Volume-sensitive release of organic osmolytes in the human lung epithelial cell line A549: role of the 5-lipoxygenase

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Holm JB, Grygorczyk R, Lambert IH. Volume-sensitive release of organic osmolytes in the human lung epithelial cell line A549: role of the 5-lipoxygenase. Am J Physiol Cell Physiol 305: C48–C60, 2013. First published March 13, 2013; doi:10.1152/ajpcell.00412.2012—Pathophysiological conditions challenge cell volume homeostasis and perturb cell volume regulatory mechanisms leading to alterations of cell metabolism, active transepithelial transport, cell migration, and death. We report that inhibition of the 5-lipoxygenase (5-LO) with AA861 or ETH 615-139, the cysteinyl leukotriene 1 receptor (CysLT1) with the antiasthmatic drug Zafirlukast, or the volume-sensitive organic anion channel (VSOAC) with DIDS blocks the release of organic osmolytes (taurine, meAIB) and the concomitant cell volume restoration following hypoosmotic swelling of human type II-like lung epithelial cells (A549). Reactive oxygen species (ROS) are produced in A549 cells upon hypotonic cell swelling by a diphenylene iodonium-sensitive NADPH oxidase. The swelling-induced taurine release is suppressed by ROS scavenging (butylated hydroxytoluene, N-acetyl cysteine) and potentiated by H2O2, Ca2+ mobilization with ionomycin or ATP stimulates the swelling-induced taurine release whereas calmodulin inhibition (W7) inhibits the release. Chelation of the extracellular Ca2+ (EGTA) had no effect on swelling-induced taurine release but prevented ATP-induced stimulation. H2O2, ATP, and ionomycin were unable to stimulate the taurine release in the presence of AA861 or Zafirlukast, placing 5-LO and CysLT1 as essential elements in the swelling-activated flux of VSOAC with ROS and Ca2+ as potent modulators. Inhibition of tyrosine kinases (genistein, curcubitacin) reduces volume-sensitive taurine release, adding tyrosine kinases (Janus kinase) as regulators of VSOAC activity. Caspase-3 activity during hypoxia is unaffected by inhibition of 5-LO/CysLT1, but reduced when swelling-induced taurine loss via VSOAC is prevented by DIDS excess extracellular taurine, indicating a beneficial role of taurine under hypoxia.

5-lipoxygenase: lung adenocarcinoma; cysteinyl leukotriene 1 receptor; hypoxia; ischemia; cell volume regulation; reactive oxygen species; taurine homeostasis

WITHIN RECENT YEARS it has become clear that cell volume regulation under isotonic conditions and cell volume restoration following osmotic perturbations are important for regulation of the intracellular milieu and cellular functions, such as cell metabolism, cell migration, and control of cell death (see refs. 16, 39). Cells maintain a steady cell volume by regulating the intracellular concentrations of ions (mainly Na+, K+, and Cl−) and organic osmolytes (16, 39). A consequence of hypertonic cell swelling is net loss of KCl and amino acids including the β-amino ethane sulfonic acid, taurine.

Taurine: a multifunctional amino acid. Taurine is the most abundant free amino acid of the human body and has been estimated to account for up to 38% of the total free amino acid pool in rat heart cells (17). Taurine is metabolically inert, resides freely in the intracellular compartment, and is traditionally assigned an important role as a compatible organic osmolyte in cell volume control and as a cytoprotector due to its ability to modulate calcium movement and elimination of reactive oxygen species (ROS) (20, 21, 27). Taurine deficiency during pregnancy and lactation, as seen in mice, results in lower than normal birth weight, chronic liver disease, cardiomyopathy, dysfunctional kidneys, and destruction of nerve cells (18, 70). Furthermore, deficiency of sulfur amino acids shifts the redox homeostasis towards more oxidized status, which is of importance for survival (29). Recently, taurine has been suggested to signal through GABA receptors (GABAARs) and glycine receptors (GlyRs) in tissues such as the central nervous system (CNS) and the lung (41, 71).

Taurine: cellular uptake and release mechanisms. The cellular taurine content is a balance between endogenous synthesis, active uptake via 1) the Na+ - and Cl−-dependent transporter TauT (SLC6A6) or 2) the H+ -coupled, pH-dependent, Na+ - and Cl−-independent amino acid transporter PAT1 (SLC36A1), and passive release via 3) a volume-sensitive leak pathway, activated by osmotic cell swelling, designated volume-sensitive organic anion channel (VSOAC) (34, 36); or 4) a volume-insensitive leak pathways activated during apoptosis (55), cholesterol depletion (67), and anoxia (49). The cellular taurine concentration ranges from 10 mM, e.g., in fibroblasts, to 40–50 mM in Ehrlich ascites cells (34). The total body pool is controlled by renal taurine retention via TauT involved in the proximal tubule and distal nephron. Translocation of one taurine molecule across the plasma membrane via TauT involves two to three Na ions and acute modulation of TauT activity by protein kinases (PKA, PKC, CK2) and ROS involves shift in TauT’s substrate affinity, transport capacity, and/or the Na:taurine stoichiometry (13, 14, 19, 45, 69). Passive taurine release from cells via VSOAC activation involves 1) mobilization of arachidonic acid from the nuclear envelope by cell-specific PLA2 subtypes (52, 53), ROS generation by NADPH oxidases (8, 34), and fatty acid oxidation to leukotrienes by 5-lipoxygenase (5-LO) (38); 2) increased tyrosine kinase activity (34, 51); and 3) activation of protein kinases not normally related to growth factor signaling, i.e., phosphatidylinositol 3-kinase (PI3K), Akt, and glycogen synthase kinase 3β (GSK3β) (36).

Adenosine triphosphate (ATP) is released following cell swelling, cell mechanical perturbations, and during the initial part of the apoptotic process and hypoxia (47). Furthermore, extracellular ATP can stimulate surfactant secretion from type II pulmonary epithelial cells (63). Human type II pulmonary epithelial A549 cells release ATP upon osmotic swelling, and...
the subsequent release of taurine depends on intracellular Ca\(^{2+}\) mobilization (3, 40). It is noted that the role of Ca\(^{2+}\) seems to be highly cell type specific, i.e., intracellular Ca\(^{2+}\) elevation inhibits volume-sensitive taurine loss in nonadherent cells, e.g., Ehrlich ascites tumor cells (31), whereas it stimulates loss in adherent cells (6, 10, 32).

