Polyamines directly induce release of cytochrome *c* from heart mitochondria

Claudio STEFANELLI¹, Ivana STANIC^{*}, Maddalena ZINI, Francesca BONAVITA, Flavio FLAMIGNI, Laura ZAMBONIN, Laura LANDI, Carla PIGNATTI, Carlo GUARNIERI and Claudio M. CALDARERA

Dipartimento di Biochimica 'G. Moruzzi', Università di Bologna, Via Irnerio, 48. I-40126 Bologna, Italy

Cytochrome *c* release from mitochondria to the cytosol represents a critical step in apoptosis, correlated to the activation of the caspase cascade. In this report, we show that addition of micromolar concentrations of polyamines to isolated rat heart mitochondria induces the release of cytochrome *c*. Spermine, which is effective at concentrations of 10–100 μ M, is more potent than spermidine, whereas putrescine has no effect up to 1 mM. The release of cytochrome *c* caused by spermine is a rapid, saturable and selective process that is independent of mitochondria damage. Spermine, unlike polylysine, is able to release a discrete amount of cytochrome *c* from intact, functional mitochondria. The cytochrome *c*-releasing power of spermine is

not affected by cyclosporin A, differently from the effect of permeability transition inducers. In a cardiac cell-free model of apoptosis, the latent caspase activity of cytosolic extracts from cardiomyocytes could be activated by cytochrome *c* released from spermine-treated heart mitochondria. These data indicate a novel mechanism of cytochrome *c* release from the mitochondrion, and suggest that prolonged and sustained elevation of polyamines, characteristic of some pathologies such as heart hypertrophy, could be involved in the development of apoptosis.

Key words: apoptosis, caspase, cyclosporin A, spermine.

INTRODUCTION

The polyamines spermidine and spermine are synthesized in all eukaryotic cells from the diamine putrescine which, in turn, derives from ornithine by the action of ornithine decarboxylase. Much is known about the biosynthesis, degradation and transport of polyamines [1–3], but despite several decades of extensive research, their function in cellular physiology remains unsettled, and the only established function of polyamines is their absolute requirement for cell growth [1,2]. In the heart, polyamine metabolism is affected strongly by numerous factors, both physiological and pathological, that influence cardiac function and growth [4]. An increase in heart polyamines is correlated, in particular, with hypertension [5], and, in general, with cardiac hypertrophy [4,6].

Even if polyamines are necessary for growth processes, their excessive accumulation triggers apoptosis [7–11]. Activation of caspases represents a fundamental point in apoptosis [12] and mitochondria play a central role in this process [13], since the release of an apoptogenic factor, like cytochrome *c*, into the cytosol is a crucial step for caspase activation [14].

The mechanism by which cytochrome *c* is released from mitochondria during apoptosis is poorly understood. *In vitro*, the induction of the mitochondrial permeability transition (MPT) causes the permeabilization of the outer mitochondrial membrane and the release of several solutes including cytochrome *c* [15–17]. However, cytochrome *c* leakage in apoptotic cells is evident before mitochondria damage [18]. Spermine causes the leakage of cytochrome *c* into the cytosol both in whole cells [11] and in cell extracts containing mitochondria along with cytosol [19]. In order to study the mechanism of spermine action, we tested the hypothesis that spermine could directly affect the translocation of cytochrome *c*. The present paper reports that polyamines can

promote cytochrome *c* exit from cardiac mitochondria. In addition, a cardiac cell-free model of apoptosis is described consisting of a cytosolic extract from embryonal cardiomyocytes, in which caspase activity can be triggered by the cytochrome *c* released from mitochondria by spermine.

MATERIALS AND METHODS

Materials

Spermine and other polyamines, horse heart cytochrome *c*, poly-L-lysine (molecular mass 1–4 kDa) and other biochemicals were products of Sigma. Monoclonal antibody against cytochrome *c* was obtained from Pharmingen.

Preparation of cardiac mitochondria

Mitochondria were prepared from the hearts of female Wistar rats weighing about 350 g by standard differential centrifugation followed by Percoll purification by implementation of the method used in our laboratory. Briefly, the hearts were homogenized gently with an Ultra Turrax apparatus in 3 vols (w/v) of 0.5% BSA containing 180 mM KCl and 10 mM EDTA (pH 7.2). The homogenate was centrifuged for 5 min at 1000 *g*, and the supernatant was filtered and centrifuged for 10 min at 8000 *g*. The pellet was resuspended in 0.5% BSA containing 180 mM KCl and 1 mM EDTA (pH 7.2) and centrifuged at 5000 *g* for 5 min. The final pellet was gently resuspended in 0.5 ml of mitochondria incubation buffer (MIB) consisting of 0.25 M sucrose, 1 mM KH_2PO_4 and 10 mM Hepes (pH 7.4). This crude mitochondria preparation was diluted to a concentration of 10 mg of protein/ml, as determined by Coomassie Brilliant Blue staining using BSA as a standard, and was used as such.

