Intracellular K^+ inhibits apoptosis by suppressing the Apaf-1 apoptosome formation and subsequent downstream pathways but not cytochrome *c* release

P Karki^{1,5}, C Seong^{2,5}, J-E Kim², K Hur², SY Shin¹, JS Lee³, B Cho⁴ and I-S Park^{*1}

Cellular ionic homeostasis, fundamentally K^+ homeostasis, has been implicated as a critical regulator of apoptosis. The intracellular K^+ efflux on apoptotic insult and suppression of apoptosis by high concentration of extracellular K^+ or after inhibition of this efflux by K^+ channel blockers have established the crucial role of K^+ in turning on the apoptotic machinery. Several contrasting observations have reported the antiapoptotic effect of intracellular K^+ concentration to be the result of inhibition of cytochrome *c* release from mitochondria, but the exact inhibitory mechanism remains obscure. However, here we show the blockage of K^+ efflux during apoptosis did not affect cytochrome *c* release from the mitochondria, still completely inhibited the formation of the apoptosome comprising Apaf-1, cytochrome *c*, caspase-9 and other accessories. As a consequence of this event, procaspase-9, -3, -8 and other death-related proteins were not processed. Furthermore, physiological concentrations of K^+ also inhibited the processing of procaspase-3 by purified caspase-8 or -9, the nucleosomal DNA fragmentation by purified DFF40/CAD and the nuclear fragmentation to varying extents. Altogether, these findings suggest that the efflux of K^+ is prerequisite not only for the formation of the apoptosome but also for the downstream apoptotic signal-transduction pathways.

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Apoptosis is an intrinsic cell death program that maintains tissue homeostasis and development in metazoans.¹ Among the two well-defined apoptotic pathways, receptor-mediated apoptosis is characterized by the formation of the death-inducing signaling complex (DISC).² On the other hand, chemical-induced apoptosis generally leads to mitochondrial damage resulting in the release of cytochrome *c* that associates with Apaf-1, caspase-9 and dATP to form a multiprotein complex called apoptosome.³ The DISC and apoptosome complex activate procaspase-8 and -9, respectively. Once they are activated, caspase-8 and -9 process effector caspases, including caspase-3, -6 and -7, which are responsible for most of the cleavage events observed during apoptosis.⁴ Apoptosis is repressed in healthy cells as illustrated by inactive caspase zymogens and suppression of DFF/CAD activity by its inhibitor protein. A number of apoptosis-related proteins, including AIF, cytochrome *c* and Smac/Diablo, are stored in the mitochondria and released only upon the requisite apoptotic signal.^{5–7} Furthermore, phosphorylation of Bad and procaspase-9 by Akt and nitrosylation of procaspase-3 at its catalytic cysteine residue promotes cell survival.^{8–10} Similarly, heat shock protein 70 binds to Apaf-1 and prevents the formation of the apoptosome.¹¹

In addition to protein factors, ions have been shown to play important roles in the regulation of apoptosis. Change in ionic strength due to K^+ efflux, Cl^- efflux and Ca^{2+} influx during apoptosis has been proposed as an important inducer of apoptotic processes.^{12,13} Among them, K^+ , maintained at ~150 mM, is a major ion in healthy cells. High concentrations of extracellular K^+ or K^+ channel blockers suppress apoptosis induced by anti-Fas antibody and dexamethasone.^{14,15} Elevated extracellular K^+ inhibited apoptosis induced by anti-CD95, and etoposide in Jurkat E6.1 and human monocytic THP.1 tumor cell prior to mitochondrial depolarization, cytochrome *c* release and caspase activation.¹⁶ And other line of study demonstrated that Cl^- and K^+ channel blockers prevented early-phase apoptotic volume decrease (AVD) and staurosporine (STS)-induced cytochrome *c* release.¹⁷ Both of these reports suggested the targets for anti-apoptotic effect of K^+ to be upstream of the mitochondria. However, some studies showed that Z-VAD-FMK blocks both AVD and apoptosis in anti-Fas antibody-treated Jurkat cells, highlighting the role of caspases in the process.^{12,18} These discrepancies may account for the existence of varying signal-transduction pathways among the cell types or even because of different apoptogens. Thus, more comprehensive studies are required to identify the

¹Department of Bio-Materials Engineering and Cellular and Molecular Medicine, School of Medicine, Research Center for Proteineous Materials (RCPM), Chosun University, Gwangju, Korea; ²Department of Biology, Ewha Womans University, Seoul, Korea; ³Department of Biotechnology, Chosun University, Gwangju, Korea and ⁴Department of Chemistry and Medicinal Chemistry, Yonsei University, Wonju, Korea

*Corresponding author: I-S Park, Department of Bio-Materials Engineering and Cellular and Molecular Medicine, Research Center for Proteineous Materials (RCPM), School of Medicine, Chosun University, Gwangju 501-759, Korea. Tel: +8262 230 6753; Fax: +8262 227 8345; E-mail: parkis@chosun.ac.kr

⁵These authors contributed equally to this work.

