CALL FOR PAPERS | Physical Biology of Cancer

Acquired cisplatin resistance in human ovarian A2780 cancer cells correlates with shift in taurine homeostasis and ability to volume regulate

Belinda Halling Sorensen, Unnur Arna Thorsteinsdottir, and Ian Henry Lambert

Department of Biology, Section of Cellular and Developmental Biology, The August Krogh Building, University of Copenhagen, Copenhagen, Denmark

Submitted 5 August 2014; accepted in final form 19 September 2014

Sorensen BH, Thorsteinsdottir UA, Lambert IH. Acquired cisplatin resistance in human ovarian A2780 cancer cells correlates with shift in taurine homeostasis and ability to volume regulate. Am J Physiol Cell Physiol 307: C1071–C1080, 2014. First published September 24, 2014; doi:10.1152/ajpcell.00274.2014.—Cisplatin resistance is a major challenge in the treatment of cancer and develops through reduced drug accumulation and an increased ability to avoid drug-induced cell damage, cell shrinkage, and hence initiation of apoptosis. Uptake and release of the semiessential amino acid taurine contribute to cell volume homeostasis, and taurine has been reported to have antiapoptotic effects. Here we find that volume-sensitive taurine release in cisplatin-sensitive [wild-type (WT)] human ovarian cancer A2780 cells is reduced in the presence of the phospholipase A2 inhibitor bromenol lactone, the 5-lipoxygenase (5-LO) inhibitor ETH diisothiocyanatostilbene-2,2'-disulfonate. Comparing WT and cisplatin-resistant (RES) A2780 cells we also find that evasion of cisplatin-induced cell death in RES A2780 cells correlates with an increased accumulation of taurine, due to an increased taurine uptake and a concomitant impairment of the volume-sensitive taurine release pathway, as well as an inability to reduce cell volume after osmotic cell swelling. Downregulation of volume-sensitive taurine release in RES A2780 cells correlates with reduced expression of the protein LRRC8A. Furthermore, acute (18 h) exposure to cisplatin (5–10 μM) increases taurine release and LRRC8A expression in WT A2780 cells whereas cisplatin has no effect on LRRC8A expression in RES A2780 cells. It is suggested that shift in LRRC8A activity can be used as biomarker for apoptotic progress and acquisition of drug resistance.

key word: platinum drugs; drug resistance; cell volume regulation; LRRC8A; TauT

Ovarian cancer is a leading cause of death among gynecological cancer patients, and the 5-yr survival rate has been estimated to 30% (64). The main reasons for the failure in treatment outcome are that 70% of the patients have already reached advanced stages of cancer progression at the time of diagnosis, i.e., the cancer has already spread within the pelvis and abdomen (68) and that most ovarian cancer cells rapidly develop resistance or multidrug resistance (MDR) against the chemotherapy.

Cisplatin and chemotherapeutic resistance. Cisplatin was the first member of a class of platinum-based anticancer drugs, which also includes carboplatin and oxaliplatin (7, 25), and today cisplatin is frequently used in chemotherapeutic treatment of ovarian cancer, lung cancer, and lymphomas (60). Cisplatin is administered to cancer patients intravenously as a sterile saline solution. Once circulating in the bloodstream, cisplatin is taken up by the cells and accumulated in the cell nucleus, where it causes DNA lesions by formation of DNA adducts and cross links. This eventually leads to G2 cell cycle arrest and apoptosis. Chemotherapeutic resistance towards cisplatin involves 1) reduced intracellular drug accumulation following activity shift in membrane bound cisplatin carriers [MATE (multidrug and toxin extrusion), CRT1 (copper transporter) 1, and OCT (organic cation transporter) 1–3] and ATP-binding cassette drug pumps, 2) enhanced drug detoxification, 3) improved DNA repair, 4) enhanced DNA damage tolerance, and finally 5) diminishing in the initiation/execution of the apoptotic cell death process (5, 7, 8, 16, 43).

Cisplatin-induced apoptosis—apoptotic volume decrease. Programmed cell death or apoptosis is a fundamental biological process needed for the elimination of unwanted or damaged cells (17, 29, 44). Apoptosis is initiated by drug-induced DNA damage/mitochondrial dysfunction (intrinsical pathway), ligand binding to death receptors (extrinsic pathway), and/or cell shrinkage (volume sensory pathway) (16, 17). Figure 1 illustrates intracellular elements involved in initiation of apoptosis. Cisplatin-mediated DNA damage is detected by the ATM (ataxia-telangiectasia mutated kinase) and ATR (ataxia-telangiectasia and Rad3-related kinase) kinases, which subsequently activate the tumor suppressor/transcription factor p53 through phosphorylation (52). Once phosphorylated p53 escapes inactivation by ubiquitination by the ligase MDM2 (mouse double minute 2 homolog) and promotes activation of proapoptotic members of the Bcl-2 family, including Bax (Bcl-2-like protein 4) and Bak (Bcl-2 homologous antagonist), either through increased transcription of their respective genes and/or through direct activation of the proteins. Bax and Bak localize to the outer mitochondrial membrane and dimerize, facilitating release of cytochrome c from the mitochondria into the cytosol and hence stalling the energy production. Cytosolic cytochrome c binds to the Atpaf-1 (apoptotic protease activating factor) 1 and ATP, which then recruits procaspase-9, hence creating the apoptosome. The latter cleaves procaspase to active caspases, which subsequently activates the executor caspase-3 causing cell elimination by apoptosis. As illustrated by cisplatin-induced cell death in, e.g., Ehrlich ascites tumor cells (EATC), induction of apoptosis via the intrinsic pathway involves an initial redistribution of monovalent ions followed by a terminal net loss...
of KCl as well as organic osmolytes and hence cell shrinkage (46). It appears that it is the decrease in the cellular K⁺ concentration, following activation of K⁺ channels in combination with an inadequate efficiency of the Na⁺-/K⁺-/ATPase activity, that causes activation of caspases and nucleases (32). The extrinsic pathway involves formation of the death-inducing signaling complex DISC (death-inducing signaling complex) and subsequently activation of the initiator caspase-8. Activated caspase-8 propagates the apoptotic signal by directly cleaving and activating caspase-3 and/or cleaving the Bcl-2-interacting protein, Bid. This leads to release of cytochrome c from mitochondria into the cytosol and apoptosis. Induction of cell death via the volume sensory pathway following cell shrinkage is in EATC shown to involve the monomeric GTP binding protein Rac and p38 MAPK-mediated phosphorylation/protection of p53 against ubiquitination (6). It is noted that trafficking of CD95 to the plasma membrane and caspase-3/8 activation follow osmotic cell shrinkage, and it has been suggested that an increased number of receptors in the plasma membrane contributes to sensitization of cells towards CD95 (51).

