure and that was subsequently down-regulated to normal levels upon gentamicin challenge. Ptma may therefore still represent a protein of some interest for future validation, especially given that Ptma has been shown to switch cell death mode pathways from necrosis (generally considered to be an irretrievable pathway once initiated) to apoptosis (Ueda, 2008, 2009). The apoptotic pathway is fully rescuable in vitro and in vivo by application or overexpression/release of neurotrophins or growth factors. Interestingly, Figure 6 demonstrates just such an up-regulation of a growth-factor-enriched expression cluster while also revealing a down-regulation of apoptosis-related proteins. Thus, UcnII pretreatment may render cells more capable of initiating a rapid switch between cell death modes while at the same time inducing protein expression levels favorable to surviving apoptosis. Such a response to Ptma during ischemic stroke has been demonstrated (Fujita et al., 2009). Finally, it has been previously demonstrated that Ptma blocks apoptosome formation, thereby inhibiting caspase activation (Jiang et al., 2003). Our biochemical assays also demonstrated an inhibition of caspase-3 activity following UcnII pretreatment. All of these data suggest that the ability of UcnII to up-

regulate Ptma can serve as a survival mechanism in the face of cellular stress and that CRF₂ activation may be crucial for inducing this mechanism in the inner ear.

Interestingly, mechanisms of aminoglycoside-induced hair cell death may be dependent on the model being examined. In vitro assays employing explant cultures have implicated caspase activation (Cunningham et al., 2002) and classical apoptotic pathways (Li et al., 1995; Vago et al., 1998) in cell death and have also demonstrated that caspase inhibitors prevent aminoglyco-side-induced hair cell death (Matsui et al., 2002, 2003). However, it has been suggested that aminoglycoside-induced cell death pathways in vivo are caspase independent (Jiang et al., 2006), and few data exist for classic apoptotic events being induced by chronic aminoglyco-side exposures. Our data may help shed light on this dichotomy. If an intact CRF signaling system functioning in vivo switches the death pathways away from apoptosis, this may explain the paucity of data implicating apoptosis as a cell death mechanism in vivo. This would also suggest that there is a loss of some aspect of CRF signaling under in vitro conditions, thus allowing the hair cells to undergo apoptosis when explants are analyzed. If this hypothesis is true, manipulating the in vitro models by activating the CRF system exogenously should inhibit the apoptosis pathways previously described under these conditions.

Only parathymosin and nucleolin (Ncl) exhibited statistically relevant expression changes in the opposite direction between gentamicin challenge (both down-regulated) and UcnII challenge (both up-regulated, although parathymosin did not reach original baseline values, and Ncl was lowered from the UcnII induced levels to expression levels that were not statistically different from baseline). Prothymosin a and parathymosin are essential for cell cycle progression and cellular proliferation in both normal and cancer cells (Tsitsilioni et al., 1993; Letsas et al., 2007). Parathymosin is also known to be involved in transient, cyclical binding with histone H1 and therefore has been suggested to be a modulator of histone–chromatin interactions in specific subnuclear domains (Martic et al., 2005). By extension, then, one possible effect of UcnII pretreatment is to modulate specific gene expression and initiation of protein translation, the specific products of which may be involved in apoptotic effects. Supporting this hypothesis is the number of ribosomal proteins that are also up-regulated with UcnII treatment/pretreatment as reported in Table I. Interestingly, numerous small nuclear ribonucleoproteins and heterogeneous nuclear ribonucleoproteins are up-regulated following UcnII exposure, whereas canonical RNA proteins of the 60S and 40S RNA complexes seem to be down-regulated. Nucleolin is significantly up-regulated by UcnII exposure, and this is maintained at baseline levels in spite of gentamicin exposure, making it a protein of particular interest. Although Ncl has been well recognized for its role in also regulating chromatin structure, it has recently been found to have a strong antiapoptotic role under H₂O₂ challenge (Zhang et al., 2009). Thus, rescuing Ncl from its gentamicin-induced down-regulation represents a potential future target for intervention. Only the vacuolar v-type H⁺-ATPase (Atp6v0c) maintained a statistically up-regulated expression profile when exposed to UcnII, even under gentamicin challenge (which itself caused a highly significant decrease in expression; $P = 0.002$). Gentamicin-induced loss of Atp6v0c expression may contribute to build up of, and toxic response to, lysosomal debris. Cellular toxicity following loss of Atp6v0c has been demonstrated under neurodegenerative conditions such as Alzheimer's disease (Liu et al., 2008). The ability of UcnII pretreatment apparently to inhibit this response to gentamicin may represent activation of multiple parallel pathways that together impart resistivity to otherwise ototoxic compounds.

