Permissive effect of EGFR-activated pathways on RVI and their anti-apoptotic effect in hypertonicity-exposed mIMCD3 cells

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Synopsis

Hypertonicity is a stressful stimulus leading to cell shrinkage and apoptotic cell death. Apoptosis can be prevented if cells are able to activate the mechanism of RVI (regulatory volume increase). This study in mIMCD3 cells presents evidence of a permissive role of the EGFR (epidermal growth factor receptor) on RVI, achieved for the most part through the two main EGFR-triggered signalling chains, the MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) and the PI3K (phosphoinositide 3-kinase)/Akt (also known as protein kinase B) pathways. Hyperosmotic solutions (450 mosM) made by addition of NaCl, increased EGFR phosphorylation, which is prevented by GM6001 and AG1478, blockers respectively, of MMPs (matrix metalloproteinases) and EGFR. Inhibition of EGFR, ERK (PD98059) or PI3K/Akt (wortmannin) phosphorylation reduced RVI by 60, 48 and 58% respectively. The NHE (Na\(^+\)/H\(^+\) exchanger) seems to be the essential mediator of this effect since (i) NHE is the main contributor to RVI, (ii) EGFR, ERK and PI3K/Akt blockers added together with the NHE blocker zoniporide reduce RVI by non-additive effects and (iii) All the blockers significantly lowered the NHE rate in cells challenged by an NH\(_4\)Cl pulse. Besides reducing RVI, the inhibition of MMP EGFR and PI3K/Akt had a strong pro-apoptotic effect increasing cell death by 2–3.7-fold. This effect was significantly lower when RVI inhibition did not involve the EGFR-PI3K/Akt pathway. These results provide evidence that Akt and its permissive effect on RVI have a predominant influence on cell survival under hypertonic conditions in IMCD3 cells. This role of Akt operates under the influence of EGFR activation, promoted by MMP.

Key words: Akt, apoptosis, extracellular-signal-regulated kinase (ERK), mIMCD3 cell, Na\(^+\)/H\(^+\) exchanger (NHE), volume regulation

INTRODUCTION

Hypertonicity is a stressful condition, leading to cell shrinkage, increase in intracellular ionic strength and macromolecule damage. High osmolarity disturbs a variety of cell functions such as growth cycle and proliferation, and generates oxidative stress [1]. Adaptation to hypertonicity depends on the ability of cells to activate compensatory mechanisms to counteract cell shrinkage and its associated deleterious consequences. A mechanism of cell volume control, known as RVI (regulatory volume increase) is present in numerous cell types [2,3]. This active cell volume recovery occurs in two steps, one that operates immediately after shrinkage and is accomplished by the activation of ion transporters and exchangers, which rapidly increases the intracellular content of inorganic ions, driving osmotic water. In the second step, occurring in the long term, ions are replaced by compatible osmolytes that, unlike the inorganic ions, do not perturb the macromolecule structure. The intracellular rise of organic osmolyte is mediated by up-regulation of osmolyte transporters or by synthesis of organic osmolytes [4]. Overexpression of the transporters of taurine, myo-inositol, and GABA (γ-aminobutyric acid) as well as that of the sorbitol synthetic enzyme aldose reductase, all increase the intracellular concentration of these compatible

Abbreviations used: Ac-DEVD-AMC, N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; ASK1, apoptosis-signal-regulating kinase 1; AVD, apoptotic volume decrease; BCECF, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein; DMEM, Dulbecco’s modified Eagle’s medium; EGFR, epidermal growth factor receptor; EIPA, 5-(N-ethyl-N-isopropyl)amiloride; ERK, extracellular-signal-regulated kinase; FBS, fetal bovine serum; HRP, horseradish peroxidase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MMP, matrix metalloproteinase; NHE, Na\(^+\)/H\(^+\) exchanger; NKCC, Na\(^+\)–(K\(^+\))–Cl\(^−\) co-transporter; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; RVI, regulatory volume increase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

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osmolytes and contribute to volume recovery even if the hypertonic condition persists [5].

