doi:10.4149/neo_2009_03_224

Inhibition of cell proliferation and arrest of cell cycle progression by blocking chloride channels in human laryngeal cancer cell line Hep-2

W. F. YU[1], Y. L. ZHAO[1*], K. WANG[2], M. M. DONG[1]

[1]Department of Otolaryngology, the First Affiliated Hospital of Zhengzhou University,Zhengzhou, Henan 450052, P. R. China. e-mail: albb1998@yahoo.cn, [2]Department of Obstetrics and Gynecology, Perinatal Research Laboratories, University of Wisconsin, Madison, WI 53715, USA.

Received July 27, 2008

Chloride channel (ClC) is involved in normal physiological processes and pathology of various diseases. Although it is recognized that blockade of ClC inhibits the cell proliferation, it is not well understood the potential function of ClC in laryngeal cancer. In this study, we investigated the effect of the ClC inhibitor on cell proliferation, cell cycle progression in human laryngeal cancer cell line Hep-2, as well as the effect on the phosphorylation levels of ERK1/2 and AKT1. In this study crystal violet method was used to study the effect of the ClC inhibitor, 5-nitro-2-(3-phenylpropylamino) benzoic acid, NPPB, on Hep-2 cell proliferation. The impaction of the inhibitor on the cell cycle distribution was investigated by the flow cytometry (FCM). Western blot was performed to measure the phosphorylation levels of ERK1/2 and AKT1. Our data indicated ClC played an important role in Hep-2 cell proliferation and cell cycle. NPPB inhibited Hep-2 cell proliferation when compared with the controls. Blockade of ClC arrested cell cycle progression and suppressed the phosphorylation of ERK1/2 and AKT1 in Hep-2 cells by inhibition of cell proliferation by CIC inhibitor (NPPB) could be through arresting cell cycle progression, which is probably by suppressing phosphorylation of ERK1/2 and AKT1.

Key words: laryngeal carcinoma, chloride channel, proliferation, cell cycle, ERK1/2, AKT1

It is well known that head and neck cancer is the eighth most common cause of cancer death worldwide [1]. Laryngeal cancer is one of the head and neck malignant tumors that seriously lead to health damage. It is very common in Brazil, Hong Kong, Italy and among the Black populations in parts of USA. There are also areas of low incidence in Japan, Norway, Sweden and Senegal. In each country there exist difference in incidence among urban and rural areas, the former having higher incidence [2]. Pathogenesis of laryngeal cancer is still not clear. It is becoming clear that cell ion transport proteins function as part of larger macromolecular complexes [3–6] is necessary for the cell biosynthesis, signal transduction, and function of membrane normal receptors [7–9]. Recently, some reports showed that cell ClC play a critical role in cell proliferation and differentiation. For example, in most cell types studied, cell swelling results in activation of ClC [10–14], and, particularly in mammalian cells. ClC is characterized as an outwardly rectifying current that is inhibited by a variety of nonspecific ClC inhibitors including both 5-nitro-2 (3-phenylpropylamino) benzoic acid (NPPB) and DIDS [12, 14, 15, 16], and is thought to be one of the major osmolytes involved in cancer cells. Volume-regulated chloride currents have been involved in the control of the cell cycle [17–19]. Therefore, understanding the mechanisms and functional significance of ClC is essential for the therapy of laryngeal cancer. However, the functions of ClC in laryngeal tumor cells still remains unknown. In the present study we investigated the effect of NPPB on cell proliferation and cell cycle in Hep-2 cells.

It is known that signal transduction pathways play important role in cellular proliferation and differentiation. They are often orchestrated by mitogen-activated protein kinases (MAPKs) [20]. MAPK is proline-directed serine/threonine kinase that have been classified into at least six subfamilies, of which ERK1/2, JNK1/2, and p38 are the most extensively studied [21]. ERK1/2 is normally activated by growth signals and mainly associated with cell cycle arrest, apoptosis and tumor formation [22, 23]. Another important kinase is Akt/protein kinase B. It is a central regulator of cell survival [24, 25] and Akt activation is required for many types of human tumors [26, 27].

* Corresponding author

Therefore, the purpose of this study is to determine the role of ClC in laryngeal cancer. We examined the effects of NPPB on the cell proliferation and cell cycle in Hep-2 cells, and the signaling pathway involving ERK1/2 and Akt1.

