Pro- and anti-apoptotic effects of K^+ in HeLa cells

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Abstract The present study determined the effects of osmotic stress induced by sorbitol and KCl on apoptosis. Sorbitol induced apoptosis, activated the mitogen-activated protein kinase (MAPK) family and stimulated accumulation of cytosolic cytochrome *c* and procaspase-3 cleavage. KCl (0.2 M) also activated the MAPKs and induced cytosolic cytochrome *c* accumulation but did not induce procaspase-3 cleavage or apoptosis and was protective against sorbitol-induced apoptosis. However, when cells were exposed to KCl for 1 h, washed and returned to isotonic medium, caspase-3 was rapidly cleaved and apoptosis induced. We conclude that hyperosmotic KCl initiates early events in apoptosis but blocks caspase-3 activation by preventing the loss of intracellular K^+.

Key words: Apoptosis; Osmotic stress; Caspase; K^+

1. Introduction

Recent reports have implicated intracellular K^+ as an important regulator of apoptosis in lymphocytes [1,2]. The studies found that apoptotic cells had a lower intracellular concentration of K^+, and that blocking K^+ efflux by incubating cells in a high K^+ medium inhibited apoptosis as determined by DNA fragmentation. A similar decrease in intracellular concentrations of both K^+ and Na^+ has been observed during apoptosis in HL-60 cells induced by cytotoxic drugs or UV irradiation [3]. In addition, physiological concentrations of K^+ inhibited the in vitro activation of caspase-3 and enzymes involved in DNA fragmentation. The effects on caspase activation are of particular interest. Although apoptosis can be induced by diverse signalling pathways, all appear to converge to activate the caspases, a family of aspartate-specific cysteine proteases [4,5]. The mechanisms which regulate caspase activation are therefore central to an understanding of the control of apoptosis. Recent reports have implicated cytochrome *c* and apoptotic protease activating factor-1 (Apaf-1), a probable mammalian homologue of the *Caenorhabditis elegans* death gene *ced-4*, as possible mediators of caspase activation. Cytochrome *c* is released from mitochondria during apoptosis of cells treated with a wide range of drugs including staurosporine, etoposide and actinomycin D [6–8]. In addition, cell-free systems have been developed which demonstrate a dATP- and cytochrome *c*-dependent activation of caspase-3 by cleavage of a 32 kDa precursor protein into 17 kDa and 12 kDa fragments [6–8]. One of the proteins required for cytochrome *c*-dependent activation of caspase-3 is Apaf-1 [9]. Apaf-1 physically interacts with cytochrome *c* [9], and the complex is probably involved in activating caspase-9 which in turn cleaves procaspase-3 to its active subunits [10,11]. In their study Hughes et al. [1] demonstrated that K^+ inhibited a dATP/cytochrome *c*-dependent activation of procaspase-3 in vitro using thymocyte extracts but did not inhibit the catalytic activity of already activated caspase-3. They concluded that the normally high intracellular K^+ levels (approx. 150 mM) would block caspase activation and that K^+ must be extruded from the cell before the final stages of apoptosis can occur.

We are studying the effects of osmotic shock on pathways potentially involved in apoptosis control. For example, osmotic stress has been shown to activate the stress-activated protein kinases c-Jun N-terminal kinases (JNKs) and p38 kinase as well as the extracellular signal-regulated kinases (ERKs: see [12–15]). Although the roles of these kinases may vary in different cell types [16–19], both JNK and p38 have been implicated in apoptosis triggered by a range of stress stimuli [17–21]. We therefore argued that osmotic shock induced by high extracellular K^+ concentrations may activate many of the pathways leading to cell death, but block the final stages involving caspase activation by preventing the loss of intracellular K^+.

In the present paper we show that sorbitol-induced osmotic stress induced apoptosis in HeLa cells, and that this was associated with activation of ERK, JNK and p38, the accumulation of cytoplasmic cytochrome *c* and the cleavage of procaspase-3. Osmotic stress induced by KCl also activated ERK, JNK and p38 and induced the accumulation of cytoplasmic cytochrome *c*, but did not induce apoptosis or the activation of caspase-3. However, brief exposure to high concentrations of K^+ followed by salt washout resulted in the rapid cleavage of procaspase-3 and to apoptotic cell death. We conclude that hypertonic conditions induced by KCl can 'prime' cells for apoptosis, but that the final stages of execution are blocked immediately upstream of the activation of caspase-3.

2. Materials and methods

2.1. Cell culture and stimulation

HeLa cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μl/ml streptomycin. Cells (80% confluence) were treated in 10% RPMI with 0.4 M sorbitol, 0.2 M KCl or 0.2 M NaCl.