Abbreviations used: CEHC, chick embryo heart cells; CsA, cyclosporin A; MIB, mitochondria incubation buffer; MPT, mitochondrial permeability transition; P/O ratio, ratio of ATP synthesized/ O_2 consumed.

¹ To whom correspondence should be addressed (e-mail cstefan@biocfarm.unibo.it).

Alternatively, intact mitochondria were isolated by Percoll fractionation [20] as follows. The crude mitochondria suspension (0.5 ml) was layered on top of 15 ml of a solution consisting of 30% Percoll, 0.25 M sucrose, 1 mM EDTA and 10 mM Hepes (pH 7.4). Self-generating Percoll gradient was developed by centrifugation at 35000 *g* for 30 min at 4 °C. The mitochondrial band was separated from less-dense contaminants and broken mitochondria, collected with a Pasteur pipette, and washed twice in 5 ml of MIB by centrifuging for 5 min at 8000 *g*. The final pellet was suspended in MIB at 10 mg of protein/ml. Mitochondrial function was assayed with standard oxymetric tests [21]. Oxygen consumption was monitored in an oxygen-electrode chamber (IC-Oxy, Gilson, France) at 25 °C in a final volume of 1.5 ml containing 0.5 mg of mitochondrial protein in MIB. Succinate (10 mM) was the oxidizable substrate (in the presence of 2 μ M rotenone).

Release of cytochrome *c* from isolated mitochondria

An aliquot of 10 μ l of the mitochondria preparation (0.1 mg of protein) was incubated for 15 min at 25 °C in a final volume of 50 μ l of MIB containing 10 mM succinate and 2 μ M rotenone (to give a final mitochondrial protein concentration of 2 mg/ml). Differences from these standard conditions of incubation are indicated in the text. At the end of the incubation, mitochondria were pelleted for 20 s in a benchtop centrifuge at 9000 *g*. Some of the clear supernatant (40 μ l) was added with 10 μ l of 5-fold-concentrated loading buffer and boiled for 3 min. Aliquots of 20 μ l were subject to SDS/PAGE and Western-blotting analysis of cytochrome *c*. In some experiments, to reproducibly obtain a concentrated supernatant, 0.7 ml of mitochondria (7 mg of protein) were incubated in a final volume of 0.75 ml of MIB and pelleted as above for 45 s. To determine the protein pattern in this late supernatant, aliquots of 50 μ l were analysed by SDS/PAGE (15% gel), followed by Coomassie Brilliant Blue staining.

Depletion of cytochrome *c* from supernatant of spermine-treated mitochondria was achieved by immunoprecipitation of cytochrome *c* as described by Chauhan et al. [22]. The concentration of cytochrome *c* in the samples was estimated by densitometric comparison with the bands of standard solutions of horse heart cytochrome *c* (2–20 ng) analysed together with samples. The activity of adenylate kinase, a marker of the mitochondria intermembrane space, was determined spectrophotometrically as described by Sottocasa et al. [23], assaying 25 μ l of sample in a final volume of 0.5 ml containing 2 μ g/ml oligomycin.

Cytosolic extracts and caspase activation

Cytosolic extracts for caspase activation *in vitro* were prepared from confluent cultures of chick embryo heart cells (CEHC), obtained and maintained as described in [24]. To prepare these extracts, the cells from 10–15 plates (10 cm diameter) were collected and washed in PBS. The cell pellet was then suspended in 0.4 ml of CFS buffer [25], left for 10 min on ice and gently lysed in a glass-Teflon Potter–Elvehjem homogenizer with 70 strokes at medium speed. Cell lysis was confirmed by microscopic examination. The homogenate was centrifuged for 5 min at 1000 *g* to remove whole cells and nuclei. The supernatant was recentrifuged for 15 min at 16000 *g* and the resulting supernatant finally centrifuged for 30 min at 100000 *g*. Generally, these extracts contained about 5 mg of protein/ml. For the detection of caspase activation, 10 μ l of cytosol were incubated for 1 h at 30 °C in a final volume of 40 μ l along with (when added) 1 mM MgCl₂, 1 mM dATP, and either 100 ng of cytochrome *c* or 20 μ l of supernatant obtained from incubations of mitochondria. At

the end of the incubation, an aliquot of 10 μ l in duplicate was used for the caspase assay. The activity of caspase enzymes was measured by the cleavage of the fluorogenic peptide substrate acetyl-Asp-Glu-Val-Asp-amido-4-methylcoumarin (Ac-DEVD-AMC) in a final volume of 30 μ l during a 15 min incubation at 37 °C as described previously in detail [26].