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Abbreviations: DF, nuclear DNA fragmentation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide; NF, nuclear fragmentation; PMSF, phenylmethanesulfonylchloride; STS, staurosporine; TEA, tetraethylammonium

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molecular details and clearly elucidate the mechanism of anti-apoptotic effect of intracellular K⁺.

The further biochemical studies revealed that intracellular K⁺ blocks the assembly of active apoptosome complex.^{16,19} Some bcl-2 family proteins were also reported to upregulate mitochondrial K⁺ efflux, thus affecting mitochondrial K⁺ homeostasis.²⁰ K⁺ also inhibited *in vitro* activation of procaspase-3-like enzymes in a dose-dependent manner²¹ and unlike the activity of caspase-3, that of procaspase-3 was decreased to ~50% in the presence of >25 mM K⁺.²² Similarly, caspase activity was not affected by high concentration of K⁺ (~200 mM), but it was inhibitory to nuclear DNA fragmentation (DF).²¹

The present study is carried out to examine the specific steps of the apoptotic pathway that are influenced by suppression of K⁺ efflux. We investigated the effect of elevated extracellular K⁺ concentration on crucial early events of apoptosis such as cytochrome *c* release and Apaf-1 apoptosome formation in human neuroblastoma SK-N-BE(2) and epithelial HeLa cells. We also studied the regulatory role of physiological K⁺ concentration (~150 mM) in other major downstream pathways of the apoptosome, such as activation of caspase-3, processing of procaspase-3 by caspase-8 or -9, DF and nuclear fragmentation (NF).

Results

The blockage of K⁺ efflux inhibited STS-induced apoptosis downstream of cytochrome *c* release from mitochondria and upstream of caspase-9 activation. Apoptotic and shrunken cells have much lower intracellular K⁺ concentration as compared to normal healthy cells, and inhibition of K⁺ efflux completely eliminates apoptosis.^{12,15} We conducted experiments to identify the particular events in the apoptotic cascades that are liable to be blocked by intracellular K⁺. Treatment of SK-N-BE(2) and HeLa cells with bacterial alkaloid STS, a broad-spectrum protein kinase inhibitor, resulted in ~50% apoptotic cell death, as assessed by 3-(4,5-dimethylthiazol-

2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay (Figure 1a). Inclusion of high K⁺ in the medium induced a remarkable reduction in the cell death (down to control levels) (Figure 1a), probably due to depolarization and blockage of intracellular K⁺ efflux. Simultaneous application of known K⁺ channel blockers, tetraethylammonium (TEA) and quinine also rescued the cell death in both cell lines (Figure 1a). Similar results were achieved with trypan blue exclusion experiments (data not shown). Furthermore, both high K⁺ medium and K⁺ channel blockers were effective in reducing the caspase-3-like (DEVDase) activity, induced by STS in both cells (Figure 1b). These results are consistent with previous reports, and confirm the significance of K⁺ efflux in apoptosis. High K⁺ medium was albeit better in suppression of cell death and caspase-3 activity, and higher concentrations of blockers themselves caused significant cell death (data not shown). Thus, for uniformity, high K⁺ concentration was maintained in the medium during all experiments, unless otherwise indicated.

The release of cytochrome *c* from mitochondria leads to activation of the caspase cascade via formation of the Apaf-1 apoptosome.³ A small amount of cytochrome *c* was observed in the cytosolic fraction (supernatant), as early as 2 h after STS treatment of SK-N-BE(2) cells, and significant increment was detected in the fractions after 4 h (Figure 1c, upper panel). More importantly, cytochrome *c* was released into the cytosol at comparable levels, even in the presence of high extracellular K⁺, TEA and quinine. Furthermore, Z-VAD-fmk, a broad-spectrum caspase inhibitor, did not notably block this release, ruling out the possible involvement of caspases in the process. These observations were consistent in HeLa cells as demonstrated by the release of cytochrome *c* into cytosol after STS treatment, even in the presence of high extracellular K⁺ (Figure 1c, lower panel). Our observation of cytochrome *c* release even in high K⁺ medium could not agree with earlier reports that insisted elevated K⁺ inhibits apoptosis before mitochondrial perturbation.^{16,17} This variation might have arisen due to the existence of multiple pathways of cytochrome *c* release governed by the nature of apoptotic stimuli

Figure 1 Effects of blockage of K⁺ efflux on STS-induced cell death, caspase-3-like activity and cytochrome *c* release. SK-N-BE(2) (black bars) and HeLa cells (gray bars) were treated with 5 and 1 μ M STS in normal or high K⁺ medium for 8 and 6 h, respectively, in the absence or presence of 40 mM TEA, 200 μ M quinine. (a) Cell survival rate by MTT assay. (b) Caspase-3-like (DEVDase) activity. The results are the mean $\pm$ S.D. of three independent experiments. * P < 0.05; ** P < 0.005 (versus untreated control cells). (c) SK-N-BE(2) cells (upper two panels) were treated with 5 μ M STS for 0, 2 or 4 h in normal (STS) or high K⁺ (K⁺ + STS) medium or in the normal medium for 4 h, in the presence of 100 μ M Z-VAD-fmk (V), 40 mM TEA (T) or 200 μ M quinine (Q). HeLa cells (lower two panels) were treated with 1 μ M STS for 0, 3 or 6 h in normal (STS) or high K⁺ (K⁺ + STS) medium. Cells were harvested, and cytochrome *c* release was assessed, as described in Materials and Methods. Data are representative of three independent experiments

