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Involvement of K^+–Cl^--cotransport in the apoptosis induced by *N*-ethylmaleimide in HepG2 human hepatoblastoma cells

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Abstract

The role of K^+–Cl^--cotransport in apoptosis in human cancer cells was investigated. *N*-Ethylmaleimide, a K^+–Cl^--cotransport activator, induced apoptosis in a dose-dependent manner in HepG2 human hepatoblastoma cells. *N*-Ethylmaleimide induced Cl^--dependent K^+ efflux, indicating that K^+–Cl^--cotransport is functionally present in HepG2 cells. Calyculin-A and genistein, inhibitors of K^+–Cl^--cotransport, significantly prevented both K^+–Cl^--cotransport activation and apoptosis induced by *N*-ethylmaleimide. These results demonstrate, for the first time, a novel role for K^+–Cl^--cotransport in apoptosis in human hepatoma cells. These results further suggest that K^+–Cl^--cotransport may be a valuable target for therapeutic interventions for human hepatoma.

Keywords: K^+–Cl^--cotransport; *N*-ethylmaleimide; Apoptosis; HepG2 cell

1. Introduction

Apoptosis, a naturally occurring cell death process, is characterized by ultrastructural modifications (cytoskeletal disruption, cell shrinkage, and membrane blebbing), nuclear alterations (chromatin condensation and internucleosomal DNA cleavage), and biochemical changes (activation of proteases) (Kidd, 1998). Recently, K^+ ions have been shown to play an important role in the regulation of apoptosis. Apoptotic cells appear to have a much lower intracellular K^+ concentration ($[K^+]_i$) than normal cells (Barbiero et al., 1995; Hughes et al., 1997). A bacterial pore-forming cytolysin, staphylococcal toxin that selectively permeabilizes plasma membranes for K^+ ions, has been found to induce apoptosis (Bhakdi et al., 1989). Apoptosis of thymocytes is suppressed by an increase in extracellular K^+ concentration in a dose-dependent manner (Hughes et al., 1997). However, the exact pathways responsible for changes in $[K^+]_i$ associated with apoptosis are not completely understood. Thus, the purpose of the present study was to investigate the role of K^+–Cl^--cotransport as a major K^+ transport pathway that mediates apoptosis using HepG2 human hepatoblastoma cells as a model cell system.

2. Materials and methods

2.1. Cell culture

The HepG2 human hepatoblastoma cell line was purchased from American Type Culture Collection (Rockville, MA). HepG2 cells were grown at 37°C in a humidified incubator under 5% CO_2/95% air in an Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 200 IU/ml penicillin, 200 μg/ml of streptomycin and 1 mM sodium pyruvate. Culture medium was replaced every other day. After confluence, the cells were subcultured following trypsinization.

2.2. Flow cytometric analysis of apoptosis

For flow cytometric analysis, HepG2 cells were collected and washed twice with phosphate-buffered saline

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(PBS) buffer (pH 7.4). After fixation in 80% ethanol for 30 min, cells were washed twice and resuspended in PBS buffer (pH 7.4) containing 0.1% Triton X-100, 5 μg/ml propidium iodide and 50 μg/ml ribonuclease A for DNA staining. Cells were then analyzed with a FACScan (BIO-RAD, Hercules, CA, USA). At least 20,000 events were evaluated. All histograms were analyzed using WinBryte software (BIO-RAD, Hercules, CA, USA) to determine the percentage of nuclei with a hypodiploid content indicative of apoptosis (Bombeli et al., 1997).

The normal lipid organization of the plasma membrane is altered soon after apoptosis is initiated. Thus, annexin V-binding was also used as an indicator of apoptosis to demonstrate the loss of phospholipid asymmetry and the presence of phosphatidylserine on the outer layer of the plasma membrane (Vermes et al., 1995). It was analyzed using a commercial kit (Boehringer Mannheim Biochemicals, Mannheim, Germany). Cells were washed in cold PBS and resuspended in binding buffer. A portion of the cell suspension (500 μl) was exposed to fluorescein isothiocyanate (FITC)-conjugated annexin V. The cells were gently vortexed, incubated at room temperature for 20 min in the dark, and then analyzed by FACScan within 1 h of staining.