Materials and methods

Cell culture. Human laryngeal cancer Hep-2 cells were purchased from the Cell Resource Center of Shanghai Academy of Life Science of Chinese Academy of Science. Cells were grown in RPMI-1640 with 5%(v/v) decomplemented fetal bovine serum (FBS) (Gibco Life Technologies, Grand Island, NY, USA), at 37°C in a humidified atmosphere containing 5% (v/v) CO_2. The medium was supplemented with 300 μg/ml L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were seeded in 75 cm^2 flasks, and the growth medium was renewed every second day. For the experiments, the cells were subcultured in 35 mm petri dishes (Nunc) and used after 3–5 days.

Cell Proliferation. Cell proliferation was assayed as described [28, 29]. Hep-2 cells were cultured in 96-well plates (4000-6000 cells/well) for 24h, starvation for overnight. Added 5%(v/v) decomplemented fetal bovine serum, cells were treated without(control) or with NPPB at 50, 100, 150, and 200μM(6 wells/dose). After another 48 hr of culture, the number of cells was determined. Briefly, wells were rinsed with PBS, fixed in methanol, air-dried, and stained with 0.1% (w/v) crystal violet. Wells were rinsed with distilled water, and air-dried again. Once dried, cells were lysed with 2% (w/v) sodium deoxycholate solution with gentle agitation. Absorbance was measured at 570 nm on a microplate reader (Bio Tek Instrument, Winooski, VT). Wells containing known cell numbers (0, 1000, 2000, 5000, 10000, 20000 or 40000 cells/well; 6 wells/cell density) were treated in the similar fashion to establish standard curves. Cell proliferation studies were run in five independent experiments.

*Cell viability.*The cell viability was detected by trypan blue rejection. After Hep-2 cells stained by trypan blue, the number of Hep-2 cells unstained by trypan blue was accounted by microscope, and then we computed cellular survival rate.

Cell cycle progression. Hep-2 cells were inoculated into culture bottles when the cells in the mid-log phase of growth. They were divided into four groups, including of control group; 50μM-NPPB group, 100μM-NPPB group and 150μM-NPPB group. There were three repeated bottles for each group. The cells were then cultured in RPMI-1640 alone or in RPMI-1640 with the corresponding NPPB concentration when the cells were in the denary logarithmic growth condition. Cells were collected from each bottle after 24 hrs and 48 hrs. A unicellular suspension was prepared and the cell concentration was adjusted to 1×10^6/ml, the agent was then added according to the kit instructions. The cell cycle of each treatment group was measured using FCM to determine.

Western blotting analysis. Western blot analysis was performed as described [28, 29]. After 16 hours of serum-deprivation, cells were treated with 50, 100 and 150μM of NPPB (Pepro Tech, Rocky Hill, NJ) for 0-8 hours. To determine changes in total and phosphorylated ERK1/2 and AKT1 protein levels, cells were washed twice with cold PBS, harvested and then lysed by sonication in buffer (4 mM sodium pyrophosphate; 50 mM HEPES, pH 7.5; 100 mM NaCl; 10 mM EDTA; 10 mM sodium fluoride; 2 mM sodium orthovanadate [Na3VO4]; 1 mM PMSF; 1% Triton X-100; 5 mg/ml leupeptin; and 5 mg/ml of aprotinin). The protein concentrations in supernatants of the lysates were determined. Proteins (15-20 μg/lane) were subjected to Western blot analysis. Proteins were separated on 10% SDS-PAGE gels and electroblotted onto Immobilon-P membrane (Millipore, Bedford, MA), Proteins on the membranes were probed with an antibody against total or phospho-specific ERK1/2 (1:2000; Cell Signaling Technology, Beverly, MA), total (1:2000; Cell Signaling Technology) or phospho-specific AKT1 (1:1000; Cell Signaling Technology). The membranes were reprobed for GAPDH as described above. Changes in total and phosphorylated ERK1/2and AKT1 protein levels were quantified. Data on phosphorylated ERK1/2 and AKT1 were normalized to total ERK1/2 and AKT1. Data on total ERK1/2 and AKT1 were normalized to GAPDH. The kinase studies were run in at least three independent experiments.