The 5-lipoxygenase pathway: role in cell volume control. Leukotrienes, produced by the 5-LO pathway, are often associated with chronic inflammatory diseases, particularly asthma, rhinitis, and atherosclerosis (56). It has also been demonstrated in several cell lines that inhibition of the 5-LO blocks volume restoration following osmotic cell swelling (30, 43) and the concomitant release of taurine (34, 36). We have demonstrated that, in Ehrlich ascites tumor cells, leukotriene D\(_4\) plays a key role in the activation of volume-sensitive transporters for K\(^+\) (15) and amino acids (31) and hence restoration of the cell volume following osmotic cell swelling (16, 22, 39). The 5-LO is a non-heme iron containing enzyme that is inactive when the iron is in the ferrous state (Fe\(^{2+}\)) and active when the iron is in its oxidized ferri state (Fe\(^{3+}\)). 5-LO is responsible for the two-step process that oxidizes arachidonic acid to form 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and subsequently dehydrates the 5-HPETE to the unstable leukotriene A\(_4\) (LTA\(_4\)). Ca\(^{2+}\) is thought to facilitate binding of 5-LO to phosphatidylcholine on the nuclear membrane whereas ATP is thought to increase the 5-LO activity by stabilizing the protein structure through the C2-like domain (56). 5-LO inhibitors have been reported to improve cell survival, to protect against ischemia in vitro (61), and to be anti-inflammatory in vivo (74). The leukotrienes synthesized by 5-LO signal through at least two classes of G protein-coupled receptors, named cysteinyl leukotriene 1 (CysLT\(_1\)) and 2 (CysLT\(_2\)) receptors, which are widely expressed in cells of the immune system but in the case of CysLT\(_1\) are also expressed in endothelial cells and the lung. Two CysLT\(_1\) receptor antagonists, Montelukast and Zafirlukast, are licensed worldwide for treatment of asthma and allergic rhinitis (28, 54).

Taurine: role during hypoxia. The role of taurine during hypoxia and ischemia has been a focus of attention as taurine seems to lower the degree of cell damage caused by ischemia (57). Taurine together with other free amino acids is released during ischemia (24), and anion channel blockers including DIDS partially reduce the ischemia-induced amino acid release (59), indicating that VSOAC may be involved in taurine release. Explanted hearts from rats fed on a diet with high taurine content showed increased resistance to ischemic damage (58). Furthermore, storage of fresh rat hearts in a solution with taurine significantly improved preservation of the rat hearts by lowering DNA oxidative stress and apoptosis (48). These results indicate that taurine might have protective effects against hypoxic/ischemic damage. Within the lung, hypoxia can be a consequence of insufficient oxygen supply (e.g., airway obstruction or high altitudes), but hypoxia also plays a role in the progression of diseases such as hypoxic pulmonary vasoconstriction, cystic fibrosis, and chronic obstructive pulmonary diseases (COPD) (68). Increased 5-LO activity and ROS levels are a potential outcome of hypoxia and are thought to be largely involved in the disease progression. Treatment with the antioxidant N-acetyl cysteine (NAC) has shown beneficial effects against COPD (65), and likewise administration of MK886, a 5 LO activity inhibitor, or MK571, a selective CysLT\(_1\) receptor antagonist, protects against damage caused by hypoxic pulmonary vasoconstriction (4). As 5-LO activity and ROS are reported as regulators of taurine release, we also speculate whether restriction of VSOAC activity and/or taurine release could have protective effects during hypoxia.

In the present work we focus on 1) requirement of 5-LO and CysLT\(_1\) in volume regulation and volume-dependent activation of taurine and alanine release via VSOAC; 2) modulation or regulation of VSOAC by reactive oxygen species; 3) modulation of VSOAC by ATP and Ca\(^{2+}\); and 4) putative impact of hypoxia on taurine release and cell viability. We use the human pulmonary adenocarcinoma A549 cells which have many features consistent with alveolar type II (AT2) epithelial cells. Morphologically they retain a cuboidal shape and bear a number of the ultrastructural characteristics, including lamellae bodies, consistent with those reported in AT2 cells in situ. They also manifest similar phospholipid biosynthetic properties and have been found to release and/or express a variety of cytokines and growth factors (7, 60). We find that swelling-induced activation of the putative organic osmolyte permeability pathway VSOAC is potently suppressed by the pharmacological inhibitors of 5-LO (AA861) and the cysteinyl leukotriene receptor CysLT\(_1\) (Zafirlukast), but potentiated by reactive oxygen species, and ATP / extracellular Ca\(^{2+}\). Finally, we demonstrate that inhibition of the VSOAC mediated taurine release could protect against apoptosis during prolonged hypoxia.

EXPERIMENTAL PROCEDURES

Reagents. Unless otherwise stated, reagents were analytical grade and purchased from Sigma (St. Louis, MO). The following stock solutions were prepared: DIDS (4,4′-disothiocyano-stilbene-2,2′-disulfonate, diH\(_2\)O), W7 [N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide, diH\(_2\)O], DPI (diphenylene iodonium, EtOH), AA861 [2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadienyl)-1,4-benzoquinone, EtOH], ETH (ETH 615-139, donated by Dr. I. Ahnfelt-Roene, Lovens Kemiske Fabrik, Denmark, EtOH), BHT (butylated hydroxytoluene, EtOH), curcubatin I hydrate (DMSO), NAC (2.5 mM in DMEM), DPI (diphenylene iodonium chloride, DMEM), Zafirlukast [N-[3-[2-methoxy-4-[[1-(methylphenyl)sulfonyl]amino]carbonyl]phenyl]methyl]-1-methyl-1H-indol-5-yl][carbamic acid cyclopentyl ester, DMSO], ATP (adenosine-5′-triphosphate, diH\(_2\)O), ionomycin (EtOH), NS3728 (kind gift from Dr. P. Christophersen, Neurosearch, Ballerup, Denmark, medium), genistein [5,7-di-hydroxy-3-(4-hydroxyphenyl)chromen-4-one, EtOH], Dulbecco’s modified Eagle’s medium [DMEM, Sigma-Aldrich (D5796)], trypsin-EDTA solution (Invitrogen, 5 mg porcine trypsin, 2 mg EDTA/ml PBS), [1,2-\(^{3}H\)N]-taurine (Perkin Elmer, NET1173250UC, specific activity: 17 Ci/mmol), [1-\(^{14}C\)]meAIB (Perkin Elmer, NEC671050UC, specific activity: 0.04–0.06 Ci/mmol), scintillation cocktail, Ultima Gold (Perkin Elmer), SuperScript II Reverse Transcriptase (Invitrogen), carboxy-H:\(H^{+}\)DCFDA [5-(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; Molecular Probes, Leiden, The Netherlands, EtOH], probenecid, pluronic F127 (Invitrogen), and MTT (5-(and 6)-bromo-2′-5′-diphenyltetrazolium bromide, 5 mg/ml in sterile PBS). When not otherwise stated, inhibitors and stimulants were added 8 min before and were present during the experiments.

