Activation of volume-sensitive Cl⁻ channel is involved in carboplatin-induced apoptosis in human lung adenocarcinoma cells

Wei He,¹ Hui Li,¹,* Xianjun Min, Jie Liu,² Bin Hu,¹ Shengcai Hou¹ and Jun Wang²,*

¹Department of Thoracic Surgery; Beijing Chao-Yang Hospital; Beijing, China; ²Department of Physiology; Capital Medical University; Beijing, China

Key words: volume-sensitive Cl⁻ channel, apoptosis, apoptotic volume decrease, carboplatin, staurosporine, human lung adenocarcinoma cells

The purpose of the present study is to observe the role of volume-sensitive Cl⁻ channels in carboplatin-induced apoptosis in the human lung adenocarcinoma cell line A549 cells. Using patch clamp and apoptosis assays, we found that A549 cells underwent the process of apoptotic volume decrease (AVD) and apoptosis when treated with carboplatin or staurosporine (STS). This AVD and apoptosis process were blocked by chloride channel blockers, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) and 5-nitro-2-(3-phenyl propylamino)-benzoate (NPPB). Both carboplatin and STS treatment activated a Cl⁻ current, which shows similar properties to hypotonicity-induced volume-sensitive Cl⁻ current in A549 cells. In addition, carboplatin pretreatment augmented the magnitude of the hypoosmotic-induced volume-sensitive Cl⁻ current. These results suggest that volume-sensitive Cl⁻ channels may be responsible for the carboplatin-induced apoptosis in A549 cells by inducing the AVD process.

Introduction

It has been reported that a major hallmark of cell apoptosis is normotonic cell shrinkage, known as apoptotic volume decrease (AVD), which is an early prerequisite for apoptotic events leading to cell death.¹⁻⁴ AVD is an upstream event to known biochemical apoptotic events such as cytochrome c release, caspase-3 activation and DNA laddering. Following prevention of AVD in a variety of cell types, subsequent apoptotic biochemical and morphological events are also prevented and cells are rescued from death.¹ Efflux of K⁺ and Cl⁻ from the cell through K⁺ and Cl⁻ channels is the major pathway responsible for AVD. Both mitochondrion-mediated intrinsic, and death receptor-mediated extrinsic apoptotic stimuli have been reported to rapidly activate volume-sensitive, outward rectifying Cl⁻ conductance (VSOR) in a wide variety of cell types,¹,⁵⁻⁸ which is crucial to AVD happening.

The platinum-based drug carboplatin has been widely used as an anticancer drug which acts mainly by forming adducts with DNA that cause the induction of apoptosis. But the exact mechanisms by which carboplatin induces cancer cell apoptosis are as yet not completely understood. Thus, the present study was designed to explore whether the VSOR Cl⁻ channel is involved in carboplatin-induced apoptosis in A549 cells. The results show that carboplatin pretreatment induces AVD, apoptotic cell death and augmentation of Cl⁻ channel activity. Treatment with VSOR Cl⁻ channel blockers inhibited carboplatin-induced AVD and cell apoptosis.

Results

Carboplatin treatment induces AVD which is sensitive to Cl⁻ channel blocker in A549 cells. Treatment of A549 cells with carboplatin (15 µM) or STS (4 µM) for 8 h resulted in an obvious reduction in mean cell volume (Fig. 1). Cell volume decrease started as early as 1 h after application of carboplatin or STS. This normotonic cell shrinkage caused by apoptotic inducers is known as apoptotic volume decrease (AVD).

Previous studies have suggested the involvement of the VSOR Cl⁻ channel in the AVD process in human epithelial HeLa, lymphoid U937, rodent neuronal PC12 and NG108-15 cells.¹⁹ As shown in Fig. 1, Carboplatin and STS treatment induced AVD process was almost completely abolished by volume sensitive Cl⁻ channel blockers, NPPB (100 µM) and DIDS (100 µM). These results suggest that the volume sensitive Cl⁻ channel is involved in the AVD process in A549 cells.