Hypertonicity induces cell death in a variety of cell types [6–16]; however, the underlying mechanisms of this effect are still unclear. An intriguing hypothesis points to the persistence of the hypertonic shrinkage as a pro-apoptotic factor, due to the link between AVD (apoptotic volume decrease) and RVI dysfunction [17]. Cell shrinkage is a hallmark of apoptotic cells and this decrease in cell volume, known as AVD, is considered to play a key role in the sequence of the death programme [18,19]. The step of cell shrinkage is accomplished by activation of K\(^+\) and Cl\(^-\) efflux and the concomitant decrease in intracellular ionic strength [19]. The persistence of these conditions requires that the process of cell volume recovery is overcome or prevented to avoid re-entry of K\(^+\) and Cl\(^-\). Therefore, it is presumed that RVI suppression results in apoptotic death [17]. This possibility is supported by studies in HeLa cells showing that RVI blockade enhances apoptotic cell death [12]. It is also reported that hypertonicity induces apoptosis in lymphoblastoid cells lacking RVI, but not in other cells with the mechanisms for an active volume recovery [20].

The present study in mIMCD3 was devised to further explore this link between RVI and apoptotic death and the possible influence of signalling cascades initiated by the EGFR (epidermal growth factor receptor) acting as a permissive factor on RVI. Ligand-independent tyrosine kinase phosphorylation of EGFR occurs in response to a variety of stressful stimuli, including UV radiation, ROS (reactive oxygen species) and cytotoxic agents [21,22,23]. EGFR activation by hyperosmolality has been described in HeLa cells [21], fibroblasts of the NIH 3T3 cell line [24,25], in human keratinocytes [26] and in rat hepatocytes [27]. Under these conditions, EGFR may act as an anti-apoptotic agent, as has been shown in a variety of cell types, although it is reported as a pro-apoptotic factor in hepatocytes, promoting phosphorylation of the death receptor CD95 [23,27]. In the present study, in mIMCD3 cells, we present evidence in favour of an anti-apoptotic effect of EGFR in cells challenged by high osmolarity, mediated through a permissive action on RVI involving essentially the EGFR/P3K (phosphoinositol 3-kinase)/Akt (also known as protein kinase B) signalling pathway.

**MATERIALS AND METHODS**

**Materials**

FBS (fetal bovine serum), DMEM (Dulbecco’s modified Eagle’s medium)/F12, penicillin–streptomycin were obtained from Gibco/Invitrogen. Primary antibodies against EGFR, phospho-EGFR (Tyr\(^{1173}\)), Akt 1/2/3, phospho-Akt 1/2/3 (Ser\(^{473}\)) and secondary antibody [HRP (horseradish peroxidase)-conjugated anti-(rabbit IgG)] were from Santa Cruz Biotechnology. Fluorescent secondary antibody [HRP (horseradish peroxidase)-conjugated anti-(rabbit IgG)] were from Santa Cruz Biotechnology. Fluoro-genic substrate Ac-DEVD-AMC (N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) and zoniporide dihydrochloride were from TOCRIS Bioscience. BCECF [2,7’-bis(carboxyethyl)-5- (and -6)-carboxyfluorescein]-AM (acetoxyethyl ester) was from Molecular Probes, Invitrogen. Primary antibodies against ERK1/2 (extracellular-signal-regulated kinase 1/2), phospho-ERK1/2 (Thr\(^{202}/\text{Ty}^{204}\)), N-methyl-d-glucamine chloride, Hepes and Complete™ protease inhibitor cocktail were from Roche. Salts for the preparation of medium solutions were from J.T. Baker. Nitrocellulose membrane and molecular-mass standards were from Bio-Rad. BioMax Light Film was obtained from Kodak. EIPA [5-(N-ethyl-N-isopropyl)amiloride], wortmannin, bumetanide and all other reagents were from Sigma Chemical. DeadEnd™ Fluorometric TUNEL System was from Promega.

**Cell culture and treatments**

mIMCD3 cells were routinely incubated at 37 °C in a humidified atmosphere with 95% air and 5% CO\(_2\), and grown in DMEM/F12 medium supplemented with FBS (10%) and antibiotics. For experimental assays, cells were cultured for at least 48 h and used at confluence. Cells were washed with isosmotic medium (135 mM NaCl, 5 mM KCl, 1.7 mM KH\(_2\)PO\(_4\), 1.17 mM MgSO\(_4\), 1 mM CaCl\(_2\), 5 mM glucose and 10 mM Heps; 320 mosM, pH 7.4) and incubated in isosmotic or hypertonic media under the condition indicated for each assay. Osmolarity was raised by the addition of NaCl or urea. All drugs were pre-incubated during 30 min and were present throughout the experiments.