Figure 2 Effects of blockage of K^+ efflux on the activation of caspases and their downstream substrates. SK-N-BE(2) (a) and HeLa (b) cells were incubated in the absence or presence of 5 and 1 μ M STS in normal or high K^+ containing medium for 4 and 6 h, respectively, and processing of caspases (-9, -3, -8), Bid and DFF45/35 were analyzed by Western blotting. The molecular size of uncleaved proteins and their corresponding cleavage products are indicated. Actin was employed as a loading control

and cell type.²³ A recent study has shown that 80 mM extracellular K^+ markedly attenuated though never completely prevented the cytochrome *c* release from mitochondria in human neutrophils.²⁴ Furthermore, experimental variations have also been raised on question for the results of cytochrome *c* dissociation from mitochondria, as the process was found to be highly dependent on ionic strength with a requirement of 50–80 mM NaCl, KCl or LiCl.²⁵ Considering these discrepancies, it was thought that the mitochondrial perturbation should not be the only loci to be targeted by intracellular K^+ and thus the ensuing pathways were taken into further investigation.

The activation of caspase-9, -3 and -8 induced by STS in both SK-N-BE(2) and HeLa cells was abolished at high K^+ concentrations (Figure 2a and b). Accordingly, the processing of Bid and DFF45/ICAD, downstream substrates of caspase-8 and -3, respectively, was prevented in both cell lines (Figure 2a and b).

Assembly of the Apaf-1 apoptosome was prevented in high K^+ medium. Since the above results suggest that the inhibitory effect of K^+ occurs between cytochrome *c* release and caspase-9 activation, we next examined the consequences of high K^+ in the assembly of Apaf-1 apoptosome, comprising Apaf-1, dATP, cytochrome *c* and caspase-9. The extract, prepared from cells treated with STS in normal or high K^+ media, was fractionated using gel filtration chromatography. The majority of Apaf-1 in the extract isolated from control cells eluted as a single peak centered around fraction 31 (relative molecular mass ~150 kDa), corresponding to its monomeric form, while both caspase-9 and -3 were eluted as unprocessed proenzymes (Figure 3a). In cells treated with STS at normal K^+ concentrations, Apaf-1 was detected in earlier (23–26) as well as in later (30–34) fractions. Apaf-1, observed in the early fractions, is possibly a part of the apoptosome complex, as reported previously.²⁶ Processed caspase-9 (p35) and caspase-3 (p17) were also detected

Figure 3 Effect of the blockage of K^+ efflux on the formation of the Apaf-1 apoptosome. SK-N-BE(2) cells were incubated in the absence (a) or presence of 5 μ M STS in normal (b) or high K^+ medium (c) for 4 h. The extract prepared from cells was fractionated by gel filtration chromatography as described in Materials and Methods. Fractions were analyzed by immunoblotting for Apaf-1, caspase-9 and -3. The standard molecular markers are indicated above the scale with elution fractions

(Figure 3b). In contrast, processed caspases and earlier Apaf-1 fractions present in STS-treated cells were not observed in extracts from cells treated with STS at high K^+ concentrations (Figure 3c). These data indicate that K^+ inhibits the formation of the Apaf-1 apoptosome, and consequently inhibits the processing of caspases.

High concentrations of K^+ suppressed Apaf-1 apoptosome formation induced by dATP/cytochrome *c* in the cell-free system. The effects of K^+ on apoptosome formation and the downstream pathway in the cell-free system upon activation by dATP and cytochrome *c* were examined. High concentrations (> 100 mM) of K^+ inhibited cleavage of procaspase-9 initiated by dATP and cytochrome *c* (Figure 4a). Consequently, the processing of procaspase-3, downstream substrate of caspase-9, was suppressed (Figure 4a) and DEVDase activity decreased with increasing K^+ concentration in dose-dependent manner (Figure 4b, open circle). Furthermore, processing of DFF45/ICAD, an essential step in the activation of DFF40/CAD, was prevented by K^+ at 100 mM or higher (Figure 4a); well correlating with the suppression of DF and NF by high K^+ concentrations (Figure 4c and d). To define whether the inhibitory effect of K^+ is specific or general salt effect *in vitro*, the same experiments were conducted by replacing K^+ with Na^+ . For Na^+ , the inhibitory effect on cleavage of procaspase-9 initiated by dATP and cytochrome *c* was observed in the similar pattern with K^+ (Figure 4a, lower panel). Consistently, the processing of downstream of caspase-9 was also suppressed (data not shown) and DEVDase activity also decreased with Na^+ in dose-dependent manner (Figure 4b, closed circle). It was speculated that suppression of procaspase-9 processing and downstream proteins may be due to the inhibition of apoptosome formation in the system. To confirm this, cell extracts incubated with dATP /cytochrome *c* in the presence

