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ORIGINAL ARTICLE

A pro-survival effect of polyamine depletion on norepinephrine-mediated apoptosis in cardiac cells: role of signaling enzymes

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Abstract Recent studies report that the primary transmitter of sympathetic nervous system norepinephrine (NE), which is actively produced in failing human heart, is able to induce apoptosis of rat cardiomyocytes. Apoptotic cell death of cardiomyocytes is involved in several cardiovascular diseases including ischemia, hypertrophy and heart failure, therefore representing a potential therapeutic target. The natural occurring polyamines, putrescine, spermidine and spermine, are biogenic amines involved in many cellular processes, including apoptosis. Thus, we have studied the involvement of polyamines in the apoptosis of cardiac cells induced by the treatment with NE. The results indicate that NE caused an early induction of the activity of ornithine decarboxylase (ODC), the first enzyme in polyamine biosynthesis, followed by a later increase of apoptotic cell death. This effect was prevented in the presence of α-difluoromethylornithine, an irreversible inhibitor of ODC. Moreover, the study of some key signal transduction pathways revealed an involvement of AMP-activated protein kinase, AKT and p38 mitogen-activated protein kinases, in the modulation by polyamines of the response of cardiomyocytes to NE. In fact, polyamine-depleted cells showed an altered activation pattern of these kinases that may contrast apoptosis and appeared to result from a differential effect on the specific phosphatases that dephosphorylate and switch off these signaling proteins. In conclusion, these results indicate that in cardiac cells polyamines are involved in the execution of the death program activated by NE, and suggest that their apoptosis facilitating action is mediated by a network of specific phosphatases and kinases.

Keywords Norepinephrine · Cardiac cells · Apoptosis · Polyamines · Kinases · Phosphatases

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Introduction

Accumulating evidence from in vivo and in vitro studies strongly suggest that apoptosis may play an important role in the pathogenesis of several cardiovascular diseases, such as ischemia/reperfusion, infarction, heart failure and aging (Czerski and Nuñez 2004). In response to pathophysiological stresses, cardiac myocytes undergo hypertrophic growth and/or apoptosis, responses associated with the development of cardiac pathologies. There has been much effort expended in order to understand the intracellular signaling pathways that are activated and potentially involved in these responses. The main pathways implicated in cardiac myocyte apoptosis or survival are those involving the family of the mitogen-activated protein kinases (MAPKs), and the phosphatidylinositol 3-kinase (PI3K)/AKT (also known as PKB) pathway (Clerk et al. 2007). In particular, the JNK and p38 subfamilies of the MAPKs were originally identified as "stress responsive" and pro-apoptotic MAPKs (Wang et al. 1998a), whereas activation of the PI3K/AKT pathway is generally believed to be protective and pro-survival (Vanhaesebroeck and Alessi 2000). JNKs and p38 MAPKs are activated by oxidative stress, ischemia/reperfusion or hypoxia/reoxygenation in isolated cardiac

myocytes or intact hearts, but they are also activated by hypertrophic stimuli including ET-1 (Sugden and Clerk 1998); whether or not they are associated with hypertrophy or cell death may depend on the precise context and the functional status of the cell. On the contrary PI3K/AKT signaling has been shown to provide protection against ischemia/reperfusion injury during preconditioning (Hausenloy et al. 2005) and AKT activation has been shown to decrease apoptosis, as well as to reduce infarct size and improve cardiac function after ischemia/reperfusion injury (Song et al. 2009).

Another pathway activated during metabolic stress, including hypoxia and ischemia, is the AMP-activated protein kinase (AMPK), which facilitates ATP production to meet energy demand. The role of AMPK in apoptosis is not clear, with both anti-apoptotic and pro-apoptotic actions being reported (Dyck and Lopaschuk 2006). In the heart, some studies have suggested that AMPK activation exerts an anti-apoptotic effect, but a recent work in neonatal cardiomyocytes has showed that AMPK can mediate the pro-apoptotic translocation of Bax to mitochondria during simulated ischemia (Capano and Crompton 2006). In other cell types, including vascular smooth muscle cells, AMPK activation has been shown to be pro-apoptotic (Meisse et al. 2002; Kefas et al. 2003; Igata et al. 2005). This pro-apoptotic effect of AMPK has been attributed to inhibition of cell cycle progression, activation of the JNK pathway and caspase-3 activation, and up-regulation of the pro-apoptotic p53 protein (Igata et al. 2005).

Norepinephrine (NE), a major regulator of cardiovascular function, has been known for a long time to have the potential to induce cardiac cell damage (Haft 1974), and the cardiotoxic effect of NE has been a focus of intensive study with accumulating evidence suggesting the possible association between NE-induced cardiac cell death and the pathophysiology of heart failure (Colucci et al. 2000). NE-induced cardiomyocyte cell death has been consistently observed (Communal et al. 1998; Fu et al. 2004a, b), and a few events leading to NE-induced apoptosis in cardiomyocytes have been identified. These pathways include the activation of a cAMP dependent-protein kinase A through stimulation of G protein-coupled adrenergic receptors (Communal et al. 1998), activation of TNF secretion (Fu et al. 2004a), generation of reactive oxygen species (Gupta et al. 2006), caspase activation (Fu et al. 2004b); however, the molecular mechanisms involved in NE-mediated effects on cell viability are not completely defined and are currently being studied.