Taurine transporters—cell volume restoration. Taurine (ß-amino-ethane sulfonic acid) is a metabolic inert amino acid that plays an important role in cell volume control, cellular metabolism, antioxidative defense, and initiation/progression of the apoptotic process (17, 23, 27–29, 31, 33, 54, 56, 57). Cellular taurine content is generally a balance between active uptake via the Na⁺– and Cl⁻–dependent taurine transporter (TatT), passive release via volume-sensitive and volume-insensitive transporters (28), and in some cells (liver, pancreas, and testis) synthesis from cystein/methionine (31). Acute modulation of TatT activity involves Ser/Thr kinases (PKA, PKC, and CK2) and reactive oxygen species (ROS) (12, 13, 22, 31, 40, 41, 63), whereas long-term regulation of TatT activity involves transcriptional regulation by cellular osmo-sensing proteins (TonEBP, tonicity end-binding protein) and amino-sensing kinases (mTOR, mammalian target of rapamycin) (9, 27, 30, 31). In contrast to the active taurine uptake system involving TatT, taurine release involves 1) volume-sensitive leak pathways, activated by osmotic cell swelling [termed the volume-sensitive organic anion channel (VSOAC), volume-regulated anion channel (VRA)], and leucine-rich repeat-containing protein 8A (LRRC8A)], which through a reduction in cell volume feedback to the volume sensory pathway. A putative connection between DNA damage and channels activation as well as a role of p53 in channel regulation is to be revealed.
nonadherent EATC, chemoresistance correlates with a reduced nuclear cisplatin accumulation and an increased TauT activity (59). In line with this, Han and colleagues (9, 10) demonstrated that TauT overexpression protects kidney cells against cisplatin-induced cell death through p53 activation. More recently, Yasunaga and Matsumura (66) recently demonstrated that TauT promotes survival and multidrug resistance in colorectal cancer cells.

In this study we investigate whether acquired cisplatin resistance in human ovarian A2780 cells correlates with an increased expression/activity of TauT and/or reduced VSOAC (LRRC8). We also test whether enzymes involved in the activation of VSOAC are affected by acquisition of resistance.

**EXPERIMENTAL PROCEDURES**

**Cell culture.** WT and cisplatin-resistant human ovarian A2780 were grown in 75-cm² culture flasks (CellStar, Gienro Bio, Germany) in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 2 mM L-glutamine. Both cell lines were kept at 37°C, 5% CO₂, and 100% humidity. Cells were passed on every 3-4 days using 0.25% trypsin in phosphate-buffered saline (PBS). To maintain resistance, cisplatin-resistant A2780 cells were treated with 1 μM cisplatin between every third passage. Penicillin/streptomycin, RPMI 1640 medium, fetal bovine serum, L-glutamine, and trypsin were from Sigma-Aldrich.

**Inorganic media.** PBS contained 137 mM NaCl, 2.6 mM KCl, 6.5 mM Na₂HPO₄, and 1.5 mM KH₂PO₄. Isotonic NaCl medium (300 mosM) contained 143 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄, 1 mM CaCl₂, 1 mM MgSO₄, and 10 mM N-2-hydroxethyl piperazine-N'-2-ethanesulfonic acid (HEPES). Hypotonic NaCl solution was prepared by reducing the NaCl concentration in the isotonic solution to 50% (150 mosM, cell volume determination) or 64% (200 mosM, efflux determination) without changing the other components. Isotonic sucrose media were prepared by adjusting the osmolarity of the hypotonic NaCl 150 mosM solution to 274 mosM with sucrose. pH was in all media adjusted to 7.4.

**High-pressure liquid chromatography.** Cells grown to ~80% confluence in T75 culture flasks were gently washed twice in PBS and subsequently lysed in 1.8 ml of 4% sulfosalicylic acid. The lysates were scraped of the flasks with a rubber policeman and transferred to Eppendorf tubes. Lysates were homogenized by passing them several times through a syringe needle (1.2-mm diameter). Aliquots were hydrolyzed with 2 M NaOH and proceeded for determination of the protein contents by the Lowry method using bovine serum albumin (BSA) as a standard (38). The residual homogenates were centrifuged (20,000 g, 10 min), and the supernatants were filtered (Millipore-GV; 0.22 μm) for determination of the amino acid content. Samples and standards were OPA-derivatized, separated, and quantified by reverse phase high-pressure liquid chromatography (HPLC; Gilson: 322-Pump, 234-Autoinjector, 155-UV/VIS) using a Nucleosil column (Macherey-Nagel, C18, 250×4; 5 μm), an acetonitrile/phosphate (12.5 mM pH 7.2) buffer gradient, and ultraviolet detection (330 nm). Taurine (1.0 mM), alanine (1.0 mM), and glycine (1.0 mM) standards were used for the quantification, and the cellular amino acid content (μmol/g protein) was calculated from the protein and the amino acid content.