2.3. Measurement of $[K^+]_i$

Intracellular K^+ levels were monitored with the K^+-sensitive fluorescent dye, K^+-binding benzofuran isophthalate aceoxylmethyl ester (PBFI/AM) (Minta and Tsien, 1989). Cells were washed and resuspended at a density of 4×10^5 cells/ml in Krebs–Ringer buffer. The cells were loaded with 5 μM PBFI/AM in Krebs–Ringer buffer containing 0.02% pluronic F-127, a nonionic surfactant, for 2 h at 37°C. Unloaded dye was removed by centrifugation at $150 \times g$ for 3 min. The dual-wavelength excitation method for measurement of PBFI fluorescence was used. Fluorescence was monitored at 500 nm with excitation wavelengths of 340 and 380 nm in a stirred quartz cuvette. In the results, relative changes in $[K^+]_i$ are reported as the 340:380 fluorescence ratios.

2.4. Materials

The powders for MEM, trypsin solution, sodium pyruvate, calyculin-A, genistein, *N*-ethylmaleimide and all salt powders were obtained from Sigma (St. Louis, MO). PBFI/AM was from Molecular Probes (Eugene, OR). Fetal bovine serum and antibiotics (penicillin and strepto-

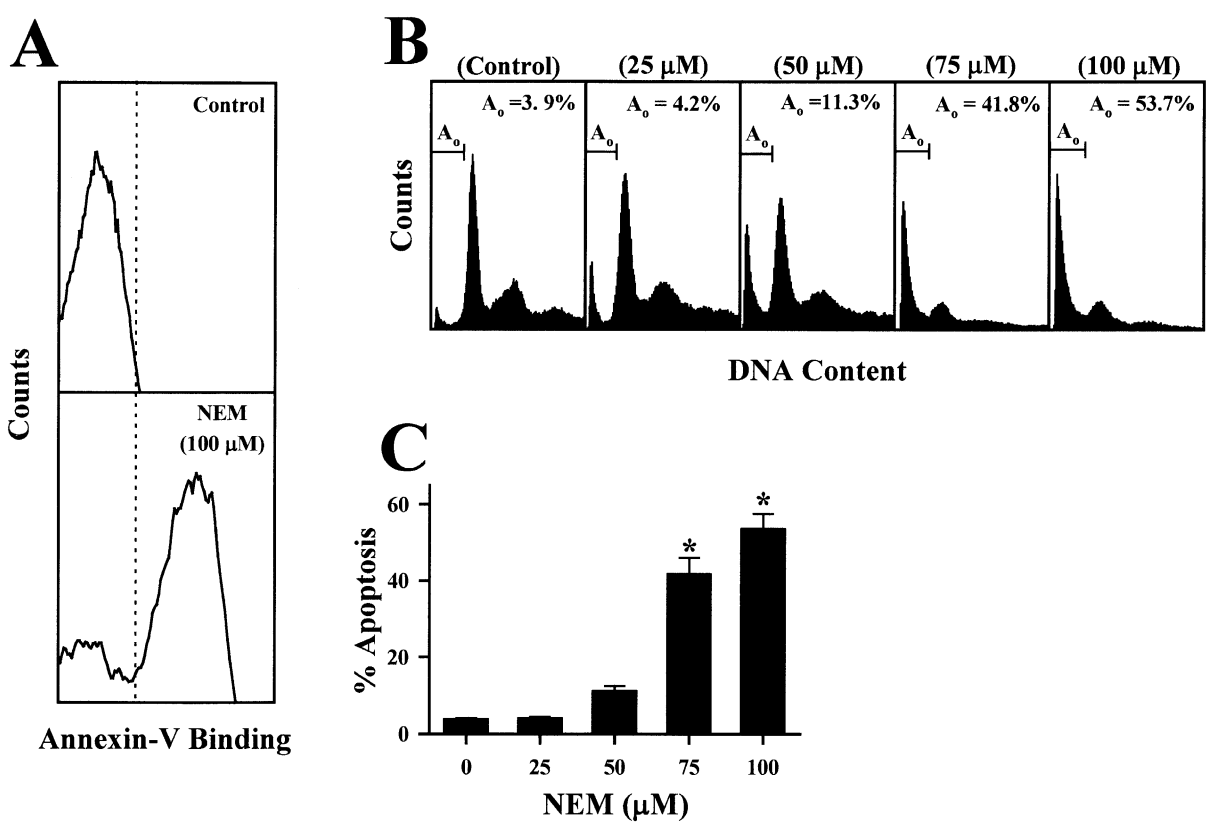

Fig. 1. *N*-Ethylmaleimide induces apoptotic cell death in HepG2 human hepatoblastoma cells. In the experiments in (A), after cells were treated with or without *N*-ethylmaleimide (NEM; 100 μM) for 4 h, the cells were stained with FITC-conjugated annexin V and analyzed by flow cytometry. In the experiments in (B), the cells were incubated with *N*-ethylmaleimide for 4 h at each designated concentration. The number of apoptotic cells was measured by flow cytometry. The region to the left of the G_o/G_1 peak, designated A_o, was defined as cells undergoing apoptosis-associated DNA degradation. In bar graphs (C), the data represent the mean values of four replicates with bars indicating S.E.M. $^*P < 0.05$ compared to control.