The polyamines spermine, spermidine, and the diamine putrescine are intrinsic constituents of all eukaryotic cells. Increasing evidence indicates that polyamines are not only essential factors for cell growth, but play a role even in other cell responses, including apoptosis (Pignatti et al. 2004; Flamigni et al. 2007; Averill-Bates et al. 2008) and in various aspects of the remodeling occurring in cardiac diseases, such as growth, death and differentiation of cardiac cells (Flamigni et al. 2009). We have recently shown that the inhibition of polyamine biosynthesis by α-difluoromethylornithine (DFMO) confers resistance to simulated ischemia-induced apoptosis in H9c2 cardiomyoblasts (Tantini et al. 2006) and is also able to inhibit the hypertrophic, pro-fibrotic and pro-apoptotic effects of aldosterone on cardiac cells (Cetrullo et al. 2010).

In the present study, we have examined the effect of polyamine depletion on NE-induced apoptosis in rat neonatal cardiomyocytes and H9c2 cardiomyoblasts. We have used DFMO to inhibit ornithine decarboxylase (ODC), the first and rate-limiting enzyme in polyamine biosynthesis, and deplete cell of polyamines. We have found that polyamine depletion promotes resistance to NE-induced apoptosis in cardiac cells. Moreover, the study of key signal transduction pathways governing cell death and survival in our models, revealed an involvement of p38 MAPK, AMPK and AKT kinases in the modulation by polyamines of cardiomyocyte response to NE. In fact, polyamine-depleted cells show an altered pattern of p38 MAPK, AMPK and AKT activation that may contrast apoptosis and appears to result from a differential effect on the specific phosphatases that dephosphorylate and switch off these signaling proteins.

Materials and methods

Materials

α-difluoromethylornithine was a generous gift of Patrick M. Woster, Wayne State University, Detroit, MI. NE and all other biochemical reagents were obtained from Sigma Chemical Company.

Polyclonal anti-survivin, anti-protein-phosphatase-2A and secondary antibody anti-mouse were purchased from Santa Cruz. Monoclonal anti-β-actin antibody and polyclonal anti-protein-phosphatase-2C were from BD and from Chemicon, respectively. Primary antibodies rabbit anti-phospho-AKT, anti-AKT, anti-phospho p38, anti-p38, anti-phospho-AMPK, anti-AMPK and HRP-conjugated secondary anti-rabbit were all from cell signaling.

Cell cultures

Cardiomyocyte cultures were prepared from 1- to 3-day-old neonatal Wistar rat hearts, as described previously (Cetrullo et al. 2010). The cardiac myocytes were plated at a density of 1×10^6 cells/ml in DMEM supplemented with 10% heat inactivated FBS, 100 IU/ml penicillin and

0.1 μg/ml streptomycin on gelatinized cell culture dishes. For experiments, cells were serum-starved for 20 h and then treated with NE.

H9c2 embryonal rat-heart derived cells were cultured in DMEM supplemented with 10% heat inactivated FBS, 100 IU/ml penicillin and 0.1 μg/ml streptomycin. For experiments, subcultured cells were grown for 24 h before NE treatment.

Polyamine depletion was obtained by culturing the cells in the presence of 0.1 mM DFMO.

Enzymatic assays and polyamine content

Caspase activity was measured by the cleavage of the fluorogenic peptide Ac-Asp-Glu-Val-Asp-7-amido-4-methyl-coumarin (Ac-DEVD-AMC), which represents a substrate for caspase-3 and other effector caspases, as previously described (Stefanelli et al. 2001; Stanic' et al. 2006).

ODC activity was measured by estimation of the release of $^{14}C–CO_2$ from ^{14}C-ornithine (Tantini et al. 2006) ODC activity was calculated as units/mg protein, where 1 unit (U) corresponds to 1 nmol of CO_2/h incubation.

Polyamines were separated and quantified in acidic cellular extracts by HPLC after derivatization with dansyl chloride (Tantini et al. 2006). Polyamine content is expressed as nmol/mg of protein.

Immunoblot analysis

The proteins were detected in cell extracts by Western blotting: cell lysates were prepared, analyzed by SDS-PAGE (10% gel) and immunoblotted essentially as described (Stanic' et al. 2009). Representative images of visualized immunoreactive bands were shown in figures.

Real-time PCR

At the end of the incubation cells were extracted with 1 mL Trizol (Invitrogen), according to manufacturer's instructions. The RNA pellets were resuspended to a 5 μl volume of RNAse inhibitor. The RNA was reverse transcribed with oligo-dT and Superscript First-Strand Synthesis System for RT-PCR (Invitrogen), according to manufacturer's instructions. Primer sequences used in RT-PCR are reported in Table 1. The various cDNA belonging to the same experiments were quantified by means of the PicoGreen double-stranded DNA quantification reagent (Molecular Probe, Eugene, OR) and then diluted to the same concentration (5 ng/μl), in order to exploit the same range of amplification efficiency. Real-time PCR analysis was run in a LightCycler instrument (Roche Molecular Biochemicals) by means of the SYBR Premix Ex Taq (TaKaRa) with the following protocol: initial activation of TaqDNA polymerase at 95°C for 15 min, followed by amplification (45 cycles: 94°C for 15 min, appropriate annealing temperature for each target gene as detailed below kept for 20 min and then 72°C for 10 min). Two microliters of each sample were processed for each gene under study. The annealing temperature were 56°C for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 58°C for phosphatase 2A and phosphatase 2C. The progressive accumulation of PCR products was monitored at each amplification cycle by measuring the increase in fluorescence due to the binding of SYBR Green dye to dsDNA. The crossing point values (i.e., the cycle number at which the detected fluorescence exceed the threshold value) were determined for each sample, and these values were used for comparative gene expression analysis.