**Real-time-quantitative polymerase chain reaction.** Real-time quantitative polymerase chain reaction (RT-qPCR) was performed to quantify the mRNA accumulation/gene expression of LRRC8A, TauT, and Actin related protein 2/3 ARP2/3 (reference gene, actin related protein 2 and 3). RNA was isolated from cells grown in 10-cm petri dishes (90% confluence), using the Nucleospin RNA II kit (Macherey Nagel). Reverse transcription was performed on 1 μg RNA (determined from the 260 nm/280 nm absorbance ratio), first incubated at 65°C for 5 min in nucleotide solution (dNTP mix) plus oligo dT primer and subsequently for 2 min at 42°C with strand buffer plus dithiothreitol. Superscript II reverse transcriptase was added and mixture incubated for 50 min at 42°C. The reaction was terminated and inactivated by raising the temperature to 70°C for 15 min. To quantify the mRNA accumulation, we used Stratagene Mx4000 or Mx3000P and Brilliant II SYBR Green QPCR master mix plus the following predesigned and validated KICqStart (TauT) and PrimePCR (LRRC8A) SYBR Green Primers from Sigma-Aldrich and Bis-Rad, respectively: TauT: forward: CTC TTT GAC TAC TAT GAT GAC and reverse: AAA CAT CCA ACA CAG AGA AC; and ARP forward: CGA CCT GGA AGT CCA ATA C and reverse: ATC TGC TGC ATC TGC TTT. RT-qPCR was performed in triplicate in the following conditions: 10 min at 95°C followed by 40–50 cycles of 30 s at 95°C, 1 min at 59°C, and 30 s at 72°C and single step consisting of 1 min at 95°C, 30 s at 55°C, and 30 s at 95°C. Mean Ct value was calculated. LRRC8A and TauT were for each sample normalized to ARP2/3.

**Western blotting.** Western blotting was used to quantify the protein levels of LRRC8A (94 kDa), TauT (70 kDa), and the house-holding protein β-actin (42 kDa). Protein extraction and blotting were performed on cells grown to 80% confluence in 10-cm petri dishes. Cells were washed gently twice in time ice cold PBS and subsequently lysed in lysis buffer containing 1% SDS, 150 mM NaCl, 20 mM HEPES, 1 mM EDTA, 0.5% Triton X-100, 1 mM Na₂VO₄, and 1% protease inhibitor mix before sonication. Lysates were centrifuged 5 min at 5°C and 20,000 rpm to separate the proteins extracts from insoluble cell debris. Protein content was estimated by a DC assay (Bio-Rad), and lysates with equivalent amount of protein (25 μg per lane) were mixed with NuPAGE sample buffer plus dithiothreitol and proceeded for SDS-PAGE gel electrophoresis (NuPAGE precast 10% Bis-Tris gels) in NOVEX chambers under reducing and denaturing conditions (NuPAGE MOPS SDS running buffer; Invitrogen). Benchmark protein ladder was used for indication of molecular weight. NuPAGE transfer buffer (Invitrogen) was used for protein transfer to nitrocellulose membranes, and protein transfer was verified by Ponceau staining. Membranes, blocked at 37°C in TBST (0.01 M Tris-HCl, 0.15 M NaCl, and 0.1% Tween 20 pH 7.4) containing 5% nonfat dry milk, incubated overnight at 4°C with primary antibodies, were washed in TBST and subsequently incubated with secondary antibodies for 1 h at room temperature. The monoclonal anti-human-LRRC8A and polyclonal anti-human-β-actin antibodies, both produced in mouse and from Sigma-Aldrich, were used in a dilution of 1:250 and 1:1,000, respectively. TauT antibody from Yorkshire-Bioscience was used in a dilution 1:10,000. The secondary AP-conjugated anti-mouse and anti-rabbit antibody (Sigma) was used in a dilution of 1:5,000. Following wash in TBST, membranes were developed using BCIP/NBT (KPL, Gaithersburg, MD). Membranes were scanned and bands were quantified using UN-SCAN-IT (Silk Scientific).

**Cell volume measurements.** Changes in cell volume upon hypotonic exposure were measured by electric cell sizing. WT and resistant A2780 cells, grown to 80% confluence in T75 flasks, were gently washed twice in PBS and once in 2 mM EDTA in PBS. The EDTA solution was quickly removed, and the cells were left to detach in 1 ml PBS. Following detachment, the cells were resuspended in growth media (~5 ml). Absolute cell volumes were estimated after 50-fold dilution in either isotonic sucrose or hypotonic NaCl solution in a Beckmann Multisizer III (Coulter, Luton, UK) using an aperture size of 100 μm and calibrated with latex beads (diameter: 20.47 μm; Coulter). All solutions (except growth media) used for cell volume measurements were microfiltered (Millipore filters; 0.45 μm) before the experiments.

**Estimation of taurine uptake and efflux.** [³H]taurine (PerkinElmer, Waltham, MA) was used to estimate the uptake of taurine via TauT as well as the efflux under isotonic and hypotonic conditions as previously described (19, 61). All the experiments were performed at room temperature.

