Apoptosis induced by hypertonicity in Madin Darby canine kidney cells: protective effect of betaine

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Abstract

Background. In mammals, the renal medulla is in a hypertonic environment related to the renal concentrating mechanism. Renal cells accumulate osmolytes such as betaine to protect cells from the perturbing effect of high concentration of electrolytes. Hypertonicity-induced cell death and the effect of betaine were investigated in Madin Darby canine kidney (MDCK) cells.

Methods. Cell viability was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. DNA fragmentation was determined by FACS analysis, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) staining and agarose gel electrophoresis. Activities of caspase-1, -3, -8, and -9 were measured.

Result. When the cells were exposed to 700 mOsm medium for 24 h, 40% of the cells were detached. TUNEL staining showed that about 20% of detached cells were apoptotic, indicating that both necrosis and apoptosis contributed to the hypertonicity-induced cell death in MDCK cells. DNA laddering was demonstrated in hypertonic cells. Caspase-3, -8, and -9 activities of the adherent cells exposed to 700 mOsm for 24 h increased approximately 20-, 3-, and 4-fold the value of isotonic cells, respectively. However, there was no significant change in caspase-1 activity. Addition of 1 mM betaine into the medium protected the cells against the hypertonicity-induced cytotoxicity and apoptosis. Betaine prevented the induction of caspase-3, -8, and -9 activities after hypertonic exposure to about 50%.

Conclusions. The present study demonstrates that (i) apoptosis is involved in the hypertonicity-induced cell death in MDCK cells; (ii) caspase-3, -8, and -9 may contribute to the apoptosis; and (iii) betaine has protective effect on the hypertonicity-induced apoptosis.

Keywords: apoptosis; betaine; hypertonicity; MDCK cell

Introduction

Necrosis and apoptosis are two types of cell death with different morphological and biochemical changes [1,2]. Apoptosis is an active process of programmed cell death and has been observed in developmental biology, in maintenance of the steady state in normal tissues, and in many types of cells after stimulation by a variety of chemical, pharmacological, and physical stresses [3,4]. Hypertonicity is one of the physiological stresses inducing cell damage. Cell death is the eventual endpoint of the injury. In mammals, the renal medulla undergoes broad shifts in osmolality related to the renal concentrating and diluting mechanism [5]. Therefore, the mechanisms of hypertonicity-induced cell death and apoptosis in the kidney, and kidney-derived cells have been of the interest. Hypertonicity-induced apoptosis in Madin Darby canine kidney (MDCK) cells have been reported. Hizōh et al. detected apoptosis by an increase in the percentage of hypodiploid nuclei [6]. Santos et al. reported hypertonicity-induced apoptosis, and that a combination of NaCl and urea enhances the survival rate to hyperosmolality [7]. They showed the detailed studies of the effects of hyperosmolality. However, little has been known about the participation of caspases, cysteine proteases that have been shown to play key roles in apoptosis in mammalian cells [8,9]. To address this issue, we investigated activities of the caspase family in MDCK cells. We demonstrated that apoptosis was involved in the hypertonicity-induced cell death.
death in MDCK cells, and caspase-3, -8, and -9 were activated in hypertonic cells.

Many kinds of cells respond to extracellular hypertonicity by the accumulation of high concentrations of small organic solutes, which are called ‘compatible osmolytes’, that protect cells from the perturbing effects of high intracellular concentrations of electrolytes [10]. Betaine is one of the major compatible osmolytes in the kidney medulla and MDCK cells [11,12]. Therefore, we investigated the effect of betaine on hypertonicity-induced apoptosis. Betaine prevented the inductions of caspase-3, -8, and -9 activities after hypertonic exposure, and protected the cells from the hypertonicity-induced apoptosis.

Methods

Cell culture

MDCK cells were donated by the Japanese Cancer Research Resources Bank (National Institute of Health, Tokyo, Japan) and grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal-calf serum (FCS), 50 U/ml of penicillin, and 50 µg/ml of streptomycin equilibrated with 5% CO₂ 95% air at 37°C. Cells were subcultured by trypsinization. When the cells were grown to subconfluence, the cells were rinsed twice with DMEM without FCS, and switched to isotonic or hypertonic medium without FCS. Hypertonic (400–700 mOsm/kg) medium was made by addition of NaCl. Betaine (Wako Chemical Co., Osaka, Japan) and other osmolytes were added simultaneously when the cells were exposed to hypertonic condition.