**Western blot analysis**

For immunoblotting assays, cells treatments was terminated by rapid removal of medium on ice, and cells were drained and scraped into lysis buffer containing 20 mM Tris/HCl, pH 7.4, 1 mM EDTA, 50 mM NaCl, 1 mM EGTA, 1% Triton X-100, 0.5 mM Na\(_3\)VO\(_4\) and 1 mM 2-glycerophosphate. Cell homogenates were sonicated and clarified by centrifugation for 5 min at 11000 g and protein concentration determined by the Bradford method. Then, 80 μg of protein was separated by SDS/PAGE (10% gel) and transferred to nitrocellulose membranes. Membranes were blocked with TBS-T [Tris-buffered saline/Tween 20; 100 mM Tris/HCl, 150 mM NaCl and 0.1% Tween 20, pH 7.5] containing 5% (w/v) non-fat dried milk and incubated with the primary antibody (1:1000) overnight at 4 °C. After further washing, blots were incubated with HRP-conjugated secondary antibody (1:5000) for 1 h at room temperature (21–25 °C). Chemiluminescent reaction was assayed using ECL®-Plus Western Blot Detection Reagents (GE Healthcare) according to the manufacturer’s recommendations and bands were visualized with exposition to Kodak BioMaxLight Films. Quantification was performed by densitometry using ImageJ v1.44p software (NIH) and the values for phosphorylated proteins were normalized by the values for total proteins.

**Cell volume measurements**

Volume measurements were performed by estimating the changes in relative cell volume with a large-angle light-scattering system [28,29]. mIMCD3 cells cultured on coverslips were placed at a...
Measurement of NHE (Na\(^{+}\)/H\(^{+}\) exchanger) activity

NHE activity of mIMCD3 cells was estimated by Na\(^{+}\)-dependent pH\(_l\) recovery after an NH\(_4\)Cl-induced acid load. BCECF \([2'7'\text{-bis-(2-carboxyethyl)}-5'6'\text{-carboxyfluorescein}]-\)labelled cells (1.2 \(\mu\)M for 30 min) cultured in glass coverslips were incubated with isosmotic control medium containing 20 mM NH\(_4\)Cl for 5 min, then gently washed in Na\(^{+}\)-free equiosmolar medium substituted with N-methyl-D-glucamine chloride and placed at a 50° angle relative to the excitation light path in a cuvette filled with Na\(^{+}\)-free medium in a Fluoromax-3, Horiba luminescence spectrometer. \(\lambda_{ex}\) was alternated between 445 and 495 nm and fluorescence intensity was monitored at 525 nm. Basal fluorescence was recorded for 3 min and then mIMCD3 cells were re-exposed to isosmotic control medium (135 mM NaCl) to initiate NHE. The values obtained through this procedure were used to calculate the ratio of fluorescence intensity (fluorescence at 445 nm/fluorescence at 495 nm). The rate of rise in fluorescence ratio is proportional to pH\(_l\), the slope was calculated by linear regression using the data over the first 1 min.

Caspase 3 activity assay

Cell treatment was completed by rapid removal of medium on ice and cells were homogenized in 100 mM Hepes, 20 mM EGTA, 1 mM EDTA, 1 M DTT (dithiothreitol), 10% sucrose, 0.1% CHAPS and 20 \(\mu\)l/ml complete proteases-inhibitor cocktail, pH 7.5, diluted 1:1 (v/v) with glycerol. Caspase 3 activity was assayed by a fluorometric method [30] in a Fluoromax-3 Horiba luminescence spectrometer, using the synthetic peptide Ac-DEVD-AMC as substrate. Fluorescence was recorded for 10 min at 360–440 nm after adding 2 ml of lysis buffer, 10 \(\mu\)M Ac-DEVD-AMC and cell homogenate (30 \(\mu\)g). Results are expressed as relative activity given by the change in fluorescence intensity during the whole assay.