2.2. Quantification of apoptosis

After treatment, cells were fixed in 1% formaldehyde in phosphate-buffered saline (PBS) and stained with 5 μl/ml Hoechst 33258 (Calbiochem) to visualise nuclear morphology. Apoptotic cells were identified as those with brightly stained, condensed nuclei.

2.3. DNA fragmentation

Chromosomal DNA was extracted to assess the extent of oligonu-

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Abbreviations: Apaf-1, apoptotic protease activating factor-1; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis

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cleosomal cleavage [22]. Briefly, cells (2×10^6) were fixed overnight in 70% ethanol in PBS. Cells were pelleted at $800\times g$ for 5 min, washed with PBS and resuspended in 40 μl phosphate-citrate buffer (192 parts 0.2 M Na_2HPO_4, 8 parts 0.1 M citric acid) at room temperature for 30 min. Cells were centrifuged at $1000\times g$ for 5 min and the supernatant transferred to a new tube, dried in a SpeedVac and reconstituted in 15 μl distilled water. DNA extract was incubated with 3 μl 0.25% NP-40 and 3 μl (1 mg/ml) RNase (Type III-A, Sigma) at 37°C for 30 min. The extract was incubated with 3 μl (1 mg/ml) proteinase K (Sigma) for a further 30 min. The samples were then analysed on a 2% agarose gel.

2.4. Preparation of cell extracts

For ERK, JNK or p38 analysis, cells (2×10^6) were washed with PBS and scraped into 200 μl buffer A (25 mM Tris, pH 7.4, 25 mM NaCl, 2 mM EGTA, 1 mM Na_3VO_4, 10 mM NaF, 1 mM dithiothreitol, 40 mM *p*-nitrophenyl phosphate, 0.2 mM phenylmethylsulfonyl fluoride) and sonicated (2×10 s bursts). Cell lysates were cleared by centrifugation at $100\,000\times g$ for 20 min at 4°C.

For cytochrome *c* and caspase-3 analysis, cells (4×10^6) were harvested and washed with ice cold PBS and resuspended in 200 μl extraction buffer (20 mM HEPES KOH, pH 7.5, 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride). Cells were lysed with 10 strokes of a Dounce homogeniser, the homogenate was centrifuged at $750\times g$ for 10 min at 4°C. The pellet was re-extracted and the pooled supernatants were centrifuged at $100\,000\times g$ for 20 min at 4°C [7].

2.5. Immunoblot analysis

Aliquots (50 μl of protein) were separated by SDS-PAGE and protein transferred to nitrocellulose (0.2 μm, Schleicher and Schuell). Membranes were blocked in a Tris-buffered Tween solution containing 5% skim milk and 0.1% Tween 20 before being probed with antibodies directed against the phosphorylated forms of ERK (Promega), JNK (SantaCruz) or p38 (New England Biolabs), CPP32 (caspase-3) antibody (Transduction Laboratories) or anti-cytochrome *c* (clone 7H8.2C12, Research Diagnostics Inc.) followed by a horseradish peroxidase-conjugated secondary antibody (Silenus). Immunoreactive proteins were detected using chemiluminescence according to the manufacturer's instructions (DuPont).

3. Results and discussion

Figs. 1 and 2 show the effects of osmotic stress on apoptosis in HeLa cells. Addition of 0.4 M sorbitol resulted in the rapid onset of apoptosis as determined by Hoechst staining or DNA laddering. However, supplementing the medium with 0.2 M KCl did not induce apoptosis and strongly inhibited apoptosis induced by sorbitol. Similar results were obtained when 0.2 M NaCl was added instead of KCl. However, although KCl did not induce apoptosis when present continuously in the culture medium, brief exposure to KCl followed by KCl washout resulted in the rapid onset of apoptosis (Figs. 1 and 2). In these experiments, cells were exposed to high concentrations of KCl for 1 h before washing and returning to isotonic conditions. These data suggested that KCl initiated the early steps in the apoptotic pathway, but imposed a block at some point which could be reversed by lowering the salt concentration.

As reported previously [12–15], both sorbitol and KCl caused a rapid and sustained activation of ERK1, ERK2, JNK and p38 protein kinase cascades (Fig. 3). Activation was determined using antibodies which detect the dual phosphorylated, active forms of these protein kinases, and in the case of ERK and JNK this was confirmed by direct measurement of enzyme activity ([23,24]; data not shown). When cells were exposed to medium containing 0.2 M KCl for 60 min and then washed and returned to isotonic medium, all three kinases remained active for at least 60 min (data not shown).

