Spermine triggers the activation of caspase-3 in a cell-free model of apoptosis

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Abstract Polyamines are ubiquitous organic cations required for cell proliferation. However, some evidence suggested that their excessive accumulation can induce apoptosis. We show here that, in a post-nuclear extract from U937 cells, the addition of spermine triggers the death program, represented by cytochrome *c* exit from mitochondria, the dATP-dependent processing of pro-caspase-3 and the onset of caspase activity. Spermine is more effective than spermidine, whereas putrescine has no effect. Polyamine acetylation abolishes their pro-apoptotic power. These data demonstrate a direct mechanism responsible for polyamine toxicity and also suggest that an excessive elevation of free polyamines could be involved in the transduction of a death signal.

Key words: Apoptosis; Caspase; Cytochrome *c*; Polyamine; Spermine

1. Introduction

The polyamines spermine, spermidine and putrescine are common components of all eukaryotic cells and are absolutely required for cell growth [1–3]. Despite their abundance within the cell, polyamine levels are tightly regulated. In fact, polyamine accumulation results in cytotoxicity [4,5] and several data have suggested that an excessive level of polyamines may be coupled to the induction of apoptosis [6–14].

Activation of caspase proteases represents a fundamental point in the induction of apoptosis [15] and mitochondria play a central role in this process since the release of an apoptogenic factor, like cytochrome *c*, into the cytosol is a crucial step for caspase activation [16–18]. We reported that exposure of leukemia cells to spermine causes caspase activation and leakage of cytochrome *c* into the cytosol [14]. In order to study the mechanism of spermine action, we tested the hypothesis that polyamines could directly affect these processes. In the present report, we used a cell-free model to study some intermediate stages of apoptosis. In this model, consisting of a post-nuclear extract, the addition of exogenous spermine directly triggers the activation of the cell death program.

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Abbreviations: Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-amido-4-methylcoumarin

2. Materials and methods

2.1. Materials and cells

Spermine and other polyamines as well as acetyl-Asp-Glu-Val-Asp-amido-4-methylcoumarin (Ac-DEVD-AMC), dATP and all other biochemicals were products of Sigma. Monoclonal antibodies against caspase-3 were from Transduction Laboratories and anti-cytochrome *c* was obtained from Pharmingen. U937 human leukemia cells were set up at 4×10^5 cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum and 100 U/ml of both penicillin and streptomycin.

2.2. Cell-free models

Cell-free extracts for in vitro caspase activation were prepared from U937 cells. Two extracts were used, a post-nuclear fraction containing mitochondria (S-3) and a cytosolic soluble fraction (S-100). The first extract was substantially similar to that obtained by Ellerby et al. from neuronal cells [19] and contains mitochondria along with pieces of plasma membranes and other light membranes, but not whole cells or nuclei. To prepare this extract, 10^8 cells were suspended in 0.4 ml CFS buffer [19,20], left 10 min in ice and then gently lysed in a glass-teflon Potter-Elvehjem homogenizer. Cell lysis was confirmed by light microscopy. The homogenate was centrifugated for 5 min at $1000\times g$ to remove whole cells and nuclei. The supernatant was re-centrifuged for 5 min at $3000\times g$, obtaining the S-3 fraction. To obtain the cytosolic S-100 fraction, the S-3 extract was centrifuged for 15 min at $16\,000\times g$ and the supernatant finally centrifuged for 30 min at $100\,000\times g$. Generally, S-3 extracts contained 12–15 mg of protein/ml, that was reduced by 40% in the S-100 fraction.

In order to trigger caspase activation, aliquots of 25 μl extract were incubated at 30°C in a final volume of 40 μl along with 1 mM $MgCl_2$ and 1 mM dATP. In some experiments, 100 ng of cytochrome *c* was added. At the end of the incubation, samples were centrifugated for 15 min at $16\,000\times g$. An aliquot of 4 μl of the supernatant in duplicate was used for the assay of caspase activity as described above, while the remainder was added to 8 μl of concentrated loading buffer and boiled. Aliquots of 20 μl were subjected to Western blotting analysis. To measure the polyamine content in the extracts, 10 μl of sample was diluted in 0.3 ml 0.3 M perchloric acid and analyzed by HPLC as described [21], except that a linear gradient from 35 to 75% of solvent B over 40 min was applied. The mean concentration of endogenous polyamines in S-3 extracts was approximately 10 μM putrescine, 20 μM spermidine and 30 μM spermine.

Isolated mitochondria were prepared from rat heart [22]. In order to detect cytochrome *c* leakage, 0.1 mg of mitochondria was suspended in 50 μl of a medium consisting of 0.25 M sucrose, 10 mM HEPES, pH 7.4, 2 μM rotenone and 20 mM succinate and incubated 15 min at room temperature in the absence or presence of spermine. Mitochondria were then pelleted by spun centrifugation and 20 μl of the supernatant was analyzed by Western blotting.