mycin mixture) were purchased from GIBCO (Grand Island, NY). The stock solutions of drugs were sterilized by filtration through 0.2-μm disc filters (Gelman Sciences: Ann Arbor, MI).

2.5. Data analysis

All experiments were performed four times. Data are expressed as means ± standard error of the mean (S.E.M.) and were analyzed using a one-way analysis of variance (ANOVA) and Student–Newman–Keul's test for individual comparisons. *P* values less than 0.05 are considered statistically significant.

3. Results

3.1. Induction of apoptotic cell death by N-ethylmaleimide

The effect of *N*-ethylmaleimide on apoptosis of HepG2 cells was examined using two independent flow cytometric analyses. *N*-Ethylmaleimide induced a loss of phospholipid asymmetry, resulting in the appearance of phosphatidylserine on the outer layer of the plasma membrane detected by annexin V-binding, as depicted in Fig. 1(A). In addition, *N*-ethylmaleimide also induced DNA fragmentation in a concentration-dependent manner, as assessed by determining the hypodiploid DNA content stained with propidium iodide (Fig. 1(B)). The lowest concentration of *N*-ethylmaleimide inducing significant apoptosis was 75 μM. Taken together, these results indicate that *N*-ethylmaleimide induced apoptotic cell death in HepG2 cells.

3.2. Activation of K^+–Cl^--cotransport by N-ethylmaleimide in HepG2 cells

To examine whether K^+–Cl^--cotransport is functionally present in HepG2 cells, we measured changes in $[K^+]_i$ using the PBFI/AM fluorescent probe. As shown in Fig. 2(A) and (B), *N*-ethylmaleimide (100 μM) markedly de-