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) assay

The TUNEL assay was carried out using the DeadEnd™ Fluorimetric TUNEL System. After the different treatments, cells were detached (trypsin/EDTA and trypsin inhibitor) and plated in DMEM/F12 medium on to 96-well plates precoated with poly-L-lysine 0.25 mg/ml (50 \(\times\)10\(^{4}\) cells/well). Cells were fixed in 4% (w/v) paraformaldehyde in PBS at room temperature, permeabilized with 0.2% Triton X100 in PBS by 5 min and incubated in darkness with the TUNEL reaction mixture (equilibration buffer, terminal deoxynucleotidyl transferase and nucleotide mix) for 60 min at 37°C. Nuclei were counterstained with 2 \(\mu\)g/ml Hoechst diluted in PBS. Each step was followed by three washes with PBS. TUNEL-positive cells were observed in an Olympus IX71 epifluorescence microscope and the QCapture Pro 6.0 software. The percentage of TUNEL-positive cells against Hoechst-labelled cells was calculated.

Statistical analysis

Statistical differences between experimental groups were determined by ANOVA followed by a post-hoc Tukey’s test. Statistically significant differences were considered at \(P < 0.05\). All results are given as means \(\pm\)S.E.M.

RESULTS

Hyperosmolality and EGFR phosphorylation in mIMCD3 cells: upstream and downstream signalling elements

The effect of hyperosmotic solutions on EGFR phosphorylation was examined in mIMCD3 cells exposed to isosmotic or hyperosmotic solutions during 1, 2 and 4 h. Osmolarity was raised from 320 to 450–900 mosM by the addition of NaCl or urea. Figure 1(A) shows that hyperosmotic solutions made with NaCl increased the phosphorylation level of EGFR to an extent related to the magnitude of hyperosmolarity. This effect was abolished by AG1478, the specific blocker of EGFR tyrosine kinase phosphorylation (Figure 1A). The time course of EGFR phosphorylation evoked by a 450 mosM NaCl solution is shown in Figure 1(B). Maximal phosphorylation occurred after 2 h of treatment with the hyperosmotic solution. Thereafter, the magnitude of phosphorylation decreased. The hyperosmotic increase in EGFR phosphorylation was markedly reduced by the metalloproteinase blocker GM6001 (Figure 1C). Solutions made hyperosmotic with urea similarly increased EGFR phosphorylation, which was reduced by AG1478 (Figure 1D).

Two main signalling cascades diverge from EGFR phosphorylation: the PI3K/Akt and the Ras/MEK [MAPK (mitogen-activated protein kinase)/ERK kinase/ERK] pathways. The effect of hyperosmolarity on elements of these pathways was next examined. Cells treated with hyperosmotic solutions (450–900 mosM) made by the addition of NaCl showed increased phosphorylation of Akt, which was prevented by the PI3K blocker wortmannin and by the EGFR blocker AG1478 (Figure 2A). Solutions made hyperosmotic with urea also increased Akt phosphorylation, sensitive to AG1478 (Figure 2B). Hyperosmolarity
also increased phosphorylation of ERK1/2, which was abolished by PD98059, the MEK/ERK blocker and reduced by the EGFR inhibitor AG1478 (Figure 2C). These results show that EGFR is the upstream activator of the hyperosmotic phosphorylation of ERK and Akt, and that its own activation results from MMP (matrix metalloproteinase) activity.

**EGFR and RVI in hyperosmotic conditions**

In most cell types so far investigated, the fast initial step of RVI is accomplished by osmotic water fluxes driven from an increase in cell ion concentration. The intracellular ion accumulation commonly results from the activity of the electroneutral co-transporter NKCC \([\text{Na}^+\text{–}(\text{K}^+\text{–})\text{Cl}^−\text{co-transporter}]\) and the NHE exchanger, coupled with other exchangers or transporters. In IMCD cells, increasing the medium osmolarity to 450 or 750 mosM led to cell volume reduction of 48 and 55% respectively. Maximal shrinkage was attained within few seconds, and thereafter cells initiate RVI (Figure 3A). In the 450 mosM condition, cell volume was recovered up to 46%. In the 750 mosM condition, RVI was impaired and cell volume recovery was only of 15% (Figure 3A). RVI in mIMCD3 cells in a medium buffered with HCO\(_3^−\) was slightly more efficient (Figure 3A). Exposure to solutions made hyperosmotic with urea showed no cell shrinkage, but rather a 60% increase in cell volume was observed (Figure 3B).