Western blotting

Aliquots of each sample obtained from incubation of mitochondria were analysed for cytochrome *c* by SDS/PAGE on a 15% polyacrylamide gel. Standard protein markers were used for molecular-mass 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 chemiluminescence kit (Amersham).

RESULTS

Under normal conditions cytochrome *c* is found only in the mitochondrial intermembrane space. In order to assess whether polyamines could induce its exit, a crude preparation of rat heart mitochondria was incubated with increasing concentrations of the polyamines spermine or spermidine or the diamine putrescine. Supernatants were collected and subjected to Western-blot analysis with a monoclonal antibody to cytochrome *c*. In untreated mitochondria, the release of cytochrome *c* was very low or not detectable, depending on the mitochondrial preparation, for at least 60 min. In contrast, cytochrome *c* was present in the supernatants of mitochondria incubated for 15 min with polyamines (Figure 1A). Spermine was the most effective, and the increase in cytochrome *c* was largely stimulated at the concentration of 10 μ M and maximal at 100 μ M. Higher polyamine concentrations did not further increase the leakage of cytochrome *c*. Spermidine was reproducibly less effective than spermine at low concentration (10 μ M), but the release of cytochrome *c* was clearly evident when its concentration was raised to or above 100 μ M. Putrescine had no effect up to 1 mM (results not shown).

Under standard conditions represented by a 15 min incubation in the presence of 100 μ M spermine, the amount of cytochrome *c* found in the supernatant of mitochondrial incubations was only dependent on the concentration of mitochondria. Based on the comparison with cytochrome *c* standards of known concentration and densitometric analysis of the bands, a release of about 0.2 μ g of cytochrome *c*/mg of protein could be estimated.

To determine whether the leakage of cytochrome *c* in spermine-treated mitochondria was a selective process, a concentrated supernatant was obtained (see the Materials and methods section). Equal volumes (50 μ l) of supernatants from mitochondrial incubations were analysed by SDS/PAGE followed by Coomassie Brilliant Blue staining (Figure 1B). The protein pattern in the supernatants of control and spermine-treated mitochondria were similar, and probably due to contamination of the mitochondrial preparation. In contrast, massive and generalized leakage of mitochondrial protein has been described when the release of cytochrome *c* was caused by MPT [16]. The sensitivity of the Coomassie Brilliant Blue staining was too low to allow a clear visualization of the minute amount of released cytochrome *c*, but a shading in the position expected for cytochrome *c* was present in polyamine-treated samples. In order to obtain quantitative data for released proteins, aliquots of the

Figure 1 Spermine induces the specific release of cytochrome *c* from isolated mitochondria

(A) Mitochondria (2 mg/ml) were incubated for 15 min in the absence (Ctrl) or presence of the indicated concentration of polyamines, followed by centrifugation and recovery of the supernatant, which was subject to cytochrome *c* analysis by SDS/PAGE and immunoblotting. The first lane (Cyt.*c*) represents the analysis of a standard solution of horse heart cytochrome *c* (4 ng). (B) A concentrated suspension of mitochondria (7 mg/0.75 ml) was incubated for 15 min in the absence (Ctrl) or presence of 100 μM spermine (Spm). After centrifugation, equal volumes (50 μl) of the supernatants were separated by SDS/PAGE and visualized by Coomassie Brilliant Blue staining. Horse heart cytochrome *c* (25 μg) was loaded where indicated. (C) Aliquots of the supernatants shown in (B) were assayed for cytochrome *c* content by Western blotting/densitometry and adenylate kinase activity (AdK) as described in the Materials and methods section. Results shown are representative of three independent experiments, except for (C), in which the means ± S.E.M. obtained for three separate preparations are shown.

supernatants were assayed for cytochrome *c* by Western blotting followed by densitometry, and for adenylate kinase activity, which is a marker of the intermembrane space [23] and is released following mitochondrial damage [16,27]. Figure 1(C) shows that the large increase in cytochrome *c* concentration in the supernatant of spermine-treated mitochondria was not accompanied by any increase in adenylate kinase activity.