Cell culture: A549 cells. The PBS contained (in mM) 137 NaCl, 2.6 KCl, 6.5 Na\(_2\)HPO\(_4\), and 1.5 KH\(_2\)PO\(_4\). Isotonic Ringer solution contained (in mM) 159 NaCl, 5 KCl, 1 Na\(_2\)HPO\(_4\), 0.1 MgCl\(_2\), 1 CaCl\(_2\), and 10 HEPES. Hypotonic Ringer solution contained (in mM) 87

\(^{1}\) This article is the topic of an Editorial Focus by Alexander A. Mongin (45a).
Ischemic medium contained (in mM) 139 NaCl, 5 KCl, 1 Na2HPO4, 0.1 MgCl2, 1 CaCl2, and 10 HEPES. Ischemic medium contained (in mM) 139 NaCl, 5 KCl, 1 Na2HPO4, 0.1 MgCl2, 1 CaCl2, 10 HEPES, and 20 Na-lactate. All media were adjusted to pH 7.4 with NaOH. A549 cells, a human lung adenocarcinomomic alveolar epithelial cell line, were purchased from American Type Culture Collection (ATCC, Manassas, VA) and grown in Greiner Bio-one tissue culture flasks (T75, 75-cm² growth area) in DMEM supplemented with 10% fetal bovine serum (vol/vol) and penicillin-streptomycin (100 units penicillin, 100 μg streptomycin per milliliter). Cells were passaged every 3–4 days. Cells were incubated at 37°C, 5% CO2, 100% humidity and natural oxygen levels. For the MTT cell viability assay the cells were seeded in black 96-well plates (34-mm² growth area). Cells for estimation of amino acid release by tracer technique were grown in six-well plates (9.6-cm² growth area/well). Cells for Western blot analysis and RNA extraction were grown in 94-mm dishes (64-cm² growth area). Anoxic conditions were simulated by keeping the cells in standard DMEM medium (hypoxia) or the custom-made glucose-free ischemic medium and incubation at 37°C, 1% O2, 94% N2, 5% CO2, 100% humidity and natural oxygen levels. For the MTT cell viability assay the cells were seeded in black 96-well plates (34-mm² growth area). Amino acid concentration gradient and k is a fractional rate constant (min⁻¹). The latter was estimated as the sum of

\[ A(t) = A(0) - k t e^{-kt} \]

where \( A(t) \) and \( A(0) \) designate cellular amino acid content (cpm) at each of the samples using a liquid scintillation counter. At the end of the efflux experiment the cells were treated with 1 ml 1 M NaOH and the amount of [³H]taurine or [¹⁴C]meAIB remaining inside the cell was estimated.

The amino acid release is unsaturable and proportional with the prevailing concentration gradient. Assuming that a single transport flux can be expressed as

\[ \text{flux} = -\frac{d[C]}{dt} = k \Delta C \]

where \( \Delta C \) is the concentration gradient and \( k \) is a fractional rate constant (min⁻¹).

Following the experimental procedure the extracellular level of taurine and meAIB is kept close to zero during the experiments and by integration the equation above can be described as

\[ \Delta C(t) = \Delta C(t = 0) e^{-kt}, \]

where \( C(t) \) and \( C(t = 0) \) are the cellular amino acid concentration at time \( t \) and \( t = 0 \), respectively. This equation describes how the concentration difference across the membrane will decrease exponentially over time and can be rearranged into

\[ \ln[A(t)/A(0)] = -kt \]

where \( A(t) \) and \( A(0) \) designate cellular amino acid content (cpm) at time \( t \) and \( t = 0 \), respectively. The latter was estimated as the sum of the activity (cpm) in the efflux samples, NaOH plus 3 ddH₂O washouts. \( \ln[A(t)/A(0)] \) was plotted versus time (see Fig. 3A), and the rate constant \( k \) at a given time point was estimated as the negative slope of the plot between the actual and proceeding time points.

\[ \text{Estimation of absolute cell volume by coulter counter. A Coulter counter (Beckman Multisizer III Coulter Counter) was used in this project to estimate absolute cell volume under ischemic conditions and following hypotonic shock in the absence or presence of different inhibitors. All the inhibitors were added 8 min before the experiment and during the experiments. Approximately 50,000 cells/ml were used in the experiments. Cell volume distribution curves were obtained at given time points, and absolute cell volume was estimated from distribution curve medians.} \]

**Estimation of cell volume, surface area, and cell height by 3-D imaging.** The cell volume, surface, and height of single substrate-attached A549 cells were estimated using the DISUR technique previously described by Boudreault and Grygorczyk (3). With the dual image surface reconstruction (DISUR) we created digital three-dimensional (3-D) reconstructions of the cells based on the digital side-view and top-view images of the cells. The images were taken through a modified Nikon TE300 inverted microscope with two perpendicular orientated microscope objectives (×10 for top view and ×20 for side view). The photos were recorded with 100-ps exposure time using two independent digital cameras attached to a microscope.

The resolution was 0.14 μm/pixel and 0.18 μm/pixel for the side-view and top-view images, respectively. The side-view and top-view outline of the cell were digitized using the MATLAB (MathWorks)-based DISUR software. A topographical reconstruction of the cell was created, and cell volume, height, and surface area were calculated. Here we illustrate the changes in cell morphology as the relative change compared with the initial values (captured over 10 min) of the given cell in isotonic solution. Before the experiments, A549 cells were seeded onto coverslips and mounted in a small custom-made quartz cell chamber. The cell chamber was perfused with a constant flow of 37°C isotonic or hypotonic solution (1 ml/min). Initially, the chamber was perfused for 10 min with isotonic solution (335 mosM) to estimate the basal values of cell volume, height, and surface area. The release was followed by a change to hypotonic solution (160 mosM).

**Radioactive tracer technique for amino acid release.** [1,2-³H(N)]-taurine and α-[¹⁴C]methylamino-isobutyric acid (MeAIB) were used to characterize amino acid release. Taurine and MeAIB are metabolically inert and not incorporated into proteins. Cells were grown to 90% confluence in six-well plates, and the efflux was measured at room temperature (22°C) and at 37°C for the normoxia experiments. The hypoxia and ischemia experiments were performed at 37°C. Cells were incubated at 37°C for 2 h before the experiment in DMEM or ischemic media containing [³H]taurine (50 nCi/ml) or [¹⁴C]meAIB (50 nCi/ml).

Before the efflux experiments the cells were washed three times in isotonic Ringer (335 mosM) and left with 1 ml isotonic Ringer. To initiate the release experiment (time, \( t = 0 \)) the solution in the wells was gently transferred to scintillation vials and replaced by 1 ml of experimental solution. This procedure was repeated with 2-min intervals for 30 min. Only isotonic solution was used during the first 8 min; thereafter, the solutions were kept either isotonic or hypotonic (200 mosM). The radioactivity was measured as counts per minute (cpm) for each of the samples using a liquid scintillation counter. At the end of the efflux experiment the cells were treated with 1 ml 1 M NaOH and the amount of [³H]taurine or [¹⁴C]meAIB remaining inside the cell was estimated.