Four mitochondrial proteins involved in ATP synthesis were significantly up-regulated following gentamicin exposure. Although these were also up-regulated following UcnII pretreatment, three of the four were expressed at lower levels than were induced by gentamicin. Most significantly, Atp5h, a protein involved in generating the pore structure of the ATP channel, is held to values insignificantly different from baseline. One may postulate that the

gentamicin-induced up-regulation of these proteins represents an imbalanced or stressed mitochondrial fraction. If this is true, the ability of UcnII to negate even some of the overexpression across this short time frame may represent a key mechanism whereby UcnII/CRF₂ activity establishes its protective effects. Because the mitochondria play an important role in the consequences following oxidative stress, this may still represent a fertile avenue to pursue.

It is possible that the expression level of any single protein could change in a small, yet still statistically meaningful, manner, making it more difficult to assign functional significance to such a change. However, large ensembles of such small changes clustered along major signaling pathways could represent major effectors for CRF₂-based protection. Thus, we carried out a hierarchical clustering of hand-annotated functional groups to examine the dynamics of differential expression (Fig. 6). The major processes that are affected by CRF₂ pretreatment in the face of gentamicin-induced oxidative stress were split into two ensembles of proteins moving with opposite trajectories. Expression of proteins related to apoptosis pathways and nucleotide binding were lower following UcnII pretreatment relative to levels induced by gentamicin exposure. Signal transduction pathways that are especially enriched for growth factors and adhesion and also protein degradation pathways were up-regulated by UcnII pretreatment with respect to gentamicin-only exposure. Thus, one may use such data to continue probing these pathways with novel drugs/substances in order to define better the efficacy of novel inhibitors of oxidative stress-related damage to the inner ear in vivo.

This work has demonstrated that CRF₂ activation induces a protective effect against ROS production and associated signaling in cochlear-derived immortalized cells. The inner ear is composed of numerous cell types, and, insofar as it was unknown whether and by what mechanisms CRF₂ activation might exert a protective effect against ROS-induction and associated damage, the OC-K3 cell line was used in this first report of CRFR-mediated protection. The cell line is useful for assessing potential signaling cascades induced by CRF₂ activation, because the line is composed of a homogeneous cell population. However, it should be recognized that direct generalization of the results obtained here to the in vivo state should be made with caution until further experiments designed to define whether these processes are present in the intact cochlea, and which cell types are involved, are performed. However, the general use of cell lines as a tool for elucidating novel signaling cascades cannot be ignored and represents the best way to define novel protein interactions prior to establishing them in any in vivo state.

In conclusion, we have demonstrated that CRF₂ activity provides significant protection from cytotoxic (oxidative) stress in an inner ear-derived cell line. We have gone on to show that the mechanisms whereby such protection is expressed are complex and multifaceted but are likely to involve protection of mitochondrial homeostasis and modulation of nuclear function that is at least partially involved with translational activity. Additionally, a switch from a necrotic to an apoptotic cell death pathway, coupled with an overexpression of a number of antiapoptotic proteins/pathways stimulated by UcnII, is likely to be of importance in the protective capacity of CRF₂ activity. Our data suggest that activation of the endogenous CRF system of the inner ear (Vetter et al., 2002; Graham et al., 2010) may represent a novel avenue by which protection from oxidative stress can be further explored and underscore the potential importance of alternate signaling pathways that are distinct from the commonly exploited purely antioxidant pathways in establishing protection against cytotoxic agent exposures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. CRF-related peptides protect OC-K3 cells from H₂O₂-induced ROS formation. Reactive oxygen species formation was monitored by using DCF fluorescence and expressed as fluorescent signal per milligram protein. H₂O₂ induced a significant increase in fluorescence, indicating a spike in ROS formation. Urocortin (Ucn) pretreatment protected against H₂O₂-induced ROS formation (fourth bar), as did UcnII (eighth bar). Antalarmin, a CRF₁ antagonist, was ineffective in blocking the effect of UcnII on its own (last bar). However, astressin2B, a CRF₂ antagonist, completely blocked the protective effects of UcnII, even when Ucn was added to the CRF₂ activation cocktail. Interestingly, both Ucn and UcnII also demonstrated a significant decrease in basal ROS levels (third and seventh bars, respectively). ***P* < 0.01.

Fig. 2.

UcnII protects against gentamicin-induced ROS formation. Gentamicin, an aminoglycoside and well-known ototoxic agent, induces significant ROS generation over background (second bar). Pretreatment for 1 hr with UcnII, which specifically activates CRF₂, protects cells against gentamicin-induced ROS formation (third bar), by normalizing the ROS levels to control values. * $P < 0.05$, ** $P < 0.01$.

Fig. 3.

UcnII protects against gentamicin-induced inhibition of superoxide dismutase (SOD) activity. SOD activity is significantly inhibited upon challenge with gentamicin (second bar). Although UcnII application does not alter baseline SOD activity (third bar), pretreatment with UcnII significantly blocked gentamicin inhibition of SOD activity (thus lessening the formation of WST1 formazan dye), suggesting that the cellular ROS response induced by gentamicin is lessened by CRF₂ activity. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 4.