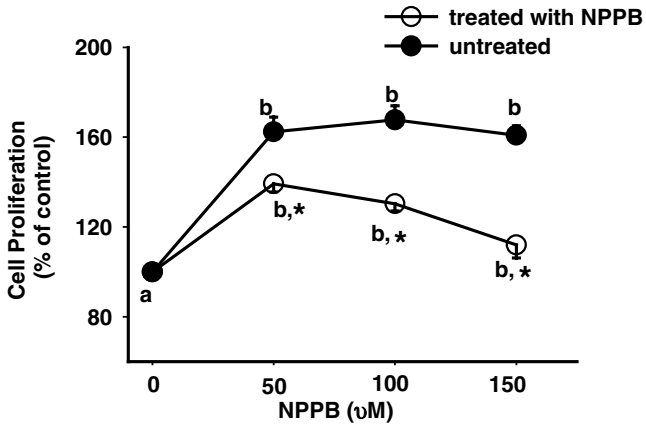

Figure 1. Effects of NPPB on Hep-2 cell proliferation. Added 5%(v/v) fetal bovine serum. Cells after treated without (control) or with NPPB were seeded in 96 well plates (4000 cells/well) for 48 hr. Data are expressed as means ± SEM percent of the control from eight independent experiments. Numbers of cells per well in the control were 6039 ± 558. Letter b indicate significant ($p < 0.05$) differences from the control. Asterisks denote significant ($p < 0.05$) differences at the corresponding dose of NPPB.

Statistical analysis. The data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc analysis. Statistical significance was defined as $p < 0.05$. SPSS 13.0 for Windows was used for all the analyses.

Results

Blockade of ClC significantly inhibited Hep-2 cell proliferation. NPPB inhibited ($p < 0.05$) (t[1]=3.24) Hep-2 cell

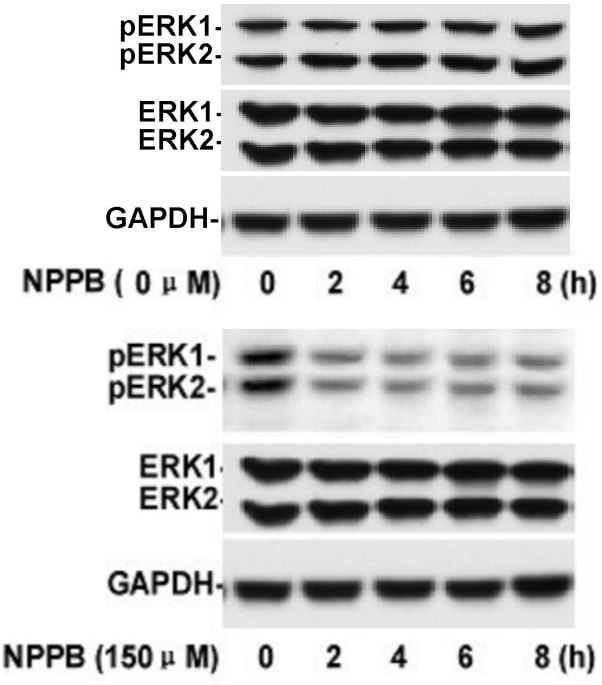

Figure 2. The effects of NPPB on the phosphorylation of ERK1/2 in Hep-2 cells. Cells were treated with NPPB (150 μM). Proteins (15μg/lane) are subjected to Western blot analysis. Data normalized to total ERK1/2 are expressed as means ± SEM fold of the controls (time 0) from three independent experiments. 150 μM-NPPB group is significantly ($p < 0.05$) different from the corresponding 0μM-NPPB group.