The amino acid release is unsaturable and proportional with the prevailing concentration gradient. Assuming that a single transport

\[ \text{flux} = -d[C]/dt = k \Delta C \]

where \( \Delta C \) is the concentration gradient and \( k \) is a fractional rate constant (min⁻¹). Following the experimental procedure the extracellular level of taurine and meAIB is kept close to zero during the experiments and by integration the equation above can be described as

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where \( A(t) \) and \( A(0) \) designate cellular amino acid content (cpm) at time \( t \) and \( t = 0 \), respectively. The latter was estimated as the sum of the activity (cpm) in the efflux samples, NaOH plus 3 ddH₂O washouts. \( \ln[A(t)/A(0)] \) was plotted versus time (see Fig. 3A), and the rate constant \( k \) at a given time point was estimated as the negative slope of the plot between the actual and proceeding time points.

**Fluorescence microscopy of ROS production.** Cells were grown to 80% confluence on 15-mm round glass coverslips. These were mounted in a closed bath imaging chamber (chamber volume of 36 μl, no. RC-20 Warner Instruments) on the stage of an inverted microscope (Nikon Eclipse TE300). A constant flow (0.2 ml/min) of solution through the chamber was applied. The isotonic or hypotonic solution contained the ROS-sensitive fluorescence probe carboxy-H₂DCFDA (10 μM), probenecid (2.5 mM), and pluronic F127 (0.2%). The experiments were carried out at room temperature (22°C). The cells were exposed to illumination at 495 nm, and fluorescence images were recorded through a 535-nm filter at 3-min intervals. The images were collected with MetaFluor (Molecular Devices) and analyzed with ImageJ (National Institutes of Health, Bethesda, MD). The analysis was carried out by outlining 10 cells and then measuring the increase in fluorescence intensity over time compared with the background. Four areas without cells were measured as

\[ \text{Fluorescence microscopy of ROS production.} \]
background and subtracted from the data. The mean fluorescence increase compared with auto-fluorescence of the cells before addition of the ROS probe was plotted and the rate of increase was estimated in the initial 3 min following hypotonic swelling.

**Determination of amino acid content.** Amino acid content was estimated by ortho-phthalaldehyde (OPA) derivatization and reversed phase-high pressure liquid chromatography (RP-HPLC, Gilson HPLC Pump 322, 234-Autoinjector, 155-UV/VIS). Cells were treated with trypsine and centrifuged at 14,000 g for 45 s. The cell pellet was resuspended and the cells were lysed in 4% sulfosalicylic acid. An aliquot of the lysed cells was taken to estimate the protein concentration by the Lowry method. The sulfosalicylic acid lysates were mixed with an OPA-test solution containing OPA, methanol, potassium-borate buffer (pH 10.4) and 3-mercaptopropionic acid. After 3 min, OPA-derived amino acids were separated on a C18 column (C18 column, CC 250/4 Nucleosil 100-5 C18, Macherey-Nagel) with a “stationary phase” consisting of silica with C18H37. The mobile phase was initially a 12.5 mM H3PO4 (pH 7.2) phosphate buffer, potassium-borate buffer (pH 10.4) and 3-mercaptopropionic acid. The mobile phase was initially a 12.5 mM H3PO4 (pH 7.2) phosphate buffer and flow rate of 1 ml/min. Elution and detection of OPA-derivatives were performed by increasing the mobile phase concentrations of acetonitrile and following the absorbance at 330 nm. Amino acids were identified and quantified using Gilson Unipoint software.

**Cell viability: the MTT assay.** MTT solution (5 mg/ml sterilized PBS) was added to 80% confluent cells in 96-well plates and incubated for 3 h (37°C, 5% CO2). SDS-HCl solution (5 ml 0.01 M HCl, 0.5 g SDS) was added to each well to lyse the cells. The samples were measured at 570 nm (FLUOstar OPTIMA, Bmg LabTechnologies, Offenburg, Germany). The percentage of living cells was calculated using the measured absorbance: (treated cells – background)/(untreated cells – background) × 100% = percent surviving cells.

**Apoptosis: caspase-3 activity.** Cells were grown in 75-cm² flasks at a density of 2–5·10⁵ cells/ml. Following the experimental challenge, cells were trypsined and centrifuged at 1,000 g for 6 min at 4°C. Pellets were washed once in PBS and lysed in ice-cold lysis buffer, thoroughly resuspended, and stored at −80°C overnight. Cell lysates were subjected to 3 freeze-and-thaw cycles, 2 × 10 s sonication before centrifugation at 20,000 g for 5 min. The supernatants were transferred to new Eppendorf tubes, and protein content was estimated and adjusted with lysis buffer to equal concentration for activity measurements (4 µg/µl). Caspase-3 activity in cell lysates was estimated in 96-well plates using the ApoTarget caspase-3/CPP32 colorimetric assay (Protease BioSource International) according to the manufacturer’s protocol. Absorbance was measured at 405 nm using the microplate reader. All experiments were performed in triplicate.

**Osmolality measurements.** The osmolality of the solutions used in the experiments was estimated on an Osmometer Automatic (Knauer).

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Fig. 1. Effect of the 5-lipoxygenase (5-LO) inhibitor AA861, the leukotriene receptor antagonist Zafirlukast, and the channel blocker DIDS on regulatory volume decrease (RVD) following osmotic cell swelling. Data in A were obtained by electronic cell sizing at room temperature (22°C), whereas data in B-D were obtained by three-dimensional imaging technique at 37°C. A: absolute cell volume was followed with time in the absence of inhibitors (control) and in the presence of AA861 (20 µM), Zafirlukast (60 µM), or DIDS (50 µM). RVD rates were estimated as the reduction in cell volume (10⁻¹³ l/min) obtained within the first 2 min following maximal cell swelling in cells exposed to the hypotonic solution. RVD rates are given relative to control cells and represent 3 sets of paired experiments. B: relative change in cell volume was followed over time with single cells attached to a coverslip. At time 0 the isotonic solution was changed to hypotonic solution (160 mosM) and the cell volume followed with time. The time traces for control and AA861 (20 µM)-treated cells are representative of 4 sets of experiments. C: maximal increases in cell volume, cell surface area, and cell height were estimated under hypotonic conditions in the absence (control) or presence of AA861 (20 µM). Control values, obtained under hypotonic conditions, were 5.8 ± 0.3 pl (volume), 2.882 ± 141 µm² (surface area), and 11.3 ± 0.4 µm (height). Data represent 4 sets of paired experiments. D: RVD in AA861-treated cells compared with control cells. The initial slope in the RVD phase in B was estimated by linear regression and is given relative to control cells (39 ± 5 µm³/min). Data represent 4 sets of paired experiments. *Significantly different from control.
Statistics. In bar- and scatterplots the error bars indicate standard error of the mean. Statistically significant differences (P < 0.05) from control or between groups were analyzed by the use of Student’s t-test or two-way ANOVA.