For the influx experiments, WT and resistant A2780 cells were grown to 80% confluence in six-well polyethylene culture plates (9.6 cm² per well) in complete growth medium. Five of the six wells were used to determine the taurine uptake and the residual well was used to determine the representative protein content (Lowry method). Before
the initiation of the experiment, each well was washed three times in 1 ml isonicotic NaCl medium to remove the growth media. Following the final wash to each well was added 600 μl isonicotic NaCl medium. The experiment was initiated by adding 50 μl [3H]taurine stock solution containing 37,000 Bq/ml (0.005 μM taurine) to wells 1–5 at time 0, 2, 4, 6, and 8 min, respectively. Taurine uptake was terminated at 10 min by removal of the extracellular medium and rapid rinsing cells by addition and aspiration of 1 ml ice-cold MgCl2, followed by cell lysis with 200 μl 96% ethanol. After evaporation of EtOH, 600 μl ddH2O was added wells 1–5 and the plate was incubated 20 min on a shaking table to dissolve/extract the isotope. The dissolved isotope was transferred to scintillation vials, and each well was washed twice in 600 μl ddH2O. These washings were likewise transferred to scintillation vials. To all vials 3.5 ml Ultima Gold were added, and activity was determined in a PerkinElmer scintillation counter. The total [3H]taurine (counts/min) taken up by the cells/wells at a given time point was estimated as the sum of 3H activity in the cell extract and two water washouts. Cellular taurine activity (counts·min⁻¹·well⁻¹) was converted to nmol/g protein, using the extracellular specific activity (counts·min⁻¹·nmol⁻¹) and the protein content (mg protein/well), and finally plotted vs. time. Taurine uptake (nmol-g protein⁻¹·min⁻¹) was determined by linear regression.

Swelling-induced taurine efflux was estimated on cells grown to 80% confluence in six-well polyethylene culture plates. Cells were loaded in complete growth medium supplemented with [3H]taurine (18,500 Bq/well) for 2 h (37°C, 5% CO₂, 100% humidity). Efflux experiment was measured at room temperature with each well representing one experiment. Before the experiment the growth media were removed and each well washed three times with 1 ml isonicotic NaCl medium (300 mosM) per well to remove remaining extracellular isotope and growth media. The efflux experiment was performed by transferring the NaCl medium from the well to vials and replacing it with new medium at 2-min intervals for a total time period of 30 min, in the absence of presence of inhibitors, i.e., the 5-LO inhibitor ETH 615–139 (donated by Dr. I. Ahnfelt-Rønne, Løvens Kemiske Fabrik, Denmark; dissolved in EtOH), the CysLT1 antagonist zafluraxat [N-[3-[2-methoxy-4-[[2-methylphenyl]sulfonyl]amino]carbonyl]phenyl]-methyl]-1-methyl-1H-indol-5-ylcarbamic acid cyclopentyl ester, dissolved in DMSO), the PLA₂ inhibitor bromoacetoxymethyl (BEL; dissolved in DMSO), and 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS; dissolved in ddH₂O). At time 12 min isonicotic medium was replaced by hypotonic medium (200 mosM). Isoptote remaining inside the cells in each well at the end of the experiment was determined by addition of 1 ml 1 M NaOH, gently shaking for 1 h, and subsequently transfer of NaOH and to timed washouts (ddH₂O) to vials. Scintillation liquid was added to all vials, which were proceeded for 3H activity determination. The sum of 3H activity released during the efflux experiment and in the NaOH/water washouts represent the total 3H activity in the cell system. The fractional rate constant (k, min⁻¹) for taurine release was calculated from the following equation: 

\[
k = \frac{\ln(X_1) - \ln(X_2)}{t_1 - t_2}
\]

where X₁ and X₂ are the fraction remaining in the cell at time t₁ and t₂, divided by the time interval.

**Determination of cell viability**—3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The colorimetric MTT assay, i.e., conversion of the yellow soluble tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into a blue insoluble formazan, was, as previous described (12), used to determine cell viability after treatment of WT and resistant cells with 5–20 μM cisplatin (48 h). A2780 cells were seeded to a density of 20,000 cells per 200 μl media per well in 96-well plates and incubated for 72 h (37°C, 5% CO₂, 100% humidity). Addition of TNF-α (Sigma-Aldrich) or hypertonic exposure by addition of 81.5 mM NaCl was supplemented the treatment during the last 18 h. At the end of the incubation period, 100 μl were removed, 25 μl of a MTT solution (5 mg/ml MTT in sterilized PBS) were added well, and the plate was incubated in the cell culture incubator for 3 h. Following incubation 100 μl fresh-made 10 mM HCl containing 1% SDS was added to each well and incubated in fume hood overnight to solubilize the colored formazan crystals. Samples were measured at 540 nm using Wallac Envision Multilabel plate reader (PerkinElmer). The absorbencies measured were assumed to be directly proportional to the number of viable cells. Data were represented relative to the absorbance from untreated control cells. Each experiment was performed in triplicate.

**Statistics.** All data were statistically tested (SigmaPlot version 12) by Student’s t-test or one-way ANOVA with Bonferroni’s test as posttest. Statistical significant indicates *P < 0.05. In bar and scatter plots, the error bars signify SE.