The contribution of NKCC and NHE to RVI in mIMCD3 cells was assessed by measuring the effect on volume regulation of the NKCC or NHE blockers, bumetanide, zoniporide and EIPA. Bumetanide \((10 \mu\text{M})\), reduced RVI by 28% and zoniporide and EIPA decreased RVI by 57 and 48% respectively (Figure 3C). The effects of bumetanide and zoniporide were additive and reduced RVI by 82% (Figure 3C).

The influence of EGFR phosphorylation on cell shrinkage and RVI was examined in cells pre-incubated during 30 min with 10 \(\mu\text{M}\) AG1478, a condition which is shown in Figure 1, fully prevents the hyperosmotic EGFR phosphorylation in mIMCD3.
Figure 2  Effect of hyperosmotic solutions on Akt and ERK phosphorylation in mIMCD3 cells
(A) Akt phosphorylation by hyperosmotic (H) solutions (450 mosM) made with NaCl in the presence (+) or absence (−) of AG1478 (10 μM) or wortmannin (100 nM). (B) Effect of hyperosmotic urea solutions on Akt phosphorylation and its reduction by AG1478 (10 μM). (C) Effect of hyperosmotic NaCl solution (450 mosM) on ERK1/2 phosphorylation. Hyperosmotic effect was prevented by PD98059, the MEK/ERK phosphorylation blocker, and reduced by the EGFR blocker AG1478. Cell treatment, immunoblot analysis and results expression as in Figure 1. Representative blots are shown with bars corresponding to densitometric analysis of 4–8 separate experiments. Results are expressed as means ± S.E.M. *Significantly different from isosmotic control; **significantly different from the 450 mosM condition, P < 0.05.

Figure 3  Shrinkage and RVI of mIMCD3 cells exposed to hyperosmotic conditions and the effect of ion transport blockers
(A) Representative traces of the relative cell volume change of mIMCD3 cells exposed to NaCl solutions (450 or 750 mosM) buffered with HEPES or HCO₃⁻. (B) Representative trace of the relative cell swelling induced by hyperosmotic urea solution (450 mosM). RVI was calculated as the volume recovered (%) at the end of the experiment. (C) Effects on RVI efficiency of bumetanide (bum, 10 μM), EIPA (10 μM) and zoniporide (zon, 10 μM), and the effect of zoniporide (10 μM) plus bumetanide (10 μM). Results are expressed as means ± S.E.M. for 4–12 separate experiments. *Significantly different from the control condition (hypertonic, 450 mosM) δ significantly different from the zoniporide or bumetanide alone condition; P < 0.05.

cells. AG1478 did not affect cell shrinkage (results not shown) but reduced RVI by 67%, with respect to controls (Figure 4A). The metalloproteinase blocker GM6001 reduced RVI to the same extent (68%) as the EGFR blocker (Figure 4A). Blockade of ERK by PD98059 (50 μM) and PI3K/Akt by wortmannin (100 nM) decreased RVI by 57 and 62% respectively (Figure 4A). AG1478 and GM6001 added together had no additive effects reducing RVI (Figure 4A). Cell treatment with AG1478 together with the ERK blocker PD98059 or with wortmannin showed no additive effects on RVI inhibition (Figure 4A). These results confirmed that ERK and Akt actions sustaining RVI are under the influence of EGFR, which is in turn dependent on MMP activity.

To investigate the mechanisms of RVI affected by the pharmacological inactivation of EGFR and its derived signalling pathways, the effect of the NHE blocker zoniporide was compared with that induced by AG1478, PD98059 or wortmannin. Results in Figure 4(B) show that zoniporide added together with the blockers showed no additive effects, suggesting an action of EGFR, ERK and the PI3K/Akt pathway on RVI, mediated by NHE (Figure 4B). This was further confirmed by measuring the effect of EGFR, Akt and ERK blockers on NHE activity, monitored by the pH recovery from cell acidification induced by a pulse of NH₄Cl. Figure 5(A) shows representative traces of pH recovery in controls and the effect of zoniporide, compared with that of the EGFR, ERK and PI3K/Akt blockers. Zoniporide abolished NHE activity, and all three blockers markedly reduced its rate and efficiency (Figures 5A and 5B).

