Effects of Phosphocreatine on Apoptosis in a Cell-free System*

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The characteristic morphological and biochemical changes during caspase-mediated apoptosis can be reproduced to a large extent in a Xenopus laevis egg extract cell-free system by addition of mouse liver nuclei and exogenous cytochrome c. We show that in this system phosphocreatine accelerated the apoptotic morphological changes of the nuclei, but selectively inhibited DNA fragmentation. Western blot showed that the degradation of lamins A and C is accelerated, which is possibly responsible for the nuclear changes during cell apoptosis. However, the degradation of ICAD/DFF45-like protein in the egg extracts is inhibited in a time-dependent manner. Exogenous creatine, ATP, and several organic acids have no effect on DNA fragmentation, excluding the possibility that creatine, ATP, or acidic conditions resulting from phosphocreatine are responsible for inhibiting DNA fragmentation. Lithium chloride, a kinase inhibitor, can overcome the phosphocreatine effects and can restore DNA fragmentation. Our results indicate that phosphocreatine protects ICAD/DFF45-like protein from proteolysis, probably through kinase actions, resulting in its resistance to caspase cleavage and leading to an inhibition of DNA fragmentation.

Apoptosis is a highly genetic-determined cell suicide program that can be induced by a variety of extracellular and intracellular stimuli and is executed through a series of signal transduction pathways (1, 2). Balance between cell death and cell proliferation ensures a controlled provision of fresh cells. Defects in apoptosis can therefore result in development abnormalities, cancer, and a number of other diseases (3, 4). Cells undergoing apoptosis display distinctive morphological changes, including the condensation of nuclei and cytoplasm, blebbing of cellular membranes, and fragmentation of the cell into apoptotic bodies that are rapidly phagocytosed by the neighboring cells (5, 6). The extensive degradation of chromosomal DNA into nucleosomal units (that can be seen as the DNA ladder in electrophoresis) is a biochemical hallmark of apoptosis (5), even though such a DNA ladder is not observed in some forms of apoptosis (7).

A protease family called caspase plays a central role in regulating and executing apoptosis, as caspases can degrade many cellular proteins leading to characteristic morphological and biochemical changes (8). For example, activated caspase 6 can degrade lamins, the major protein subunits of the nuclear envelop, thus resulting in chromatin condensation and margination (9, 10). Activated caspase 3 can cleave ICAD/DFF45, an inhibitor of CAD/DFF40, thus allowing the latter to cause DNA fragmentation (11–13). The homologues of this DNase/inhibitor pair are known to exist in human, mouse, Drosophila, and Xenopus laevis (13, 14), suggesting a conserved and important biological function of this pair in evolution. Caspase can be regulated by phosphorylation. In human cells, the phosphorylation of procaspar 9 inhibited apoptosis (15), although this step is species-dependent (15–17). Also, the phosphorylation of death substrates can protect them from being degraded by activated caspase 3 (18). In addition, lithium chloride, an inhibitor of protein kinases, inhibited the phosphorylation (19, 20). On the other hand, apoptosis is known to be energy-dependent (21); without a proper energy source, cells will undergo necrosis, instead of apoptosis, in response to apoptotic signals (22).

Phosphocreatine has been used as an energy source to convert ADP to ATP in cell-free systems during studying the mechanism of nuclear assembly (23, 24). To study the mechanism of in vitro apoptosis, we have developed a cell-free system consisting of a mixture of mouse liver nuclei and X. laevis egg extracts (14, 25–27). This system duplicates the natural apoptotic process quite well, because cytochrome c can induce the mouse liver nuclei to undergo characteristic apoptotic changes; the DNase activities in this system correlate well with apoptosis, and the ICAD/DFF45-like proteins are involved in mediating apoptosis (14). Since apoptosis is an energy-dependent process, we investigated the effects of phosphocreatine on the cytochrome c-induced apoptosis in this cell-free system. To our surprise, we found that phosphocreatine accelerates nuclear changes; however, it effectively inhibits the formation of DNA ladders in a time-dependent manner. Although phosphocreatine can produce creatine, increase the concentration of ATP, and decrease the pH, these did not inhibit DNA fragmentation. Western blot revealed that although phosphocreatine can accelerate lamin degradation, it suppresses the degradation of the DFF45-like DNase inhibitor. Lithium chloride, a kinase inhibitor, can overcome this inhibition, thus overriding the phosphocreatine-induced inhibition of DNA degradation. These results indicate that phosphocreatine inhibits DNA fragmentation through its effects on the degradation of DFF45-like proteins.