**RESULTS**

Chemotherapeutic resistance can be either an acquired property (extrinsic resistance) following chemotherapy or an innate property (intrinsic resistance) caused by cell differentiation or genetic changes occurred during the early stages of tumor formation. The phenotype of the cisplatin-resistant (RES) human ovarian cell line A2780 reflects extrinsic resistance and in Fig. 2A, it is seen that 48-h exposure to 5 and 10 μM cisplatin...
has no significant effect on cell viability in cisplatin RES A2780 cells whereas WT A2780 cells show a 40–50% reduction in viability under the same conditions. Increasing the cisplatin concentration to 20 μM elicits a significant increase in cell death in RES A2780 cells, i.e., induction of cell death in RES A2780 is observed although at a high cisplatin dose. To test whether cisplatin-induced cell death via the intrinsic pathway in WT A2780 cells involves other cell death pathways (see Fig. 1), we exposed WT and RES A2780 cells to cisplatin plus TNF-α (intrinsic plus extrinsic pathway) or cisplatin plus hypertonicity obtained by increasing the extracellular NaCl concentration (intrinsic plus volume sensory pathway). To ensure minimal independent effects of TNF-α and hypertonicity, we limited the exposure time to 18 h compared with the 48-h cisplatin exposure. From Fig. 2B, no significant effect of TNF-α or hypertonicity on cell viability is observed in WT and RES cells in the absence of cisplatin. However, TNF-α increases the toxicity of cisplatin in both WT A2780 and RES A2780 cells, whereas incubation in hypertonic medium increases the toxicity of cisplatin radically in WT A2780 and to a less extent RES A2780 cells (Fig. 2B). These data are taken to indicate that RES A2780 cells are less sensitive to induction of cell death via the volume sensory pathway compared with WT A2780 cells.

A hallmark in apoptosis is shift in TauT activity and/or a decrease in the volume-sensitive leak permeability towards taurine. The HPLC technique revealed that the cellular content of taurine in WT A2780 cells is 0.019 ± 0.003 μmol amino acid/mg protein (Fig. 3A), which is lower than taurine content in, e.g., ELA (0.06 - 0.07 μmol/mg protein; Refs. 59, 61) and NIH3T3 fibroblasts (0.025–0.055 μmol/mg protein; Refs. 42, 63). With the use of average values for cellular water and protein content, i.e., 3.24 ml cell water/g cell dry wt (18) and 0.78 g protein/g cell dry wt (15), the taurine content in A2780 cells corresponds to a cellular taurine concentration at 4.5 ± 0.7 mM. Plasma taurine concentration is 10–100 μM (20), indicating that the intracellular taurine concentration, due to TauT activity, is presumably 400-fold higher than the extra-

---

Fig. 3. Cellular amino acid content, taurine accumulation, volume-sensitive taurine release and ability to perform regulatory volume decrease (RVD) in WT and RES A2780 cells. Measurements were performed on WT and RES A2780 cells. A: cellular amino acid content (μmol/mg protein) was determined by high-pressure liquid chromatography following OPA derivatization. Data represent 6 sets of data for WT A2780 cells (open bars) and RES A2780 cells (closed bars). *Significantly changed compared with WT cells. B: increase in cellular taurine content (nmol/g protein) was determined under isotonic conditions as a function of time using tracer technique in WT A2780 (○) and RES A2780 cells (●). Curves represent 1 set out of 4 sets of experiments. C: taurine influx (nmol·gram protein⁻¹·min⁻¹) was determined from the slope of time traces illustrated in B. Values for RES A2780 cells (closed bars) are given relative to WT A2780 cells (open bars) and represent means of 4 sets of experiments. *Significantly increased compared with WT cells. D: fractional rate constant (min⁻¹) for taurine release was determined from release of ¹H-labeled taurine and plotted vs. time under isotonic and hypertonic conditions (shift in tonicity indicated by the arrow) in WT A2780 cells (○) and RES A2780 cells (●). The curves represent 10 (WT) and 9 (RES) sets of experiments. E: cell volume in WT A2780 cells (○) and RES A2780 cells (●) was followed with time after transfer to hypotonic solution using electronic cell sizing. Values are given relative to the cell volume under isotonic conditions, measured prior to hypotonic exposure. Time traces for each cell line represent 1 out of 4 independent sets of experiments. F: cell volume recovery is given in percentage recovery at time 6 min, where total recovery from the maximal values to the initial value equals 100%. Data represent 4 sets of data. *Significantly reduced compared with WT cells.
cellular concentration in A2780 cells grown in 10% serum. From Fig. 3A, it is seen that the cellular content of the organic osmolyte taurine and alanine is significantly increased in RES A2780 cells compared with WT A2780 cells, whereas cellular glycine content is concomitantly reduced. It is noted that loss in glycine almost balances gain in taurine plus alanine. As increased taurine accumulation could reflect an increased uptake and/or a reduced release, we determined the unidirectional taurine uptake and release in WT and RES A2780 cells. From Fig. 3, B and C, it is seen that taurine uptake is linear within the initial 10 min and that acquired cisplatin resistance in RES A2780 cells is accompanied by 1.7-fold increase in taurine influx. RT-qPCR and Western blotting revealed that TauT mRNA accumulation and expression of the 70-kDa TauT protein band were unaltered, i.e., TauT mRNA relative to ARP2/3 was determined in three sets of experiments at 0.9817 ± 0.0004 (WT) and 0.9823 ± 0.00002 (RES), whereas TauT protein expression relative to β-actin was determined in five sets of experiments at 0.09 ± 0.02 (WT) and 0.08 ± 0.03 (RES). This could indicate that upregulation of TauT activity in connection with cisplatin resistance reflects shift in the regulation of TauT and/or the number of TauT transporters in the plasma membrane. This was not investigated further.