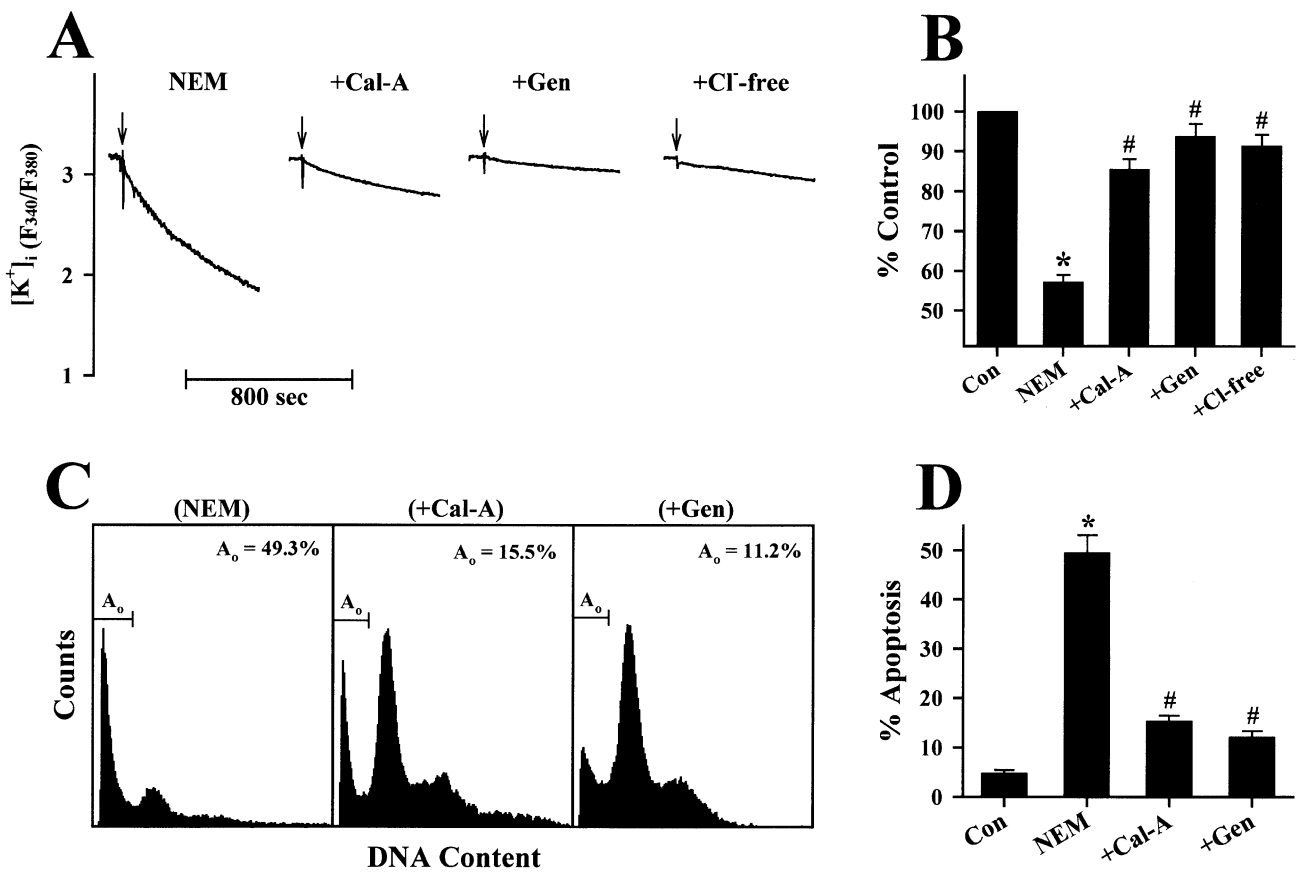

Fig. 2. K^+–Cl^--cotransport mediates the *N*-ethylmaleimide-induced K^+ efflux and apoptosis in HepG2 human hepatoblastoma cells. The data (A) show changes in intracellular K^+ concentration as a function of time, measured by using the K^+-sensitive fluorescent dye PBFI/AM. PBFI fluorescence ratios are directly proportional to the intracellular K^+ level. In all figures, the arrows show the time points for addition of *N*-ethylmaleimide (NEM; 100 μM). Calyculin-A (Cal-A; 25 nM) and genistein (Gen; 200 μM), KCC inhibitors, were added 10 min before *N*-ethylmaleimide treatment. For extracellular Cl^--free buffer solution (Cl^--free), external Cl^- was replaced with gluconate. Quantitative changes (B) are expressed as percent changes of the PBFI fluorescence ratio induced by the designated condition compared to that of control, in which the cells were treated with a drug-free vehicle. Each column represents the mean value of four replicates with bars indicating S.E.M. $^*P < 0.05$ compared to control. $^\#P < 0.05$ compared to *N*-ethylmaleimide alone. In graphs (C) and (D), data presentation is the same as in Fig. 1(B) and (C), respectively. In these experiments, calyculin-A (Cal-A; 25 nM) and genistein (Gen; 200 μM) were used. These drugs were given 30 min before *N*-ethylmaleimide (NEM; 100 μM) application. $^*P < 0.05$ compared to control. $^\#P < 0.05$ compared to *N*-ethylmaleimide alone.

creased $[K^+]_i$. To verify that this K^+ efflux is due to K^+–Cl^--cotransport activation, we examined whether the *N*-ethylmaleimide-induced decrease in $[K^+]_i$ is dependent on the Cl^- concentration, which is regarded as a functional hallmark of the presence of K^+–Cl^--cotransport (Lauf et al., 1992; Cossins and Gibson, 1997). With the Cl^--free buffer, the effect of *N*-ethylmaleimide was completely prevented, as illustrated in Fig. 2(A) and (B). These results support that the K^+–Cl^--cotransport is functionally present in HepG2 cells and that the K^+ efflux induced by *N*-ethylmaleimide is due to K^+–Cl^--cotransport activation.