RESULTS

The 5-lipoxygenase pathway controls the rate of volume recovery following osmotic cell swelling. During hypoosmotic exposure, cells swell initially as almost perfect osmometers due to their high water permeability but regain their initial cell volume due to net loss of ions, organic osmolytes, and osmotically obliged water in a process designated as regulatory volume decrease (RVD). Using electronic cell sizing and trypsinized A549 cells we found that the rate of RVD in A549 is significantly reduced in the presence of the anion channel blocker DIDS, the 5-LO inhibitor AA861, as well as the CysLT1 receptor antagonist Zafirlukast (Fig. 1A). To evaluate the effect of AA861 on cell volume regulation under more physiological conditions, we increased the temperature from 22°C to 37°C and used 3-D imaging on attached cells (Fig. 1B). Similar to what is observed in the Coulter Counter experiments, inhibition of 5-LO reduces the rate of the RVD response significantly (Fig. 1, B and D). It is noted that 5-LO inhibition actually increases the maximal cell volume following osmotic cell swelling (Fig. 1C) most probably due to a delay in the onset of the RVD response (Fig. 1B). The surface area and the height of the cells were not significantly affected by the 5-LO inhibitor. Hence activation of RVD in A549 requires 5-LO activity and signaling via the CysLT1.

The 5-lipoxygenase pathway controls swelling induced release of amino acids. In agreement with other mammalian cell types, A549 cells release amino acids following exposure to hypotonic solution and it is estimated that the cellular taurine, glycine, and alanine content is reduced to <50% within 30 min following hypoosmotic exposure and that this loss is not increased within the subsequent 30 min (Fig. 2A). Loading A549 cells with labeled taurine or labeled meAIB, which is a metabolically stable alanine analog, revealed that release of the amino acids is low under isotonic conditions but increases dramatically and transiently following osmotic swelling (Fig. 2B). Using the maximal rate constant for taurine (0.20 min⁻¹) or meAIB (0.12 min⁻¹) and the initial, isotonic content of taurine (0.093 µmol/mg protein) or alanine (0.026 µmol/mg protein) (absolute values from the legend to Fig. 2) it is estimated that maximum efflux (maximal rate constant-cellular content) for taurine is sixfold higher than for alanine, i.e., taurine release accounts for a larger part of the swelling-induced organic osmolytes release than alanine in A549 cells. From Fig. 2, C

![Fig. 2. Swelling-induced amino acid release. Amino acid loss following osmotic exposure was estimated by HPLC technique and tracer technique. A: amino acid content in A549 cells measured with HPLC under isotonic conditions (335 mosM) and following 30 min and 60 min of exposure to hypotonic conditions (200 mosM). Values are given relative to the isotonic control values (taurine: 0.09 ± 0.02 µmol/mg protein; glycine: 0.11 ± 0.02 µmol/mg protein; alanine: 0.026 ± 0.006 µmol/mg protein) and represent mean values from 5 sets of experiments. B: swelling-induced taurine and meAIB release. A549 cells were preincubated for 2 h with [3H]taurine or [14C]meAIB. The cells were washed and exposed to isotonic NaCl Ringer (335 mosM) for 8 min and subsequently to hypotonic NaCl Ringer (200 mosM) for 22 min (shift in tonicity indicated by the arrow). Samples were drawn and rate constants for amino acid release were estimated at 2-min intervals. Rates are given as mean values from 7 sets of experiments. C and D: DIDS sensitivity of the swelling-induced taurine and meAIB release. Cells were preincubated for 2 h with [3H]taurine or [14C]meAIB, and release of labeled amino acid followed with time as indicated in B in the absence (control) or presence of DIDS (10 µM/50 µM). The osmolality of experimental solution was reduced from isotonicity to hypotonicity at time (t) = 8 min as indicated by the arrow. Time traces for taurine release (C) represent 7 sets of experiments. Maximum taurine release rate constants obtained after hypoosmotic exposure (D) are given relative to rate constants from control cells with no DIDS (taurine: 0.146 ± 0.007 min⁻¹; meAIB: 0.129 ± 0.009 min⁻¹) with the number of paired sets of experiments being 7 and 11 for 10 µM and 50 µM DIDS during taurine release, respectively, and 4 for 10 µM DIDS during meAIB release. *Significantly different from control. #Significant difference between the two DIDS groups.](image-url)
and $D$, it is seen that taurine and meAIB release is reduced by DIDS at a low concentration, which is characteristic for VSOAC (34), and to a similar extent as the overall RVD response (Fig. 1A). Furthermore, the swelling-induced activation of amino acid release like the overall RVD response requires 5-LO activity and signaling via the CysLT1. This is seen from Fig. 3 where it is shown that the 5-LO inhibitors AA861 and ETH 615-139 as well as the CysLT1 receptor antagonist Zafirlukast dramatically reduce the swelling-induced taurine release (Fig. 3B) and meAIB (Fig. 3C). Adding AA861 to the A549 cells at a time where the swelling-induced taurine efflux is at its maximum rate leads to an immediate inhibition of the efflux, whereas removal of AA861 8 min after hypoosmotic exposure has only a minor effect on taurine release (Fig. 3A), indicating that 5-LO activity is involved in activation and maintenance of the transporter responsible for taurine release under hypoosmotic conditions. It is noted that the effect of AA861 on meAIB release is less potent compared with its effect on taurine release and that exposure to the anion channel blocker NS3728, which in itself blocks swelling-induced meAIB efflux, does not enhance the inhibitory effect of AA861 (Fig. 3C). The data in Figs. 2 and 3 are taken to indicate that swelling-induced release of taurine and meAIB in A549 cells is mediated by the same transport pathway that requires 5-LO and CysLT1 signaling for activation and that an additional pathway might contribute to meAIB (alanine) release under hypoosmotic stress.