UcnII induces lower basal caspase-3 activity and protects against gentamicin-induced caspase-3 activation. Control, nonstimulated caspase-3 activity is significantly elevated by application of gentamicin (bar 2). Application of UcnII not only significantly reduces basal level caspase-3 activity (bar 3) but pretreatment also protects against gentamicin-induced caspase-3 activity. * $P < 0.05$, *** $P < 0.001$.

Fig. 5.

A four-way Venn diagram reveals proteins that are contraregulated. By using only proteins reaching a 99% confidence level of identification as a database, a Venn diagram was generated to indicate with highest confidence those proteins that were differentially expressed between gentamicin and UcnII treatments. For both conditions, the majority of differentially expressed proteins were up-regulated. Only four proteins were up-regulated under UcnII treatment while also being down-regulated by gentamicin treatment. Conversely, one protein was down-regulated by UcnII treatment while being up-regulated by gentamicin. Identities are listed in Table II.

Fig. 6. Hierarchical clustering of manually curated functional groups of proteins that are differentially expressed across conditions reveals significant and concordant changes in five functional sets. Proteins were identified using the highest confidence filter for both protein identification (99%) and *P* value of expression change (≤ 0.01). Each protein was then annotated for functionality and submitted for clustering. Manual curation identified five main protein sets, as defined on the left hand margin of the heat map. The color of each heat map cell represents the Z-score of expression along the row, allowing a comparison of expression of any protein along across conditions. Both nucleotide binding/transcriptional regulation and apoptosis-related protein sets are up-regulated under gentamicin conditions (115:114) while being comparatively down-

regulated under the pretreatment with UcnII (117:114) condition. Signal transduction (especially concerning growth factors) and protein-degradation-related proteins are more highly expressed following pretreatment with UcnII than under gentamicin conditions. Identity of members of the final list is indicated on the right margin of the heat map. Column headers: 115:114, expression under gentamicin stimulation normalized to control, untreated condition; 116:114, expression under urocortin II stimulation normalized to control, untreated condition; 117:114, expression under urocortin II pretreatment stimulation followed by gentamicin challenge, normalized to control, untreated condition. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE I

Unused ProtScore ≥ 1.3 ; $0.8 \geq X:114 \geq 1.2$; Any P Value for Any Protein ≤ 0.05

Name	Symbol	Unused	115:114	P	116:114	P	117:114	P
Acetyl-coenzyme A acyltransferase 2	Acaa2	3	1.19	0.10655	1.16	0.04989	1.25	0.02612
Alpha-actinin-1	Actn1	10.07	0.72	0.00520	0.97	0.56941	0.86	0.01405
Splice Isoform 2 of alpha-adducin	Add1	2.19	1.23	0.12389	1.24	0.02382	0.92	0.10495
Adenosylhomocysteinase	Ahcy	7.3	1.28	0.00857	1.21	0.01460	1.04	0.48866
Testis-specific SSeCKS	Akap12	14.83	1.35	0.00047	1.13	0.08924	1.07	0.23754
Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial precursor	Aldh4a1	4.59	1.42	0.00405	1.02	0.74304	1.08	0.03851
Annexin A3	Anxa3	20.05	0.77	6.68E-09	0.88	0.00001	0.90	0.00055
Annexin A4	Anxa4	22.79	0.71	8.04E-07	0.92	0.08543	0.87	0.00284
Amyloid-like protein 2, isoform 751	Aplp2	3.05	0.77	0.03011	0.91	0.02647	0.96	0.01915
Splice Isoform APP751 of Amyloid beta A4 protein precursor	App	14.74	0.77	0.00072	0.80	0.00025	0.95	0.27227
ADP-ribosylation factor 5	Arf5	6	1.29	0.00633	1.11	0.23170	1.03	0.72005
ADP-ribosylation factor-like 10B	Arl8a	2	0.94	0.18331	0.75	0.00380	0.85	0.02373
ARMET protein precursor	Armet	10.25	0.80	0.00717	0.90	0.01279	0.97	0.49759
Actin-related protein 2/3 complex subunit 3	Arpc3	2.15	0.97	0.86568	1.29	0.03126	1.15	0.12455
ATP synthase alpha chain, mitochondrial precursor	Atp5a1	19.49	1.30	3.56E-07	1.18	0.02213	1.19	0.00002
ATP synthase gamma chain, mitochondrial precursor	Atp5c1	1.4	1.38	0.00818	1.36	0.00843	1.34	0.01042
ATP synthase B chain, mitochondrial precursor	Atp5f1	6.01	1.33	0.00001	1.20	0.01632	1.25	0.00034
ATPase, F0 complex, subunit D, mitochondrial	Atp5h	4.61	1.26	0.01414	1.14	0.19651	1.20	0.08039
V-type proteon ATPase 16 kDa proteolipid subunit	Atp6v0c	2	0.49	0.00166	1.30	0.04380	1.26	0.04152
ATPase, H ⁺ transporting, V1 subunit B, isoform 2	Atp6v1b2	4.15	0.66	0.06271	0.62	0.01397	0.75	0.09479
B-cell receptor-associated protein 31	Bcap31	2	0.55	0.02015	0.84	0.15366	1.01	0.87081
Biglycan precursor	Bgn	5.7	0.78	0.05639	0.78	0.03182	0.92	0.28058
Splice isoform 2C of dihydropyridine-sensitive L-type, calcium channel alpha-2/delta subunits precursor	Cacna2d1	2	0.72	0.00060	0.81	0.01788	0.91	0.06214
Cullin associated and neddylation disassociated 1	Cand1	8.41	1.22	0.01944	1.18	0.00333	1.08	0.10822
Capping protein (actin filament) muscle Z-line, beta isoform a	Capzb	2	1.38	0.01499	1.31	0.02294	1.22	0.01440
Splice Isoform Short of H-2 class II histocompatibility antigen, gamma chain	Cd74	3.15	0.59	0.02162	0.90	0.54316	1.10	0.59026
Cytoskeleton-associated protein 4	Ckap4	8.97	1.04	0.53204	1.22	0.01480	1.18	0.02673
UMP-CMP kinase homolog	Cmpk1	4	1.37	0.07191	1.28	0.02922	1.07	0.23327