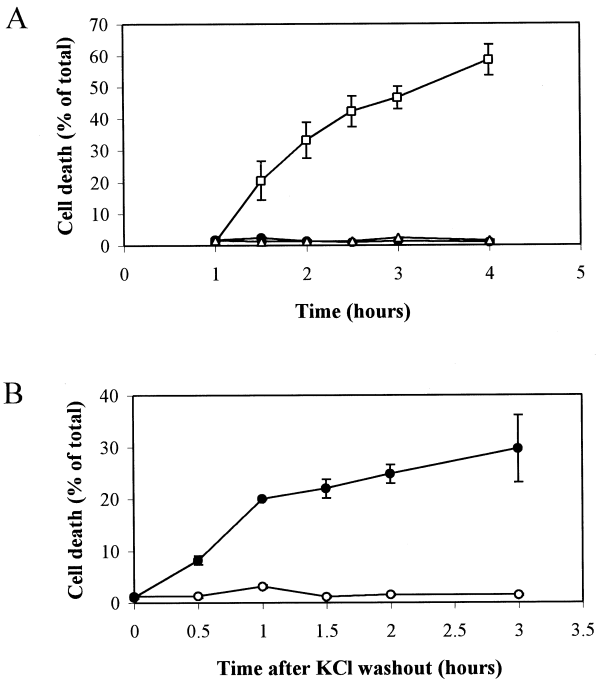

Fig. 1. Effects of osmotic stress on apoptosis in HeLa cells. A: HeLa cells (4×10^5) were incubated with 0.4 M sorbitol (□), 0.2 M KCl (●) or 0.2 M KCl+0.4 M sorbitol (△) for the times indicated. B: Cells were incubated with 0.2 M KCl for 60 min, washed and incubated in isotonic medium (●) or returned to 0.2 M KCl (○) for the times indicated. Apoptosis was assessed by Hoechst 33258 staining. The data points shown represent the mean ± S.E.M. of three independent experiments.

In addition, both KCl and sorbitol stimulated the accumulation of cytosolic cytochrome *c* (Fig. 4A). However, only sorbitol caused the formation of a cleavage product of procaspase-3 and the formation of this product was inhibited when 0.2 M KCl was added to the medium (Fig. 4B). Finally, when cells were exposed to 0.2 M KCl for 1 h, washed, and then incubated in isotonic medium for a further 30 min, cleavage of procaspase-3 was readily detected (Fig. 4B). The appearance of a fragment could be observed within 5 min of returning the cells to an isotonic medium (Fig. 4C) and after 10, 20 or 30 min incubation a lower molecular weight fragment was also detected. A similar pattern of cleavage has been reported during apoptosis induced by Fas ligand in Jurkat cells [25].

Our data support and extend the model put forward by Hughes et al. [1]. This model proposes that the normally high intracellular concentrations of K^+ are sufficient to block activation of caspases and enzymes involved in the fragmentation of DNA. Consequently, triggering of the diverse apoptotic pathways is not sufficient to ensure apoptosis unless associated with a reduction in intracellular K^+. In their experiments Hughes et al. [1] blocked this reduction by stimulating cells in isotonic medium in which the concentrations of KCl and NaCl had been reversed (i.e. K^+ high, Na^+ low). This prevented the final stages of apoptosis presumably by preventing the efflux of K^+ down its concentration gradient. In our experiments apoptosis was blocked at a point between the accumulation of cytosolic cytochrome *c* and caspase-3 activation by either high external K^+ or Na^+ indicating that introduction of the block was not ion-specific. Clearly identification of the channel(s) involved in K^+ efflux during apop-

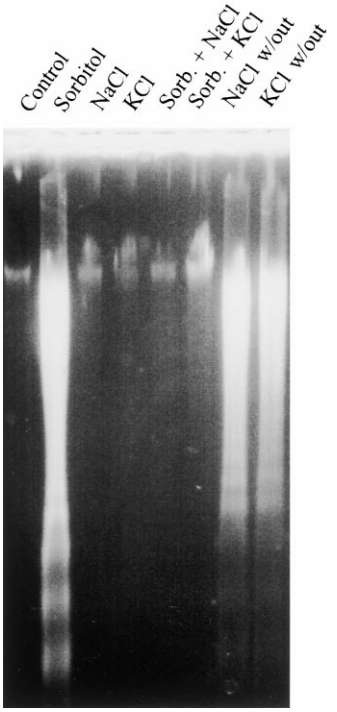

Fig. 2. Effects of osmotic stress on DNA fragmentation in HeLa cells. HeLa cells (2×10^6) were incubated with 0.4 M sorbitol, 0.2 M NaCl/KCl ± 0.4 M sorbitol for 2 h, or with 0.2 M NaCl/KCl for 1 h followed by washing and incubation for a further 2 h in isotonic medium before fragmented DNA was extracted as described in Section 2 and analysed on a 2% agarose gel.