Table 1 Primer sequences used in reverse transcription-polymerase chain reaction

Gene	Primers	Sequence
GAPDH	Forward	GACCTCAACTACATGGTCTACA
	Reverse	ACTCCACGACATACTCAGCAC
PP2A	Forward	AGACACACTGGATCACATCC
	Reverse	CCATGATTGCAGCTTGGTTA
PP2C	Forward	TGTCAATGGCTCTCTGGCTGTATC
	Reverse	ACACTCATGTTGTCTCGACTTCCC

The comparative C_t method is also known as the $2^{-[\mathrm{delta}][\mathrm{delta}]C_t}$ method, where

$$[\mathrm{delta}][\mathrm{delta}]C_t = [\mathrm{delta}]C_{t,\mathrm{sample}} - [\mathrm{delta}]C_{t,\mathrm{reference}}$$

Here, $[\mathrm{delta}]C_{t,\mathrm{sample}}$ is the C_t value for any sample normalized to the endogenous housekeeping gene and $[\mathrm{delta}]C_{t,\mathrm{reference}}$ is the C_t value for the calibrator also normalized to the endogenous housekeeping gene.

RNA interference of AMPK expression

Post-transcriptional gene silencing was performed using two chemically synthesized duplex RNAs oligonucleotides against rat AMPKα1 and AMPKα2 (Sigma-Proligo), and against Luciferase as a negative control. H9c2 cells were transfected with 200 nM siRNA by Transfection reagent (Santa Cruz) according to manufacturer's instructions. siRNA sequences used are reported in Table 2.

Statistical analysis

All the data presented as graphs or tables are expressed as means ± SEM of the indicated numbers of determinations, and analyzed for statistical significance by one-way or two-way ANOVA. Differences were considered significant for $P < 0.05$.

Table 2 siRNA sequences

siRNA target	Sequence
AMPKα1	
Sense	CUUAUUGGAUUUCCGAAGUTT
Antisense	ACUUCGGAAAUCCAAUAAGTT
AMPKα2	
Sense	GACAUUAUGGCGGAGGUGUTT
Antisense	ACACCUCCGCCAUAAUGUCTT
LUC	
Sense	AACUUACGCUGAGUACUUCTT
Antisense	GAAGUACUCAGCGUAAGUUTT

Results

ODC and polyamine content in NE-treated neonatal rat cardiomyocytes

In order to ascertain whether NE treatment may affect polyamine metabolism, we have evaluated the ODC activity in NE-treated neonatal rat cardiomyocytes. Figure 1 shows that in response to 3 h treatment with 50 μM NE, ODC activity increased in cardiomyocyte cells to 300% of the basal value, after which it declined to control levels by 6 h. This early induction of ODC was completely prevented when the cells were pretreated with DFMO.

The pretreatment with DFMO not only abolished ODC induction, but also led to a strong reduction of intracellular polyamines. As shown in Table 3 in fact, DFMO treatment reduced putrescine content to undetectable level and spermidine and spermine levels by about 50 and 20%, respectively.

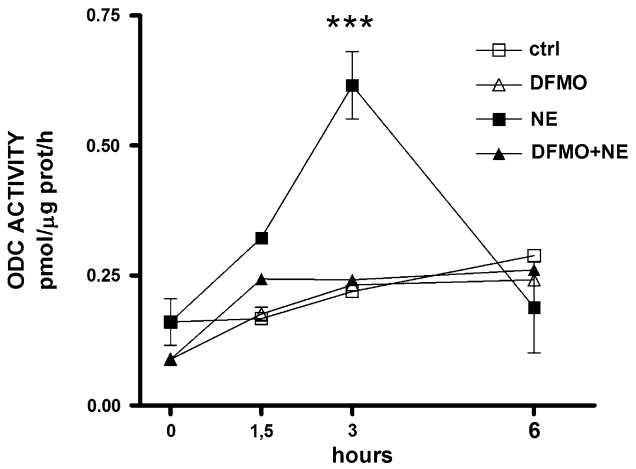

Fig. 1 ODC activity in NE-treated neonatal rat cardiomyocytes. Confluent cultures, grown for 72 h after seeding in the absence or presence of 100 μM DFMO, were then incubated for 6 h in the presence or absence of 50 μM NE. Results are means ± SEM of three determinations. ***$P < 0.001$ versus control cells

Table 3 Polyamine content in DFMO-treated neonatal rat cardiomyocytes

Pretreatment	Putrescine	Spermidine	Spermine
None	2.15 ± 0.5	2.95 ± 0.3	3.51 ± 0.9
DFMO	<0.3*	1.61 ± 0.5	2.83 ± 0.8

Cardiomyocytes were grown for 72 h in the presence or absence of 100 μM DFMO. Data are expressed as nmol/mg of protein and are the mean ± SEM of three different experiments

* $P < 0.05$ versus control cells

NE-induced caspase activation and down regulation of survivin expression in neonatal rat cardiomyocytes were counteracted by polyamine depletion

Several previously published papers have shown that NE induces a dose- and time-dependent apoptosis in similar experimental conditions and have reported evidence of this effect by a variety of analysis (Communal et al. 1998; Fu et al. 2004a, b; Gupta et al. 2006; Kniewald et al. 2007; Zaugg et al. 2000). In particular, Fu and collaborators have demonstrated that 50 μM NE causes an increased DNA fragmentation estimated after 48 h treatment by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay in neonatal rat cardiomyocytes. After this treatment of cardiomyocytes with NE, a 39% apoptotic cells was detected, whereas only 9% apoptotic cells was observed in control cultures. Apoptosis was characterized by an increase in caspase activities (Fu et al. 2004a). Therefore, to determine the effects of NE on myocardial cell apoptosis, neonatal rat cardiomyocytes were treated with 50 μM NE and caspase activity was assayed as a marker of apoptosis.