Figure 4 Effects of K^+ on dATP/cytochrome *c*-induced *in vitro* apoptosis. *In vitro* apoptosis was induced by the addition of dATP and cytochrome *c* to a mixture of rat liver nuclei and cell extracts, prepared from healthy cells, in the presence of indicated concentrations of K^+ or Na^+ for 2 h. The mixture was immunoblotted for caspase-9, -3 and DFF45 (a) or assayed for DEVDase activity (b), DF (c) and NF (d), as described in Materials and Methods. In (b), closed and open circles indicate DEVDase activity in the presence of K^+ and Na^+ respectively

of 20 mM or 150 mM K^+ were fractionated, as described above. In the presence of 20 mM K^+ , Apaf-1 eluted in two peaks, corresponding to the monomeric form (fractions 30–32) and the multimeric complex (fractions 20–26). Processed caspase-9 and -3 proteins were also detected (Figure 5b). However, at 150 mM K^+ , Apaf-1 was recovered as a single monomeric peak, and no processing of procaspase-9 and -3 was observed (Figure 5a and c). These results together with cellular experiment confirm that K^+ blocks Apaf-1 apoptosome formation. But as data shown, when K^+ was replaced with Na^+ *in vitro* system, the suppression of subsequent events corresponding to the downstream of apoptosome including procaspase-9 processing was observed in similar modes. Therefore, the inhibitory effect of blockage of K^+ efflux on apoptosome formation and its downstream events might be explained in terms of ionic strength rather than K^+ specificity. This view is in agreement with the previous observation that the interaction of cytochrome *c* and Apaf-1 highly depends on ionic strength²⁷ and efficacy of some cations along with K^+ to inhibit the cleavage of procaspase-9 and -3.²⁸ However, being the predominant ion *in vivo*, the efflux of K^+ may handle the major regulatory role during apoptosis.

The effect of K^+ on caspase activities for its synthetic and natural substrates. It is reported that K^+ (up to 200 mM) does not inhibit caspase activity.²⁹ We also obtained comparable results showing that at 0–150 mM

Figure 5 Effect of K^+ on the formation of Apaf-1 apoptosome in the cell-free system. The cell extract was incubated without (a) or with dATP and cytochrome *c* in the presence of 20 mM (b) or 150 mM (c) K^+ . The reaction mixture was fractionated by gel filtration chromatography and analyzed as in Figure 3

Figure 6 Effect of K^+ on the cleavage of downstream substrates by caspase-9 and -8. (a) Activities of recombinant caspase-9 (closed circle and rectangle), caspase-8 (open circle and rectangle) and caspase-3 (closed triangle and open triangle) were measured in the presence of indicated concentrations of K^+ (closed symbols) or Na^+ (open symbols) with synthetic substrates. (b) The cell extract was incubated with recombinant caspase-9 (closed circle and rectangle) or caspase-8 (open circle and rectangle) in the presence of indicated concentrations of K^+ (closed symbols) or Na^+ (open symbols), and DEVDase activity was measured. Additionally, cleavage of procaspase-3 (Casp-3) by caspase-9 (c) and that of procaspase-3 (Casp-3) and Bid by caspase-8 (d), were examined by Western blotting

K^+ , purified recombinant caspase-3, -8 and -9 displayed similar levels of activity on synthetic tetrapeptides (Figure 6a). And as expected, the influence of Na^+ on caspase activity was also observed in consistent pattern with that of K^+ (Figure 6a). However, DEVDase activity induced by recombinant caspase-9 in the cell-free system decreased with increasing concentrations of K^+ (Figure 6b), and the processing of endogenous procaspase-3 in the extract was significantly inhibited (Figure 6c). Similarly, processing of

procaspase-3 induced by recombinant caspase-8, and resulting DEVDase activity, was reduced by K^+ in a dose-dependent manner (Figure 6b and d). And the cleavage of Bid, a substrate of caspase-8, was also inhibited at high concentrations of K^+ , although the degree of suppression was relatively low, compared to that of procaspase-3 (Figure 6d). Caspase-8 and -9 displayed similar reduced activity with purified procaspase-3 and Bid in high salt conditions (data not shown), confirming the cell extract data. The inhibitory effects on DEVDase activity induced by caspase-9 or -8 were reproduced when K^+ was replaced with Na^+ (data not shown). Moreover, Na^+ also reduced the processing of caspase-3 induced by recombinant caspase-9 or caspase-8 in dose-dependent manner (data not shown). These results imply that K^+ might not affect caspase activity *per se*, but rather act on the caspase activation process, and it also indicates that the inhibitory effect of K^+ and Na^+ on caspase activation can be generalized as the effect of ionic strength. These observations support that ionic strength must be maintained at a permissive level for the time required for the entire apoptosis to occur. Again, this ionic regulation of apoptosis should be largely due to intracellular K^+ , that is the major intracellular osmole with one to several orders of higher magnitude than other ions concentrations.