3.3. Effects of K^+–Cl^--cotransport inhibitors on the N-ethylmaleimide-induced K^+–Cl^--cotransport activation and apoptosis

To confirm that *N*-ethylmaleimide-induced apoptosis is due to K^+–Cl^--cotransport activation, we investigated the effects of K^+–Cl^--cotransport inhibitors, calyculin-A (Kaji and Tsukitani, 1991) and genistein (Weaver and Cossins, 1996), on the K^+ efflux and apoptosis induced by *N*-ethylmaleimide. These structurally different K^+–Cl^--cotransport inhibitors significantly prevented the K^+ efflux (Fig. 2(A) and B) and apoptosis induced by *N*-ethylmaleimide (100 μM) (Fig. 2(C) and (D)). These results strongly suggest that *N*-ethylmaleimide-induced apoptosis may be due to activation of K^+–Cl^--cotransport in HepG2 cells.

4. Discussion

K^+–Cl^--cotransport has been reported to have many physiological and pathophysiological functions. K^+–Cl^--cotransport appears to contribute to cell shrinkage following swelling, and has therefore been implicated in the regulation of volume homeostasis (Lauf et al., 1992; Cossins and Gibson, 1997). Additionally, K^+–Cl^--cotransport is involved in transepithelial salt absorption (Amlal et al., 1994), myocardial K^+ loss during ischemia (Yan et al., 1996), regulation of neuronal Cl^- concentration (Rivera et al., 1999), and renal K^+ secretion (Ellison et al., 1985). Interestingly, a recent report has shown that human cervical carcinogenesis is accompanied by up-regulation of K^+–Cl^--cotransport transcripts (Shen et al., 2000). In red blood cells, inappropriate activation of K^+–Cl^--cotransport leads to excessive KCl loss, cell shrinkage and elevation of hemoglobin concentration, leading to deleterious rheological effects, including increased vascular resistance (Stuart and Ellory, 1988). However, the role of K^+–Cl^--cotransport in apoptosis has not been reported yet.

The results of the present study clearly show, for the first time, that K^+–Cl^--cotransport is functionally present and involved in the regulation of apoptosis in HepG2 human hepatoma cells. These conclusions are based on (i) *N*-ethylmaleimide, an alkylating agent that has long been known to cause strong activation of K^+–Cl^--cotransport activity in red blood cells (Lauf et al., 1992), induced Cl^--dependent K^+ efflux, a functional hallmark of the presence of K^+–Cl^--cotransport (Lauf et al., 1992; Cossins and Gibson, 1997), as shown in Fig. 2(A) and (B); (ii) *N*-ethylmaleimide induced apoptosis in a dose-dependent manner, as evaluated by two independent methods, detection of phosphatidylserine translocation to the outer layer of the plasma membrane by annexin V-binding (Fig. 1(A)) and measurement of the hypodiploid DNA content by propidium iodide staining (Fig. 1(B)); and (iii) K^+–Cl^--cotransport inhibitors (calyculin-A and genistein) significantly prevented both K^+–Cl^--cotransport activation and apoptosis induced by *N*-ethylmaleimide (Fig. 2(A) and (B)).

In this study, we did not determine the downstream mechanism linking K^+ efflux to the final induction of apoptosis, and it can only be speculated upon. Since accumulating evidence implies that the loss of cell volume is a fundamental feature of apoptosis (Bortner and Cidlowski, 1998), cell shrinkage induced by K^+ efflux is probably involved in the *N*-ethylmaleimide-induced apoptosis observed in this study. Considering the results of previous studies, activation of caspases may be a plausible target linking the loss of intracellular K^+ to apoptosis. In human monocytes, pro-interleukin-1β is processed by the interleukin-converting enzyme (also known as caspase 1) via intracellular K^+ depletion (Walev et al., 1995). Caspase 3-like activation during apoptosis is dependent on the loss of intracellular K^+ (Hughes et al., 1997).

In conclusion, *N*-ethylmaleimide induced apoptosis in HepG2 human hepatoblastoma cells, and the mechanism of action of *N*-ethylmaleimide includes intracellular K^+ loss through the activation of K^+–Cl^--cotransport. These results further suggest that K^+–Cl^--cotransport may be a valuable target for the therapeutic interventions for human hepatoma.

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