Name	Symbol	Unused	I15:I14	P	I16:I14	P	I17:I14	P
Calponin-2	Cnn2	2	7.10	0.00984	3.05	0.01954	2.78	0.01604
Calponin-3	Cnn3	6.02	1.36	0.04934	0.97	0.71265	0.98	0.73881
Collagen alpha-1(XII) chain, isoform 4	Coll2a1	13.26	0.79	0.03761	0.75	0.00963	1.13	0.15020
Procollagen, type I, alpha 2	Col1a2	42.23	0.71	4.0E-17	0.71	2.4E-20	0.93	0.01803
Collagen alpha-1(III) chain precursor	Col3a1	49.83	0.53	1.2E-13	0.59	5.0E-15	0.85	0.00015
Collagen alpha-1(IV) chain precursor	Col4a1	2.02	0.59	0.02488	0.77	0.10088	0.85	0.111560
Collagen alpha-1(IV) chain	Col5a1	13.66	0.68	0.00254	0.82	0.00911	0.96	0.62754
Coatomer subunit beta	Copb1	4.16	1.55	0.00167	1.41	0.00067	1.19	0.03385
Coatomer subunit beta	Copb2	3.22	1.42	0.02954	1.33	0.00297	1.23	0.06778
Cytochrome c oxidase subunit VIb isoform 1	Cox6b1	2	1.32	0.00053	1.15	0.02654	1.17	0.00400
Cp protein	Cp	2	1.20	0.16834	1.45	0.02559	1.32	0.00547
Cystatin C precursor	Cst3	6	0.78	0.10577	0.74	0.00278	0.94	0.29031
Doublecortin and calcium/calmodulin-dependent protein kinase-like 1	Dclk1	2.01	1.36	0.02664	1.10	0.14404	1.07	0.22534
2,4-Dienoyl-CoA reductase, mitochondrial precursor	Deer1	2	1.42	0.05570	1.14	0.08451	1.47	0.02531
Cytoplasmic dynein heavy chain	Dync1h1	11.32	1.33	0.00330	1.08	0.34341	0.97	0.55667
Dynein, cytoplasmic, intermediate chain 2	Dync1i2	2	1.26	0.00278	1.14	0.03052	0.82	0.09319
EGF-like repeats and discordin I-like domains 3	Edil3	2.59	0.66	0.00606	0.73	0.00157	0.97	0.62209
Elongation factor 1-alpha 1	Eef1a1	16.59	1.44	4.37E-11	1.15	0.00033	1.03	0.60766
Elongation factor 1-beta	Eef1b2	2.96	1.58	0.00405	1.54	0.00099	1.26	0.00316
Elongation factor 1B gamma	eEF1bg	6	1.29	0.00202	1.12	0.02556	0.95	0.03239
Elongation factor 2	Eef2	19.7	1.23	0.00282	1.11	0.05674	0.95	0.27293
Eukaryotic translation initiation factor 3, subunit 8	Eif3c	2.02	1.14	0.25951	1.23	0.05069	1.20	0.03923
Eukaryotic translation initiation factor 3, subunit 6 interacting protein	Eif3eip	2.01	1.17	0.04418	1.39	0.00859	1.25	0.02601
Splice isoform 2 of eukaryotic initiation factor 4A-II	Eif4a2	2	1.41	0.10876	1.27	0.03575	1.11	0.14509
Eukaryotic translation initiation factor 5A	Eif5a	1.7	1.30	0.00755	1.04	0.34764	0.89	0.12779
Alpha-enolase	Eno1	18.9	1.22	0.00002	1.08	0.09906	0.97	0.45577
Type 1 tumor necrosis factor receptor shedding aminopeptidase	Erap1	9.35	0.77	0.00440	0.94	0.20115	0.89	0.04079
ERO1-like protein alpha precursor	Ero1a	5.05	0.80	0.00259	0.81	0.00479	0.97	0.34587
Coagulation factor III (fragment)	F3	4	0.73	0.00373	0.86	0.01499	0.86	0.05201
Fatty acid synthase	Fasn	10.01	1.32	0.03305	1.09	0.22451	0.94	0.54579
FK506-binding protein 10 precursor	Fkbp10	10.04	0.74	0.00349	0.83	0.00003	0.92	0.00582
Flotillin 2	Flot2	2.2	1.43	0.05516	1.55	0.00575	1.51	0.03622