2.3. Determination of caspase activity

The activity of caspase enzymes was measured by the cleavage of the fluorogenic peptide substrate Ac-DEVD-AMC, that represents a substrate for caspase-3 and other members of the caspase family. A sample of 4 μl obtained from in vitro incubations was assayed in a final volume of 30 μl during a 15 min incubation at 37°C. A detailed description of the caspase assay is given elsewhere [23]. One unit is defined as the amount of enzyme activity cleaving 1.0 nmol of substrate per minute. The activity is expressed as U/mg cytosolic protein.

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2.4. Western blotting

Aliquots of each sample were analyzed by SDS-PAGE on a polyacrylamide gel (12% for caspase-3 or 15% for cytochrome *c*). Standard protein markers were used for molecular weight calibration. After blotting on a nitrocellulose membrane, this was blocked with 5% non-fat milk, washed with Tris-buffered saline and probed for 30 min with specific primary antibody. After a further washing, the membrane was incubated for 30 min with the secondary antibody (horseradish peroxidase-conjugated anti-mouse IgG). After washing, immunoreactive bands were visualized with an enhanced chemoluminescence kit (Amersham).

3. Results

The reproduction of key events of apoptosis in cell-free models has proved a very useful approach for the understanding of molecular mechanisms involved in cell death. It is established that apoptosis may be initiated in vitro by the addition to cytosolic extracts of cytochrome *c* together with an adenylic nucleotide triphosphate (dATP or ATP), that results in the formation of the apoptosome leading to activation of the initiator caspase-9, that triggers the proteolytic activation of pro-caspase-3 into the active caspase-3, the best characterized of the effector caspases [16,24,25].

Spermine accumulation triggers caspase activation in whole cells [14]. To test the hypothesis that spermine could directly activate the death program, a cell-free extract containing mitochondria was prepared from U937 cells. This simple model consists of a post-nuclear extract obtained at 3000×*g* (S-3) and is similar to that utilized by Ellerby et al. [19]. Addition of spermine to the S-3 extract triggered the dATP-dependent induction of caspase activity, that progressively increased for at least 3 h (Fig. 1A). A low degree of caspase activation was also caused by dATP alone and was probably due to the presence of small amounts of cytochrome *c* in the preparations (see below). In the absence of dATP, spermine did not affect the caspase activity at all. The increase in caspase activity triggered by spermine in dATP-primed extracts was accompanied by the processing of the p32 pro-caspase-3 and the p20 cleaved fragment became detectable after 1 h and increased thereafter (Fig. 1B).

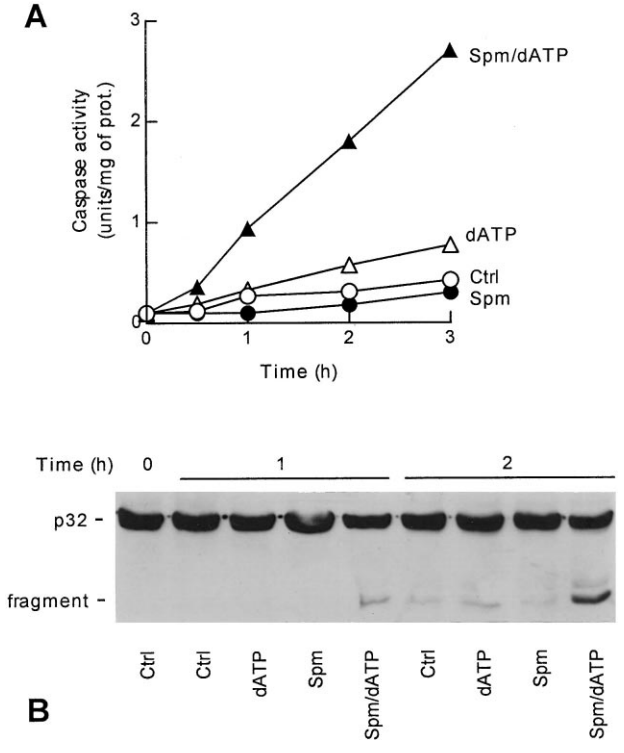

Fig. 1. Spermine causes the dATP-dependent caspase activation in a cell-free extract. A: Time course of caspase activation in post-nuclear S-3 extract. The extract was incubated without any treatment (Ctrl) or in the presence of 1 mM dATP (dATP) or 1 mM spermine (Spm) or 1 mM spermine plus 1 mM dATP (Spm/dATP). The caspase activity was assayed at the indicated time. B: The processing of the p32 pro-caspase-3 was determined at the beginning of the experiment and after 1 and 2 h of incubation. The data reported in the figure are representative of at least three experiments.