MATERIALS AND METHODS

Preparation of Cytosol—The crude extracts of Xenopus eggs were prepared as described by Forbes et al. (23) with some modifications. Briefly, Xenopus eggs were dejellied and rinsed with egg extract buffer (50 mM HEPES-KOH, pH 7.4, 50 mM KCl, 2 mM MgCl2). After the crude extracts were ultra-speed centrifuged at 200,000 × g for 2 h (Hitachi 55p-72, RPS 50-II rotor), aprotinin and leupeptin were added at the final concentrations of 6 and 8 μg/ml, respectively. The cytosol was frozen in aliquots under liquid nitrogen.

Preparation of Nuclei—Mouse liver nuclei were prepared according to Ref. 28. In brief, minced mouse liver was homogenized in nuclear
buffer (250 mM sucrose, 10 mM HEPES-KOH, pH 7.4, 15 mM KCl, 2 mM MgCl₂, 5 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM β-mercaptoethanol, 2 mM cytochalasin B). After filtration through a layer of 200-mesh silk screen, the filtrate was added to 2 volumes of homogenization buffer containing 2.3 mM sucrose, mixed thoroughly, and centrifuged (Hitachi 55p-72, RPS 50–2 rotor, 4°C, 30 min, 124,000 × g). The sediment was re-suspended in nuclei stock solution (10 mM PIPES, 80 mM KCl, 20 mM NaCl, 250 mM sucrose, 5 mM EGTA, 0.5 mM spermidine, 0.2 mM spermine, 50% glycerol) at a concentration of 5 × 10⁵ nuclei/μl and stored in liquid nitrogen.

Assay of in Vitro Apoptosis—The reaction mixture, containing 50 μl of egg extract, approximately 1 × 10⁵ mouse liver nuclei, and cytochrome c at a final concentration of 2 μM, was incubated at 23°C for indicated time. The process of nuclei apoptosis in a cell-free system was observed under a fluorescence microscope (Leica DMRB) by placing 4-μl aliquots of the samples on a microscope slide and mixed with 1 μl of sample buffer containing 200 mM sucrose, 30% formaldehyde, and 0.1 mg/ml DAPI. To assay for DNA fragmentation, 10 volumes of Buffer D (100 mM Tris-Cl, pH 8.0, 5 mM EDTA, 0.2 M NaCl, 0.4% SDS, 0.2 mg/ml proteinase K) was added into each reaction and incubated at 37°C overnight. The DNA was prepared and loaded onto a 1.5% agarose gel for electrophoresis.

Western Blot—Samples were electrophoresed for lamin protein and ICAD/DFF45-like protein in 12 and 10% SDS-polyacrylamide gel electrophoresis, respectively, then transferred onto nitrocellulose membranes. After blocking with 5% bovine serum albumin in Tris-buffered saline-Tween buffer (0.5% Tween 20) at 37°C for 1 h, the membranes were probed with anti-lamins A and C (Serotec Corp.) or anti-DFF45 antibodies (kindly provided by Dr. Xiao Dong Wang, University of Texas Southwestern Medical Center), then reacted with alkaline phosphatase-conjugated secondary antibodies for detection in detection buffer.