From Fig. 3D, it is seen that taurine is released from WT A2780 cells under isotonic conditions and that release increases dramatically and transiently upon hypotonic exposure, reaching a maximal rate constant for taurine release within 8 min. However, in RES A2780 cells the volume-sensitive taurine release was absent (Fig. 3D). It is noted that rate constant for taurine release under isotonic conditions in A2780 cells was not affected by acquisition of resistance, i.e., the fractional rate constant was determined at 0.0033 ± 0.0002 min⁻¹ (n = 10) for WT A2780 cells and 0.0039 ± 0.0004 min⁻¹ (n = 9) for RES A2780 cells. However, with the use of values for the cellular taurine content (Fig. 3A), and the fractional rate constant for taurine release, it is seen that the actual taurine release under isotonic conditions is almost tripled in RES A2780 (1.7·10⁻⁴ μmol·mg prot⁻¹·min⁻¹) compared with WT A2780 cells (0.6·10⁻⁴ μmol·mg prot⁻¹·min⁻¹) due to the steeper cellular to extracellular taurine gradient. It is noticed that values for taurine efflux under isotonic conditions are larger than values for taurine influx (Fig. 3B), primarily due to the experimental setups, i.e., influx is determined at a low extracellular taurine concentration (0.005 μM), which is significantly lower than the concentration required for half saturation of TauT (20, 27, 31) and the concentration in the growth medium, and secondly because the specific activity, used for the calculations of taurine influx, might be smaller than the specific activity determined from the added isotope due to taurine release during the experiment. It is assumed that the taurine leak pathway, evoked during apoptosis, represents taurine loss via the volume-sensitive leak pathway designated VSOAC (17). Hence, the increased taurine content (Fig. 3A) in resistant cells reflects an increased taurine uptake via TauT and impaired taurine release via the volume-sensitive leak pathway VSOAC.

Osmotic cell swelling normally elicits a volume regulatory response [regulatory volume decrease (RVD)], which reflects net loss of K⁺, Cl⁻, and the organic osmolytes (17). From Fig. 3, E and F, it is seen that WT A2780 swell within 2 min following exposure to hypotonic environment where after they regulate their volume towards its original value. RES A2780 cells likewise swell as WT A2780 cells but show no RVD (Fig. 3, E and F). Previous findings with EATC indicate that the volume-sensitive Cl⁻ and K⁺ conductances are also impaired during acquisition of drug resistance (46). In the case of A2780, the cellular taurine concentration (4.5 mM, see above) is low compared with the cellular K⁺ and Cl⁻ concentration and taurine loss as well as loss of glycine and alanine will only account for a minor fraction of the total loss of osmolytes during RVD. Hence, acquired resistance in A2780 cells just like resistance in EATC most probably implies reduction in the volume-sensitive transporters for organic osmolytes as well as for ions. Downregulation of the volume-sensitive ion transporters was not investigated further.

Reduced VSOAC activity could reflect either reduced activation and/or expression of the transporter. From Fig. 4A, it is seen that activation of the volume-sensitive taurine release from WT A2780 cells in congruence with findings from EATC (27) and human lung cells (19) is significantly reduced following inhibition of PLA2 activity (iPLA2 inhibited by BEL), 5-LO activity (inhibited by ETH), and blockage of the CysLT1 receptor with the antagonist zafirlukast. VSOAC activity is also inhibited following acute exposure to the anion channel blocker DIDS (Fig. 4A). As inhibition of the channel in WT A2780 cells with DIDS reduces the VSOAC activity to an even lower value than the activity in RES A2780 (Fig. 4A), we tested for the expression of LRRC8A, which has recently been identified as a VSOAC component (49, 62). From Fig. 4, B and C, it is seen that the expression of LRRC8A is significantly reduced although not eliminated in RES A2780 cells compared with WT A2780 cells. In congruence we find that acute DIDS exposure reduces VSOAC activity in RES A2780 cells by 18 ± 3% compared with WT A2780 cells. Using QPCR we find in three sets of experiments that LRRC8A mRNA relative to ARP2/3 mRNA is reduced from 0.00045 ± 0.00004 (WT) to 0.00033 ± 0.00005 (RES). Hence, reduced expression of LRRC8A (Fig. 4C) correlates the reduced volume-sensitive taurine release (Fig. 3D) and ability to volume regulate after hypotonic exposure (Fig. 3F). It is noted that 5-LO and CysLT1 mRNA/protein expression could also be reduced as part of acquirement of drug resistance but that the effect on taurine release would be masked by the concomitant reduction in LRRC8A expression/activity.

Loss of amino acids in, e.g., EATC, is in full progress within the initial 20 h following exposure to cisplatin (46). Similarly we find that exposure of WT A2780 cells for 18 h to 10 μM cisplatin reduces the cellular content of taurine, glycine, and alanine significantly to 62 ± 11, 78 ± 5, and 86 ± 3% of the initial value (data from 3 sets of experiments). From Fig. 5, A and B, it is seen that acute exposure (18 h) of WT A2780 cells to 5 or 10 μM cisplatin induces a significant increase in LRRC8A expression. In contrast, acute exposure of RES A2780 cells to cisplatin had no effect on LRRC8A expression, i.e., the LRRC8A to β-actin protein expression ratio, when given relative to the ratio in control RES A2780 cells, was in three sets of experiments determined at 1.04 ± 0.13 and 0.84 ± 0.08 after 18-h treatment with 5 and 10 μM cisplatin, respectively. Hence, induction of taurine loss during the initial stage of apoptosis correlates with increased LRRC8A expression whereas cisplatin resistance correlates with reduced LRRC8A expression.
from the release of 3H-labeled taurine under hypotonic conditions in WT (open
protein expression were determined in A2780 cells by tracer technique, and
with reduced LRRC8A expression. Volume-sensitive taurine release, and
Fig. 4. Reduced volume-sensitive taurine release in RES A2780 cells correlates
become clear that cisplatin-induced cell shrinkage [apoptotic
In recent years it has
DISCUSSION