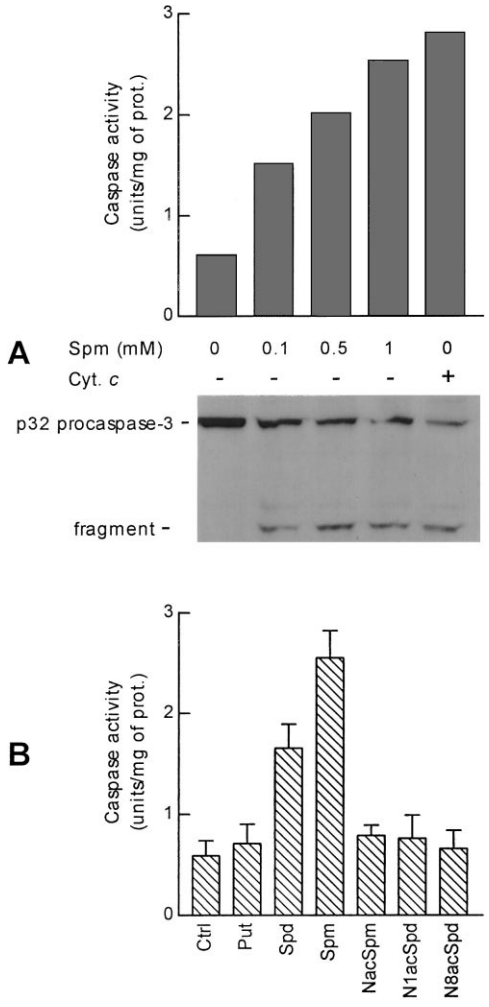

Fig. 2. Caspase activation by polyamines in S-3 extracts. A: Caspase activity (up) and the processing of the p32 precursor of caspase-3 (down) were determined as described in Setion 2 in aliquots of S-3, incubated 3 h in the presence of the indicated concentration of spermine (Spm) or with 100 ng of cytochrome *c* (Cyt.*c*). This experiment was performed twice with similar results. B: S-3 extracts were incubated 3 h without polyamines (Ctrl) or in the presence of a 1 mM concentration of putrescine (Put), spermidine (Spd), spermine (Spm), N^1-acetylspermine (NacSpm), N^1-acetylspermidine (N1acSpd), N^8-acetylspermidine (N8acSpd). The data are means ± S.E.M. of three determinations. dATP (1 mM) was always present in all incubations.

The effect of spermine on caspase-3 activation as well as the onset of caspase activity in S-3 extracts was well evident when a 100 μM concentration of the polyamine was added and increased thereafter in a dose-dependent manner, mimicking

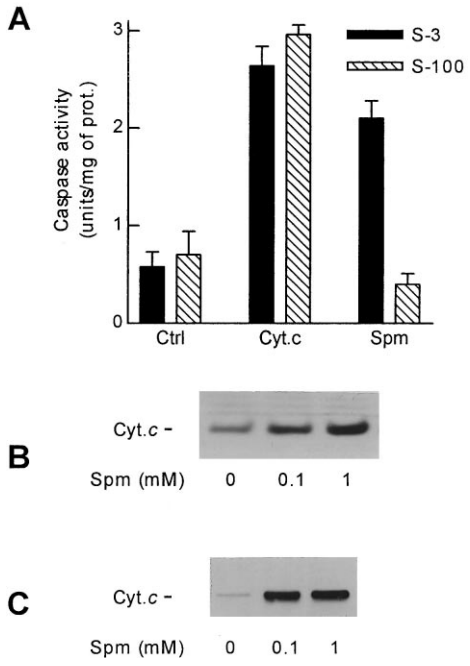

Fig. 3. Spermine activation of the death program in a cell-free model requires mitochondria. A: The ability of spermine or cytochrome *c* to activate caspase enzymes was compared in post-nuclear (S-3) and cytosolic (S-100) fractions obtained from U937 cells. The extracts were incubated for 2 h in the presence of 1 mM dATP without any additional treatment (Ctrl) or with 100 ng of cytochrome *c* (Cyt.*c*) or 1 mM spermine (Spm), then, the caspase activity was assayed. In the absence of dATP, there was no significant activation in any sample. Data are means ± S.E.M. of three determinations. B: Aliquots of S-3 extract were incubated 15 min in the presence of the indicated concentration of spermine (Spm). To measure the release of cytochrome *c* from mitochondria, the samples were centrifugated and cytochrome *c* was assayed in the supernatant by Western blotting. C: Rat heart mitochondria (0.1 mg) were incubated 15 min in the presence of the indicated concentration of spermine. Afterwards, mitochondria were pelleted and the supernatant was analyzed for the cytochrome *c* content.

the effect of added cytochrome *c* (Fig. 2A). Note that the effective concentration of spermine was slightly higher than that indicated, because the non-particulate part of S-3 contained about 30 μM of endogenous spermine. Fig. 2B shows that spermidine was less effective than spermine in the induction of caspase activation, whereas putrescine was completely ineffective. Acetylation of polyamines inhibited their ability to activate caspases. Caspase activation by spermine in S-3 extracts was not counteracted by 1 mM of amine oxidase inhibitor aminoguanidine or 10 mM of the antioxidant *N*-acetylcysteine (data not shown).