As shown in Fig. 2a, NE markedly increased effector caspase 3-like activity after 48 h of treatment.

Figure 2a, b also shows that pre-treatment of cell cultures with DFMO reduced the increase of caspase activity observed after NE treatment. Moreover, the effect of DFMO was polyamine specific, since addition of 100 μM putrescine to DFMO-treated cardiomyocytes that completely restored polyamine content (not shown), allowed the marked activation of caspases by NE (Fig. 2b).

The treatment with DFMO not only counteracted the activation of caspases, but also the NE-mediated decrease of survivin expression, a protein member of the apoptosis-inhibitor protein family that inhibits the execution of apoptosis. In fact, as shown in Fig. 2c, the survivin level was significantly decreased in cardiomyocytes treated for 24 h with NE, as assessed by western blot analysis. This effect was prevented in DFMO pretreated cell cultures, suggesting that polyamine depletion may exert a protective effect in the execution of apoptotic program in these cells.

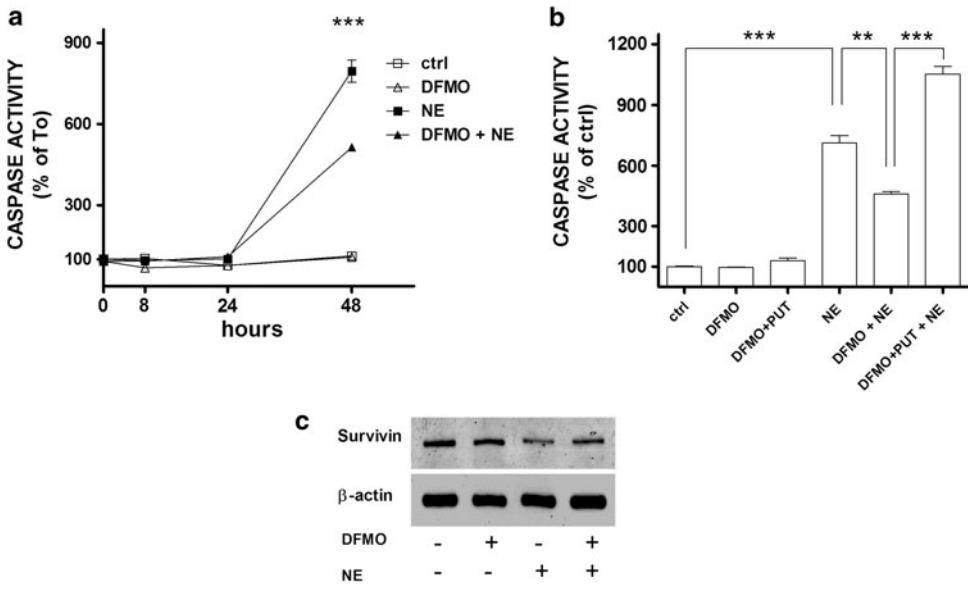

Fig. 2 Effect of polyamines depletion on caspase activity and survivin expression in NE-treated neonatal rat cardiomyocytes. Cardiomyocytes, grown for 72 h after plating in the presence or absence of 100 μM DFMO or 100 μM DFMO plus 100 μM putrescine (PUT), were then incubated for 8, 24, 48 h (**a**), for 48 h (**b**) or for 24 h (**c**), in the presence or absence of 50 μM NE. Results are means ± SEM of three determinations. ***$P < 0.001$; **$P < 0.01$

Modulation of p38 MAPK, AKT and AMPK phosphorylation by NE. Effect of polyamine depletion

The MAPK signaling cascades play an important role in the regulation of cardiomyocyte apoptosis. A pro-apoptotic role has been proposed for p38 MAPK (Wang et al. 1998a), whereas the role of AMPK in apoptosis is not clear with both anti-apoptotic and pro-apoptotic actions being reported for cardiac cells (Dyck and Lopaschuk 2006). The PI3K/AKT pathway, instead, promotes cell survival in many different cell types (Vanhaesebroeck and Alessi 2000). We have thus evaluated whether or not these pathways are activated upon NE treatment. An early increase of AKT phosphorylation was observed in cardiomyocytes after 15 min of treatment with NE (Fig. 3), after which it declined to control levels by 5 h. A more delayed increase of phospho-AMPK, i.e., at 1 h, was observed in the presence of NE, but, differently from AKT, it was largely maintained up to 8 h of treatment. Cell extracts were also analyzed for active phosphorylated p38 MAPK. No change in protein phosphorylation was detected at any time up to 5 h of NE treatment, but increased p38 phosphorylation was appreciated after 8 h. The total amounts of AKT, AMPK, p38 MAPK were not changed during NE treatment (Fig. 3). These results indicate that 50 μM NE can activate critical kinases with different timing and suggest that AMPK, p38 MAPK and AKT dependent pathways may modulate or be involved in the apoptotic response of cardiac cells to NE.