Preparation of Samples of Transmission Electron Microscope—The samples were taken after incubation at 23°C for the indicated time, then fixed at 4°C for 1 h in a final concentration of 0.5% (v/v) glutaraldehyde. The sediments were collected at 500 × g for 3 min, then fixed at 4°C for 1 h in the final concentration of 1% OsO₄. After dehydration in a graded series of ethanol and acetone (15 min each) and embedding in Epon 812, the samples were cut into the silver grey or white sections using a Leica Ultracut R cutter. After staining with uranyl acetate and lead citrate, the sections with apoptotic nuclei were observed and photographed under a JEM-1010 transmission electron microscope.

Preparation of Samples of Scanning Electron Microscope—The fixation method of the samples was followed as described above. After dehydration in a graded series of ethanol, for the preparation of scanning electron microscope, the samples were treated in the graded series of mixed solution of ethanol and isomyl acetate (2:1, 1:1, 15 min each, then treated with pure isomyl acetate twice, 15 min each). Dried in CO₂ at a critical point, the samples of apoptotic nuclei were observed and pictures taken under an Amray 1910FE scanning electron microscope.

RESULTS

Effects of Phosphocreatine on DNA Fragmentation and Apoptotic Morphological Changes—To investigate the effects of an energy source on DNA fragmentation, we added various concentrations of phosphocreatine to our in vitro apoptosis-inducing system consisting of a mixture of cytosol, ~1 × 10⁵ nuclei, and cytochrome c at a final concentration of 2 μM. As a control, Ac-DEVD-CHO, the special inhibitor of caspase 3, was introduced into the cytosol at different final concentrations with cytochrome c. The results indicated that phosphocreatine at a final concentration of 0.1 mM partly, and 0.2 mM completely, inhibited the formation of DNA ladder (Fig. 1B, lane 1, 50 μM; lane 2, 0.1 mM; lane 3, 0.2 mM; or lane 4, without phosphocreatine. On Ac-DEV-D-CHO at different final concentrations was introduced into this apoptosis inducing system: lane 5, 0.5 μM; lane 6, 5 μM; lane 7, control, no Ac-DEV-D-CHO. After incubation at 23°C for 4 h, DNA was purified. The results suggested that phosphocreatine at a final concentration of 0.1 mM partly, and 0.2 mM completely, inhibited the formation of DNA fragmentation. In this egg extract apoptosis-inducing system, Ac-DEVD-CHO also inhibited the formation of DNA fragmentation. After phosphocreatine at a final concentration of 0.2 mM was introduced into this apoptosis-inducing system at different times and incubated at 23°C for 4 h with nuclei and cytochrome c at a final concentration of 2 μM, DNA was purified and electrophoresed. The results showed when phosphocreatine and cytochrome c were added into cytosol together, the formation of DNA fragmentation was inhibited (lane 1). After cytosol had been incubated with cytochrome c at 23°C for 10 min (lane 2) or 20 min (lane 3), the formation of DNA fragmentation was also inhibited by addition of phosphocreatine. If the addition of phosphocreatine was delayed for 40 min, the inhibition of DNA fragmentation was only partial (lane 4); a delay of 1 h resulted in no inhibition (lane 5). When nuclei were incubated with 2 μM cytochrome c alone in cytosol, the typical DNA ladder appeared (lane 6). Creatine at a final concentration of 0.2 mM with cytochrome c inhibited the formation of the DNA ladder (lane 7). This result indicated that the inhibition of phosphocreatine on the formation of DNA fragmentation was time-dependent, and it was not due to the formation of creatine.

We also examined the effects of phosphocreatine on apoptotic morphological changes. Although it took 30 min before the cytochrome c-induced nuclear changes became evident (not shown), phosphocreatine greatly accelerated this process so that chromatin condensation and margination became apparent within 10 min (Fig. 2b). Moreover, the nuclear changes became more pronounced than cytochrome c alone induced, showing the formation of not only numerous apoptotic-like bodies (Fig. 2, j and k), but also some “strings” released from the nuclei (Fig. 2, g and h), a feature not seen in the presence of cytochrome c alone. With cytochrome c alone, apoptotic nuclei underwent normal chromatin condensation and margination after incubation for 40 min (Fig. 2a), and the appearance of apoptotic-like bodies occurred after the nuclei were incubated for more than 2 h in this condition (not shown). When phosphocreatine was introduced into the apoptosis-inducing system with cytochrome c, the apoptotic morphological changes had no differences compared with what that of cytochrome c alone evoked (not shown).