Name	Symbol	Unused	I15:I14	P	I16:I14	P	I17:I14	P
Fibronectin precursor	Fn1	16.79	1.08	0.16536	1.17	0.07149	1.27	0.00253
Fascin	Fscn1	4	1.32	0.00156	1.15	0.06462	0.93	0.16715
Mannosyl-oligosaccharide glucosidase	Gcs1	2	1.41	0.00834	1.31	0.16586	1.26	0.02932
Hypothetical glycosyl transferase, family 25	Gl25d1	4.64	0.77	0.04454	0.77	0.01608	0.86	0.04285
Predicted: similar to ribosomal protein L27 isoform 1	Gm6301	4	1.17	0.00009	1.27	0.00007	1.11	0.00140
Predicted: similar to 40S ribosomal protein S7 (S8) isoform 1	Gm6472	2.01	1.16	0.03182	1.23	0.01406	1.15	0.06964
Glypican-4 precursor	Gpc4	10.91	0.69	0.00077	0.96	0.51617	1.01	0.83763
Gelsolin precursor	Gsn	10.06	1.36	0.00008	1.36	0.00021	1.15	0.02775
histone 1, H2ab	H2a1	4	1.44	0.00396	1.46	0.00027	1.06	0.13184
Histocompatibility 2, class II, locus DMa	H2Dma	2	1.16	0.15268	1.23	0.11280	1.27	0.02078
Hemoglobin alpha subunit	Hba-a2	2.52	1.52	0.03220	1.27	0.15750	1.08	0.54494
Hexosaminidase A	Hexa	6.01	0.75	0.00063	0.76	0.00153	0.87	0.07372
Beta-hexosaminidase beta chain precursor	Hexb	2.04	1.80	0.03116	1.33	0.02409	1.59	0.02675
Hypothetical twin-arginine translocation pathway signal containing protein	Hgsnat	2	0.88	0.47902	0.74	0.00251	0.92	0.36047
Histone H1.2	Hist1h1c	4.01	1.27	0.00946	1.50	0.00358	1.04	0.63660
Histone H2B	Hist3h2ba	4.02	1.27	0.00333	1.40	0.01046	1.04	0.10255
Hematological and neurological expressed 1-like protein	Hn11	2	1.37	0.01644	1.30	0.02738	1.17	0.14991
Heterogeneous nuclear ribonucleoprotein C	Hnrnc	2.92	1.10	0.48055	1.22	0.04580	1.18	0.18811
Heterogeneous nuclear ribonucleoprotein F	Hnrpf	5.7	1.01	0.51723	1.20	0.02655	1.12	0.01691
Heterogeneous nuclear ribonucleoprotein M	Hnrpm	9.16	2.77	0.00048	1.51	0.00073	1.45	0.00077
Haptoglobin precursor	Hp	2	6.51	0.02087	6.33	0.03657	3.30	0.00731
Heat shock protein HSP 90-alpha	Hsp90aa1	8.18	1.29	0.00031	1.19	0.00952	1.00	0.98177
Heat shock 70 kDa protein 4	Hspa4	2.22	2.20	0.02308	1.74	0.01329	1.54	0.11746
Isocitrate dehydrogenase 1 (NADP ⁺), soluble	Idh1	4.05	1.42	0.00091	1.48	0.00276	1.30	0.01552
Interferon-Inducible protein homolog	Ifitm3	10	1.48	0.01991	1.33	0.02416	1.43	0.04082
Insulin-like growth factor binding protein 7	Igfbp7	4	0.64	0.00086	0.64	0.00012	0.99	0.24127
Predicted similar to RAB6A, member PvAS oncogene family	IP100458129	2	2.04	0.01115	1.35	0.20837	1.40	0.05857
Predicted similar to murinoglobulin 1	IP100458628	2.22	1.18	0.24269	1.47	0.03579	1.55	0.01363
Predicted similar to 60S ribosomal protein L26-like 1	IP100461916	2.96	1.20	0.00986	1.23	0.01941	1.07	0.05728
Predicted similar to 40S ribosomal protein S2 isoform 1	IP100462315	4.12	1.13	0.11148	1.23	0.01681	1.03	0.74056
Predicted similar to cortactin isoform B	IP100624988	2	1.23	0.00759	1.13	0.15730	1.01	0.74524
Predicted similar to syndecan binding protein	IP100658860	1.7	0.78	0.17799	0.84	0.01026	0.76	0.00081