Figure 3 also shows that NE-mediated AKT and AMPK phosphorylations were not affected by the pre-treatment of DFMO, at 15 min and 1 h, respectively. However, polyamine-depleted cells retained increased amounts of phospho-AKT even up to 8 h after NE. On the contrary, in DFMO-treated cells, AMPK phosphorylation declined to control levels already after 5 h with NE. These data suggest that polyamines do not influence the activation of these pathways after NE, but they affect the duration of the signal. Finally, the increase in p38 MAPK phosphorylation seen 8 h after NE was prevented in polyamine-depleted cells.

Since the occurrence of apoptosis has been largely demonstrated also in H9c2 cardiomyoblasts (Chun and Pratt 2005; Tantini et al. 2006), this cell line was used, together with rat neonatal cardiomyocytes, to investigate the apoptotic action of NE.

In order to determine whether AMPK and AKT pathways could trigger apoptosis or survival in cardiac cells, we treated cardiac cells with the AMPK activator AICAR or with the PI3K/AKT pathway inhibitor LY294002 (LY). LY and AICAR significantly increased cell death in H9c2 cardiomyoblasts (Fig. 4a), and LY treatment enhanced NE-induced caspase activity in neonatal rat cardiomyocytes (Fig. 4b). Moreover, to confirm the involvement of AMPK in apoptosis induction, we knocked-down AMPK using gene silencing in H9c2 cardiomyoblasts. The levels of phosphorylated and total AMPK were strongly decreased in cells treated with AMPK siRNAs (siAMPK in Fig. 4c), compared to cells treated with a control siRNA. Figure 4a shows that cell death induced by NE was significantly reduced in AMPK knocked-down cells. As far as the p38 MAPK pathway is concerned, caspase activation was significantly lower in rat cardiomyocytes pretreated with the specific inhibitor SB203580 (SB) before NE, with respect to cells treated with NE (Fig. 4b).

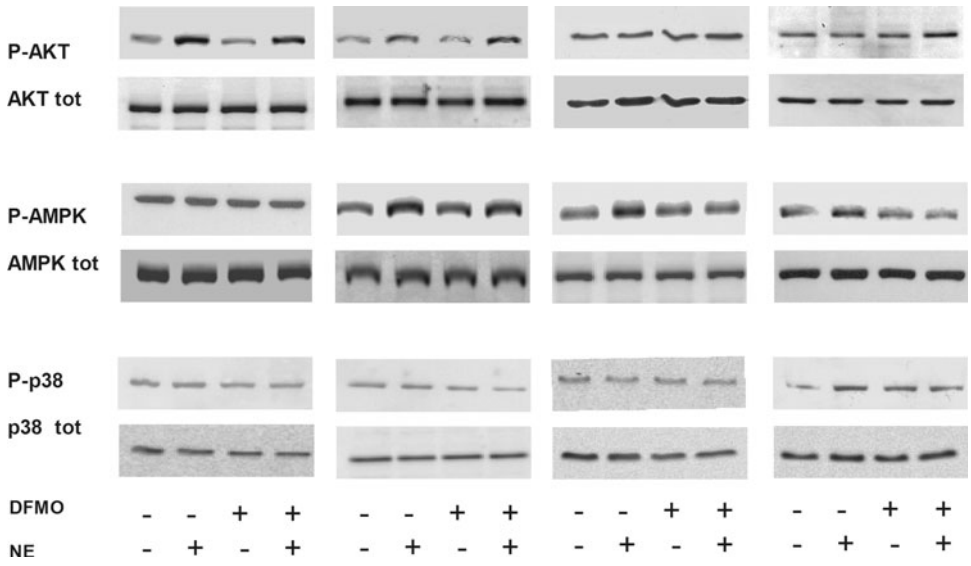

Fig. 3 AKT, AMPK, p38 MAPK phosphorylation following NE treatment in neonatal rat cardiomyocytes and effect of polyamine depletion. Cardiomyocytes, grown for 72 h after plating in the presence or absence of 100 μM DFMO, were then incubated for 15 min, 1, 5, 8 h (columns from left to right, respectively) in the presence or absence of 50 μM NE