Carboplatin treatment induces A549 cell apoptosis which is inhibited by Cl⁻ channel blockers. Treatment of A549 cells with carboplatin (15 µM) for 24 h, 36 h and 48 h, resulted in a profound reduction in cell number (Fig. 2) and increase in the percentage of apoptotic cells (Fig. 3), but these effects were inhibited by simultaneous application of DIDS (100 µM). Flow cytometry measurement results further confirmed that carboplatin-induced increase in apoptosis rate was blocked by DIDS and NPPB (Fig. 4).

Apoptotic stimuli activate Cl⁻ currents which have similar properties to volume-sensitive Cl⁻ currents in A549 cells.
Previous patch-clamp studies have shown activation of anion currents in a wide variety of cell types in response to various apoptotic stimuli. Most anion currents studied in relation to apoptotic cell death resemble VSOR Cl- channel currents, which exhibit outward rectification, time dependent inactivation kinetics at large positive potential and are sensitive to NPPB and DIDS.

In order to compare the properties of apoptosis stimuli induced Cl- currents with volume-sensitive Cl currents in A549 cells, whole cell patch clamp recording was employed and a hypotonic challenge was used to induce the volume-sensitive Cl- currents. As shown in Fig. 5A, under isotonic conditions, no obvious current was observed in A549 cells. However, hypotonic solution reversibly induced increases in cell membrane currents. The hypotonicity-induced currents exhibited moderate outward rectification and time dependent inactivation kinetics at large positive potential. The pharmacological properties of volume-sensitive currents recorded in A549 cells were also almost identical to those of VSOR Cl- channels. Addition of 100 µM NPPB in the bath solution inhibited swelling-activated currents at both outward (71.37% ± 3.811%) and inward directions (62.01% ± 5.949%), respectively (p < 0.05). DIDS (100 µM) also suppressed the currents to about (67.71% ± 6.51%) at +100 mV and (46.08 ± 15.3%) at -100 mV. To confirm that this current is Cl selective, we observed reversal potentials by changing the Cl- concentration in bath solution (extracellullar side) from 110 mM to 50 mM. The reversal potential was shifted from -4.02 ± 4.07 mV to 12.48 ± 4.07 mV. These reversal potentials are close to the equilibrium potentials for Cl- (0 and 20.55 mV at 110 mM to 50 mM respectively). Perfusion of A549 cells with carboplatin and STS in isotonic solution, both induced VSOR-like currents, which also exhibited outward rectification, time dependent inactivation kinetics at large positive potential and sensitivity to DIDS or NPPB (Figs. 6 and 7). These results suggest that the properties of apoptosis stimuli-induced Cl- currents are similar to those of VSOR Cl- channel currents.

Carboplatin pretreatment increases hypooosmotic-induced VSOR channel activity in A549 cells. Following pretreatment of A549 cells with carboplatin (15 µM) for 12 h, VSOR Cl- currents were recorded under hypotonic conditions after washout of carboplatin. As summarized in Fig. 8, compared with control cells, the VSOR Cl- channel current density was profoundly greater in carboplatin-pretreated A549 cells.

Discussion

This study demonstrates for the first time that VSOR Cl- channel activity is associated with carboplatin-induced apoptotic cell death in A549 cells. The evidence comprises: (a) Carboplatin treatment of A549 cells induced AVD and apoptotic cell death, which was sensitive to Cl- channel blockers. (b) Apoptotic stimulation activated VSOR Cl- currents. (c) Carboplatin pretreatment increased VSOR Cl- channel activity.

The results of these experiments further expound the close relationship between VSOR Cl- channel activity and apoptosis, which are linked by apoptotic volume decrease. AVD, as an early prerequisite to apoptotic events leading to cell death, is induced by activation of K+ and Cl- channels, which are also involved in physiological regulatory volume decrease (RVD). VSOR Cl- channel activity has been shown to be essential for AVD in mouse cardiomyocytes, NG108-15 cells and epithelial Hela cells. Here we demonstrate that VSOR Cl- channel activity is also responsible for the AVD process in A549 cells.

Previous patch-clamp studies have shown activation of anion currents in a wide variety of cell types in response to different apoptotic stimuli, including the bacterial alkaloid staurosporine, the death receptor ligand TNFα, Fas ligand, lipopolysaccharide, Pseudomonas aeruginosa and H2O2. Here the results demonstrate that in A549 cells apoptotic inducers also activate a VSOR-like current, and that carboplatin pretreatment increases this channel activity.