In order to confirm that cytochrome *c* is released from intact mitochondria and not from unspecific binding sites, mitochondria were purified further by Percoll fractionation, which separates intact mitochondria from lighter, damaged, ones [20,21]. Actually, centrifugation on a self-generating Percoll gradient (Figure 2A) separated heavier mitochondria from lighter contaminants, consisting of damaged mitochondria and membrane fragments, represented on Figure 2(A) by the broad fraction B and the very light fraction A, respectively. Mitochondria in fraction C accounted for more than half of the protein content of crude preparations ($59 \pm 4\%$, $n = 11$) and were fully functional in standard oxymetric tests. They were strictly coupled (differently from bands A and B) and exhibited a respiratory control ratio of 3.8 ± 0.2 using succinate (+rotenone) as substrate and a P/O ratio (the ratio of ATP synthesized/O₂ consumed) of 1.59 ± 0.02 , which are good values for heart mitochondria [21].

To determine the contribution of each fraction to the release of cytochrome *c* by spermine we introduced a polylysine test. This test is based on the ability of basic peptides, such as polylysine, to detach cytochrome *c* bound to artificial membranes [28]. A commercial preparation of polylysine consisting of

Figure 2 Spermine is able to induce cytochrome *c* release from intact mitochondria

(A) Rat heart mitochondria were fractionated on a self-generating Percoll gradient. The resulting three fractions were collected and washed twice in MIB. Each fraction was assayed for protein content and functionally characterized by standard oxymetric tests. (B) Comparison of the cytochrome *c*-releasing power of spermine (Spm) and polylysine (pLys) in the mitochondrial fractions obtained from Percoll fractionation (see A). Aliquots of each fraction corresponding to 0.1 mg of protein were incubated for 15 min without any treatment (C, control), or in the presence of 100 μM spermine or 100 μg/ml polylysine. After centrifugation, cytochrome *c* was detected in supernatants by Western blotting.

peptides of different chain lengths with molecular masses from 1 to 4 kDa was used. In preliminary experiments (results not shown) we determined that polylysine at a concentration of 10 μg/ml (10–40 μM) released virtually all of the bound cytochrome *c* from a membrane model consisting of phospholipid large unilamellar vesicles. A concentration of 100 μM spermine was required to obtain a similar effect. Next we compared the effects of polylysine and spermine on the fractions obtained from Percoll fractionation (Figure 2B). The fractions were washed and suspended in MIB (10 mg/ml). Aliquots of 0.1 mg of protein of each fraction were then incubated for 15 min with 100 μM spermine, or 100 μg/ml polylysine, or vehicle alone (controls). In the very light fraction A, spermine and polylysine slightly increased the amount of cytochrome *c* in the supernatant. In fraction B, both spermine and polylysine caused a marked accumulation of cytochrome *c* in the soluble fraction. In fraction C, consisting of functional coupled mitochondria, only spermine and not polylysine induced cytochrome *c* release from the particulate portion. The release of cytochrome *c* by polylysine means that cytochrome *c* is electrostatically linked to membrane fragments, or is located in mitochondria with the damaged external membranes, allowing the passage of polylysine. Thus the negativity of fraction C to the polylysine test is a proof of the integrity of mitochondria, and demonstrates that the effect of spermine is specific. Percoll-purified mitochondria of fraction C were used in all the following experiments.

Several reports have shown that induction of MPT results in the release of cytochrome *c* from the intermembrane space of the mitochondrion [15–17,26,27,29]. It seems improbable that spermine utilizes a similar mechanism to induce cytochrome *c* leakage, because the polyamine is a powerful inhibitor of MPT in heart mitochondria [30]. To confirm this prediction, we compared the effects of cyclosporin A (CsA), an MPT inhibitor [31], on the release of cytochrome *c* caused by spermine or MPT induction. A high level of Ca²⁺ in combination with a triggering agent, such as elevated inorganic phosphate concentration, represents a classical inducer of MPT, which causes a CsA-

Figure 3 The release of cytochrome *c* from spermine-treated mitochondria is independent of the permeability transition

(A) Percoll-purified mitochondria (2 mg/ml) were incubated for 15 min without any treatment (Ctrl) or in the presence of 100 μ M spermine (Spm), 200 μ M Ca^{2+} plus 15 mM phosphate (Ca/P), 5 mM atractylsode (Atr) or 100 μ M *t*-butyl hydroperoxide (tBOOH). Following centrifugation, cytochrome *c* was determined by Western blotting in the supernatant. (B) CsA (1 μ M) was added, where indicated, to mitochondria incubated with Ca^{2+} plus phosphate, or spermine, as described above. These experiments were repeated twice with similar results.