EGFR, RVI and apoptotic death
Exposure of mIMCD3 cells to media made hyperosmotic (450 and 750 mosM) with NaCl, significantly increased apoptotic
Figure 4  Effects on RVI of EGFR upstream and downstream signalling molecules

(A) Effects of blockers of MMP (GM6001, 10 μM), EGFR (AG1478, 10 μM), ERK1/2 (PD98059, 50 μM) and PI3K/Akt (wortmannin (100 nM; white bars) and the non-additive effect of AG1478 added together with each one of these blockers (grey bars). (B) Effect on RVI of the NHE blocker zoniporide, tested alone (white bars) or together (grey bars) with AG1478, PD98059 or wortmannin. Results expressed as in Figure 3 and represent means ± S.E.M. for 3–5 experiments. *Significantly different P < 0.05 from hyperosmotic 450 mosM condition (black bars); values in grey bars not significantly different from the corresponding values in white bars.

cell death. Apoptosis was evaluated by caspase 3 activity and by the TUNEL assay (Figure 6). The 450 mosM NaCl hyperosmotic solution led to a mild increase in apoptosis, which was particularly remarkable at 750 mosM (Figure 6A). Apoptotic death was not observed when cells were treated with hyperosmotic solutions (450 and 750 mosM) made of urea (Figure 6A). The effect of RVI blockers on cell death evoked by NaCl 450 mosM is shown in Figures 6(B)–6(D). Measuring apoptosis by caspase 3 activity, the NKCC blocker bumetanide, did not modify the extent of the hyperosmolarity-induced apoptotic death. NHE blockers zoniporide and EIPA increased by 43% (zoniporide) and 57% (EIPA) apoptosis observed in the 450 mosM condition (Figure 6B). EGFR inhibition, either directly by AG1478 or by the MMP blocker GM6001 had a remarkable effect increasing apoptotic cell death by 2.4-fold over the hyperosmotic value. The ERK blocker PD98059 increased 66% the hyperosmotic cell death. Akt inhibition by wortmannin resulted in a 2-fold increase of hyperosmotic cell apoptosis. Exposure to higher osmolarity (750 mosM, NaCl) increased considerably the apoptotic cell death (3.2-fold) and, under these conditions, EGFR blockade with AG1478 did not further increase apoptosis (Figure 6A). Some quantitative differences were found with the effect of AG1478 and zoniporide when apoptosis was evaluated by the number of TUNEL-positive cells as compared with results using caspase 3 activity. AG1478 increased apoptosis by 3.7-fold (as compared with 2.4-fold) and zoniporide by 103% (as compared with 43%; Figure 6D). All other conditions did not show significant differences between the two methods used to measure apoptosis.

DISCUSSION

The present study showed the ability of mIMCD3 cells to regulate volume after shrinkage induced by moderate hyperosmolarity. This cell line retains many features of the renal inner medullary collecting tubule cells, which are normally exposed to variable and often high extracellular levels of NaCl and urea during the process of urinary concentration. Primary cultures of inner medullary cells exposed to high (600–900 mosM) hyperosmolar solutions exhibit RVI only in the presence of the anti-diuretic hormone or of analogues of cAMP [31,32]. We found the same inability for volume regulation in mIMCD3 cells exposed to 700 mosM, but at 450 mosM, cells activate regulatory mechanisms and recover 46% their original volume. RVI in mIMCD3 cells, in agreement with reports in other cell types [14] is accomplished by the activity of the electroneutral co-transporter NKCC and the NHE, the exchanger contribution being far more important than that of the co-transporter. Some contribution of cationic channels activated by hypertonicity cannot not excluded, but could not be

Figure 5  Effect of EGFR, ERK1/2 and PI3K/Akt blockers on NHE activity

(A) Representative traces of cell pH recovery from an acid load by an NH₄Cl pulse in the absence (control) or presence of zoniporide (zon, 10 μM), AG1478 (AG, 10 μM), PD98059 (PD, 50 μM) and wortmannin (wort, 100 nM). Results are expressed as the ratio of BCECF fluorescence intensity (445 nm/495 nm). (B) In bars, the percentage of the rate recovery for each inhibitor over the control relative activity. Results are expressed as means ± S.E.M. for 8–12 separate experiments. *Significantly different from the control condition; P < 0.05.
accurately in mIMCD3 cells using the present pharmacological approach, since both NHE and these channels are blocked by the amiloride derivative EIPA [33].