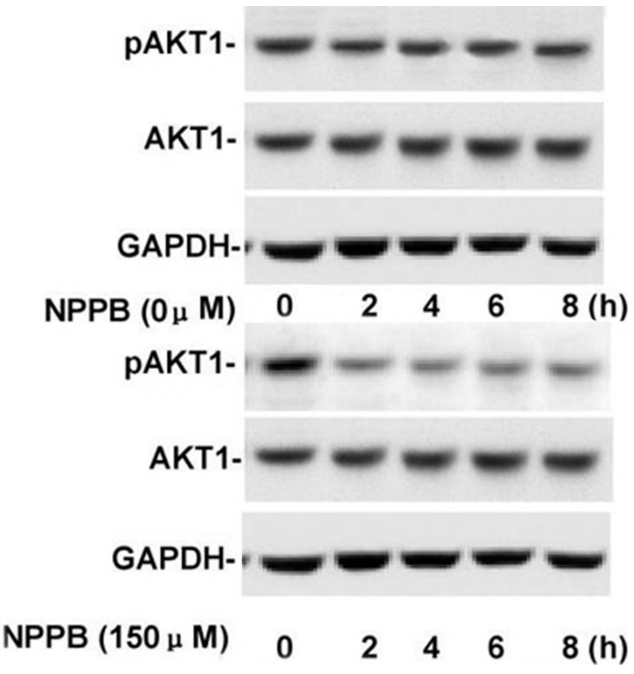

Figure 3. The effects of NPPB on the phosphorylation of AKT1 in Hep-2 cells. Cells were treated with NPPB (150 μM). Proteins (15μg/lane) are subjected to Western blot analysis. Data normalized to total AKT1 are expressed as means ± SEM fold of the controls (time 0) from three independent experiments. 150 μM-NPPB group is significantly ($p < 0.05$) different from the corresponding 0μM-NPPB group.

proliferation in a dose-dependent manner (Figure 1). NPPB at the concentration of 50μM, 100μM and 150μM inhibited Hep-2 cell proliferation, but did not significantly alter cell viability. As compared with the control group, the cellular survival rate was not changed significantly ($p > 0.05$) (t[1]=0.18) (97.2±0.2% vs. 97.6±0.5% for the concentration of 50μM: untreated group, 96.9±0.7% vs. 97.6±0.5% for the concentration of 100μM: untreated group, 96.3±0.4% vs. 97.6±0.5% for the concentration of 150μM: untreated group). But, NPPB significantly decreased ($p < 0.001$) (t=6.76) cell viability (76.5±0.8% vs. 97.6±0.5% for the concentration of 200μM: untreated group). Thus we used 50μM, 100μM and 150μM NPPB to block ClC in our experiments.

Effects of ClC blockers on Hep-2 cell cycle. The cell ratio of G0/G1-phase increased ($p < 0.05$) (t[1]=2.84) the cell ratio of S-phase and G2/M-phase decreased ($p < 0.05$) (t[1]=3.05) correspondingly after various levels of NPPB were added for 24 hours. After 48 hours, the cell ratio of G0/G1 phase reached a peak then decreased especially in the 150μM-NPPB group (Table 1). Further problem studied was the effect of ClC blockers on phosphorylation of ERK1/2 and AKT1 in Hep-2 cells**.** Cells were treated with 50, 100 and 150μM of NPPB (Pepro Tech, Rocky Hill, NJ) for 0-8 hours. To determine whether blocking ClC affected activation of ERK1/2 and AKT1, the phosphorylation of ERK1/2 (Figs. 2) and AKT1 (Figs. 3) was assessed by Western blot analysis. As compared with the control group, blockade of CLC did not significantly ($p > 0.05$) (t[1]=0.26) change levels of total ERK1/2(total ERK1/2/GAPDH: 1. 47 ± 0.142 vs. 1.32 ± 0.212 for the concentration of 150μM: untreated group) and total AKT1 (total AKT1/GAPDH: 1.39 ± 0.205 vs. 1.25 ± 0.189 for the concentration of 150μM: untreated group). But blockade of CLC significantly ($p < 0.05$) (t[1]=2.63) changed phosphorylation levels (at time 2 hour) of ERK1/2 (phospho/total ERK1/2: 1.13 ± 0.030 vs. 0.53 ± 0.019 for the concentration of 150μM: untreated group) and AKT1 (phospho/total AKT1: 1.07 ± 0.093 vs. 0.52 ± 0.062 for the concentration of 150μM: untreated group) as well as other time point (4, 6 and 8 hours) examined (Figs. 2 and 3; quantitative data not shown).