Figure 4 Spermine causes the rapid release of a definite amount of cytochrome *c* from mitochondria

(A) Percoll-purified mitochondria (2 mg/ml) were incubated for 15 min in the presence of the indicated concentrations of spermine. Following centrifugation, cytochrome *c* was determined by Western blotting in both the mitochondrial pellet and the supernatant. (B) Percoll-purified mitochondria (2 mg/ml) were incubated for the indicated times in the presence of 100 μ M spermine prior of centrifugation and cytochrome *c* determination in the supernatant.

inhibitable release of cytochrome *c* [16,17]. Actually, MPT induction by Ca^{2+} plus phosphate, as well as by atractylsode or *t*-butyl hydroperoxide, caused the release of cytochrome *c* to an extent similar to spermine (Figure 3A). However, the leakage of cytochrome *c* triggered by Ca^{2+} plus phosphate was inhibited by CsA, a classical inhibitor of MPT, whereas CsA did not block the effect of spermine (Figure 3B).

Altogether, the data presented in Figures 2 and 3 show that spermine triggers the leakage of cytochrome *c* from intact mitochondria, without causing any apparent damage. This was also confirmed by the lack of any significant effect of spermine on the mitochondrial function measured by oxymetric tests (respiratory control ratio 3.7 ± 0.3 and P/O ratio 1.53 ± 0.07 in the presence of 100 μ M spermine).

The correlation between spermine concentration and cytochrome *c* release in intact mitochondria of fraction C was similar to that previously observed in crude mitochondria. At a concentration of 100 μ M spermine, maximum release of cytochrome *c* was achieved (Figure 4A). The released cytochrome *c* was a small fraction of the overall protein, accounting for 5–10% of the total mitochondrial cytochrome *c*, as determined by densitometry. The release of cytochrome *c* was a rapid event, and

Figure 5 Spermine triggers caspase activation in a cardiac cell-free model of apoptosis

(A) Cytosolic extracts from CEHC were incubated for 1 h without any treatment (Ctrl), or in the presence of 1 mM dATP and/or 100 ng of cytochrome *c* (Cyt. *c*), after which caspase activity was assayed. (B) Percoll-purified mitochondria (5 mg/ml) were incubated for 15 min with or without 100 μ M spermine. After centrifugation, 20 μ l of the supernatants obtained from control or spermine-treated (Spm) mitochondria was incubated for 1 h in the presence of 10 μ l of cytosolic extract from CEHC and 1 mM dATP. An aliquot of the supernatant from spermine-treated mitochondria was also subjected to immunoprecipitation of cytochrome *c* (Spm, ppt) prior to incubation with cytosol. At the end of the incubation, samples were assayed for caspase activity. The cytochrome *c* present in an aliquot of 10 μ l of each of the supernatants is also shown. Results in both panels are means $\pm$ S.E.M. of four determinations.

a 30 s incubation in the presence of 100 μ M spermine was sufficient to cause the leakage of virtually all of the releasable cytochrome *c* (Figure 4B).

Caspase cascade can be initiated *in vitro* in cytosolic extracts from several cell lines by the addition of cytochrome *c* together with dATP [14]. In order to establish a cardiac cell-free model of apoptosis, we obtained cytosolic extracts from CEHC. These non-transformed heart cells can be easily obtained in large quantities, and express a high caspase activity, albeit usually latent [24]. Figure 5(A) shows that cytosolic extracts from CEHC represent a good model, with a very low degree of spontaneous caspase auto-activation. Caspase activity, however, can be triggered if dATP and cytochrome *c* are both added to cytosol. Thus the ability of supernatants from spermine-treated mitochondria to trigger caspase activation in this extract was assessed (Figure 5B). When CEHC cytosols enriched with dATP were mixed with supernatants from untreated control mitochondria, not containing cytochrome *c*, caspase activity remained latent. In contrast, cytochrome *c*-containing supernatants from spermine-treated mitochondria gained the ability to trigger caspase activity, and this effect was abolished by immunodepletion of cytochrome *c* from the supernatants.

DISCUSSION

The interaction of polyamines with mitochondria has been known about for many years. Polyamines are specifically transported into heart [32] and liver [33] mitochondria, where they appear to exert several functions, generally protective. For example, spermine regulates calcium transport [34], ATP synthesis [35], the ATP/ADP carrier [36], glutathione extrusion [37], transport of cytosolic enzymes [38,39] and is a strong inhibitor of MPT [30,34,37]. In the current report we have described a novel and

unexpected effect of polyamines: the induction of cytochrome *c* release from heart mitochondria. Similarly to other mitochondrial actions of polyamines, spermine is more potent than spermidine, whereas putrescine has no effect.