Deregulation of proapoptotic, volume-sensitive taurine release correlates with drug resistance. In recent years it has
KCl via K⁺, Cl⁻ channels, as well as organic osmolytes and
and that drug resistance in cancer cells implies downregulation of
K⁺ (16, 46) and Cl⁻ (34, 39, 48) channel activities. In addition,
prevention of KCl loss through inhibition of K⁺ channels (36, 46) and Cl⁻ channels (39, 46) is found to limit or postpone
apoptosis in WT EATC. The Na⁺-K⁺-2Cl⁻ cotransporter
NKCC1, the Na-K-ATPase, cation channels, and the Na⁺/H⁺
exchanger NHE1 have been designated antiapoptotic transport-
ers as they compensate for loss of osmolytes and hence limits
cell shrinkage and unset of apoptosis (16). On the other hand,
K⁺ and Cl⁻ channels, which are responsible for AVD, as well as
Ca²⁺ channels, which are involved in Ca²⁺ influx and modulation of Ca²⁺-sensitive steps during apoptosis, have
been designated proapoptotic ion channels (16). It is noted that
inhibition of Cl⁻ loss not only limits cell shrinkage but also prevents DNA degradation in human Jurkat cells following
intrinsic activation of the apoptotic process (14). Inhibition of
K⁺ loss will likewise limit the initial cell shrinkage but presumably also restrict decay of the membrane potential,
which would normally favor Ca²⁺ influx and activation of
apoptotic enzymes (32, 35, 67).

In the present study we find that hyperosmotic exposure, i.e.,
cell shrinkage, obtained by increasing the NaCl concentration
in the RPMI growth medium, increases the toxicity of cisplatin
in WT A2780 cells but has only a minor effect in RES A2780

A

TAURINE HOMEOSTASIS AND DRUG RESISTANCE
C1077

B

A

A2780 WT

LRRC8A

β-actin

Control 5μM 10μM cisplatin

LRRC8Aexpression, relative scale

Control

5μM

10μM
cisplatin

0.00

0.50

1.00

1.50

2.00

2.50

3.00

LRRC8Aexpression, relative scale

C

Fig. 5. Short-term/acute exposure to cisplatin increases LRRC8A expression in
WT A2780 cells. LRRC8A protein expression in WT A2780 was determined by
Western blotting following acute (18 h) exposure to cisplatin. A: Western blot
illustrating LRRC8A and β-actin expression in WT A2780 cells exposed to 5
and 10 μM cisplatin for 18 h. Control cells were not treated with cisplatin. Blot
represents 1 out of 3 sets of experiments. B: quantification of LRRC8A
expression in WT A2780 cells following acute (18 h) cisplatin exposure from
3 sets of paired experiments was performed using UN-SCAN-IT. The
LRRC8A/β-actin was determined and shown relative to the ratio in untreated
control cells. *Significantly increased compared with control.

AJP-Cell Physiol • doi:10.1152/ajpcell.00274.2014 • www.ajpcell.org

Fig. 4. Reduced volume-sensitive taurine release in RES A2780 cells correlates
with reduced LRRC8A expression. Volume-sensitive taurine release, and
protein expression were determined in A2780 cells by tracer technique, and
Western blot, respectively. A: volume-sensitive taurine release was determined
from the release of 3H-labeled taurine under hypotonic conditions in WT (open
bars) and RES (black bar) A2780 cells in the absence (control) or presence of
bromoenol lactone (BEL; 10 μM, 30-min preincubation), ETH (10 μM, present
during the efflux experiment), zafirlukast (60 μM, present during the efflux
experiment), and DIDS (50 μM, present during the efflux experiment) as
illustrated in Fig. 3D. The maximal rate constant (min⁻¹) for taurine release
was obtained 8 min after hypotonic exposure. Data for WT A2780 cells
represent 18, 6, 4, 6, and 3 sets of experiments for control, BEL, ETH,
zafirlukast, and DIDS, respectively. Data for RES A2780 represent 9 sets of
experiments. *Significantly reduced compared with WT control cells. B:
Western blot illustrating LRRC8A and β-actin expression in WT and RES
A2780 cells. Blots representing 5 separate sets of paired preparations (set 1 to
3 are shown). C: quantification of LRRC8A expression in WT and RES A2780
cells from 5 sets of paired experiments (3 of which are shown in B) was
performed using UN-SCAN-IT. Values are presented relative to the house
holding protein β-actin. *Significantly reduced compared with WT cells.

Downloaded from http://ajpcell.physiology.org/ by 10.220.33.6 on August 14, 2017
cells. In pig LLC-PK1 kidney cells, it has been shown that an increase in the extracellular NaCl concentration per se has no effect on cisplatin accumulation although a dramatic effect on cisplatin toxicity (11). In the case of EATC, the osmotic cell shrinkage has previously been shown to induce apoptosis via activation of a volume sensory pathway, which like the cisplatin-induced intrinsic pathway involves phosphorylation and activation of the transcription factor p53 and subsequently caspase-3 (see Fig. 1; Ref. 6). Hence, cisplatin-induced cell death in WT A2780 cells most probably reflects activation of the intrinsic pathway and a concomitant activation of the volume sensory pathway due to activation of transporters for ions/organic osmolytes (LRRC8A) and a subsequent reduction in cell volume. In RES A2780 cells, reduced sensitivity to cisplatin could partly reflect reduced activation of the volume sensory pathway due to the reduced LRRC8A expression and the inability of RES A2780 cells to activate their volume-sensitive transporters for ions, which in the present study is seen as an inability to perform RVD. A shift in p53 activation/downstream signaling in RES A2780 cannot be excluded from the present study.