3-(4,5-dimethylthiazo-2-yl)-2,5-Diphenyl tetrazolium bromide assay

Cell viabilities were assessed by measuring 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cytotoxicity assay [13]. 10⁶ cells/well were seeded in a 96-well plates. After 24 h, the medium was replaced by a 100 ml hypertonic medium. After 24 or 48 h incubation, 20 ml of 5 mg/ml MTT was added to the medium and the cells were incubated at 37°C for 4 h. The MTT formazan reaction product was dissolved by 100 ml of 20% sodium dodecyl sulfate, 50% dimethylformamide, 2% acetic acid, and 2.5% 1 N HCl. The optical density at 570 nm was measured using 96-well multisclanner.

Agarose gel electrophoresis

DNA extraction and the detection of DNA fragmentation by agarose gel electrophoresis were performed by using the Apoptosis Ladder Detection Kit (Wako Chemical Co., Osaka, Japan). This contained a protein digestion enzyme, RNase A, and DNA extraction solution. Detached cells floating in the medium were centrifuged at 200 g for 10 min. Adherent cells were trypsinized, washed with phosphate-buffered saline (PBS), and pelleted. The detached cells and the adherent cells were processed together. The pellet was incubated in protein digestion enzyme and RNase A at 50°C for 30 min. After addition of DNA extraction solution, DNA was obtained by isopropanol and ethanol precipitation.

DNA (10 µg) was examined on a 2% agarose gel stained with SYBR Green I (Molecular Probes, Eugene, Oregon, USA).

TUNEL staining

Detection of DNA fragmentation using terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay [14] was performed by using a kit (Apoptosis in situ Detection Kit, Wako Chemical Co., Osaka, Japan). This kit employs 3’-OH end-extension of fragmented DNA using terminal deoxynucleotidyl transferase (TdT) and labelled the DNA fragmentation with fluorescent tagged deoxyuridine triphosphate nucleotides (FITC-dUTP). FITC-dUTP was detected by an anti-fluorescent antibody conjugated with peroxidase. Cells were seeded into Lab-Tek Permanox slide chambers (NUNC, Naperville, IL, USA) or 10 cm culture dishes and grown to subconfluence. Adherent cells in a chamber slide were rinsed with PBS and fixed with 1% paraformaldehyde for 10 min on ice. The cells were then incubated in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice. After rinsing with PBS, apoptotic cells were detected by in situ TUNEL assay and counterstained using methyl green. Detached cells cultured in 10 cm dish were collected and rinsed with PBS by centrifugation at 200 g for 10 min. The cells were fixed with 1% paraformaldehyde and dried on a slide glass. The cells were stained by TUNEL method as described above.

Flow cytometric analysis

TUNEL positive cells were also detected by using a flow cytometry kit for apoptosis (APO-DIRECT, Phoenix Flow Systems, San Diego, CA, USA); 10⁶ MDCK cells exposed to hypertonicity were trypsinized and suspended in PBS. The cells were fixed by 1% paraformaldehyde in PBS on ice for 15 min. DNA fragmentation was labelled with F-dUTP. The cells were also stained with propidium iodide (PI) to assess the DNA content of the cells. Analysis was performed on a flow cytometer (Becton-Dickinson) equipped with a 488 nm argon laser as the light source. The non-clumped cells were gated in the display with DNA area signal and DNA width signal (Becton-Dickinson). The second display was generated with PI (DNA) signal on the X-axis and the F-dUTP (apoptotic cells) signal on the Y-axis.