To study the details of these special morphological changes, transmission and scanning electron microscopes were used. The results showed that phosphocreatine enhanced chromatin condensation, accumulation of nuclear membrane (Fig. 3, b–e), formation of strings extending from nuclear surface (Fig. 4), can prevent DNA fragmentation in Xenopus egg extract cell-free system, and that this inhibition is time-dependent. The effect of phosphocreatine on DNA fragmentation is not due to the creatine.

FIG. 1. The effects of phosphocreatine on the formation of DNA fragmentation. A, cytochrome c at a final concentration of 2 μM transforms Xenopus laevis egg extract into an apoptosis-inducing system. Approximate 1 × 10⁵ nuclei and a different final concentration of phosphocreatine were added into 50-μl apoptotic extracts: lane 1, 50 μM; lane 2, 0.1 mM; lane 3, 0.2 mM; or lane 4, without phosphocreatine. On Ac-DEV-D-CHO at different final concentrations was introduced into this apoptosis system: lane 5, 0.5 μM; lane 6, 5 μM; lane 7, control, no Ac-DEV-D-CHO. After incubation at 23°C for 4 h, DNA was purified. The results suggested that phosphocreatine at a final concentration of 0.1 mM partly, and 0.2 mM completely, inhibited the formation of DNA fragmentation. In this egg extract apoptosis-inducing system, Ac-DEVD-CHO also inhibited the formation of DNA fragmentation. B, after phosphocreatine at a final concentration of 0.2 mM was introduced into this apoptosis-inducing system at different times and incubated at 23°C for 4 h with nuclei and cytochrome c at a final concentration of 2 μM, DNA was purified and electrophoresed. The results showed when phosphocreatine and cytochrome c were added into cytosol together, the formation of DNA fragmentation was inhibited (lane 1). After cytosol had been incubated with cytochrome c at 23°C for 10 min (lane 2) or 20 min (lane 3), the formation of DNA fragmentation was also inhibited by addition of phosphocreatine. If the addition of phosphocreatine was delayed for 40 min, the inhibition of DNA fragmentation was only partial (lane 4); a delay of 1 h resulted in no inhibition (lane 5). When nuclei were incubated with 2 μM cytochrome c alone in cytosol, the typical DNA ladder appeared (lane 6). Creatine at a final concentration of 0.2 mM with cytochrome c inhibited the formation of the DNA ladder (lane 7). This result indicated that the inhibition of phosphocreatine on the formation of DNA fragmentation was time-dependent, and it was not due to the formation of creatine.

The abbreviations used are: PIPES, 1,4-piperazinedietanesulfonic acid; DAPI, 4,6-diamidino-2-phenylindole; Ac-DEVD-CHO, N-acetyl-Asp-Glu-Val-Aasp-CHO (aldehyde).
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Approximately $1 \times 10^5$ nuclei were added into 50 µl of cytosol with 2 µM cytochrome c and 0.2 mM phosphocreatine. Aliquots of nuclei were taken out at different times, dyed with DAPI, and examined under fluorescence microscopy. After incubation for 10 min, the chromatin condensation and margination appeared in nuclei; c and d, after incubation for 20 min, the chromatin condensation and margination in nuclei become clearer; e and f, after 30 min, more intensive condensation of chromatin occurred in the nuclei. g and h, after 40 min, there were strings released from the nucleus (arrows). After 1 h (i) or 1.5 h (j and k), a great number of apoptotic-like bodies were visible. In cytosol with cytochrome c alone, chromatin condensation and margination appeared later (a, after incubation for 40 min), and the strings were never seen. In cytosol with phosphocreatine alone, the nuclei did not undergo chromatin condensation and margination after incubation for 2 h (not shown). This result indicated that phosphocreatine enhanced the apoptotic morphological changes, which were induced by cytochrome c. Bar = 2 µm.