Name	Symbol	Unused	I15:I14	P	I16:I14	P	I17:I14	P
Predicted similar to 60S ribosomal protein L18	IP00660168	2.01	1.36	0.00576	1.33	0.01801	1.20	0.00237
Predicted similar to peroxiredoxin 6	IP00667059	2	1.31	0.01308	1.14	0.10505	1.09	0.41735
Predicted: similar to heterogeneous nuclear ribonucleoprotein A3	IP00668080	12.39	0.45	0.00413	0.82	0.02460	0.74	0.04749
Predicted: similar to 40S ribosomal protein S6	IP00671512	2.22	1.53	0.03667	1.57	0.09780	1.36	0.11542
Predicted: similar to 60S ribosomal protein L22	IP00672745	2	0.99	0.31630	1.33	0.02302	1.11	0.03785
Similar to aldo-keto reductase family 1, member B3 (aldose reductase) isoform 3	IP00672751	6.52	1.24	0.00038	1.04	0.33160	0.92	0.04130
Predicted: similar to nuclear mitotic apparatus protein 1 isoform 8	IP00679098	4.31	1.73	0.00666	1.71	0.00980	1.33	0.08625
Ras GTPase-activating-like protein IQGAP1	Igap1	4.55	1.27	0.07468	1.24	0.03558	1.17	0.07167
Interalpha trypsin inhibitor, heavy chain 2, full insert sequence	Ith2	4.8	0.81	0.15388	0.84	0.00319	0.77	0.00269
Kinesin family member 5B	Kif5b	1.34	0.37	0.00131	0.29	0.00033	0.40	0.00097
Lysosomal-associated transmembrane protein 4A	Laptm4a	4.16	0.74	0.01955	0.91	0.18230	0.87	0.23043
LIM and SH3 domain protein 1	Lasp1	1.87	1.28	0.00081	1.02	0.82691	0.89	0.07143
Splice Isoform 1 of leukemia inhibitory factor receptor precursor	Lifr	2	0.73	0.07785	0.79	0.02379	0.83	0.05927
Splice isoform A of lamin-A/C	Lmna	8.74	1.42	0.00177	1.48	0.00429	0.90	0.02039
Lamin-B1	Lmnbl	2.02	2.05	0.01306	1.82	0.00986	0.85	0.18476
DNA replication licensing factor MCM6	Mcm6	4.14	1.76	0.01764	1.68	0.00668	1.00	0.92590
72 kDa type IV collagenase precursor	Mmp2	3.57	0.69	0.02808	0.71	0.02033	0.95	0.49834
Moesin	Msn	15.87	1.23	0.00184	1.06	0.20770	1.06	0.21205
Microtubule-associated protein 4, isoform 1	Mtap4	10.01	1.46	0.04986	1.14	0.22768	1.07	0.44384
Nicotinamide phosphoribosyltransferase	Nampt	2	1.35	0.02439	1.07	0.24788	0.87	0.17672
Nucleosome assembly protein 1-like 4	Nap1l4	4	1.18	0.06649	1.22	0.00632	0.98	0.80709
Beta-soluble NSF attachment protein	Napb	2	1.07	0.65191	1.55	0.04053	1.24	0.17255
Neural cell adhesion molecule 2	Ncam2	2	1.38	0.01051	0.91	0.27592	0.91	0.31025
Nucleolin	Ncl	8.03	0.64	0.02786	1.25	0.00204	0.96	0.36012
N-myc downstream regulated 1	Ndrg1	4	1.55	0.00619	1.34	0.00082	1.34	0.00041
Predicted: similar to Nucleophosmin (NPM) isoform 2	Npm1	7.31	1.04	0.70252	1.25	0.04476	1.02	0.83235
Splice Isoform 2 of Prolyl 4-hydroxylase alpha-1 subunit precursor	P4ha1	14.27	0.82	0.00054	0.80	0.00002	0.93	0.02886
Splice Isoform 1 of Programmed cell death 6-interacting protein	Pdc6ip	5.69	1.31	0.01627	1.24	0.05127	1.23	0.08927
Phosphatidylethanolamine-binding protein	Pebp1	3.4	2.11	0.03143	1.77	0.03640	1.61	0.02473
Prohibitin	Phb	6	1.38	0.04142	1.21	0.12291	1.08	0.38330
Metalloendopeptidase homolog PEX	Phex	4	0.46	0.02234	0.53	0.01021	0.80	0.01815
Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2 precursor	Plod2	19.3	0.61	3.14E-10	0.69	3.21E-08	0.84	0.00002