Loss of amino acids including taurine is part of AVD, and it has been determined that EATC reduce their cellular content from 350 to 100 μmol/g dry wt within 20 h in the presence of 5 μM cisplatin (46). Cisplatin resistance was in EATC shown to correlate with a reduced activity of the volume-sensitive taurine transporter VSOAC (46), i.e., VSOAC could be characterized as a proapoptotic transporter. In daunorubicin-resistant EATC cells, it was demonstrated that TauT mRNA as well as TauT protein expression and activity was significantly reduced compared with WT EATC (47). On the other hand, taurine uptake in MDRI-transfected NIH3T3 cells was increased compared with parental fibroblasts, i.e., downregulation of taurine transporter in EATC with acquired drug resistance occurs independently of P-glycoprotein overexpression (47). In ELA cells, which have an innate resistance to cisplatin, TauT could be designated an antiapoptotic transporter as it has been demonstrated that cisplatin resistance correlated with an increased TauT expression/activity (59). Accordingly, Yasunaga and Matsumuru (66) characterize TauT as a colorectal cancer-specific cell surface marker, as TauT knockdown attenuates survival and enhanced drug sensitivity, whereas TauT overexpression increases cell survival and drug resistance. In the present work acquired resistance in A2780, similar to innate resistance in ELA, correlates with an increased TauT activity, which does not reflect shift in TauT mRNA or protein but more likely increased activity or translocation of TauT protein to the plasma membrane.

Taurine accumulates in A2780 cells as they develop resistance, and taurine per se has previously been reported to play a role in progression of apoptosis, i.e., taurine supplementation reduces apoptosis induced by drug exposure, ischemia, hypoxia, and glucose supplementation through mechanisms that involve 1) prevention of mitochondrial permeability transition/dysfunction and reduction in cytochrome c release (3); 2) prevention in drug-induced upregulation of Bak and Fas (37); 3) prevention of p53 activity; ROS generation, and Ca2+ mobilization (4, 65); 4) elevation in cellular Bcl-2 (55); 5) suppression of Apaf-1/caspase-9 apoptosis assembly (57); as well as 6) reduction in caspase-8 and caspase-9 expression (58).

Reduced LRRC8A expression and volume-sensitive taurine release correlate with acquisition of cisplatin resistance. Members of the LRRC8 family have been found to be important entities of the swelling activated Cl− and taurine channel(s) (49, 62). Sequence comparisons have revealed that LRRC8 shares a common ancestor with pannexines, a protein family considered to form hexameric channels like LRRC8 and known to be involved in leakage of Ca2+ from the endoplasmic reticulum and ATP-dependent cell death (1, 50). The LRRC8 proteins are like pannexines composed of four transmembrane segments located at the NH2-terminal half of the protein and a leucine-rich repeat domain (LRRD) carrying up to 17 leucine-rich repeats located at the COOH-terminal end (1). Furthermore, LRRC8 contains putative sites that could be regulated through phosphorylation and ubiquitinylation (1). To date not much is known about the LRRC8 family and its cellular functions. The LRRC8 family was first described in a girl with congenital agamaglobulinemia and minor facial anomalies due to lack of B cells in peripheral blood (53). The patient was found to carry a chromosomal translocation on chromosome 9 causing a truncated LRRC8 gene, and the truncation was found to result in pro-B-cell arrest and thereby prevents B-cell maturation (53). In the present context it is of interest that LRRC8A has been demonstrated to be an essential component of VSOAC, i.e., LRRC8A knockout almost blunted RVD and the concomitant swelling-induced taurine release in cancer cells (49, 62). In congruence we find that the impairment of swelling-induced taurine efflux and ability to volume regulate observed in cisplatin resistance in A2780 cells correlate with a decreased protein level of LRRC8A. LRRC8A mRNA is also reduced in RES A2780 cells compared with WT A2780 cells although not to the same extent as the LRRC8A protein expression, indicating that posttranslational ubiquitination and degradation of LRRC8A could be increased during acquisition of cisplatin resistance in A2780 cells as indicated by Bradley et al. (2). It is assumed that the functional VSOAC transport pathway is formed by six proteins from the LRRC8 family and that shift in stoichiometry of LRRC8A and other LRRC8 members affects channel activity (62). According to Qiu et al. (49) and Voss et al. (62), LRRC8 hexamers constitute a common volume leak pathway for Cl− and amino acids including taurine. If this is the case, the differences observed with respect to time frame for activation and inactivation of volume-regulated anion channel (VRAC) and VSOAC (27) and sensitivity to cholesterol depletion (24, 61) following osmotic cell swelling could represent heterogeneity in the stoichiometry of LRRC8 components and/or that the substrate selectivity is differently regulated. Qui et al. (49) show that mutation of T44 at the external part of TM1, which lines the permeability pore, has a considerable impact on the anion permeability.

In conclusion our data indicate that induction of apoptosis, i.e., cisplatin-induced loss of osmolytes in WT A2780 cells correlates with increased LRRC8A expression whereas cisplatin resistance in RES A2780 cells correlates with reduced LRRC8A expression. Hence, shift in LRRC8A activity could be used as biomarker for apoptotic progress and acquisition of drug resistance. It is noted that other LRRD-containing proteins have been associated with induction of cell death, i.e., Nod1, which is a Apaf-1 like, LRRD-containing protein, was found to regulate apoptosis through caspase-9 activation (in-
trinic pathway) but not caspase-8 (extrinsic pathway) (21). Likewise leucine-rich repeat and Ig domain containing 1 (LINGO-1) potentiates neuronal apoptosis by inhibiting with no lysine kinase 3 (WNK3) activity (69).

ACKNOWLEDGMENTS

Dorthe Nielsen is acknowledged for technical assistance. Peter E. Nielsen and Mia Henriksen are acknowledged for data included in Fig. 3, A and D. We thank COST Action CM1105—Functional metal complexes that bind to biomolecules for stimulating discussions.

GRANTS

The present work was supported by “Fabrikant Einar Willumsens Mindel-egat” and “Læge Sofus Carl Emil Friis og hustru Olga Doris legat.”

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