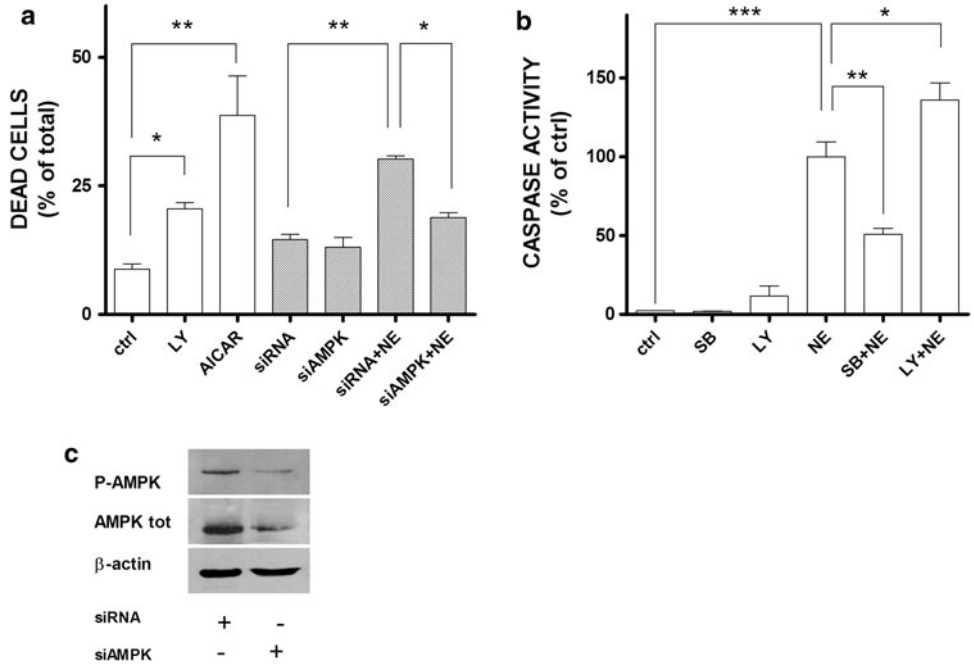

Fig. 4 Anti-apoptotic role of AKT and pro-apoptotic role of AMPK and p38 MAPK in cardiac cells. **a** Cell death was measured by trypan blue exclusion test. H9c2 cardiomyoblasts were cultured for 48 h and then incubated with 20 μM LY294002 (LY) or 4 mM AICAR for 48 h; H9c2 cells were also transfected with AMPK-silencing RNAs or control RNA (striped bars) and after 48 h treated with or without 50 μM NE and incubated for further 48 h. **b** The cells were assayed for caspase activity. Rat cardiomyocytes were treated with 50 μM NE for 48 h LY294002 (LY, 20 μM) or SB203580 (SB, 10 μM) was added 30 min before NE. **c** H9c2 cells were transfected with AMPK-silencing RNAs or control RNA and analyzed by western blotting for phospho-AMPK, total AMPK and β-actin as internal control. Results are means ± SEM of three determinations. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$

An increase in phosphorylation of AMPK was also observable in H9c2 cardiomyoblasts upon AICAR treatment (not shown), whereas basal AKT and p38 MAPK phosphorylation were completely inhibited in the presence of LY and SB, respectively (not shown). These findings strongly suggest that AMPK and p38 MAPK activation and AKT inhibition play an important role in favoring apoptosis of cardiac cells.

Polyamine depletion decreases PP2A and increases PP2C levels in cardiac cells

The observed persistent phosphorylation of AKT in polyamine-depleted cardiac cells treated with NE prompted us to evaluate the levels of protein phosphatase 2A (PP2A), the enzyme responsible for dephosphorylation/inactivation of AKT (Ivaska et al. 2002). Figure 5a shows that the