The effect of K^+ on apoptotic DF. In the same cell-free system, processing of DFF45/ICAD by recombinant caspase-3 was examined. The protein was completely cleaved within 1 h, regardless of K^+ concentration (up to 150 mM, data not shown). To further examine this event, extracts were incubated with caspase-3 in the presence of 40 or 120 mM K^+ , for various time points. As shown in Figure 7a, processing of DFF45/ICAD was complete within

Figure 7 Effect of K^+ on DF induced by caspase-3. (a) Cell extracts were incubated with recombinant caspase-3 for indicated time periods, in the presence of 40 or 120 mM K^+ , and immunoblotted for DFF45/35. (b) The mixture of cell extracts and rat liver nuclei was incubated with caspase-3 in the presence of fixed concentrations of K^+ , and DF was analyzed. (c) Plasmid DNA was incubated with recombinant CAD in the presence of different concentrations of K^+ , and DF was analyzed

40 min of incubation with 40 mM K^+ . With 120 mM K^+ , processing was slightly delayed, but completed within 1 h. In contrast to these results, DF was suppressed by K^+ concentrations higher than 100 mM (Figure 7b). Similar results were obtained by replacing K^+ with Na^+ (data not shown). This observation suggests that the activity of DFF40/CAD released from the protein inhibitor, not the processing of DFF45/ICAD by caspase-3, is influenced by high salt concentrations. To verify this hypothesis, we examined the effect of K^+ on the digestion of plasmid DNA by purified recombinant CAD. As expected, high concentrations of K^+ inhibited degradation of plasmid DNA (Figure 7c), indicating that K^+ regulates the activity of the nuclease.

The effect of K^+ on apoptotic NF. Finally, it was determined whether K^+ regulates factor(s) responsible for NF, another apoptotic hallmark. To exclude the known chromatin condensation and NF activity of DFF40/CAD, an ICAD mutant (D117E) was employed in the reaction that inhibits DFF40/CAD activity, even in the presence of caspase-3. When the caspase cascade was induced, either by dATP/cytochrome *c* or by caspase-8 in the cell-free system, the ICAD mutant effectively blocked NF as well as DF induced by DFF40/CAD (data not shown). Thus, an *in vitro* condition in which NF readily occurred in the presence of the ICAD mutant was required. An effective way involved the combined treatment of dATP/cytochrome *c* and caspase-8, although the mechanism behind this process was unclear. Under these conditions, the addition of ICAD mutant abolished DNA ladder formation (Figure 8a, lane 2), but did not block NF (Figure 8b, panel 2). High concentrations of K^+ effectively inhibited NF (Figure 8b), suggesting that intracellular K^+ also negatively regulates apoptotic NF.

Discussion

In the present study, the apoptosis induced by STS in SK-N-BE(2) and HeLa cells was significantly reduced by high K^+ ,

Figure 8 Effects of K^+ on NF. Cell extracts were pre-incubated for 40 min with dATP, cytochrome *c* and caspase-8 in the presence of 20 mM K^+ , following which rat liver nuclei were added to the reaction mixture and incubated for 2 h in the absence (1) or presence of 300 ng ICAD mutant (D117E) with 20, 50, 75, 100, 120 or 150 mM K^+ (2–7). Examinations of DF (a) and NF (b) were followed. C represents the control group incubated without dATP, cytochrome *c* or caspase-8

while cytochrome *c* release was unaffected (Figure 1). These observations led us to conclude that the apoptotic pathway upstream of cytochrome *c* release from the mitochondria is not extensively influenced by K⁺. The results are inconsistent with others, which propose that blockage of K⁺ efflux also suppresses cytochrome *c* release.^{16,17} The reasons for this discrepancy are presently unclear. A huge variation regarding the effect of K⁺ efflux on apoptosis depending upon cell type and nature of apoptotic insult³⁰ and similar diversities in the pathways of cytochrome *c* release might have contributed to these contrasting findings. But it should be noted that when cells were incubated for more than 8 h in high K⁺ medium, the incubation itself could induce cell death. For this reason, apoptotic triggers needed for relatively longer time could not be used here and only STS served as a good choice. Nevertheless, the differing effectiveness of K⁺ channel blockers to inhibit apoptosis also precludes the universal inhibition mechanism of K⁺ efflux.³⁰