Name	Symbol	Unused	I15:I14	P	I16:I14	P	I17:I14	P
Peptidyl-prolyl cis-trans isomerase, mitochondrial precursor	Ppif	2.36	1.55	0.09794	1.35	0.02459	1.11	0.40978
Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	Ppp1ca	4	1.15	0.11370	1.23	0.01697	1.22	0.19022
Proteasome (prosome, macropain) subunit, alpha type 2	Psm2	6	0.79	0.00584	0.85	0.01403	0.82	0.00292
Proteasome subunit alpha type 6	Psm26	7.3	1.04	0.63170	1.20	0.17251	1.21	0.02916
Proteasome subunit beta type 4 precursor	Psm24	1.7	0.67	0.00041	0.85	0.14877	0.84	0.08104
Proteasome subunit, beta type 9	Psm29	2	0.73	0.00017	0.81	0.00302	0.86	0.02689
26S proteasome non-ATPase regulatory subunit 12	Psm212	2.01	1.89	0.01565	1.91	0.01172	1.46	0.01089
Polypyrimidine tract binding protein 1	Ptbp1	6.8	1.29	0.01348	1.34	0.00353	1.39	0.00342
Prothymosin alpha	Ptma	2	0.78	0.10205	1.72	0.00193	1.04	0.74595
Parathymosin	Ptms	2.21	0.47	0.00653	1.21	0.02743	0.80	0.03355
Pituitary tumor-transforming gene 1 protein-interacting protein precursor	Ptglip	2.4	0.70	0.05267	0.79	0.00607	0.92	0.56299
Pentraxin-related protein PTX3 precursor	Ptx3	9	0.72	0.01151	0.87	0.02842	0.77	0.00052
Ras-related protein Rap-1b	Rap1b	6.01	0.78	0.00019	0.80	0.00002	0.88	0.00139
Heterogeneous nuclear ribonucleoprotein G	Rbm3	2.25	1.53	0.00002	1.30	0.03901	1.40	0.00004
Reticulocalbin-3 precursor	Rcn3	8.96	0.77	0.01407	0.83	0.00019	0.84	0.00147
Predicted: similar to 60S ribosomal protein L11	Rpl11	2.01	1.41	0.00013	1.38	0.00005	1.25	0.00527
60S ribosomal protein L13	Rpl13	9.36	1.39	0.07805	1.45	0.02174	1.24	0.11835
Ribosomal protein L14, cytosolic homolog	Rpl14	6	1.39	0.00465	1.45	0.00063	1.34	0.00957
60S ribosomal protein L15	Rpl15	2.09	1.17	0.31704	1.31	0.01177	1.19	0.00001
60S ribosomal protein L34	Rpl34	2.51	1.93	0.00475	2.43	0.00218	1.80	0.00601
60S ribosomal protein L7	Rpl7	8.13	1.16	0.03492	1.30	0.00114	1.12	0.01597
Ribosomal protein, Large P2	Rplp2	6	1.21	0.04420	1.25	0.01254	1.18	0.00833
40S ribosomal protein S27	Rps27	2	1.24	0.00371	1.24	0.01741	1.15	0.06352
40S ribosomal protein S4, X isoform	Rps4x	5.7	1.49	0.00200	1.53	0.00107	1.31	0.01517
40S ribosomal protein S9 homolog	Rps9	7.01	1.16	0.00244	1.27	0.00016	1.06	0.17068
Similar to ribosomal_S3Ae	RS3Ae	10.78	1.08	0.21645	1.29	0.00287	1.14	0.08070
Reticulon 3 isoform 2	Rtn3	2	0.88	0.17876	0.79	0.09679	0.74	0.04592
S100 calcium-binding protein A11 (Calgizzarin)	S100a11	4	1.45	1.27E-06	1.40	4.63E-06	1.30	4.85E-06
S100 calcium-binding protein A4	S100a4	4	1.35	0.02872	1.22	0.11344	1.04	0.72927
Lysosome membrane protein II	Scarb2	10.96	0.84	0.00314	0.78	0.00055	0.90	0.00567
Retinoid-inducible serine carboxypeptidase precursor	Sepep1	5.7	0.69	0.01942	0.80	0.00492	0.88	0.10695
Septin-2	Sept2	4	1.39	0.00221	1.33	0.00322	1.10	0.01895