Effects of Phosphocreatine on the Proteolysis of Lamins A and C—In a living cell, lamina is composed of three kinds of lamins under the nuclear membrane. It forms a fiber network that underlies the normal conformation of the nucleus (29). Lamins play an important role in the morphological changes of apoptotic cells. The proteolysis of lamin proteins, initialized by the activated caspase 6, results in morphological changes in the process of apoptosis (9, 10).

To investigate the regulation of lamin degradation, we examined the degradation of lamins A and C as triggered by cytochrome c and its possible regulation by phosphocreatine. Western blot results showed that phosphocreatine accelerated the cytochrome c-induced proteolysis of lamins A and C (Fig. 5). This might contribute to the accelerated morphological changes in apoptotic nuclei, which occurred after phosphocreatine was added into cytosol with cytochrome c.

Effects of ATP on Apoptosis in Cytosol—Considering that phosphocreatine can convert ADP to ATP, we then examined the possibility that phosphocreatine exerts its effects by increasing the ATP content of the cell. Thus we tested the effects of ATP (1 mM initial concentration with additional fresh 0.5 mM concentration added every 10 min) on DNA fragmentation and morphological changes. We found that ATP enhanced the apoptotic morphological changes, which were induced by cytochrome c (Fig. 6A). However, ATP did not affect cytochrome c-induced DNA fragmentation (Fig. 6B). These results indicated that an increased ATP content might contribute to the apoptotic morphological changes, but without affecting DNA fragmentation.

Effects of Acidity on the Formation of DNA Fragmentation—Phosphocreatine is acidic. To determine whether phosphocreatine inhibited DNA fragmentation by acidifying the cytosol, we tested the effects of several organic acids, such as citric acid, glycine, salicylic acid, and tartaric acid, at concentrations that generated a final cytosol pH identical to that produced by 0.2 mM phosphocreatine. None of these acids affected the cytochrome c-induced DNA fragmentation (Fig. 7), suggesting that the phosphocreatine inhibition of DNA fragmentation was not due to simple acidification.

LiCl Overrides the Effects of Phosphocreatine on DNA Fragmentation—Some of the caspases, such as caspase 9, are known to play a key role in regulating apoptosis, and the activities of caspase 9 are accelerated by LiCl (9, 22). To find out if LiCl can override the effects of phosphocreatine, we incubated nuclei with 0.5 mM phosphocreatine, 2.5 mM LiCl, and cytochrome c in cytosol. The results (Fig. 8) showed that LiCl nullified the inhibitory effect of phosphocreatine on caspase 9 activity. This might provide a link between the morphological changes and caspase 9 activation.

\[ \text{Phosphocreatine} + \text{Cytochrome c} + \text{Cytosol} \rightarrow \text{ATP} + \text{Cytosol} \]

and no or little membrane on the surface of apoptotic-like bodies (Fig. 3f). These results indicate that phosphocreatine can enhance apoptotic morphological changes of nuclei induced by cytochrome c.
A and C proteins became extreme (18). When incubation time was extended to 1.5 h, the proteolysis of lamins degraded. After incubation for 40 min (lane 5), lamins A and C proteins began to be degraded from 88- and 64-kDa to 44- and 37-kDa segments. When incubation time was extended to 1.5 h, the proteolysis of lamins A and C proteins became extreme (lane 6). B, approximately $1 \times 10^8$ nuclei were added into 50 μl of cytosol with 2 μM cytochrome c alone. After incubation at 23 °C for a determined time, samples were taken. Using anti-lamins A and C antibody, the samples were examined by Western blot. The results suggested that after incubation for 0 min (lane 1), 5 min (lane 2), 10 min (lane 3), and 20 min (lane 4), the lamins A and C proteins were not degraded. After incubation for 40 min (lane 5), lamins A and C proteins began to be degraded from 88- and 64-kDa to 44- and 37-kDa segments. After incubation for 10 min, however, the lamins began to be degraded (18). Phosphocreatine created a phosphorylation of certain proteins. To see whether phosphocreatine accelerated the proteolysis of lamin proteins that were aroused after cytochrome c was introduced into cytosol.