The assembly of apoptosome and processing of caspase-9, -3 and -8 was completely abolished by high K⁺ (Figures 2 and 3). Apoptosome formation induced by dATP and cytochrome *c* in the cell-free system was also suppressed (Figure 5), consistent with the above results and the previous study.¹⁹ Although there is controversy whether Apaf-1 interacts with cytochrome *c* or dATP first,^{31,32} a reconstitution experiment with purified Apaf-1 and cytochrome *c* showed that the former protein bound the latter with high affinity (association constant = $\sim 10^{10} \text{ M}^{-1}$).²⁷ The same study demonstrated that Na⁺ ($\sim 200 \text{ mM}$) decreased the association constant by an order of 1000. Due to the similarity between two cations, it can be speculated that K⁺ may similarly affect the formation of apoptosome. However, K⁺ inhibition of apoptosome formation was shown to be reversible with the increasing concentrations of cytochrome *c*.¹⁹ It did not hold true in the case of cytochrome *c*-mediated procaspase-3 activation where both alkaline pH and normocellular K⁺ concentration inhibited the activation, but only the inhibition by alkaline pH could be overcome by increasing amounts of cytochrome *c* and not that by K⁺.²⁸ In our case, release of cytochrome *c* was not affected by higher concentration of K⁺ but still the apoptosome formation was inhibited, suggesting a different scenario of inhibition mechanism. Similar results were reported for *S*-nitroso-*N*-acetyl-penicillamine (SNAP)-treated Jurkat cells where mitochondrial membrane depolarization and cytochrome *c* release remain unaffected but apoptosome formation was inhibited, accompanied by attenuation of caspase activation.³³ These observations suggested the regulatory point of SNAP inhibition to be the apoptosome rather than upstream of mitochondria, possibly due to the inhibition in the interaction between procaspase-9 and Apaf-1, and this explanation appears to be true in our case, too. The apoptosome formation is a crucial step in cellular commitment to apoptosis, and if this event is the target point for K⁺ to safeguard the cell against inappropriate caspase activation, the inhibition mechanism should not be expected as simple as to be determined by the amount of cytochrome *c* release. Considering the inevitable accidental cytochrome *c* release along with the occurrence of diverse pathways to release it from mitochondria, the idea of specific inhibition of apoptosome by K⁺ to solely depend on this

process seems to be quite unconvincing. Recently, calcium was found to block the formation of apoptosome by preventing nucleotide exchange in Apaf-1.³⁴ So, it is too early to conclude any specific mechanism for K⁺ inhibition of apoptosome assembly without exploring the details of complex interplay between ionic homeostasis and apoptosis.

Consistent with the earlier reports, we also observed a little effect of K⁺ on activity of mature caspases for their synthetic substrates²⁹ but caspase-8 and -9 processed their natural substrates procaspase-3 and Bid, respectively, less efficiently at high concentrations of K⁺. The dramatic specific effect of K⁺ on procaspase-3 activity²² and somewhat suppression in processing of natural substrates suggest that the effect of K⁺ is associated with initial caspase activation process rather than activity itself. This notion is bolstered by a previous report that elevated K⁺ inhibited cytochrome *c*-dependent activation of caspase-3 but not the activity of once activated caspase-3.²⁸ The variation of K⁺ effect on caspase activity with natural and synthetic substrates may be the outcome of attaining a different structure by protein substrates at high intracellular K⁺ concentrations and thus becoming resistant to cleavage by upstream caspases.²¹

DFF40/CAD is considered as the main apoptotic DNase, since DF was largely abolished in DFF45-deficient mouse.³⁵ The DNase is complexed with its inhibitor protein, DFF45/ICAD that is cleaved by caspase-3, ensuring the release of active DNase.³⁶ In our experiments, the reaction was completed within 1 h at varying concentrations of K⁺ (0–200 mM; data not shown). However, DF induced by caspase-3 in the cell-free system was significantly suppressed by high concentrations of K⁺ (Figure 7). This is attributed to the inhibition of DFF40/CAD activity by high concentrations of the ion. Several reports indicate that DFF40/CAD also induces CC and NF.³⁷ We aimed to achieve experimental conditions in which only NF occur, and ascertained that both caspase-8 and dATP/cytochrome *c* were necessary for NF-independent of DF, although the mechanism is not currently known. In these conditions, high concentrations of K⁺ suppressed NF (Figure 8). Finally, on the basis of all these observations, a model indicating the potential regulatory points of intracellular K⁺ is proposed (Figure 9).

In summary, the current study demonstrated that one of the main steps in STS-induced apoptosis sensitive to the blockage of K⁺ efflux is the formation of Apaf-1 apoptosome. In addition, downstream pathways including the processing of

Figure 9 Proposed model for the negative regulation of staurosporine-induced apoptosis by intracellular K⁺. NFF, nuclear fragmentation factor

caspase-3 and other apoptotic proteins, DF and NF are also suppressed by high intracellular concentrations of K^+ . Taken together, these data suggest that the apoptotic machinery is active only in the presence of relatively low concentration of K^+ that ensures the safety of cells from unexpected onset of apoptosis in healthy cells.

Materials and Methods

Reagents. STS, *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl-coumarin (Ac-DEVD-AMC), *N*-acetyl-Leu-Glu-His-Asp-7-amino-4-methyl-coumarin (Ac-LEHD-AMC), *N*-acetyl-Ile-Glu-Thr-Asp-7-Amino-4-methyl-coumarin (Ac-IETD-AMC) and benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) were purchased from Alexis Biochemicals (Laufelfingen, Switzerland). TEA, MTT, quinine, cytochrome *c*, bovine serum albumin (BSA), Hoechst 33258, 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfate (CHAPS) and digitonin were purchased from Sigma. Monoclonal antibodies to Apaf-1, caspase-9 and DFF45 were purchased from R&D Systems (Minneapolis, MN, USA), Medical & Biological Laboratories (Nagoya, Japan) and Transduction laboratories (Lexington, KY, USA), respectively. Polyclonal antibodies to cytochrome *c*, caspase-3, -8 and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Pharmingen (San Diego, CA, USA) and Leehyo (Seoul, Korea), respectively. Polyclonal antibody against Bid was generated by injecting mice with purified recombinant Bid. All other materials were purchased from Sigma.