Recently, VSOR Cl- channel blockers were found to inhibit apoptotic cell death in cancer cells treated with the anticancer drug cisplatin, in cardiomyocytes subjected to ischemia-reperfusion, and in hippocampal neurons subjected to ischemia-reperfusion in vivo. In the present study, the apoptotic events examined in A549 cells were found to be prevented by VSOR Cl- channel blockers NPPB or DIDS. Thus, the results suggest that in A549 cells, VSOR Cl- channel activity may be a prerequisite for carboplatin-induced apoptosis to occur. This would explain why carboplatin-treated A549 cells showed increased VSOR Cl- currents, and why A549 cells with impaired VSOR Cl- channel activity did not undergo AVD and apoptosis following carboplatin treatment. It is likely that the augmentation of VSOR Cl- channel activity is not specific to carboplatin-induced apoptosis in A549 cells, as activity of this channel has been observed in other types of apoptotic cells.

Reduced sensitivity of cancer cells to drug treatment is a major problem in cancer therapy and has been the subject of intense study for a long time. Elucidation of drug-resistance mechanisms will lead to the development of more effective treatments for cancer. Recent research indicated that impaired activity of
measurements, cells were detached from the plastic substrate and suspended, as described previously. 9 Cell suspensions (0.5 ml) were placed in a chamber and, after attachment to the glass bottom, perfused with bath solution at a flow rate of approximately 2

the VSOR Cl⁻ channel is involved in the cisplatin resistance of KCP-4 cancer cells.8 Similarly, the results of present study also suggest that VSOR Cl⁻ channel activity is very important for the carboplatin sensitivity of A549 cells. Impairement of VSOR Cl⁻ channel may reduce the effectiveness of carboplatin treatment. Therefore, it is reasonable to expect that a large increase in the functional expression of VSOR Cl⁻ channels would lead to a corresponding increase in carboplatin-induced apoptosis. These results imply the importance of further studies on the effect of Cl⁻ channel and its activation mechanisms in cancer therapy.

**Materials and Methods**

**Cell culture.** Human lung adenocarcinoma cell line, A549 cells (Peking Union Medical College, Beijing, China) was cultured in monolayer in RPMI medium 1640 (Gibco, Gran Island, NY, USA) supplemented with 10% newborn bovine serum (Hyclone Laboratories, Inc., Logan, UT, USA.) For patch clamp and volume measurements, cells were detached from the plastic substrate and suspended, as described previously.9 Cell suspensions (0.5 ml) were placed in a chamber and, after attachment to the glass bottom, perfused with bath solution at a flow rate of approximately 2

**Figure 2.** Carboplatin-induced A549 cell number change and its sensitivity to Cl⁻ channel blocker. Cell number was determined by cell counting after pretreatment with CBP (15 µM) for 12 h, 24 h, 36 h and 48 h with or without DIDS (100 µM). Each symbol represents the mean ± SEM (vertical bar). *p < 0.05 CBP vs. control groups, #p < 0.05 CBP vs. CBP + DIDS groups (n = 8).

**Figure 3.** Carboplatin-induced apoptotic rate change and its sensitivity to Cl⁻ channel blocker. A549 cell apoptotic rate was assessed by DAPI staining after pretreatment with CBP (15 µM) for 24 h and 48 h with or without DIDS (100 µM). DIDS (100 µM) showed blockade effect on carboplatin-induced cell apoptotic rate increase. Each symbol represents the mean ± SEM (vertical bar). *p < 0.05 CBP vs. control groups, #p < 0.05 CBP vs. CBP + DIDS groups (n = 8).
an inverted microscope (Eclipse TE2000-U, Nikon, Japan) with a high-resolution electronic camera (Spot RF/SE, Diagnostic). Cell images were captured every 60 s throughout the experiment and analyzed with a medical digital image analysis system (HMIAS-2000, WuHan, China). The cells keep remaining spherical for the length of time that measurements, so the relative cell volume was calculated as follows and with each cell used as its own control:

\[
\frac{v(t)}{v_0} = \left(\frac{4/3 \pi r_t^3}{4/3 \pi r_c^3}\right)
\]

(t: apoptosis inducer or/and blocker group; c: control).