Discussion

*Blockade of ClC inhibited Hep-2 cells proliferation and increased cell cycle arrest.*ClC has been referred to as "the problem of chloride ion channels" [30], and the field harbors considerable controversies. The role of ClC in human laryngeal cancer is poorly defined. In this study, for the first time in human laryngeal cancer Hep-2 cells, we have demonstrated

successfully that blockade of ClC with NPPB dose-dependently significantly inhibited Hep-2 cell proliferation. These data implicate a direct link between ClC and Hep-2 cell proliferation. This conclusion is in agreement with the previously published data. For example, it has been demonstrated that cell division could be inhibited by channel blockers [31–33]. Similarly, the proliferation of C6 glioma cells, rat aortic smooth muscle cells, and mouse liver cells is inhibited after ClC knockdown [34–36]. The reduction in cell proliferation observed in this study may be mediated by a number of factors. It is well known that cell cycle analysis was carried out to elucidate the antiproliferative activity. Increasing cell cycle arrest in the G0/G1 phase of the cell cycle may mediate its antiproliferative effects via its impact on cell cycle progression. These results are similar to data obtained using the human cervical epithelial cell-lines SiHa and HeLa [37, 38]. In both studies, NPPB increased the cells arrested in G0/G1. On the other hand, increased chloride channels activity has been shown to coincide with the entry into the cell cycle of human cervical cancer cells [39].

What is the mechanism of NPPB dose-dependently inhibited-cell proliferation with arrested cell cycle progression? Did NPPB kill Hep-2 cells directly? To answer these questions, Hep-2 cells viability was determined.Blockade of CIC with NPPB (50, 100 and 150μM), the cell survival rates did not change remarkably. This indicated that inhibition of Hep-2 cells proliferation and arrest of cell cycle by blocking ClC has other reasons.

Blockade of ClC decreased the phosphorylation of ERK1/2 and AKT1 It is well known that ERK1/2 and AKT1 are actively involved in the regulation of cell function. Firstly, ERK1/2, a member of the MAP kinase family, is activated by upstream kinases, resulting in its translocation to the nucleus where it phosphorylates nuclear targets. This pathway is constitutively active in several human malignancies and may be involved in the pathogenesis of these tumors [40]. The ERK1/2 pathway mediates ligand-stimulated signals for the induction of cell proliferation, differentiation, and cell survival [41, 42]. It has been shown that MAP kinases Erk1/2 was involved in the activation of volume-sensitive chloride channels in astrocytes [43]. In this study, we examined the phosphorylation of ERK1/2 before and after blockade of ClC. We have demonstrated successfully that NPPB (50, 100 and 150μM) inhibited the phosphorylation of ERK1/2 in Hep-2 cells. The reasons may be that the volume-sensitive chloride channel appears to be a cytoplasmic target for the MAP kinase signaling pathway [43]. When chloride channels were blocked by NPPB (50, 100 and 150μM), the MAP kinase signaling pathway was influenced. Therefore, the phosphorylation of ERK1/2 was inhibited. This conclusion agreed with those studies. For example, the MAP kinase kinase inhibitor PD 98059 reversibly inhibited activation of the chloride current by hypo-osmotic solution [43].

Secondly, protein kinase B (PKB/Akt) is a pivotal regulator of diverse metabolic, phenotypic, and antiapoptotic cellular controls, and also well known to be involved in transformation and cell survival. It is a critical kinase that regulates events downstream of growth factor receptors. AKT has been shown to be a key player in cancer progression, where its overexpression induces malignant transformation. It affects prosurvival proteins as well. Blocking the flow-activated chloride current abolished flow-induced Akt phosphorylation in BAECs [44]. It was also reported that volume-sensitive chloride currents $I_{(Cl,vol)}$ inhibitors in preventing doxorubicin-induced apoptosis and subsequent contractile dysfunction through AKT in cardiomyocytes [45]. In the current study, we examined the phosphorylation of AKT1 before and after blockade of ClC. We have demonstrated successfully that NPPB(50, 100 and 150μM) inhibited the phosphorylation of AKT1.

Table 1. The ratios of different cell cycles in Hep-2 cells at different time points (%, mean ± SD). The cell cycle of each treatment group was measured using FCM to determine.