Cell culture. Human neuroblastoma SK-N-BE(2) and epithelial HeLa cells were cultured either in normal Dulbecco's modified Eagle's medium (DMEM) or in high K^+ DMEM medium (containing 81.3 mM KCl, 5.4 mM NaCl, 44 mM $KHCO_3$ instead of 5.4 mM KCl, 81.3 mM NaCl, 44 mM $NaHCO_3$ in the normal media) with 10% heat-inactivated calf serum, 100 units of penicillin and 100 μ g/ml streptomycin in 5% CO_2 at 37°C. Calf serum for human neuroblastoma SK-N-BE(2) used in the high K^+ medium was dialyzed against multiple changes of the high K^+ medium. And dialyzed fetal bovine serum was used in the high K^+ medium for HeLa cells.

Cell death and caspase-3-like activity assay. Cells were seeded at the density of 2×10^4 cells/well in 96-well microtiter plates and incubated overnight. After replacing with fresh medium, the cells were treated with indicated chemicals. The cell viability was assessed by colorimetric MTT assay at 570 nm on Spectra Max 190 (Molecular Devices, CA, USA). For caspase-3 activity (DEVDase) assay, treated cells were washed twice with ice-cold phosphate-buffered saline (PBS). Then, 40 μ l of buffer containing 20 mM Hepes-NaOH, pH 7.0, 1 mM EDTA, 1 mM EGTA, 20 mM NaCl, 1 mM DTT, 1 mM phenylmethanesulfonylchloride (PMSF), 10 μ g/ml leupeptin, 5 μ g/ml pepstatin A, 2 μ g/ml aprotinin, 25 μ g/ml ALLN was added into each well and incubated on ice for 20 min. Caspase assay buffer (final 20 mM Hepes-NaOH, pH 7.0, 20 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA and 10 mM dithiothreitol) was added and the release of AMC was monitored for 2 h in 2 min interval with final 10 μ M DEVD-AMC at excitation and emission wavelengths of 360 nm and 480 nm, respectively, using micro plate spectrofluorometer (Molecular Devices). The results were expressed as a slope of total readings versus time. The statistical analysis was performed with the Student's *t*-test and considered as significantly different from control at $P < 0.05$.

Preparation of cell extract and rat liver nuclei. To prepare the cell extract, harvested cells were washed with ice-cold PBS, resuspended in buffer A (20 mM HEPES-KOH, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM KCl, 1 mM β -mercaptoethanol, 0.1 mM PMSF, 10 μ g/ml leupeptin, 5 μ g/ml pepstatin A, 2 μ g/ml aprotinin, 25 μ g/ml ALLN) and dounce-homogenized. Supernatant was obtained by centrifugation at $100\,000 \times g$ and stored at $-80^\circ C$. Nuclei were prepared by rinsing rat liver with ice-cold PBS, homogenization in four volumes of buffer B (10 mM HEPES-KOH, pH 7.5, 2.4 M sucrose, 15 mM KCl, 2 mM EDTA, 0.15 mM spermine, 15 mM spermidine, 1 mM β -mercaptoethanol and 0.5 mM PMSF) and centrifugation through a 10 ml cushion of the buffer B at 25 000 r.p.m. for 1 h in a Beckman SW28 rotor at 4°C. The nuclei pellets were resuspended in buffer C (10 mM HEPES-KOH, pH 7.5, 10 mM KCl, 250 mM sucrose, 1 mM EDTA and 1 mM β -mercaptoethanol) at 1×10^9 nuclei/ml and stored at $-80^\circ C$.

Production of recombinant proteins. Active caspase-3, -9 or -8, which lack prodomain, caspase recruitment domain or death effector domain, respectively,

in bacterial expression vector pET-22b (Novagen), pET-28b or pET-15b, and CAD/ICAD in the vector pRSETB were overexpressed in *Escherichia coli* BL21(DE3)pLysS, as described previously.³⁸ ICAD (D117E) mutant protein, which is cleaved at Asp224 by caspase-3 but is still able to inhibit the activity of CAD,^{36,39} was constructed using overlap polymerase chain reaction techniques, subcloned into pET-28b and overexpressed in *E. coli* BL21(DE3)pLysS. The caspases were purified by three successive chromatographic procedures. Initially, bacterial cell lysates from 2 l culture were prepared by sonication and centrifugation at 20 000 r.p.m. for 1 h in buffer D (20 mM Tris-HCl, pH 8.0, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM PMSF, 1 mM β -mercaptoethanol). The supernatant was loaded onto a DEAE-sepharose column (70 ml) equilibrated with buffer E (20 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1 mM β -mercaptoethanol, 10% glycerol) and eluted with a 10–500 mM NaCl gradient. The active fractions were applied onto a Ni-NTA column (Qiagen). The column (3.8 ml) was washed with two column volumes of buffer E and three column volumes of 60 mM imidazole-containing buffer E. The proteins were then eluted with 250 mM imidazole-containing buffer E. The active fractions were diluted, applied to a HiTrapQ sepharose column (Amersham Pharmacia Biotech) equilibrated with buffer F (20 mM HEPES-NaOH, pH 7.5, 10 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol) and eluted with 1–400 mM NaCl gradient. ICAD (D117E) was purified as described above without DEAE column step. To obtain pure CAD protein, cell lysates containing CAD/ICAD were incubated with active caspase-3 supplemented with 5 mM DTT at 30°C for 1 h, and the reaction mixture was loaded onto a HiTrapSP sepharose column (Amersham Pharmacia Biotech) equilibrated with buffer G (20 mM HEPES-NaOH, pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 10% glycerol) and eluted with a 10–500 mM NaCl gradient. The active fractions were applied onto a HiTrapQ sepharose column equilibrated with buffer G and eluted with a 10–500 mM NaCl gradient. The purified proteins were stored in 30–40% glycerol at $-80^\circ C$.