such caspases can be regulated by phosphorylation (15–17). At the same time, phosphorylation of caspase substrates also regulated their degradation (18). Phosphocreatine created a phosphate radical in the presence of kinase, which might lead to phosphorylation of certain proteins. To see whether phosphocreatine inhibited DNA fragmentation by promoting the phosphorylation of some of the key components in the apoptotic pathways, we studied the effects of LiCl, a general kinase inhibitor. We found that LiCl at a final concentration of 2 mM can completely restore cytochrome c-induced DNA fragmentation that was inhibited by phosphocreatine (Fig. 8A). Control experiments showed that: 1) 2 mM LiCl alone did not induce DNA fragmentation (Fig. 8B), and 2) when added 40 min after the nuclei had been incubated with cytochrome c and phosphocreatine, 2 mM LiCl did not override the inhibition of phosphocreatine on DNA fragmentation. This result suggested that phosphocreatine inhibited DNA fragmentation through phosphorylation.

CAD/DF445 is the direct executor of DNA fragmentation. Normally, it is in complex with ICAD/DF445, which serves as an inhibitor; ICAD/DF445 was degraded during apoptosis, releasing CAD/DF445 to cleave DNA. We then sought to determine whether phosphocreatine prevented ICAD/DF445-like protein from being degraded to inhibit DNA fragmentation and whether this process was regulated by phosphorylation. Western blot showed that 0.2 mM phosphocreatine indeed inhibited the cytochrome c-induced degradation of ICAD/DF445-like protein (Fig. 9). Interestingly, this inhibition of DNA fragmentation, as well as nuclear morphological changes, was overridden by 2 mM LiCl (Figs. 9 and 10). LiCl at a final concentration of 2 mM alone did not induce the apoptotic morphological changes of the nuclei after incubated for 2 h in our cell-free system (not shown). These results indicated that phosphocreatine might phosphorylate ICAD/DF445-like protein to prevent it from degradation by caspase 3, resulting in the inhibition of DNA fragmentation.

**DISCUSSION**

Cytochrome c is liberated from mitochondria during the initiation of apoptosis and binds to Apaf-1 in cytosol, which needs dATP or ATP's cooperation to alter the transformation of Apaf-1, then Apaf-1 recruits procaspase 9 and results in its self-activation (30, 31). Activated caspase 9 in turn cleaves and activates procaspase to cleaves its substrates, such as ICAD/DF445 and PARP (32). Caspase 3 also activates caspase 6 to cleaves its substrate, lamin proteins, which contributes to the morphological changes in apoptosis (9, 10).

Upon activation of apoptosis, ICAD/DF445 is cleaved by caspase-3, liberating CAD/DF440 from the DFF40/45(CAD/ICAD) complex, then cleaves DNA into oligonucleosomal size fragments, showing a DNA ladder in electrophoresis (11–13). Previous studies have demonstrated that the DFF40/45(CAD)/ICAD-like protein also exists in Xenopus egg extracts and can be activated in apoptosis induced by cytochrome c (14). We have been working on the apoptotic mechanism in this Xenopus egg extract cell-free system (14, 25–27), and the results prove that it is a good in vitro system for studying apoptosis. Nutrient-rich materials are stored in Xenopus eggs to maintain the cell proliferation after fertilization, among which dATP is abundant, so no additional dATP is required to induce apoptosis in the system.