Groups		24 hr	48 hr	72 hr
Control	G0/G1	61.53±1.29	62.12±1.28	65.45±1.16
	S	25.16±1.53	23.37±1.69	18.35±1.71
	G2/M	14.89±1.24	15.57±1.52	17.08±1.45
50μM-NPPB	G0/G1	74.68±2.12	75.58±1.79	74.12±1.78
	S	17.87±1.25	19.56±1.36	15.24±1.42
	G2/M	10.34±0.35	5.26±0.32	12.96±0.17
100μM-NPPB	G0/G1	80.52±1.68	81.78±1.35	82.57±1.47
	S	10.45±1.08	12.67±1.22	10.57±1.49
	G2/M	9.23±0.34	7.58±0.36	7.98±0.26
150μM-NPPB	G0/G1	85.57±1.23	87.82±1.26*	84.59±1.49
	S	5.28±1.42	6.75±1.04	5.89±1.36
	G2/M	10.12±0.82	6.04±0.28	10.04±1.82

* The comparison between the 150μM-NPPB group and control group, the 100μM-NPPB group, and 50μM-NPPB group, $p < 0.05$, respectively.

As shown above, blockade of ClC inhibited both the Hep-2 cells proliferation and the phosphorylation of ERK1/2 and AKT1. We examined further the relationship between NPPB inhibited-cell proliferation and the phosphorylation of ERK1/2 and AKT1. The data indicated that the concentration dependence of NPPB inhibition of cell proliferation was in excellent agreement with the phosphorylation of ERK1/2 and AKT1. The inhibition of the cell proliferation by NPPB was positively related to phosphorylation of ERK1/2 and AKT1. These results are in agreement with those studies above.

Taken together, our current study has shown for the first time that blockade of ClC inhibited Hep-2 cell proliferation and arrested cell cycle progression by inhibiting ERK1/2 and AKT1 activation. Further experiments are required using different types of tumor cells. Future studies are needed to dissect the mechanism, which may provide fundamental information for ClC.

In conclusion, in Hep-2 cells ClC played an important role in cell proliferation and cell cycle. ClC inhibitor (NPPB) doesdependly inhibited cell proliferation and arrested cell cycle progression, which is probably by suppressing phosphorylation of ERK1/2 and AKT1.

We thank Dr. Yang Yunlie from Mount Sinai School of Medicine for critically reading this manuscript.

References

[1] tcher ED, Yarney J, Gyasi RK et al. Laryngeal cancer at the korle bu teaching hospital Accra Ghana. Ghana Med J 2006; 40: 45–49.

[2] Blot WJ, McLaughlin JK, Winn DM et al. Smoking and drinking in relation to Oral and pharyngeal cancer. Can Res 1988; 48: 3282–3287.

[3] Guggino WB, Stanton BA. New insights into cystic fibrosis: molecular switches that regulate CFTR. Nat Rev Mol Cell Biol 2006; 7: 426-436. doi:10.1038/nrm1949

[4] Bulenger S, Marullo S, Bouvier M. Emerging role of homo-and heterodimerization in G-protein-coupled receptor biosynthesis and maturation. Trends Pharmacol Sci 2005; 26: 131-137. doi:10.1016/j.tips.2005.01.004

[5] Engelman DM. Membranes are more mosaic than fluid. Nature 2005; 438: 578-580. doi:10.1038/nature04394

[6] Prinster SC, Hague C, Hall RA. Heterodimerization of protein-coupled receptors: specificity and functional significance. Pharmacol Rev 2005; 57: 289–298. doi:10.1124/pr.57.3.1

[7] Shenolikar S, Voltz JW, Minkoff CM et al. Targeted disruption of the mouse NHERF-1 gene promotes internalization of proximal tubule sodium-phosphate cotransporter type IIa and renal phosphate wasting. Proc Natl Acad Sci USA 2002; 99: 11470–11475. doi:10.1073/pnas.162232699

[8] Guggino WB, Banks SP. Macromolecular interactions and ion transport in cystic fibrosis. Am J Respir Crit Care Med 2004; 170: 815–820. doi:10.1164/rccm.200403-381WS

[9] Scott K, Zuker CS. Assembly of the Drosophila phototransduction cascade into a signalling complex shapes elementary responses. Nature 1998; 395: 805–808. doi:10.1038/27448

[10] Bond TD, Ambikapathy S, Mohammad S et al. Osmosensitive Cl– currents and their relevance to regulatory volume decrease in human intestinal T84 cells: outwardly vs. inwardly rectifying currents. J Physiol 1998; 511: 45–54. doi:10.1111/j.1469-7793.1998.045bi.x

[11] Hoffmann EK. Cell swelling and volume regulation. Can J Physiol Pharmacol 1992; 70: S310–S313.