Activities of caspases

Caspase-1, -3, -8, and -9 activities were detected by using a fluorometric assay kit (Caspase-1 and -3: Promega, Madison, WI, USA; Caspase-8 and -9: MBL, Nagoya, Japan). Ac-YVAD-AMC, Ac-DEVD-AMC, Ac-IETD-AMC, and Ac-LEHD-AMC were used as substrates for caspase-1, -3, -8, and -9, respectively. Fluorochrome 7-amino-4-methyl coumarin (AMC) or 7-amino-4-trifluoromethyl coumarin (AF) is released from these substrates upon cleavage by caspases. Caspase-1 and -3 activities were measured in the presence and absence of selective inhibitors for either caspase-1 (Ac-YVAD-CHO) and -3 (Ac-DEVD-CHO). The differences between the substrate cleavage activity levels in the presence and absence of inhibitors were used as the enzyme activities. Inhibitors were not used for assays of caspase-8 and -9. The production of AMC upon cleavage was measured with fluorometer at an excitation of 360 nm and an emission of 460 nm. The production of AFC upon cleavage
was measured at an excitation of 400 nm and an emission of 505 nm.

Adherent MDCK cells were scraped and washed with PBS by centrifugation. The cells were resuspended in hypotonic cell lysis buffer (25 mM HEPES (pH 7.5), 5 mM MgCl₂, 5 mM EDTA, 5 mM DTT, 2 mM phenyl methyl sulfonyl fluoride, 10 μg/ml peptatin A, 10 μg/ml leupeptin) and were lysed by four cycles of freezing and thawing. The cells were centrifuged at 16 000 g for 20 min at 4°C and the supernatants were used as the sample for caspase assay. The pellets were dissolved in 0.5 N NaOH, and protein contents were assayed using the Bio-Rad protein assay.

**Data analysis**

The results shown are mean ± standard deviation (SD). When no error bars are shown in a figure, the SD is smaller than the symbol. The data were statistical analysed by the one-way ANOVA and Dunnett multiple comparison. Statistical significance was set at P < 0.05.

**Results**

MTT assay of MDCK cells exposed to 400, 500, 600, and 700 mOsm for 48 h, were 94 ± 5%, 23 ± 4%, 10 ± 3%, and 3 ± 1% of the value of isotonic cells, respectively (Figure 1). The value of MTT assay in the cells exposed to 400 mOsm at 48 h was higher than that at 24 h, indicating that the cells can proliferate in this condition. On the other hand, the values of MTT assay of the cells exposed to over 500 mOsm decreased in a time-dependent manner indicating the hypertonicity-induced cell death in these conditions. Addition of 1 mM betaine, myo-inositol, taurine, and neutral amino acids prevented the hypertonicity-induced cell damage evaluated by MTT assay (Figure 2). Acidic and basic amino acids had no protective effect on the hypertonicity-induced cell damage. Betaine had the most protective effect on the cell damage. Therefore, the effect of betaine on hypertonicity-induced cell death was investigated in the following experiments. The addition of 1 mM betaine significantly prevented the damage of cells exposed to 500 or 700 mOsm, while it had no significant effect on MTT assay in isotonic cells (Figure 3).

When MDCK cells were exposed to 700 mOsm medium, the percentage of detached cells increased with time after 8 h. The percentage of detached cells was 38.7 ± 16.6% in the cells exposed to 700 mOsm for

**Fig. 2.** Protective effect of osmolites on hypertonicity-induced cytotoxicity in Madin Darby canine kidney (MDCK) cells. Cells in 96-well dishes were exposed to isotonic or hypertonic (500 mOsm) medium for 48 h. Betaine (1 mM), myo-inositol, taurine, and amino acids were added simultaneously when the cells were exposed to hypertonic condition. Cell viabilities were assessed by 3-(4,5-dimethylthiazolo-2-yl)-2,5-diphenyl tetrazolium (MTT) assay. Data are shown as mean ± SD of isotonic cells (n = 8). a; P < 0.01 vs hypertonic (500 mOsm) cells. b; P < 0.05 vs hypertonic (500 mOsm) cells.