At present, we can only speculate about the mechanism used by polyamines to cause cytochrome *c* translocation out of the mitochondrion. However, the exit of cytochrome *c* is not caused by damage to the mitochondrial membranes. Unlike other models in which cytochrome *c* leakage from mitochondria is induced by caspases [40], the pro-apoptotic protein Bax [27,29] or various treatments targeting mitochondria [15–17], MPT is not involved, as expected on the basis of the inhibitory effect of spermine on MPT itself. To our knowledge, the present report is the first to describe the release of cytochrome *c* by a small physiological and ubiquitous molecule that does not cause mitochondrial damage. Spermine is acknowledged to be a protector of mitochondrial function. Evidently, the limited amount of cytochrome *c* released from spermine-treated mitochondria does not preclude mitochondrial respiration. In this way spermine may allow the ATP production necessary for the completion of the apoptotic program [13].

The effect of spermine is selective, rapid and saturable, suggesting the presence of a binding site specific for its action. Spermine is already known to influence the movement of proteins across the external membrane of mitochondria [38,39], but one intriguing question may be why only a small fraction of cytochrome *c* is released. This finding suggests the existence of an exchangeable sub-population of the protein. Actually, a small cytochrome *c* pool with this characteristic has been described [41]. Further work is required to clarify this point.

The physiological relevance of our findings remains to be determined. However, it is intriguing that the translocation of cytochrome *c* into the cytosol represents a central point in many forms of apoptosis, since cytochrome *c* is a fundamental component of the apoptosome, responsible for caspase activation [42]. Recently, this mitochondrial pathway of apoptosis has been found also in intact cardiomyocytes [43]. We have described here a cardiac cell-free model of apoptosis: caspase activity in cytosol of embryonal cardiomyocytes is triggered by the cytochrome *c* released from mitochondria by spermine. The described cell-free model can directly explain the toxicity of an excessively high level of intracellular spermine [11,44]. In fact, spermine and spermidine are present in high amounts within the cells, but are acknowledged to be almost totally bound to anionic structures, leaving only low concentrations of free polyamines [45]. In addition, our data also suggest a possible involvement of polyamines in some pathological forms of apoptosis. Cardiac hypertrophy, either pharmacologically induced or consequent to hypertension, is characterized by a large and prolonged increase in polyamine level [4–6], and is coupled to excessive apoptosis [46,47]. The abnormal accumulation of polyamines in cells stimulated to growth, but which cannot divide, may contribute to induction of apoptosis by causing the release of cytochrome *c* that could trigger caspase activation directly or by lowering the threshold of sensitivity to other pro-apoptotic stimuli.

This research was supported by Compagnia di San Paolo and by grants from the Italian Ministry of University and Scientific Research (fondi MURST ex 40% and 60%).