For cell volume measurement, isotonic (310 mosmol/kg-H2O) bathing solution contained (in mM) 90 NaCl, 4.5 KCl, 0.5 MgCl2, 2 CaCl2, 110 mannitol, 10 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) (pH 7.4).

Patch clamp recordings. Volume-sensitive Cl- currents were recorded using the whole cell patch clamp technique at room temperature.15 Patch pipettes were pulled from borosilicate thin-wall glass capillaries using a micropipette puller (PC-10: Sutter instruments, Novato, CA) and had a resistance of 3–5 MΩ when filled with electrode solution. Data were acquired using an EPC-10 amplifier and Pulse software (HEKA Electroniks, Lambrecht, Germany). Current signals were low-pass filtered at 2.9 kHz using a four-pole Bessel filter. Sampled data were analyzed using an original software application called Pulsefit and Origin 6.1 (Origin Lab, Northampton, MA). In all experiments, a grounded Ag-AgCl pellet electrode was placed in the perfusion solution. In whole cell recordings, series resistance (<10 MΩ) was compensated 70%. In order to monitor the voltage dependence of the current, step pulses were applied from a pre-potential of -100 mV to test potentials of -100 to +100 mV in 20 mV increments.

To eliminate K+ currents, Cs-rich solution was employed. Pipette solution consisted of (in mM): 110 CsCl, 2 Na2ATP, 10 HEPES, 2 MgSO4, 1 EGTA and 50 mannitol (295 mosmol (kg-H2O)-1, pH 7.3). Isotonic (330 mosmol (kg-H2O)-1) or hypotonic (250 mosmol (kg-H2O)-1) bathing solution contained (in mM) 110 CsCl, 10 HEPES, 2 MgSO4 and 80 or 0 mannitol (pH 7.4). To prevent spontaneous cell swelling after attaining whole cell mode,16 the osmolality of the pipette solution was set lower than that of the isotonic bathing solution.

Apoptosis rate assays. A549 cells were seeded at a density of 1 x 10^5 cells/well in six-well plates exposed to medium containing carboplatin (15 µM), with
or without DIDS (100 μM) or NPPB (100 μM) for different time periods. The MEBSTAIN Apoptosis TUNEL Kit Direct (MBL, Nagoya, Japan) was used and analysis was performed using a FACScan flow cytometer (Beckman Coulter, CA, USA). Four parallel samples were measured and more than 1 x 10^5 cells were tested in each sample.

**Cell counting and DAPI staining assay.** To determine whether carboplatin treatment induced A549 cell apoptosis is inhibited by Cl⁻ channel blockers, A549 cells were seeded in 96-well plates at a density of 5 x 10^3 cells/well and exposed to medium containing carboplatin (15 μM), with or without DIDS (100 μM) for different time periods. Cell number was directed counted with a hemocytometer using 0.45% trypan blue (Sigma-Aldrich, St. Louis, MO, USA). The apoptotic cell number was determined by DAPI staining assay as described previously. In brief, the morphology of the cells’ nuclei is observed using a fluorescence microscope at excitation wavelength 350 nm. Nuclei are considered to have the normal phenotype when glowing bright
and homogenously. Apoptotic nuclei can be identified by the condensed chromatin gathering at the periphery of the nuclear membrane or a total fragmented morphology of nuclear bodies.

**Drugs and reagents.** Carboplatin and staurosporine, as well as Cl- channel blockers (DIDS and NPPB), were obtained from Sigma-Aldrich (Shanghai, China). Stock solutions of DIDS, NPPB and STS were prepared in dimethyl sulfoxide (DMSO). The concentration of DMSO in the experimental solutions was <0.1%.

**Statistical analysis.** Data are presented as means ± SEM of n observations. Statistical differences in data were evaluated by student’s paired or unpaired t-test and considered significant at p < 0.05.

**Acknowledgements**

This study was supported by the Natural Science Foundation of China (No.30670765), Natural Science Foundation of Beijing (7072010) and the Scientific Research Common Program of Beijing Municipal Commission of Education (No. KM200710025019).

**References**