[12] Pasantes MH, Alavez S, Sanchez OR et al. Contribution of organic and inorganic osmolytes to volume regulation in rat brain cells in culture. Neurochem Res 1993; 18: 445–452. doi:10.1007/BF00967248

[13] Schmidt NB. Comparative physiology of cellular ion and volume regulation. J Exp Zool 1975; 194: 207–219. doi:10.1002/jez.1401940114

[14] Stobrawa SM, Breiderhoff T, Takamori S et al. Disruption of ClC-3, a chloride channel expressed on synaptic vesicles, leads to a loss of the hippocampus. Neuron 2001; 29: 185–196. doi:10.1016/S0896-6273(01)00189-1

[15] D'Anglemont de Tassigny A, Souktani R, Ghaleb B et al. Structure and pharmacology of swelling-sensitive chloride channels, ICl,swell. Fundam Clin Pharmacol 2003; 17: 539–553. doi:10.1046/j.1472-8206.2003.00197.x

[16] Mountian I, Declercq PE, Van Driessche W. Volume regulation in rat brain glial cells: lack of a substantial contribution of free amino acids. Am J Physiol Cell Physiol 1996; 270: C1319–C1325.

[17] Wang L, Chen L, Zhu L et al. Regulatory volume decrease is actively modulated during the cell cycle. J Cell Physiol 2002; 193: 110–119. doi:10.1002/jcp.10156

[18] Zheng YJ, Furukawa T, Tajimi K et al. Cl- channel blockers inhibit transition of quiescent (G0) fibroblasts into the cell cycle. J Cell Physiol 2003; 194: 376–383. doi:10.1002/jcp.10218

[19] Nilius B. Chloride channels go cell cycling. J Physiol 2001; 532: 581. doi:10.1111/j.1469-7793.2001.0581e.x

[20] Pearson G, Robinson F, Beers T et al. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 2001; 22:153–183. doi:10.1210/er.22.2.153

[21] Cobb MH, Goldsmith EJ. How MAP kinases are regulated. J Biol Chem 1995; 270: 14843–14846. doi:10.1074/jbc.270.25.14843

[22] Khokhlatchev AV, Canagarajah B, Wilsbacher J ei al. Phosphorylation of the MAP kinase ERK2 promotes its homodimerization and nuclear translocation. Cell 1998; 93: 605–615. doi:10.1016/S0092-8674(00)81189-7

[23] Chen DB, Davis JS. Epidermal growth factor induces c-fos and c-jun mRNA via Raf-1/MEK1/ERK-dependent and -independent pathways in bovine luteal cells. Mol Cell Endocrinol 2003; 200: 141–154. doi:10.1016/S0303-7207(02)00379-9

[24] Scheid MP, Woodgett JR. PKB/AKT: functional insights from genetic models. Nat Rev Mol Cell Biol 2001; 2: 760–768. doi:10.1038/35096067

[25] Song G, Ouyang G, Bao S. The activation of Akt/PKB signaling pathway and cell survival. J Cell Mol Med 2005; 9: 59–71. doi:10.1111/j.1582-4934.2005.tb00337.x

[26] Hsu JH, Shi Y, Hu L et al. Role of the AKT kinase in expansion of multiple myeloma clones: effects on cytokine-dependent proliferative and survival responses. Oncogene 2002; 21: 1391–1400. doi:10.1038/sj.onc.1205194

[27] Rudelius M, Pittaluga S, Nishizuka S et al. Constitutive activation of Akt contributes to the pathogenesis and survival of mantle cell lymphoma. Blood 2006; 108: 1668–1676. doi:10.1182/blood-2006-04-015586

[28] Du K, Tsichlis PN. Regulation of the Akt kinase by interacting proteins. Oncogene 2005; 24: 7401–7409. doi:10.1038/sj.onc.1209099