In this study, we demonstrated that introduction of phosphocreatine into the Xenopus egg extract cell-free system inhibited the formation of DNA fragmentation induced by exogenous cytochrome c. Since addition of phosphocreatine did not affect the formation of DNA fragmentation after the cytochrome c-induced apoptosis went on for more than 40 min (Fig. 1B), this inhibition was time-dependent. We had thought phosphocreatine would inhibit the apoptotic morphological changes of nu-
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Fig. 7. Other kinds of organic acid did not inhibit the formation of DNA fragmentation. Nuclei were incubated in cytosol with different kinds of organic acid and 2 μM cytochrome c to examine the acidic effect on the formation of DNA fragmentation. Different kinds of organic acids at definite final concentrations were added into cytosol to generate a final cytosol pH identical to that produced by phosphocreatine at a final concentration of 0.2 mM. Then nuclei were incubated in cytosol at 23 °C for 4 h. After that, DNA was purified for electrophoresis. Lane 1, citric acid at 0.223 mM; lane 2, glycine at 0.118 mM; lane 3, salicylic acid at 0.186 mM; lane 4, tartaric acid at 0.252 mM; lane 5, phosphocreatine at 0.2 mM; lane 6, without any organic acid. The results showed that other organic acids did not inhibit the formation of DNA fragmentation except phosphocreatine and suggested that the inhibition of DNA fragmentation was not due to the acidic character of phosphocreatine.

Fig. 8. LiCl overrides the effects of phosphocreatine on DNA fragmentation. A, nuclei were added into cytosol with 2 μM cytochrome c, 0.2 mM phosphocreatine and LiCl at final concentrations of 0.2 mM (lane 3), 0.5 mM (lane 4), 1 mM (lane 5), 2 mM (lane 6), 4 mM (lane 7), and 10 mM (lane 8), in cytosol with 2 μM cytochrome c alone (lane 1), or in cytosol with 2 μM cytochrome c and 0.2 mM phosphocreatine (lane 2). After incubation at 23 °C for 4 h, chromatin DNA was analyzed. It demonstrated that 2 mM LiCl overrides the inhibition of phosphocreatine on DNA fragmentation and makes the DNA fragmentation appear again. B, after the apoptotic mixture, including nuclei, 2 μM cytochrome c, and 0.2 mM phosphocreatine, was incubated at 23 °C for different time, LiCl at a final concentration of 2 mM was introduced: lane 2, 0 min; lane 3, 5 min; lane 4, 10 min; lane 5, 20 min; lane 6, 40 min; or lane 7, 1 h or nuclei were incubated in cytosol with 2 μM cytochrome c and 0.2 mM phosphocreatine (lane 1) or with 2 mM LiCl alone (lane 8). After incubation at 23 °C for 4 h, chromatin DNA was analyzed. The results showed that when cytosol was incubated with phosphocreatine and cytochrome c for more than 40 min, the introduction of LiCl into the mixture did not make DNA fragmentation appear again, and 2 mM LiCl alone did not induce the DNA fragmentation.

Fig. 9. Effects of phosphocreatine and LiCl on the proteolysis of DFF45-like protein. After the cytosol with nuclei and 2 μM cytochrome c was incubated at 23 °C for 0 min (lane 3), 10 min (lane 4), 20 min (lane 5), or 40 min (lane 6), 0.2 mM phosphocreatine was added, or nuclei were incubated in cytosol with 0.2 mM phosphocreatine alone (lane 1), with 2 μM cytochrome c alone (lane 2), with 2 mM phosphocreatine, 2 μM cytochrome c, and 2 mM LiCl (lane 7), or with 2 mM LiCl alone (lane 8). After incubation at 23 °C for 4 h, samples were taken and examined by Western blot using anti-DFF45 antibody. The results suggested that phosphocreatine prevented the proteolysis of ICAD/DFF45-like protein, which was induced by cytochrome c; LiCl overrides the effect of phosphocreatine on ICAD/DFF45-like protein. It was suggested that the inhibition of DNA fragmentation by phosphocreatine might be through phosphorylation on ICAD/DFF45-like protein.