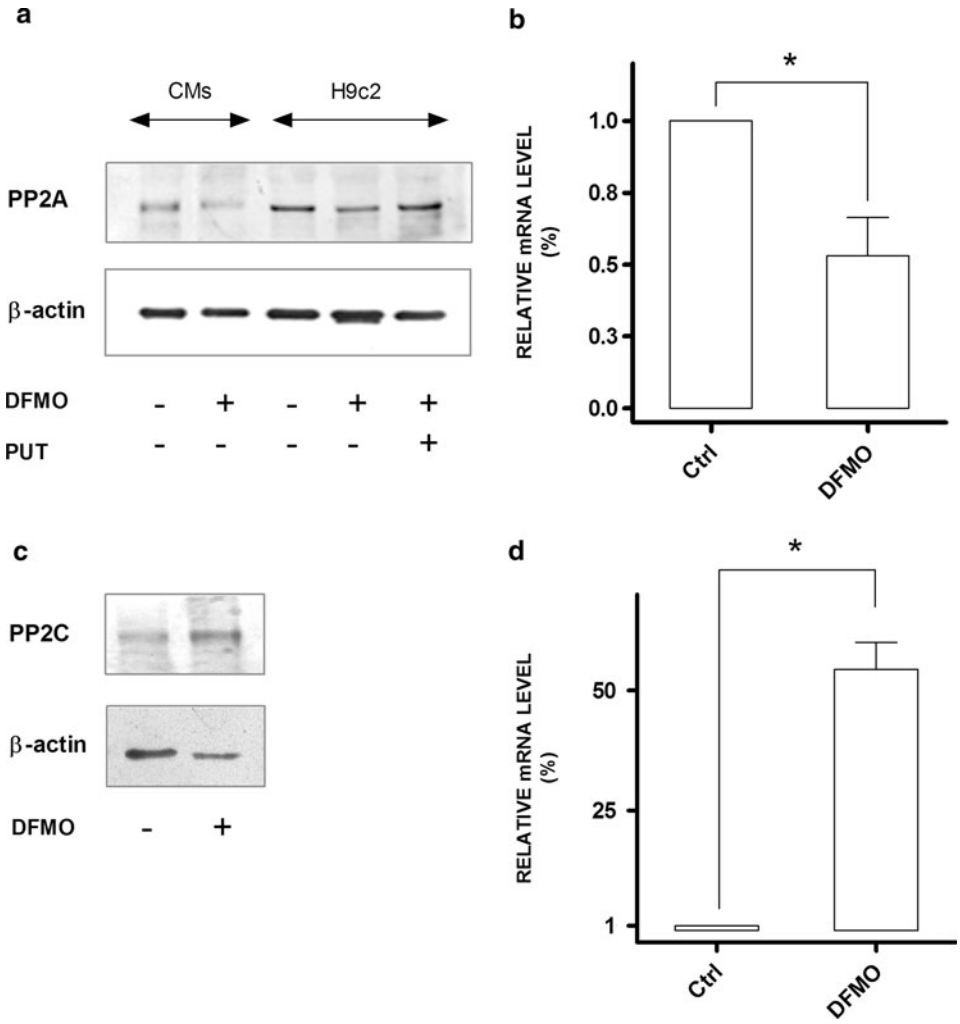

Fig. 5 Polyamine depletion reduces PP2A and increases PP2C levels**. a** Neonatal rat cardiomyocytes (CMs) were grown to confluence for 3 days in DMEM, 10% FBS with or without 100 μM DFMO; H9c2 cardiomyoblasts were grown for 24 h after plating in the presence or absence of 100 μM DFMO or 100 μM DFMO plus 100 μM putrescine. **b–d** H9c2 cardiomyoblasts were grown for 24 h after plating in the presence or absence of 100 μM DFMO. PP2A and PP2C proteins were detected by western blot (**a**, **c**). PP2A and PP2C mRNA levels were analyzed by RT-PCR (**b**, **d**). Results are means ± SEM of three determinations. $*P < 0.05$

levels of PP2A protein were significantly lower both in neonatal rat cardiomyocytes and in H9c2 cardiomyoblasts grown in DFMO containing medium compared with those grown in control or DFMO plus putrescine containing media. The decrease of PP2A expression in polyamine-depleted H9c2 cardiomyoblats, was confirmed by real-time PCR (Fig. 5b). In fact, PP2A mRNA level was noticeably reduced (by 2.0-fold) compared with control cells.

The protein phosphatase 2C (PP2C) is known to directly dephosphorylate and thus inactivate several stress-activated protein kinases including p38 MAPK, JNK and AMPK in mammals (Davies et al. 1995). Therefore, it would seem reasonable that the down regulation and reduction of active phospho-AMPK and phospho-p38 MAPK in DFMO-treated cardiac cells (Fig. 4) could be due to changes in this phosphatase. We therefore monitored the levels of PP2C protein and mRNA in control and DFMO-treated H9c2 cardiomyoblasts. The results indicated that increased PP2C expression was detected in polyamine-depleted cells by western blot (Fig. 5c), and confirmed by real-time PCR (Fig. 5d), showing that PP2C mRNA level was much higher in DFMO-treated H9c2 cells with respect to controls.

These results are consistent with a role of PP2A and PP2C in the changes of AKT, AMPK and p38 MAPK activity in polyamine-depleted cardiac cells, and thus affecting the pro-survival as well as the pro-apoptotic pathways involved in NE-induced cardiac apoptosis.

Discussion

In response to pathophysiological stresses, cardiac myocytes undergo hypertrophic growth or apoptosis, which are associated with the development of cardiac pathologies. Much effort has been expended in order to identify the extracellular stimuli that promote these responses, and the intracellular signaling pathways activated and potentially involved. One of such factors is NE, the elevation of which in plasma closely correlates with the severity and poor

prognosis of heart failure (Haft 1974). It has been shown that NE can induce apoptosis in cardiac myocytes and multiple signal transduction pathways have been proposed to be involved in the cardiotoxic effects of NE. In particular, several data suggest that NE-induced cell death is linked to the increase in ROS production (Fu et al. 2004a), and oxidative stress may occur by both endoplasmic reticulum stress and mitochondrial death pathway (Mao et al. 2006) and may influence several kinase activities (Griendling et al. 2000).

Recent experimental evidence has shown that a class of molecules involved in the responsiveness of the cells to apoptosis are the polyamines spermine, spermidine and the diamine putrescine. Increasing evidence indicates that polyamines play a role in various aspects of the remodeling occurring in cardiac diseases, such as growth, death and differentiation of cardiac cells (Flamigni et al. 2009).

The present study focuses the attention on the effect of DFMO, an inhibitor of polyamine biosynthesis, on apoptosis induced by NE in two different types of cultured cardiac cells, i.e., rat neonatal cardiomyocytes and H9c2 rat cardiomyoblasts. We first demonstrated that NE affects polyamine metabolism in rat neonatal cardiomyocytes by inducing ODC, the key enzyme of polyamine biosynthesis. The synthesis of polyamines appears to be correlated with NE-activation of programmed cell death. In fact, the depletion of the intracellular polyamines by DFMO inhibits the NE-triggered caspase activation, the main biochemical marker of apoptosis, as well as the NE-mediated decrease of survivin expression. Survivin, a protein member of the inhibitor of apoptosis protein family, plays an important role in cardiac functions (Levkau et al. 2008); in particular, its expression has been associated with reduced apoptosis and a more favorable cardiac remodeling in aging spontaneously hypertensive rats (Abbate et al. 2006) and it has been recently proposed as a possible target of the apoptosis facilitating action of polyamines (Cetrullo et al. 2010). Since we have found that the apoptosis-inhibiting effect of DFMO is circumvented by exogenous putrescine, we may conclude that the prevention of NE-induced apoptosis that occurs in DFMO-treated cardiomyocytes is indeed caused by the inhibition of polyamine synthesis.

These results are consistent with recent papers from our group reporting a similar protective effect of DFMO against apoptosis of cardiac cells treated with aldosterone (Cetrullo et al. 2010) or exposed to a condition of simulated ischemia (Tantini et al. 2006). In addition we observed that cardiomyocytes isolated from transgenic mice over-expressing ODC, thus with a four-fold increase of putrescine, show an higher caspase activation with respect to cells from control mice (Tantini et al. 2006). Polyamine depletion attenuates also isoproterenol-mediated myocardial injury in rats (Tipnis et al. 2000) and it has been reported to delay apoptosis of rat intestinal epithelial cells (Ray et al. 2000) and to prevent TNF-induced apoptosis in IEC6 cells (Bhattacharya et al. 2003).