**Fig. 3.** Protective effect of betaine on hypertonicity-induced cytotoxicity in Madin Darby canine kidney (MDCK) cells. Cells in 96-well dishes were exposed to hypertonic medium for 48 h in the absence or presence of betaine. Betaine was added simultaneously when the cells were exposed to hypertonic condition. Cell viabilities were assessed by MTT assay. Data are shown in percentage as mean ± SD of isotonic cells grown without betaine (n = 8). a; P < 0.01 vs isotonic cells without betaine. b; P < 0.01 vs hypertonic (500 mOsm) cells without betaine. c; P < 0.01 vs hypertonic (700 mOsm) cells without betaine.
24 h. Addition of 1 mM betaine reduced the detachment of the cells about 50%. We observed extensive population of TUNEL-positive apoptotic cells in both detached and adherent MDCK cells exposed to 700 mOsm for 24 h (Figure 4B, C). Counting the number of TUNEL positive cells at high power fields showed that about 20% of detached cells was apoptotic (Figure 4B), indicating that hypertonicity-induced cell death in MDCK cells is through both necrosis and apoptosis. Apoptosis was also detected in adherent cells exposed to 700 mOsm for 24 h (Figure 4C). Betaine (1 mM) reduced the population of apoptotic cells (Figure 4D). The population of apoptotic cells was quantified by flow cytometry analysis. The percentages of TUNEL-positive cells in adherent cells exposed to 300, 500, 600, and 700 mOsm for 24 h were 0.98 ± 0.04%, 1.10 ± 1.76%, 1.98 ± 2.36%, and 12.11 ± 5.11%, respectively (Figure 5). The addition of 1 mM betaine significantly reduced the percentage of the TUNEL-positive cells.

Agarose gel electrophoresis demonstrated DNA laddering of about 200-bp multiple DNA fragments occurring in the cells exposed 600 or 700 mOsm medium for 24 h, which is one of the hallmarks of apoptosis (Figure 6). The protective effect of betaine on DNA fragmentation was not clear in the experiment.

Caspase-3 activity of the cells exposed to 700 mOsm for 24 h increased the value of isotonic cells by approximately 20-fold (Figure 7A). The caspase-3 activity increased osmolality in a dependent manner. Significant increase of the activity was evident after 16 h of hypertonicity (Figure 7B). Caspase-8 and -9 activities of the cells exposed 700 mOsm medium for
Fig. 7. Caspase-3 activity in Madin Darby canine kidney (MDCK) cells after hypertonic exposure. (A) MDCK cells were incubated with 300, 500, 600, and 700 mOsm medium for 24 h. Data are shown as mean ± SD (n = 3). a; P < 0.01 vs 300 mOsm. b; P < 0.05 vs 300 mOsm. (B) Time course of caspase-3 activities in MDCK cells after hypertonic exposure. Cells were incubated with 300 mOsm medium without betaine (□), 700 mOsm medium without betaine (●), or 700 mOsm medium with 1 mM betaine (□) for various periods of time (0-24 h). Data are shown as mean ± SD (n = 3). a; P < 0.01 vs 300 mOsm medium at same period. b; P < 0.01 vs 700 mOsm medium with betaine at same period.

24 h were significantly higher than the values of isotonic cells (Figure 8A). The time courses of the activation of caspase-8 and -9 were similar to that of caspase-3 (Figure 8B). Addition of 1 mM betaine prevented the induction of caspase-3, -8, and -9 activities after hypertonic exposure to about 50% (Figures 7B and 8A). There was no significant increase of caspase-1 activity in the cells exposed to 700 mOsm medium (Figure 9).

Discussion

We used DMEM medium without serum for the hypertonic exposure. Serum deprivation may increase the susceptibility of cells and be a factor in inducing apoptosis. Therefore, the cells exposed to the hypertonic medium were compared with control cells that were cultured with isotonic medium without serum. In our experiments, serum deprivation did not induce apoptosis within 48 h in MDCK cells. MTT assay showed the continuous proliferation of the control cells that were cultured with DMEM without serum for 48 h (Figure 1).

When MDCK cells were exposed to hypertonic medium, cell detachment occurred in time and osmolality-dependent manners. Approximately 40% of the cells were detached after 24 h of 700 mOsm hypertonicity. DNA laddering, chromatin condensation and TUNEL positive cells were observed, indicating that apoptosis was involved in the hypertonicity-induced cell death in MDCK cells. Approximately 20% of detached
cells were apoptotic, showing that both apoptosis and necrosis were involved in hypertonicity-induced cell death in MDCK cells.