Reactive oxygen species are produced upon hypotonic swelling and increase volume-sensitive amino acid release. Using the ROS-sensitive fluorescent probe carboxy-H2DCFDA it was found that the intracellular ROS production increases during hypotonic swelling and that the NADPH oxidase inhibitor DPI impairs the swelling-induced ROS production. Hence, a NADPH oxidase contributes to the swelling-induced ROS production in A549 cells. In agreement with what has been found in other cell lines (34), ROS modulate swelling-induced taurine release. This is seen from Fig. 4, B–D, where it is shown that $H_2O_2$ potentiates whereas inhibition of the NADPH oxidase with DPI, or ROS trapping with the antioxidants BHT (butylhydroxytoluene, i.e., a synthetic analog of vitamin E) and NAC (i.e., a precursor for glutathione synthesis) impairs swelling-induced taurine release from A549 cells. Quantification of the cell volume using Coulter Counter illustrated that similar to AA861, BHT also affects the cells’ ability to perform RVD, i.e., the rate of RVD following hypotonic swelling is significantly inhibited by BHT (0.05 mM) to 54 ± 17% of the rate seen in control cells. ROS also seem to be involved in the swelling-induced release of meAIB, i.e., $H_2O_2$ (100 μM) increases the maximal rate constant for meAIB release under hypoosmotic conditions significantly by 3.1 ± 1.6-fold compared with control, whereas DPI (25 μM) reduced the rate constant to 68 ± 3%. From Fig. 4D it is seen that $H_2O_2$ is unable to stimulate swelling-induced taurine release in the presence of the 5-LO inhibitor AA861, indicating that ROS potentiates the 5-LO activity or an enzyme upstream to the 5-LO. Inhibition of protein tyrosine kinases was previously shown to prevent swelling-induced release of taurine (32, 51), and similarly we find that addition of the tyrosine kinase inhibitor genistein also inhibits volume-sensitive taurine release in A549 cells (Fig. 4D). Cucurbitacin is a selective Janus kinase (JAK) inhibitor with potent antitumor activity (2), and

**Fig. 3.** Effect of 5-LO inhibitors and leukotriene receptor antagonists on swelling-induced amino acid release. Cells were preincubated for 2 h with $[^1]H$taurine or $[^14]C$meAIB, and the release of labeled amino acids followed with time under isotonic and hypoosmotic conditions as indicated in Fig. 2 in the absence (control) or presence of AA861 (30 μM), ETH 615-139 (10 μM), Zafirlukast (60 μM), or NS3728 (10 μM). A: release is shown as the change in the fraction of $[^1]H$taurine remaining in the cell (ln scale) plotted vs. time. AA861 (30 μM) was present from $t = 0$ to $t = 30$ min (+/+/+), from $t = 0$ to $t = 16$ min (+/+/−), from $t = 16$ to $t = 30$ min (−/−/−), or absent (−/−/−/−). Arrow indicates shift to hypotonicity ($t = 8$ min). Lines indicate shift in the presence of AA861. Trace is representative mean of 3 sets of paired experiments. B: maximal rate constants for taurine release, obtained after hypoosmotic exposure, are given relative to control cells (taurine: 0.182 ± 0.007 min$^{-1}$) with the number of paired experiments being 9, 4, and 3 for AA861, ETH 615–139, and Zafirlukast, respectively. C: maximal rate constants for meAIB release, obtained after hypoosmotic exposure, are given relative to control (meAIB: 0.11 ± 0.01 min$^{-1}$) from 3 sets of paired experiments. *Significantly reduced compared with control. Rate constants in cells tested for AA861 plus NS3728 were not different from those tested with the inhibitors alone.
from Fig. 4D it is seen that cucurbitacin reduces the swelling-induced taurine release significantly and that H_2O_2, which is able to increase volume-sensitive taurine efflux even in the presence of DIDS (Fig. 4D), reverses the effect. This is taken to indicate a role for tyrosine kinases, e.g., JAK and hence STAT3 in the regulation of VSOAC activity.

**Calcium and ATP potentiate the swelling-induced release of taurine and meAIB.** ATP is thought to regulate ion and water transport across the membrane of pulmonary epithelial cells (40). Stimulating A549 cells with ATP has no effect on taurine release during isotonic condition (data not shown), whereas ATP significantly increases the maximum efflux rate of taurine and meAIB during hypotonic conditions (Fig. 5). Exposing the A549 cells to the calcium ionophore ionomycin at the time of swelling in the absence and presence of the NOX4 system inhibitor DPI (25 µM), or butylated hydroxytoluene (BHT; 50 µM), rate constants are given as mean values of 11 (control), 11 (H_2O_2), and 3 (BHT) sets of experiments. C and D: maximal rate constants for taurine release, obtained under hypotonic conditions in the absence (control) or presence of H_2O_2 (100 µM), or AA861 (30 µM), N-acetyl cysteine (NAC; 2.5 mM, 24 h), DPI (25 µM), or cucurbitacin (10 µM, 4 h). Values are given relative to the maximal rate constant for control cells (0.144 ± 0.011 min⁻¹) and represent 11 (100 µM H_2O_2), 6 (200 µM H_2O_2), 3 (BHT), 4 (NAC), 4 (DPI), and 11 (100 µM H_2O_2), 9 (30 µM AA861), 3 (AA861 + H_2O_2), 2 (genistein, genistein + H_2O_2), 4 (cucurbitacin, cucurbitacin + H_2O_2), 11 (DIDS), and 3 (DIDS + H_2O_2) sets of experiments (D). *Significantly different from control and cells treated with DIDS or cucurbitacin alone, respectively.

**Effect of hypoxia on swelling-induced taurine release and the 5-lipoxygenase expression.** Anoxia was previously shown to induce loss of taurine in, e.g., porcine myotubes (49), and a large decrease in the intracellular amino acid pool during severe cell stress will inevitably leave the cell with less chance for survival in situations such as hypoxia/ischemia. A549 cells incubated under hypoxia (37°C, 18 h, 1% O_2) had a slight but nonsignificant decrease in the rate constants for taurine release under isotonic (normoxia: 0.0034 ± 0.0006 min⁻¹, n = 3; hypoxia: 0.0027 ± 0.0002 min⁻¹, n = 22) as well as hypotonic (normoxia: 0.152 ± 0.005 min⁻¹, n = 3; hypoxia: 0.119 ± 0.014 min⁻¹, n = 9) conditions. Increased 5-LO activity and upregulated expression of FLAP have previously been reported during hypoxia (12, 42, 64) and as 5-LO seems to be a main player in the regulation of the volume-sensitive release pathway for organic osmolytes but is required for the agonist-induced potentiation.
expression is not affected at the mRNA level by hypoxia. Furthermore, inhibition of 5-LO with AA861, NADPH oxidase with DPI and stimulation with H2O2 affected the swelling-induced taurine release to the same extent under normal O2 levels (normoxia) as under low O2 levels (hypoxia) (Fig. 6B). Hence cell signaling, provoked by osmotic cell swelling and leading to taurine release, does not seem to be affected by 18 h of hypoxia. To test whether long-term hypoxia could affect the cells’ sensitivity toward ROS and 5-LO inhibition, we exposed cells to hypoxia for 18 h and subsequently estimated the taurine release under isotonic conditions in the presence of AA861 (30 μM, n = 3) or Zafirlukast (60 μM, n = 3). Values are given relative to the control cell with Ca2+. *,#§Significantly different from control cells, cells treated with Ca2+ agonist alone, and cells exposed to Ca2+ plus ATP, respectively.