The observed increased resistance to apoptosis of polyamine-depleted cardiac cells suggests that death as well as survival signals are modulated in these cells.

To further investigate the mechanism whereby the treatment with DFMO attenuates NE-induced cell death, we examined the modulation of AMPK, p38 MAPK and AKT pathways, which play an important role in regulating apoptosis and survival in cardiomyocytes (Wang et al. 1998a; Dyck and Lopaschuk 2006; Clerk et al. 2007).

We observed that these pathways were all activated by the treatment with NE, but with different time course as well as different duration and timing of the signal. The AKT phosphorylation was earlier and transient, whereas AMPK and p38 MAPK phosphorylations were more delayed and persistent.

In addition we have shown that sustained phosphorylation of AKT was maintained during the course of NE treatment in polyamine-depleted cells, whereas AMPK phosphorylation declined more rapidly to control levels and the increase in p38 MAPK phosphorylation was prevented in DFMO-treated cardiomyocytes. In our model, the modulation of these signal transduction pathways is correlated with the execution of apoptosis: in fact, NE-mediated caspase activation was reduced in the presence of SB, an inhibitor of p38 MAPK, and increased in the presence of the AKT inhibitor LY. Moreover, the treatment with LY as well as with AICAR, a specific AMPK activator, increased H9c2 cell death, whereas AMPK knockdown protects cells from NE-induced death. These results suggest that in DFMO-treated cardiomyocytes the permanent activation of AKT, as well as the early inactivation of AMPK and the prevention of p38 MAPK activation, might be correlated with a pro-survival effect of polyamine depletion.

An involvement of AMPK and p38 MAPK in simulated ischemia-induced apoptosis of cardiac cells has been recently suggested (Capano and Crompton 2006), whereas a protective effect of AKT activation has been proposed in other studies showing that administration of IGF-1, an upstream regulator of AKT, reduces myocardial apoptosis in response to ischemia and reperfusion in rats (Buerke et al. 1995) and acts as a survival factor for cultured cardiomyocytes (Wang et al. 1998b). A facilitation action of polyamine depletion by DFMO on modulation of AKT pathway and protection from apoptosis has been also found in a variety of other cell systems (Bhattacharya et al. 2003, 2005; Stanic' et al. 2006; Flamigni et al. 2007).

Since the activity of each of these signaling proteins is influenced by serine/threonine phosphorylation, we hypothesize that polyamine depletion either activated anti-

apoptotic proteins or deactivated pro-apoptotic proteins by altering the level of their phosphorylation. Pioneers studies of Tung et al. (1985) as well as others (Cornwell et al. 1986) have reported that polyamines increase PP2A activity. PP2A is a multifunctional protein Ser/Thr phosphatase that regulates a variety of signal pathways in eukaryotic cells (Sontag 2001) and is considered a phosphatase responsible for the dephosphorylation and inactivation of AKT (Liu et al. 2003; Ni et al. 2007). Previous reports in H9c2 cells also showed that AKT was dephosphorylated by PP2A (Yasuoka et al. 2004). Here, we have shown that the levels of PP2A protein were significantly reduced in polyamine-depleted cardiac cells compared with controls or cells grown in DFMO plus putrescine. In our experimental system, we have also found that DFMO treatment significantly increased the levels of PP2C, another serine/threonine phosphatase involved in the regulation of key signal kinases, including AMPK and p38 MAPK (Davies et al. 1995). The finding that PP2A gene expression was lowered while PP2C gene expression was increased in DFMO-treated H9c2 cells suggests that down-regulated PP2A and up-regulated PP2C proteins may be responsible for the DFMO-maintained phosphorylation of AKT and DFMO-induced loss of phosphorylation of AMPK and p38 MAPK, respectively. All these data support a pro-survival, i.e., anti-apoptotic, effect of polyamine depletion in NE-induced apoptosis of cardiac cells mediated by concerted changes in key signaling pathways.

An involvement of polyamine depletion on PP2A-regulated apoptosis has been recently reported in intestinal epithelial cells showing that DFMO prevents apoptosis in IEC-6 cells by regulating serine/threonine phosphorylation of pro- and anti-apoptotic protein (Ray et al. 2005). Furthermore, a protective effect of PP2A inhibition during reperfusion subsequent to myocardial ischemia has been shown in a recent paper (Fan et al. 2010).

In conclusion, the present study focuses the attention on the effect of DFMO, an inhibitor of polyamine biosynthesis, on apoptosis induced by NE in cultured cardiac cells, demonstrating that the ability of high levels of NE to induce cell death is contrasted in polyamine-depleted cultures by a concomitant inhibition of death signals and activation of cell survival mechanisms. The present report provides novel data suggesting an involvement of p38 MAPK, AMPK and AKT kinases in the response of cardiomyocytes to NE, and suggests that these pathways are modulated in polyamine-depleted cardiac cells as result of a differential effect on the specific phosphatases that dephosphorylate and switch off these signaling proteins.

Understanding not only the anti-apoptotic events regulated by polyamine depletion, but also the signaling pathways via which DFMO mediates cardioprotective effects may be important in developing these basic studies toward therapeutic exploitation and may represent a novel approach to identify molecular targets for treatment of cardiac diseases.

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