Fig. 10. LiCl resists the effects of phosphocreatine on apoptotic morphological changes. Approximately 1 × 10⁵ nuclei were added into 50 μl of cytosol with 2 μM cytochrome c, 0.2 mM phosphocreatine, and 2 mM LiCl. After incubation at 23 °C for 40 min, an aliquot of nuclei was taken out and dyed with DAPI, then examined under fluorescence microscope. The picture showed that normal apoptotic morphological changes were recovered compared with Fig. 2, a, g, and h. Bar = 4 μm.

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There were(a) or some factor(s) associated with DNA fragmentation activated by cytochrome c, and it(they) was(were) inhibited by the addition of phosphocreatine before activation; once it(they) was/were activated, the phosphocreatine did not affect it/them any more, and inhibition of DNA fragmentation was removed.

Meanwhile, we studied the degradation of lamins, which contributes to the morphological changes of nuclei in the apoptotic process (9, 10). The result showed that phosphocreatine did not inhibit the lamins degradation induced by cytochrome c, but accelerated this process (Fig. 5, A and B). This just explained why morphological changes were hastened and enhanced. It also implied that the activation of both caspase 3 and caspase 6, the executioners of lamin degradation, were not inhibited.

Phosphocreatine is hydrolyzed into creatine, releasing a phosphate radical, which can be transferred to ADP to form ATP (30); being acidic, phosphocreatine can decrease the pH of the system. Are these factors responsible for the effect of phosphocreatine on the apoptosis induced by cytochrome c? Our studies showed that creatine, just as the acidic character of phosphocreatine, did not influence cytochrome c-induced apoptotic process (Figs. 1B and 7), and increase of the ATP level enhanced the apoptotic morphological changes (Fig. 6A), but did not inhibit DNA fragmentation (Fig. 6B). Since many substances were stored in egg extracts, including dATP, the amount stored was enough to induce apoptosis. So, we did not need to add additional dATP with cytochrome c to induce apoptosis (14, 25–27), and when additional ATP was added, dATP content was augmented, which may increase the caspases activities and accelerate the degradation of lamins,
resulting in the enhancement of apoptotic morphological changes. This might explain why phosphocreatine enhanced the morphological changes. These results excluded the possibility that creatine and ATP production and pH reduction by phosphocreatine contribute to DNA fragmentation inhibition.

As mentioned above, inhibition of DNA fragmentation by phosphocreatine was time-dependent (Fig. 1B), suggesting that factor(s) resulting in DNA fragmentation had been activated when cytochrome c induction went on for a period of time, then addition of phosphocreatine did not affect it (them) again. So, phosphocreatine must affect it (them) before its (their) activation. CAD/DF40 is the direct executor of DNA fragmentation during apoptosis, whereas ICAD/DF45 serves as its inhibitor (11–13). If ICAD/DF45 is not degraded by activated caspase 3, the chromatin DNA will not be cleaved by CAD/DF40 at internucleosomal sites. Western blot showed that phosphocreatine inhibited the degradation of ICAD/DF45-like proteins during cytochrome c-induced apoptosis, and the inhibition was also time-dependent (Fig. 9). After cytochrome c-induced apoptosis progressed for more than 40 min, phosphocreatine contributed to DNA fragmentation inhibition.

Are the upstream factors in apoptosis pathway affected by phosphocreatine, which results in inhibition of ICAD/DF45-like protein degradation? It is now known that caspase 9 and DFF45-like protein in cytosol might be phosphorylated and was not degraded by caspase 3. Interestingly, LiCl made the morphological changes recover themselves compared with the apoptotic nuclei induced by cytochrome c alone (Fig. 10). It is suggested that LiCl overrides the effects of phosphocreatine not only on DFF45-like protein but also on the morphological changes.

In conclusion, these studies show that phosphocreatine has a special effect on apoptosis induced by cytochrome c in Xenopus egg extracts. It can accelerate the apoptotic morphological changes and inhibit the formation of DNA fragmentation. The increase of ATP content in cytosol aroused by phosphocreatine may contribute to the morphological changes, and phosphocreatine protects ICAD/DF45-like protein from proteolysis, probably through kinase actions, resulting in its resistance to caspase cleavage and leading to the inhibition of DNA fragmentation.

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