DISCUSSION

Here we demonstrate that signaling via 5-LO and the CysLT1 receptor is essential for release of taurine from human lung cells (A549) following osmotic cell swelling and hence the ability to control cell volume. As altered leukotriene signaling is involved in inflammatory and hypoxia-related diseases, our findings link regulation of VSOAC and cell volume to diseases and medical treatments interfering with taurine signaling is involved in inflammatory and hypoxia-related diseases, our findings link regulation of VSOAC and cell volume to diseases and medical treatments interfering with cell signaling through the 5-LO/CysLT1 pathway. Furthermore, we indicate a protective role for taurine against apoptosis during hypoxia.

Inhibition of the 5-LO impairs cell volume regulation in A549 cells. Inhibition of 5-LO (AA861/ETH) impairs the swelling-induced taurine release and the concomitant RVD response of the A549 cells. However, the cell swelling in the presence of the 5-LO inhibitor AA861 was slower, i.e., peak...
volume was obtained minutes later compared with control, presumably due to modulation of aquaporin activity and hence lowered water permeability. The intracellular pH and calcium concentrations play a role in the regulation of the aquaporin activity (66), and AA861 could potentially affect these factors but further investigation is needed. Using the 3-D imaging system it was demonstrated that the cell height increased ~25% in control cells, illustrating spherical-shaped morphological changes. Furthermore, the cell surface area increased with ~15% during hypotonic swelling. The maximum increase in cell volume during hypotonic conditions was higher in the AA861-treated cells than the control cells most likely due to a delayed onset of the RVD response. Altered cell volume regulation affects a wide variety of cellular responses, e.g., proliferation, cell death, hormone release, and trans-epithelial transport (see refs 16 and 39), and medical treatment with 5-LO inhibitors could potentially affect cellular homeostatic functions.

The leukotriene and ATP-activated pathways. Hypotonic conditions led to an ~50% reduction in the intracellular glycine and alanine content in A549 cells and as the pharmacological profiles of the swelling-induced release of taurine and the metabolic inert alanine-analog meAIB are similar, it is assumed that taurine and meAIB are released through the same swelling-induced pathway. Similar to observations from Ehrlich ascites tumor cells (37), we demonstrate that the volume regulatory response and activation of the volume-sensitive transport pathway for amino acids in A549 cells require 5-LO activity and signaling via cysteinyi leukotriene receptors.

Stimulation with ATP increased the swelling-induced taurine release. Chelation of the extracellular calcium with EGTA abolished the stimulating effect of ATP. This indicates that ATP potentiates the taurine release through the entrance of extracellular calcium possibly through the P2X ligand-gated calcium-permeable receptors. Treating the cells with ionomycin illustrates that extracellular calcium indeed stimulates the swelling-induced taurine release. However, in the absence of ATP stimulation, chelating the extracellular calcium had no effect on the swelling-induced taurine release. Taken together with the inhibitory effect of the calmodulin antagonist W7, this indicates that swelling-induced taurine release is mainly regulated by release of calcium from intracellular stores but can be potentiated by extracellular calcium influx.

Inhibition of 5-LO abolished the effect of ATP and ionomycin, which indicates that calcium-mediated stimulation of the taurine release depends on 5-LO activity, possibly via an upstream mechanism. As previously described, relatively low concentrations of calcium activate 5-LO through a mechanism that involves binding of 5-LO to membrane phosphatidylcholine (56). Another possibility is that calcium is not necessarily a single specific step in the pathway leading to the taurine release but instead it participates in the activation of multiple proteins involved in the pathway such as 5-LO, protein kinase C (PKC), or calmodulin, thereby potentiating the whole pathway. Involvement of Ca2+ in the RVD response has been demonstrated in various cell systems, predominantly those of epithelial origin (16). Hypotonic cell swelling of A549 cells leads to increase in intracellular calcium that elicits release of ATP (63), presumably due to a Ca2+-dependent exocytosis of adenosine and uridine nucleotide release (1). The signaling cascade in A549 cells triggered by osmotic cell swelling leading to taurine release is therefore suggested to involve sequential calcium release from intracellular stores, 5-LO activation, leukotriene production (26), activation of CysLT1 receptor, and subsequent activation of the volume-sensitive amino acid transporter and release of organic osmolytes including taurine.

ROS-sensitive targets. In accordance with previous findings in, e.g., NIH3T3 cells (32) it is shown that H2O2 potentiates the swelling-induced taurine release in A549 cells and that the antioxidants BHT and NAC are effective inhibitors of the taurine release, i.e., ROS are critical for the taurine release signal. We found that the increase in intracellular ROS becomes detectable within the first 4 min of hypotonic exposure and that this increase is abolished in the presence of the NADPH oxidase inhibitor DPI. DPI also inhibited the taurine release, indicating that the volume-sensitive ROS production and the taurine release are NADPH oxidase dependent. In NIH3T3 cells it has been demonstrated that a NOX4 isotype is the NADPH oxidase responsible for a main part of this ROS production (8). Stimulation of PKC potentiates the swelling-induced taurine release in NIH3T3 cells but as this potentiation is impaired by NOX inhibition (33), this indicates that NOX could be a downstream target for PKC in the swelling-induced taurine release pathway, but that NOX also function upstream for PKC.

It was previously shown that exogenous H2O2 besides an amplification of the swelling-induced taurine release also prolongs the open probability of the taurine release system in NIH3T3 cells (35). However, as seen in Ehrlich Lettré cells (35) the inactivation of the volume-sensitive taurine release pathway in A549 cells in the presence of H2O2 is similar to the untreated control cells. H2O2 was unable to stimulate the