Recent studies have documented that the caspase family plays an important role in apoptosis [8,9,15]. Caspases are constitutively present in most cells. Once apoptotic upstream caspases are activated in response to specific apoptotic stimuli, they activate the downstream caspases in an activation cascade. The caspase family can be divided into distinct groups based on substrate specificity. Caspases-1, -4, and -5 prefer the sequences WEHD and YVAD [16,17]. The optimal sequence for caspase-2, -3, and -7 is DEVD [16]. Caspases-6, -8, and -9 prefer the sequence (L/V)EXD [16]. The optimal sequences for caspase-8 and -9 are LETD and LEHD, respectively [16]. We used Ac-YVAD-AMC, Ac-DEVD-AMC, Ac-LETD-AFC, and Ac-LEHD-AFC as the substrates for caspase-1, -3, -8, and -9, respectively. These peptides are not totally specific for individual caspases. Therefore, we note that related caspases in the same group could interfere with the assay of individual caspases.

Two major pathways for induction of apoptosis have been identified. One pathway involves the participation of tumour necrosis factor family receptors characterized by death domain. The ligation of death receptors by their ligands activates procaspase-8. The other pathway for apoptosis involves the participation of mitochondria, which release cytochrome c that activates procaspase-9. Caspase-8 and -9 activate the downstream or effector class of caspases in an activation cascade. Caspase-3 has been thought as the downstream caspase, which is directly involved in the execution of apoptosis [15]. Caspase-3, -8, and -9 activities of the cells exposed to 700 mOsm for 24 h increased about 20-, 3-, and 4-fold the value of isotonic cells, respectively. The activities were increased significantly after 16 h of hypertonicity. Activations of caspase-8 and -9 suggested that both the mitochondria and death receptor pathways might involve in the hypertonicity-induced apoptosis in MDCK cells. There was no clear change of caspase-1 activity after hypertonic exposure, suggesting that caspase-1 may not play a required role in hypertonicity-induced apoptosis in MDCK cells. The knockout of caspase-3 and -9 had profound developmental effects [18]. Cells from these animals displayed resistance to a number of forms of apoptosis as well. In contrast, caspase-1 deficient mice have no readily demonstrable defect in apoptosis [19]. Pro-interleukin-1β is cleaved to mature IL-1β by caspase-1. Caspase-1 deficient mice have a profound defect in their capacity to produce mature IL-1β. Caspase-1 may be largely responsible for the proteolytic processing of proinflammatory cytokines, such as interleukin-1β, and may not have a necessary function in apoptosis [19].

Study about the protective effects of caspase inhibitors against apoptosis is very important. As a preliminary study we added an inhibitor of caspase-3 (Ac-DEVD-CHO), which was provided as a solution for the caspase assay kit (Promega), into the culture medium simultaneously as the cells were exposed to the hypertonic conditions. The protective effect of 10 μM of the inhibitor on the cell viability as assessed by MTT assay, was not obvious after 24 h of 700 mOsm (data not shown). Necrosis accounted for the majority of the hypertonicity-induced cell death in MDCK cells. This may be a partial explanation of why it is difficult to detect the protective effect of the caspase inhibitor on the cell survival rate. Further studies are required.

Many kinds of cells respond to extracellular hypertonicity by the accumulation of high concentrations of small organic solutes, which are called ‘compatible osmolytes’, that protect cells from the perturbing effects of high intracellular concentrations of electrolytes. Myo-inositol, betaine, taurine, and neutral amino acids are the major compatible osmolytes in the kidney medulla and MDCK cells [11,12,20]. MDCK cells accumulate myo-inositol, betaine, taurine, and neutral amino acids via the specific transporters when cultured in hypertonic medium [11,20,21]. Addition of 1 mM betaine, myo-inositol, taurine, and some neutral amino acids prevented hypertonicity-induced cell damage evaluated by MTT assay (Figure 2). DMEM medium contains originally myo-inositol and neutral amino acids such as glycine, glutamine, and threonine, but not betaine or taurine. This may explain why the addition of betaine had the most protective effect on the hypertonicity-induced cell damage evaluated by MTT assay. Betaine clearly improved cell survival rate during hypertonic exposure. It partially prevented the hypertonicity-induced apoptosis and the activation of caspase-3, -8, and -9. Betaine belongs to methylamine osmolytes, which have protein stabilizing effect on macromolecules [10]. It has a potent counteracting effect on urea perturbation of proteins [22]. The precise mechanism of the protective effect of betaine on hypertonicity-induced apoptosis is unknown, but the protein stabilizing effect on
macromolecules may contribute to the protection from hypertonicity-induced apoptosis.

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