REFERENCES

- Tabor, C. W. and Tabor, H. (1984) Polyamines. *Annu. Rev. Biochem.* **53**, 749–790
- Pegg, A. E. (1988) Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. *Cancer Res.* **48**, 759–774
- Casero, Jr., R. A. and Pegg, A. E. (1993) Spermidine/spermine N¹-acetyltransferase - the turning point in polyamine metabolism. *FASEB J.* **7**, 653–661
- Flamigni, F., Rossoni, C., Stefanelli, C. and Caldarera, C. M. (1986) Polyamine metabolism and function in the heart. *J. Mol. Cell. Cardiol.* **18**, 3–11
- Ibrahim, J., Schachter, M., Hughes, A. D. and Sever, P. S. (1995) Role of polyamines in hypertension induced by angiotensin II. *Cardiovasc. Res.* **29**, 50–56
- Pegg, A. E. and Hibasami, H. (1980) Polyamine metabolism during cardiac hypertrophy. *Am. J. Physiol.* **239**, E372–E378
- Packham, G. and Cleveland, J. L. (1994) Ornithine decarboxylase is a mediator of c-Myc-induced apoptosis. *Mol. Cell. Biol.* **14**, 5741–5747
- Poulin, R., Pelletier, G. and Pegg, A. E. (1995) Induction of apoptosis by excessive polyamine accumulation in ornithine decarboxylase-overproducing L1210 cells. *Biochem. J.* **311**, 723–727
- Tobias, K. E. and Kahana, C. (1995) Exposure to ornithine results in excessive accumulation of putrescine and apoptotic cell death in ornithine decarboxylase overproducing mouse myeloma cells. *Cell Growth Differ.* **6**, 1279–1285
- Xie, X., Tome, M. E. and Gerner, E. W. (1997) Loss of intracellular putrescine pool-size regulation induces apoptosis. *Exp. Cell Res.* **230**, 386–392
- Stefanelli, C., Bonavita, F., Stanic, I., Mignani, M., Facchini, A., Pignatti, C., Flamigni, F. and Caldarera, C. M. (1998) Spermine causes caspase activation in leukaemia cells. *FEBS Lett.* **437**, 233–236
- Thornberry, N. A. and Lazebnik, J. (1998) Caspases: enemies within. *Science* **281**, 1312–1316
- Green, D. R. and Reed, J. C. (1998) Mitochondria and apoptosis. *Science* **281**, 1309–1318
- Liu, X., Kim, C. N., Yang, J., Jemmerson, R. and Wang, X. (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. *Cell* **86**, 147–157
- Kantrow, S. P. and Piantadosi, C. A. (1997) Release of cytochrome *c* from liver mitochondria during permeability transition. *Biochem. Biophys. Res. Commun.* **232**, 669–671
- Scarlett, J. L. and Murphy, M. P. (1997) Release of apoptogenic proteins from the mitochondrial intermembrane space during the mitochondrial permeability transition. *FEBS Lett.* **418**, 282–286
- Petit, P. X., Gubern, M., Dolez, P., Susin, S. A., Zamzami, N. and Kroemer, G. (1998) Disruption of the outer mitochondrial membrane as a result of large amplitude swelling: the impact of irreversible permeability transition. *FEBS Lett.* **426**, 111–116
- Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P. and Wang, X. (1997) Prevention of apoptosis by Bcl-2: release of cytochrome *c* from mitochondria blocked. *Science* **275**, 1129–1132
- Stefanelli, C., Bonavita, F., Stanic, I., Pignatti, C., Flamigni, F., Guarnieri, C. and Caldarera, C. M. (1999) Spermine triggers the activation of caspase-3 in a cell-free model of apoptosis. *FEBS Lett.* **451**, 95–98
- Neuburger, M., Journet, E. P., Bligny, R., Carde, J. P. and Douce, R. (1982) Purification of plant mitochondria by isopycnic centrifugation in density gradients of Percoll. *Arch. Biochem. Biophys.* **217**, 312–323
- Rickwood, D., Wilson, M. T. and Darley Usmar, V. M. (1987) Isolation and characteristics of intact mitochondria. In *Mitochondria: A Practical Approach* (Darley-Usmar, V. M., Rickwood, D. and Wilson, M. T., eds.), pp. 1–16. IRL Press, Oxford
- Chauhan, D., Pandey, P., Ogata, A., Teoh, G., Krett, N., Halgren, R., Rosen, S., Kufe, D., Kharbanda, S. and Anderson, K. (1997) Cytochrome *c*-dependent and -independent induction of apoptosis in multiple myeloma cells. *J. Biol. Chem.* **272**, 29995–29997
- Sottocasa, G. L., Kulenstierna, B., Ernster, L. and Bergstrand, A. (1967) Separation and some enzymatic properties of the inner and outer membranes of rat liver mitochondria. *Methods Enzymol.* **10**, 448–463
- Stefanelli, C., Pignatti, C., Tantini, B., Stanic, I., Bonavita, F., Muscari, C., Guarnieri, C., Clo, C. and Caldarera, C. M. (1999) Nitric oxide can function as either a killer molecule or an antiapoptotic effector in cardiomyocytes. *Biochim. Biophys. Acta* **1450**, 406–413
- Ellerby, H. M., Martin, S. J., Ellerby, L. M., Naiem, S. S., Rabizadeh, S., Salvesen, G. S., Casiano, C. A., Cashman, N. R., Green, D. R. and Breddesen, D. E. (1997) Establishment of a cell-free system of neuronal apoptosis: comparison of premitochondrial, mitochondrial, and postmitochondrial phases. *J. Neurosci.* **17**, 6165–6178
- Stefanelli, C., Bonavita, F., Stanic, I., Pignatti, C., Farruggia, G., Masotti, L., Guarnieri, C. and Caldarera, C. M. (1998) Inhibition of etoposide-induced apoptosis with peptide aldehyde inhibitors of proteasome. *Biochem. J.* **332**, 661–665
- Kohler, C., Gahn, A., Noma, T., Nakazawa, A., Orrenius, S. and Zhivotovsky, B. (1999) Release of adenylate kinase 2 from the mitochondrial intermembrane space during apoptosis. *FEBS Lett.* **447**, 10–12
- Rytomaa, M. and Kinnunen, P. K. J. (1996) Dissociation of cytochrome *c* from liposomes with histone H1. Comparison with basic peptides. *Biochemistry* **35**, 4529–4539