In order to determine whether caspase activation by spermine in S-3 samples was dependent on the particulate fraction of the extract, the effect of the polyamine was also determined in the cytosolic S-100 fraction obtained by centrifugation at $100\,000 \times g$ of S-3. Fig. 3A shows that spermine did not have any effect on the caspase activity in dATP-primed S-100 extracts, where caspase activation was triggered by cytochrome *c*, as expected. Instead, in mitochondria containing S-3 extracts, spermine could substitute cytochrome *c* and triggered caspase activation. This finding suggested that in the S-3 extract, spermine could render cytochrome *c* available for caspase activation. Actually, Fig. 3B shows that spermine induced the leakage of cytochrome *c* from mitochondria in S-3 extracts. The spermine concentration required to trigger cytochrome *c* release mirrored the dose-dependence seen for caspase activation. The translocation of cytochrome *c* in spermine-treated extracts was similar in both the presence or absence of 1 mM dATP (not shown). A detectable amount of cytochrome *c* was also present in control samples incubated without added spermine. Probably, this contamination was caused by breakage of mitochondria during the preparation of the extract. Fig. 3C shows that spermine, in the concentration range used in the above-described experiments, was also able to induce the release of cytochrome *c* from isolated heart mitochondria.

4. Discussion

There is increasing evidence that, at least in some cells, excessive polyamines may induce the activation of programmed cell death [6–14]. Polyamine oxidation has frequently been implicated in their cytotoxicity, but it is evident that high polyamine levels are toxic per se [5,8,14]. The present data furnish a rationale for these findings. We have shown here that spermine is able to directly activate the death program in a cell-free model containing mitochondria, mimicking its effect in whole cells [14]. At present, we can only speculate about the mechanism used by polyamines to activate apoptosis in vitro. In the post-nuclear S-3 extract from U937 cells, addition of spermine triggers cytochrome *c* exit from mitochondria and the dATP-dependent activation of caspase-3, the best characterized of effector caspases. Since the addition of exogenous cytochrome *c* to the same extract triggers caspase activation, it seems reasonable to think that spermine directly activates the cytochrome *c* pathway of apoptosis by inducing its cytosolic re-localization. In support of this view, we have found that spermine is able to induce cytochrome *c* leakage from isolated heart mitochondria. However, it cannot be ruled out that spermine also causes the release (or the activation) of an apoptogenic molecule(s) other than cytochrome *c* [17,18,26] from mitochondria or from another particulated part of the extract.

Spermine is more active than spermidine, whereas putrescine is completely ineffective in causing caspase activation in S-3 extracts as well as in whole cells [14]. Furthermore, polyamine acetylation abolishes their pro-apoptotic activity in vitro. These data fit well within the hypothesis that polyamine acetylation represents a mean to avoid toxicity by an excessive level of free polyamines [4].

The ability of spermine to trigger caspase activation can explain the mechanism that underlies its known toxic effect. However, it can also suggest a physiological role for polyamines, spermine in particular, in the transduction of a death message. Polyamines are very ancient molecules found in all living organisms and necessary for entry and progression of the cell cycle [1–3]. Among polyamines however, spermine is ubiquitous only in eukaryotes and its function has been questioned [1,3]. It is generally assumed that, because of its four positive charges, spermine is predominantly bound to cellular components and its free concentration in the cell is very low despite the high cellular content of the polyamine [3,27–29]. Thus, spermine has the characteristics of a damage-sensing molecule, since its free concentration may rapidly and largely increase following insults to nucleic acids, membranes or other

storage sites. This increase would be proportional to the extent of the damage and could transduce a death signal to the mitochondrion. However, the principal appeal of a polyamine role in apoptosis lies in their link with the cell growth that could help to explain one of the most intriguing questions in apoptosis research, i.e. the strict correlation between cell proliferation and cell death [30,31]. Polyamine levels are markedly increased in tumor cells and oncogenic transformation is constantly coupled to an increased polyamine biosynthesis [1–3]. On the other hand, paradoxically, oncogenic changes also render tumors more susceptible to apoptosis. Cells with deregulated oncogenes appear primed for apoptosis, suggesting that cell proliferative and apoptotic pathways are coupled [31]. Polyamines could represent a part of this connection.

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