Name	Symbol	Unused	I15:I14	P	I16:I14	P	I17:I14	P
Septin 7	Sept7	4.03	1.24	0.00549	0.95	0.50655	0.98	0.59337
Placental thrombin inhibitor	Serpmb6a	11.41	1.25	0.00006	1.15	0.00209	1.12	0.03375
Glia derived nexin precursor	Serpine2	9.71	0.68	0.00036	0.74	0.00331	0.98	0.74391
Splicing factor, proline- and glutamine-rich	Sfpq	8.23	1.47	0.00645	1.38	0.00114	1.42	0.00045
SH3 domain-binding glutamic acid-rich-like protein	Sh3bgrl	2	0.93	0.08474	0.82	0.07161	0.78	0.01127
ADP/ATP translocase 2	Slc25a5	4.7	0.75	0.00768	0.81	0.02415	1.08	0.20140
Solute carrier family 29	Slc29a1	4	0.70	0.03439	0.79	0.05475	0.86	0.19471
Splice Isoform 1 of Choline transporter-like protein 2	Slc44a2	4.16	0.78	0.00775	0.78	0.00097	0.85	0.01557
Synaptosomal-associated protein 23	Snap23	2	1.35	0.01229	0.90	0.28088	1.11	0.24989
Small nuclear ribonucleoprotein Sm D3	Snrp3	2	1.08	0.08386	1.21	0.04227	1.25	0.02455
Small nuclear ribonucleoprotein E	Snrpe	2	0.78	0.02898	0.95	0.20513	1.02	0.40027
Superoxide dismutase [Mn], mitochondrial precursor	Sod2	5	0.78	0.00325	0.81	0.03148	0.86	0.05072
Hsc70-interacting protein	Stl3	5.3	1.28	0.03667	1.31	0.08439	1.12	0.38800
Transgelin	Tagln	3.1	1.24	0.03847	1.09	0.38651	0.99	0.94747
Transaldolase	Taldo1	4.26	1.39	0.00149	1.51	0.00264	1.19	0.09349
Protein-glutamine gamma-glutamyltransferase 2	Tgm2	4	0.83	0.00727	0.74	0.00072	1.00	0.82842
Trans-Golgi network integral membrane protein 2 precursor	Tgoln2	2	0.80	0.16160	0.64	0.04075	0.98	0.71384
Thioesterase superfamily member 2	Them2	4	0.71	0.00205	0.78	0.00750	1.04	0.11957
Transketolase	Tkt	10	1.29	0.00489	1.25	0.00691	1.05	0.40896
Transmembrane emp24 domain trafficking protein 2	Tmed2	2	1.37	0.06743	1.43	0.01484	1.59	0.18361
Hypothetical protein Tnem 106b	Tnem 106b	3.05	0.80	0.00724	0.88	0.03337	0.97	0.10148
Similar to elastin	Tnem43	5.7	1.10	0.00318	1.16	0.00158	1.20	0.00599
Splice isoform short of thymosin beta-4	Tmsb4x	3.51	1.32	0.00490	0.98	0.80410	1.05	0.47081
Precursor tenascin protein precursor	Tnc	8.7	1.21	0.02554	1.10	0.09750	1.16	0.04027
Testis-specific serine kinase 4	Tssk4	2	0.93	0.02715	1.25	0.00351	1.19	0.01167
Tubulin, alpha 1a	Tuba 1a	15.83	2.36	0.01315	2.05	0.01677	1.72	0.06347
Tubulin, beta 2	Tubb2a	2.02	1.00	0.99946	1.05	0.41712	0.76	0.00163
Tubulin beta-2C chain	Tubb2c	25.23	1.35	0.02188	1.40	0.01263	1.14	0.13922
Ubiquitin-activating enzyme E1 1	Uba1	8	1.34	0.00793	1.25	0.01538	1.01	0.68045
Vimentin	Vim	50.95	1.23	1.8E-10	1.16	8.9E-09	0.96	0.12721
Splice Isoform 2 of 14-3-3 protein theta	Ywhaq	17.16	1.26	0.01944	1.07	0.29184	0.97	0.49853

TABLE II

True Contraregulated Proteins (Gent vs. UcnII): Unused ProtScore ≥ 2.0

	Symbol	115:114	P	116:114	P	117:114	P
Gent down/Ucn up Prothymosin α	Ptma	0.8	0.102	1.7	0.002	1.0	0.746
Parathymosin	Ptms	0.5	0.007	1.2	0.027	0.8	0.034
Nucleolin	Ncl	0.6	0.028	1.3	0.002	1.0	0.360
v-Type proton ATPase	Atp6v0c	0.5	0.002	1.3	0.044	1.3	0.042
Gent up/Ucn down							
Synaptosomal-associated protein 23	Snap23	1.3	0.012	0.9	0.281	1.1	0.250