- 29 Marzo, I., Brenner, C., Zamzami, N., Jurgensmeier, J. M., Susin, S. A., Vieira, H. L. A., Prevost, M. C., Xie, Z., Matsuyama, S., Reed, J. C. and Kroemer, G. (1998) Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. *Science* **281**, 2027–2031
- 30 Lapidus, R. G. and Sokolove, P. M. (1992) Inhibition by spermine of the inner membrane permeability transition of isolated rat heart mitochondria. *FEBS Lett.* **313**, 314–318
- 31 Crompton, M. (1999) The mitochondrial permeability transition pore and its role in cell death. *Biochem. J.* **341**, 233–249
- 32 Toninello, A., Dalla Via, L., Testa, S., Siliprandi, D. and Siliprandi, N. (1990) Transport and action of spermine in rat heart mitochondria. *Cardioscience* **1**, 287–294
- 33 Toninello, A., Dalla Via, L., Siliprandi, D. and Garlid, K. D. (1992) Evidence that spermine, spermidine and putrescine are transported electrophoretically in mitochondria by a specific polyamine uniporter. *J. Biol. Chem.* **267**, 18393–18397
- 34 Rustenbeck, I., Loptien, D., Fricke, K., Lenzen, S. and Reiter, H. (1998) Polyamine modulation of mitochondrial calcium transport. II. Inhibition of mitochondrial permeability transition by aliphatic polyamines but not by aminoglucosides. *Biochem. Pharmacol.* **56**, 987–995
- 35 Igarashi, K., Kashiwagi, K., Kobayashi, H., Ohnishi, R., Kakegawa, T., Nagasu, A. and Hirose, S. (1989) Effect of polyamines on mitochondrial F_1 -ATPase catalyzed reactions. *J. Biochem.* **106**, 294–298
- 36 Kramer, R., Mayr, U., Heberger, C. and Tsompanidou, S. (1986) Activation of the ADP/ATP carrier from mitochondria by cationic effectors. *Biochim. Biophys. Acta* **855**, 201–210
- 37 Rigobello, M. P., Toninello, A., Siliprandi, D. and Bindoli, A. (1993) Effect of spermine on mitochondrial glutathione release. *Biochem. Biophys. Res. Commun.* **194**, 1276–1281
- 38 Gonzalez-Bosch, C., Marcote, M. J. and Hernandez-Yago, J. (1991) Role of polyamines in the transport *in vitro* of the precursor of ornithine transcarbamylase. *Biochem. J.* **279**, 815–820
- 39 Bordin, L., Cattapan, F., Clari, G., Toninello, A., Siliprandi, N. and Moret, V. (1994) Spermine-mediated casein kinase II-uptake by rat liver mitochondria. *Biochim. Biophys. Acta* **1199**, 266–270
- 40 Marzo, I., Brenner, C., Zamzami, N., Susin, S. A., Beutner, G., Brdiczka, D., Remy, R., Xie, Z. H., Reed, J. C. and Kroemer, G. (1998) The permeability transition pore complex: a target for apoptosis regulation by caspases and bcl-2-related proteins. *J. Exp. Med.* **187**, 1261–1271
- 41 Miralles, V. M., Marcote, M. J., Hernandez-Yago, J. and Grisolia, S. (1988) Loading rat liver mitochondria with apocytochrome *c* provokes exit of cytochrome *c*. *Arch. Biochem. Biophys.* **266**, 516–521
- 42 Salvesen, G. S. and Dixit, V. M. (1999) Caspase activation: the induced-proximity model. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 10964–10967
- 43 Bialik, S., Cryns, V. L., Drincic, A., Miyata, S., Wollwick, A. L., Srinivasan, A. and Kitsus, R. N. (1999) The mitochondrial apoptotic pathway is activated by serum and glucose deprivation in cardiac myocytes. *Circ. Res.* **85**, 403–414
- 44 Brunton, V. G., Grant, H. M. and Wallace, H. M. (1991) Mechanisms of spermine toxicity in baby-hamster kidney (BHK) cells. *Biochem. J.* **280**, 193–198
- 45 Davis, R., Morris, R. D. and Coffino, P. (1992) Sequestered end products and enzyme regulation: the case of ornithine decarboxylase. *Microbiol. Rev.* **56**, 280–290
- 46 Diez, J., Fortuno, M. A. and Ravassa, S. (1998) Apoptosis in hypertensive heart disease. *Curr. Opin. Cardiol.* **13**, 317–325
- 47 Teiger, E., Than, V. D., Richard, L., Wisniewsky, C., Tea, B. S., Galbourn, L., Tremblay, J., Schwartz, K. and Hamet, P. (1996) Apoptosis in pressure overload-induced heart hypertrophy in the rat. *J. Clin. Invest.* **97**, 2891–2897

Received 3 December 1999/8 February 2000; accepted 29 February 2000