![Fig. 7. Activation and potentiation of the volume-sensitive organic anion channel (VSOAC) in the human lung epithelial cell line A549. 5-LO activity, Ca2+ mobilization from intracellular stores, ROS, and signaling through the CysLT1 receptor are requisites for activation of VSOAC. The putative 5-LO product (leukotriene, LT) and the interaction between CysLT1 and VSOAC have not yet been identified. ROS, generated by a NADPH oxidase, and Ca2+, mobilized from extracellular stores by ATP/P2X-receptor signaling, potentiate the swelling-induced activation of VSOAC presumably through 5-LO. The Ca2+ signal is mediated partly by calmodulin. Tyrosine kinases, including the Janus kinase JAK2, are assumed to be involved at a step downstream to ROS.](http://ajpcell.physiology.org/issue)
Fig. 6. Effect of low O2 pressure on 5-LO expression, taurine release, cell viability, and caspase-3 activity. Cells were incubated for 18 h at 37°C under 21% O2 (normoxia), 1% O2 (hypoxia), or 1% O2 followed by 8 h in 21% O2 (reperfusion) in DMEM medium. A: 5-LO expression was estimated by RT-PCR analysis using GAPDH as house holding protein as indicated in EXPERIMENTAL PROCEDURES. The ratio between 5-LO and GAPDH under hypoxia and reperfusion is given relative to the ratio from control (normoxia) and represents 3 sets of experiments. B: regulation of swelling-induced taurine release under normal O2 (normoxia, open bars) and low O2 (hypoxia, gray bars). Cells were loaded with [3H]taurine, and taurine release followed with time at 37°C under normal oxygen or hypoxic conditions in isotonic NaCl Ringer (335 mosM) for 8 min and subsequently in hypotonic NaCl Ringer (200 mosM) for 22 min. Maximum rate constants for taurine release were estimated in the absence (control) or presence of AA861 (30 μM), H2O2 (100 μM), or DPI (25 μM). Values are given relative to the respective control, i.e., normal oxygen (0.152 ± 0.035 min⁻¹) and hypoxia (0.119 ± 0.014 min⁻¹), and represent 3 sets of experiments for normoxia and 5 (AA861), 5 (H2O2), and 3 (DPI) for hypoxia. C: rate constants for taurine release under isotonic conditions were estimated in the absence (control) and presence of H2O2 (100 μM) or AA861 (30 μM) under 1% O2. Values are given relative to the respective control and represent 3 sets of paired experiments for H2O2 and AA861. D: viability was tested by MTT assay in cells incubated for 18 h under normal O2 (normoxia) or reduced O2 (hypoxia). E: caspase-3 activity was estimated under hypoxic conditions in the absence (control) or presence of AA861, Zafirlukast, DIDS, or taurine as in C. Values are given relative to the respective control and represent 3 sets of experiments. *Significantly different from the respective control.

Taurine release in the presence of the 5-LO inhibitor AA861, i.e., ROS could be an upstream signal for the 5-LO activity. A potential role of ROS is oxidation of the non-heme iron (Fe²⁺ → Fe³⁺) in the catalytic site of 5-LO and thereby activation of 5-LO. If BHT keeps the iron molecule in the reduced ferrous state it would prevent the activation of 5-LO and explain the similar effect from the 5-LO inhibitors and the antioxidant BHT. It is noted that inhibition of the 5-LO at a time point where the taurine efflux pathway is being inactivated elicits an instant inactivation of the release pathway, indicating that inactivation of VSOAC does not solely represent inactivation of 5-LO activity.

Phosphorylation by protein tyrosine kinases, which is normally associated with growth factor-induced cellular signaling (36), is required for VSOAC activation, i.e., tyrosine kinase inhibition prevents activation whereas oxidation/inhibition of the corresponding protein tyrosine phosphatase precludes inactivation of the volume-sensitive transporter (34, 51). H2O2 is unable to stimulate the taurine release in A549 cells under isotonic conditions and has no or only a weak stimulating effect on taurine release under hypotonic conditions when tyrosine kinases are inhibited with genistein (unspecific inhibitor) or curcubitacin (JAK inhibitor), indicating that volume-sensitive kinases are activated and the ROS signal is upstream for the tyrosine kinases. The expression of the tyrosine kinases is cell type dependent, i.e., inhibition of tyrosine-phosphatases promotes VSOAC activity in fibroblasts but has no effect on VSOAC in Ehrlich Lettré cells (35). We have in the present study demonstrated that the JAK and presumably the STAT3 pathway are involved in regulation of the volume-sensitive leak pathway for organic osmolytes in A549 cells, which is in congruence with previous findings in Ehrlich ascites cells where we demonstrated JAK phosphorylation and activation of the volume-sensitive K channel TASK-2 in Ehrlich ascites tumor cells (25). Figure 7 is a summary diagram depicting a hypothetical hierarchy of the signaling of pathways and VSOAC.

Taurine release during prolonged hypoxia. The rate constant for taurine release from A549 cells under isotonic conditions and following hypotonic exposure is unaffected by 18 h of hypoxia, and the sensitivity of the volume-sensitive taurine release pathway to 5-LO inhibition (AA861), ROS exposure...
(H₂O₂), or NOX inhibition (DPI) showed no significant difference during hypoxia compared with control (normoxia). RT-PCR experiments illustrated that 5-LO expression was not affected by 18 h of hypoxia or during 8 h reperfusion at normoxia at the mRNA level.

Cell viability is reduced under hypoxic conditions, and MTT assay illustrated that inhibition of 5-LO by AA861 actually increased the cell viability during ischemia (data not shown). It is important to note that when performing an MTT assay 18 h after the treatment it is not possible to say whether it is altered cell proliferation or cell death that is affecting the viability. The caspase-3 data are taken to indicate that the volume-sensitive 5-LO/CysLT1-dependent signaling pathway leading to taurine release is intact under hypoxia and that inhibition of this pathway does not affect apoptotic activity.

The role of taurine during cerebral ischemia has been a focus of attention. Taurine together with other free amino acids are released during ischemia (24), and anion channel blockers including DIDS partially reduce the ischemia-induced amino acid release (59). We find that reduction in volume-sensitive taurine release through inhibition of the efflux pathway (addition of DIDS) or elimination of the taurine gradient (increasing extracellular taurine) protects against apoptosis in A549 cells. This is in line with our recent findings (55) in which limitation in ion loss, e.g., inhibition of ion channels and hence limitation in apoptotic cell shrinkage, limits cisplatin-induced apoptosis in Ehrlich ascites tumor cells. Rat brain cortical slices in artificial cerebrospinal fluid containing increased levels of taurine before and during ischemia and oxygen reperfusion similarly showed reduced cellular damage, estimated as release of lactate dehydrogenase, and decreased amount of edema in brain tissue (57). Adding the VSOAC inhibitor, NPPB, together with increased taurine levels further reduced release of lactate dehydrogenase; however, the edema was higher than without NPPB but still lower than control (57). Taken together these data indicate a protective role of taurine and VSOAC inhibition during ischemia. Accordingly, we have shown that knockdown of the transporter TauT, i.e., prevention of taurine reuptake, leads to a significant increase in apoptosis in Ehrlich Lettré cells following cisplatin exposure (62). A general problem in organ transplantation is to protect the explanted organ against ischemic damage. However, explanted hearts from rats treated with increased dietary taurine intake have shown to be significantly more resistant to ischemic damage (58). Addition of 10 mM taurine to the St. Thomas’ cardioplegic solution, used to store transplanted hearts during transplantation surgery, significantly improved preservation of the heart by lowering DNA oxidative stress and apoptosis (48). Thus taurine might have beneficial effects in both ischemic pathological situations such as stroke and also during organ transplantation.

ROS is thought to have an important impact on cell damage during ischemia and reperfusion. The largest increase in ROS production is seen immediately after reperfusion, and a smaller ROS production takes place during ischemia where superoxide formation is limited due to little or no oxygen availability (9). The mechanisms responsible for the large increase in ROS following reperfusion are debated, but NOX seems to play a major role (75). Inhibition or knockout of NOX4 prevents the damage caused by ischemia reperfusion (46, 75) which could potentially be through preservation of the intracellular amino acid pool.
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