Cell-free system. *In vitro* apoptosis was induced by incubating the cell extract (150 μ g) with 1 mM dATP and 10 μ M cytochrome *c*, recombinant caspase-9 (1 μ g), -8 (100 ng) or -3 (50 ng) in buffer H (20 mM HEPES-KOH, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1.5 mM $MgCl_2$, 2 mM DTT, various concentrations of KCl or NaCl) for 2 h at 30°C in the presence or absence of 5×10^5 rat liver nuclei. For the analysis of apoptosome assembly, the extract (2 mg) was reacted with 1 mM dATP and 10 μ g/ml cytochrome *c* for 1 h at 30°C in buffer H supplemented with additional 1 mM $MgCl_2$, 0.1% CHAPS and 10% glycerol. Recombinant purified CAD (4 μ l) was also incubated with 200 ng of pGEM7Z(+) plasmid (Promega) in buffer H supplemented with 1 mg/ml BSA and 2.5 mM $MgCl_2$ for 1 h at 30°C. The activated cell extract was used for Western blotting, caspase activity assay or analysis of apoptosome formation. For DF assay, DNA was extracted as described previously³ and analyzed by 1–1.5% agarose gel electrophoresis. For NF assay, nuclei were fixed with 4% paraformaldehyde, stained using 3 μ g/ml Hoechst 33258 and observed under a fluorescence microscope (Zeiss).

Measurement of caspase activity. The activities of caspase-3 (20 ng) or DEVDase of the activated cell extract, caspase-9 (1 μ g) and caspase-8 (200 ng) were measured using 10 μ M Ac-DEVD-AMC, 20 μ M Ac-LEHD-AMC and 20 μ M IETD-AMC, respectively. The fluorescence of the AMC release from the substrates was measured as described above. Activity was determined from the initial velocities of substrate hydrolysis. The velocities of hydrolysis have been normalized to the velocity of hydrolysis in the absence of KCl.

Western blot analysis. Cells were harvested, washed with ice-cold PBS and resuspended in buffer consisting of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 10 μ g/ml leupeptin, 2 μ g/ml pepstatin A and 2 μ g/ml aprotinin for 20 min on ice. The extract was obtained by centrifugation at 14 000 r.p.m. at 4°C for 15 min. The amount of protein was measured through Bradford assay. Equal amounts of proteins were separated on 10–13.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The membrane was immunoprobed with primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Pierce). The blots were visualized using ECL plus reagent (Amersham Pharmacia Biotech).

Analysis of cytochrome *c* release. Cells were washed with ice-cold PBS and resuspended in digitonin buffer (75 mM NaCl, 1 mM NaH_2PO_4 , 8 mM Na_2HPO_4 , 250 mM sucrose, 190 μ g/ml digitonin). After 5 min on ice, cells were spun for 5 min at 14 000 r.p.m. at 4°C in a microcentrifuge. Supernatants were transferred to fresh

tubes and the pellets were resuspended in buffer containing 25 mM Tris-HCl, pH 8.0 and 1% Triton X-100. Proteins from each sample were subjected to SDS-PAGE and Western blotting.

Chromatographic analysis of apoptosome assembly. After STS treatment for 4 h, the cell extract was prepared in buffer I (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM MgCl₂, 2 mM DTT, 0.1% CHAPS, 10% glycerol, 0.1 mM PMSF, 10 µg/ml leupeptin, 5 µg/ml pepstatin A, 2 µg/ml aprotinin, 25 µg/ml ALLN) as described above. The cell extract (2 mg) was loaded onto a Superose 6 HR (10/30) column (Amersham Pharmacia Biotech) pre-equilibrated with buffer I containing 50 mM KCl and proteins were eluted from the column at a flow rate of 0.2 ml/min. From each of the 0.5 ml fractions, appropriate fractions were concentrated in Microcon (Millipore) concentrators (10 kDa cutoff) and analyzed by SDS-PAGE; followed by immunoblot analysis. The column was calibrated with calibration kits from Amersham Pharmacia Biotech, including thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa) and aldolase (158 kDa).

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