Results of the present study pointed to a permissive role of EGFR on the cell volume regulation activated following the hypertonic-induced shrinkage. As in other cell types [21,24–27] high osmolarity increased EGFR phosphorylation in mIMCD3 cells. We showed here that the hyperosmotic activation of EGFR in mIMCD3 cells occurs via the action of MMP, as MMP inhibition prevents EGFR phosphorylation and its effect reducing RVI. EGFR effects sustaining RVI proceeds through the two main signalling pathways activated downstream of EGFR, i.e. the MAPK/ERK and the PI3K/Akt [34]. Both signalling chains participate in the permissive effect of EGFR on cell volume regulation. Hypertonicity increases phosphorylation of ERK and Akt and this effect was abrogated by the EGFR blocker AG1478, a result that points to EGFR as the upstream signal inducing activation of the two signalling chains. Inhibition of ERK and Akt leads to a marked reduction of RVI and this effect is not further increased by the simultaneous blockade of AG1478, a result that points to EGFR as the upstream signal inducing activation of the two signalling chains. Inhibition of ERK and Akt leads to a marked reduction of RVI and this effect is not further increased by the simultaneous blockade of EGFR, a result stressing the hierarchical link between EGFR and its signalling chains sustaining RVI. Different effects of hypertonicity on Akt have been reported. As in mIMCD3 cells, in inner medulla and in MDCK (Madin–Darby canine kidney) cells, mild hyperosmolality increases Akt phosphorylation [10,42]. Likewise, in HeLa cells hyperosmotic-induced phosphorylation of Akt 1 [35], while in Swiss 3T3 cells, Akt activation by PDGF (platelet-derived growth factor) is prevented by hypertonic sorbitol solutions [36]. The involvement of different Akt isoforms may explain these differences, since in HeLa cells only Akt 1 (and not Akt 2) responds to high osmolarity [35]. We provide here evidence of a permissive role of Akt on RVI. This is in line with recent results in HeLa cells [35], in which diverse manoeuvres directed to suppress Akt activity markedly reduce RVI. A link between Akt activation and EGFR or another growth factor receptor was not examined in these cells and rather the anti-apoptotic kinase ASK1 (apoptosis signal-regulating kinase-1) is mentioned as the Akt activator. In mIMCD3 cells, the EGFR-Akt connection in response to hypertonicity seems well supported by our present results.

The permissive effect of EGFR on RVI is likely to have NHE as a main downstream effector. Evidence consistent with this notion includes (i) the non-additive effect reducing RVI of EGFR and NHE blockers, and (ii) the EGFR action decreasing NHE rate in cells challenged by an NH4Cl pulse. The same kind of arguments, i.e., the non-additive effects reducing RVI of the ERK and the PI3K/Akt blockers added simultaneously with zoniporide and the decrease of NHE rate and efficiency, point to NHE as the mechanism responsible for the effect sustaining RVI of the EGFR-activated signalling pathways. Reports exist relating NHE to EGFR [37–40] and ERK [41] in a variety of cell types.