tosis has a high priority and represents a potential target for natural and therapeutic regulation of apoptosis. Our results also make the novel observation that hyperosmotic stress induced by K^+ has a bimodal effect on apoptosis in HeLa cells. While K^+-induced osmotic stress activates the initial events associated with apoptosis, including the accumulation of cytosolic cytochrome *c*, high K^+ blocks the conversion of procaspase-3 to caspase-3 and prevents the final stages of cell death. Finally, the almost total block of the terminal stage of apoptosis by high extracellular K^+ also provides a useful model to investigate apoptotic events which are upstream of caspase-3 activation.

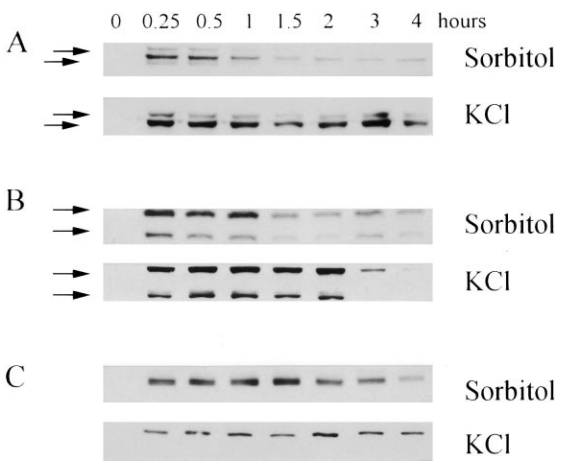

Fig. 3. Effects of osmotic stress on the activity of ERK, JNK and p38. HeLa cells (2×10^6) were incubated with 0.4 M sorbitol or 0.2 M KCl for the times indicated. Cytosolic extracts (50 μg protein) prepared as described in Section 2 were resolved by 10% SDS-PAGE and analysed by Western blot using phospho-specific antibodies. A: ERK, where arrows represent ERK 1 (p44) and ERK 2 (p42). B: JNK, where arrows represent JNK1 (p46) and JNK2 (p54). C: p38.

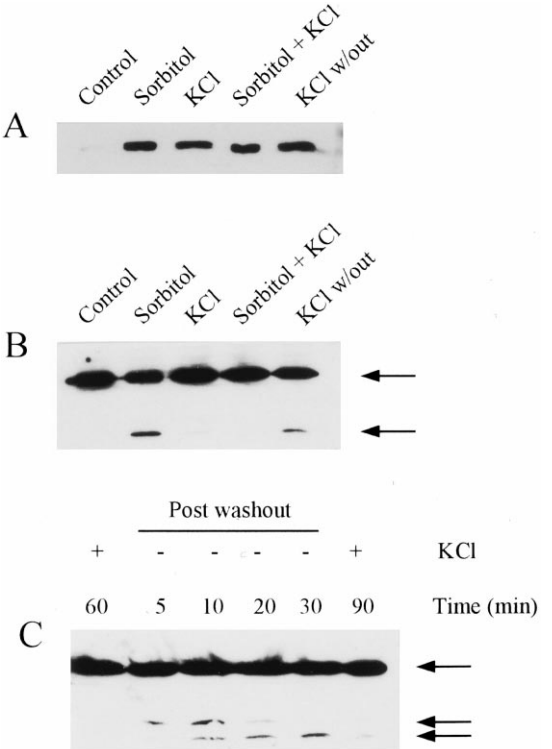

Fig. 4. Effects of osmotic stress on cytosolic cytochrome *c* accumulation and procaspase-3 cleavage. HeLa cells (4×10^6) were incubated with 0.4 M sorbitol or 0.2 M KCl ± 0.4 M sorbitol for 90 min, or incubated with 0.2 M KCl for 60 min before washing and incubating for a further 30 min in isotonic medium. Cytosolic extracts (50 μg protein) were prepared as described in Section 2 and resolved by 15% SDS-PAGE and analysed by Western blot using a cytochrome *c* antibody (A) or caspase-3 antibody (B). C: Cells were incubated with 0.2 M KCl continuously or for 60 min before washing, returning to isotonic medium and incubating for the indicated times. Cytosolic samples were analysed by Western blot with a caspase-3 antibody as described in A.

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