[29] Zheng J, Bird IM, Melsaether AN et al. Activation of the mitogen-activated protein kinase cascade is necessary but not sufficient for basic fibroblast growth factor- and epidermal growth factor-stimulated expression of endothelial nitric oxide synthase in ovine fetoplacental artery endothelial cells. Endocrinology 1999; 140: 1399–1407. doi:10.1210/en.140.3.1399

[30] Clapham D. How to lose your hippocampus by working on chloride channels. Neuron 2001; 29: 1–3. doi:10.1016/S0896-6273(01)00172-6

[31] Voets T, Szucs G, Droogmans G et al. Blockers of volume-activated Cl– currents inhibit endothelial cell pro-

liferation. Pflügers Arch 1995; 431: 132–134. doi:10.1007/BF00374387

[32] Phipps DJ, Branch DR, Schlichter LC. Chloride-channel block inhibits T lymphocyte activation and signaling. Cell Signal 1996; 8: 141–149. doi:10.1016/0898-6568(95)02039-X

[33] Schlichter LC, Sakellaropoulos G, Ballyk B et al. Properties of K+ and Cl– channels and their involvement in proliferation of rat microglial cells. Glia 1996; 17: 225–236. doi:10.1002/(SICI)1098-1136(199607)17:3<225::AID-GLIA5>3.0.CO;2-#

[34] Rouzaire-Dubois B, Milandri JB, Bostel S et al. Control of cell proliferation by cell volume alterations in rat C6 glioma cells. Pflügers Arch 2000; 440: 881–888. doi:10.1007/s004240000371

[35] Wondergem R, Gong W, Monen SH et al. Blocking swelling-activated chloride current inhibits mouse liver cell proliferation. J Physiol (Lond) 2001; 532: 661–672. doi:10.1111/j.1469-7793.2001.0661e.x

[36] Wang GL, Wang XR, Lin MJ et al. Deficiency in ClC-3 chloride channels prevents rat aortic smooth muscle cell proliferation. Circ Res 2002; 91: E28–E32. doi:10.1161/01.RES.0000042062.69653.E4

[37] Meng RS, Guy D, Jan E et al. Differential expression of volume-regulated anion channels during cell cycle progression of human cervical cancer cells. J Physiol 2000; 529: 385–394. doi:10.1111/j.1469-7793.2000.00385.x

[38] Gauthier NC, Ricci V, Gounon P et al. Glycosylphosphatidylinositol-anchored proteins and actin cytoskeleton modulate chloride transport by channels formed by the Helicobacter pylori vacuolating cytotoxin VacA in HeLa cells. J Biol Chem 2004; 279: 9481–9489. doi:10.1074/jbc.M312040200

[39] Shen MR, Droogmans G, Eggermont J et al. Differential expression of volume-regulated anion channels during cell cycle progression of human cervical cancer cells. J Physiol (Lond) 2000; 529: 385–394. doi:10.1111/j.1469-7793.2000.00385.x

[40] Fearon ER, Vogelstein BA. genetic model for colorectal tumorigenesis. Cell 1990; 61: 759–767. doi:10.1016/0092-8674(90)90186-I

[41] Nishimoto S, Nishida E. MAPK signalling: ERK5 versus ERK1/2. EMBO Rep 2006; 7: 782–786. doi:10.1038/sj.embor.7400755

[42] Marshall CJ. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 1995; 80: 179–185. doi:10.1016/0092-8674(95)90401-8

[43] Valérie C, William P, Melanie E M et al. Mitogen-Activated Protein and Tyrosine Kinases in the Activation of Astrocyte Volume-Activated Chloride Current. J Neurosci 1998; 18: 1196–1206.

[44] Mamta G, Yue S, Twanda L Flow-activated Chloride Channels in Vascular Endothelium shear stress sensitivity, desensitization dynamics, and physiological implications. J Biol Chem 2006; 281: 36492–36500. doi:10.1074/jbc.M605866200

[45] d'Anglemont de Tassigny A, Berdeaux A, Souktani R et al.The volume-sensitive chloride channel inhibitors prevent both contractile dysfunction and apoptosis induced by doxorubicin through PI3kinase, Akt and Erk 1/2. Eur J Heart Fail 2008; 10: 39–46.