The effect of EGFR and its signalling chains propitiating cell volume recovery after hypertonic shrinkage, shown in the present study, may explain its role as anti-apoptotic factor under hypertonic conditions. Hypertonicity-induced cell death has been reported in numerous cell types [6–15], including renal tubule cells [16] and mIMCD3 cells [42], and in the present study. Death is presumably related to the sustained reduction in cell volume when RVI is impaired. A link between cell shrinkage and apoptosis is well established. The early study by Bortner and Cidlowski [20] in lymphoid cells exposed to high osmolarity related the defective operation of RVI to apoptotic death. This link

Figure 6  Effect of hyperosmotic conditions and of RVI blockers on apoptotic death in mIMCD3 cells

Caspase-3 activity and TUNEL were used to evaluate apoptosis. (A) Relative caspase 3 activity in cells exposed to NaCl or urea hyperosmotic (H) solutions (450 and 750 mosM) and the effect of 10 μM AG1478. (B) Effects of bumetanide (bum, 10 μM), EIPA (10 μM) and zoniporide (zon, 10 μM) on apoptotic cell death. (C) Effects increasing apoptosis of AG1478 (AG, 10 μM), PD98059 (PD, 50 μM) or wortmannin (wort, 100 nM). (D) Apoptotic cell death evaluated by TUNEL in isosmotic, 450 mosM and in the presence of blockers as in (B, C). Black bars correspond to isosmotic (320 mosM) or hyperosmotic (450 mosM) conditions and white bars to data in the presence of the blockers. Results are expressed as means ± S.E.M. for 6–16 experiments. **Significantly different from isosmotic control. *Significantly different from hyperosmotic 450 mosM condition, P < 0.05.
was confirmed in HeLa cells exposed to high osmolarity showing that RVI dysfunction induced by NHE blockers resulted in apoptotic cell death, while restoring RVI by NHE overexpression reduced apoptotic cell death [12]. The association between shrinkage and apoptosis is also suggested by a report showing that when osmolality is modified by gradual and small changes, cell volume remains constant, and cell death is substantially reduced [43]. Our results comparing the effect of hyperosmolar solutions made with NaCl and urea are also in line with these findings. Neither cell shrinkage nor apoptotic cell death occurs by treatment with solutions made hyperosmolar with urea, while the opposite is observed in NaCl solutions. Urea is a highly permeable solute and accumulates in the cell by the activity of specific transporters. As a result of this accumulation, hyperosmolar solutions made with urea did not reduce but increased cell volume [44]. Results similar to those in the present study were found in mIMCD3 cells exposed to hyperosmolar solutions of NaCl or urea, reporting apoptotic death by hyperosmolar NaCl but not by urea, after up to 8 h of exposure to the hypersonic conditions [45]. Altogether these results stress the importance of cell volume changes as pro- or anti-apoptotic signals.

The present results are consistent with an anti-apoptotic effect of EGFR in mIMCD3 cells in hypertonic conditions, operating via a permissive role on RVI, which requires the activation of the EGFR/PI3K/Akt survival pathway. In the study in HeLa cells referred to above, a variety of manoeuvres directed to block Akt activity consistently led to RVI prevention and apoptotic cell death [35]. In these cells, the Akt hyperosmotic activation was related to the ASK1. ASK1 activates by hyperosmolarity via a ROS-dependent mechanism, and its suppression blocks hyperosmotic Akt phosphorylation, inhibits RVI and generates apoptosis. In our study, Akt activation, and its permissive effect on RVI, as well as its anti-apoptotic action, are directly under the ascendency of EGFR, and promoted by MMP. Altogether, these results contribute to establish a link between Akt, RVI and apoptosis, although different mechanisms for Akt activation may operate under different conditions and different cell types.

In mIMCD3 cells, the EGFR anti-apoptotic effect was evident only under moderate hyperosmolality. Under conditions of high osmolality, leading to severe cell shrinkage and full RVI prevention, the anti-apoptotic action of EGFR is surpassed by pro-apoptotic factors, presumably evoked by the persistent and severe cell shrinkage. All these results are consistent with a mechanism whereby the regulation and relative predominance of anti-apoptotic EGFR-mediated signals and pro-apoptotic elements define the fate of medullary cells challenged by high osmolality. Akt and cell volume changes seem to be essential cues in this process.

**REFERENCES**


**AUTHOR CONTRIBUTION**

Alejandro Ruiz-Martínez performed the Western blots and carried out the cell volume measurements, and participated in writing the paper; Erika Vázquez-Juárez performed the NHE experiments; Gerardo Ramos-Mandujano maintained the cell cultures for all of the experiments, and performed the caspase and TUNEL assays; and Herminia Pasantes-Morales supervised and directed the study.

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EGFR, RVI and apoptosis


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