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ORIGINAL ARTICLE

Difluoromethylornithine inhibits hypertrophic, pro-fibrotic and pro-apoptotic actions of aldosterone in cardiac cells

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Abstract Recent studies have shown that aldosterone may play a critical role in the transition to heart failure and that heart is a direct target of the action of aldosterone, which can provoke hypertrophy and apoptosis of isolated cardiomyocytes and also increase the expression of genes that favor tissue fibrosis. Early work from this and other laboratories has established a link between the aliphatic polyamines and cardiac hypertrophy, while more recently an involvement of polyamines even in cell death and survival has emerged. In the present study we have treated cardiac cells, i.e. rat H9c2 cardiomyoblasts and neonatal cardiomyocytes, with (D,L)-2-(difluoromethyl)ornithine, a specific inhibitor of polyamine biosynthesis, to investigate the effects of polyamines in relation to the hypertrophic, pro-fibrotic and pro-apoptotic actions of aldosterone. The results indicate that inhibition of polyamine biosynthesis may prevent or attenuate the adverse actions of aldosterone, by modulating the expression of genes related to cardiac hypertrophy and fibrosis, as well as the levels of proteins and the activities of enzymes that control apoptosis.

Keywords Aldosterone · Apoptosis · Cardiac cells · Difluoromethylornithine · Hypertrophy · Polyamines

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Abbreviations

Ac-DEVD-AMC	Ac-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin
ANF	Atrial natriuretic factor
APAO	N^1-Acetylpolyamine oxidase
DFMO	(D,L)-2-(difluoromethyl)ornithine
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
IAP	Inhibitor of apoptosis protein
MDL 72527	N^1,N^4-di(buta-2,3-dienyl)butane-1, 4-diamine
MHC	Myosin heavy chain
MR	Mineralcorticoid receptor
ODC	Ornithine decarboxylase
PAI-1	Plasminogen activator inhibitor-1
PCR	Polymerase chain reaction
SMO	Spermine oxidase
SSAT	Spermidine/spermine N^1-acetyltransferanse

Introduction

The natural polyamines, spermidine, spermine and putrescine can specifically bind to nucleic acids, proteins and phospholipids in vitro and may affect gene expression, signaling pathways and ionic transport in cells (Bachrach et al. 2001; Gerner and Meyskens 2004). The intracellular concentration of these aliphatic polycations can be modulated by enzymatic and transport systems. Ornithine decarboxylase (ODC), the first and rate-limiting enzyme in polyamine biosynthesis, is rapidly induced following growth stimuli with consequent accumulation of polyamines. On the other

hand, high polyamine levels as well as stress stimuli can up-regulate polyamine catabolism and interconversion, which involve inducible enzymes like spermidine/spermine N^1-acetyltransferanse (SSAT) and spermine oxidase (SMO) and constitutive enzymes like N^1-acetylpolyamine oxidase (APAO) (Wang and Casero 2006). Polyamine catabolism not only contributes to the fine regulation of polyamine levels, but can also result in the production of potentially cytotoxic H_2O_2 and aldehydes as by-products of either APAO or SMO activity.

The higher requirement of polyamines for tumor growth has made polyamine metabolism an attractive target in experimental cancer therapy and chemoprevention, but attempts to employ specific polyamine-related drugs have not produced significant clinical success in cancer treatment so far. Beside cancer, heart failure (Hunter and Chien 1999) is another leading cause of mortality worldwide. Heart failure may be the final outcome of a variety of cardiovascular diseases, such as myocardial ischemia and infarction, sustained pressure and volume overload of the heart. Myocardial hypertrophy is a major predictor of progressive cardiac disease resulting in heart failure. Pathological hypertrophy has been associated to the stimulation by neurohormonal, stress and inflammatory signals (Hilfinker-Kleiner et al. 2006), which also promote cardiac fibrosis and apoptosis that may contribute to the transition to overt heart failure. In particular, the renine–angiotensin–aldosterone system is considered a major player and its over-activation leads to cardiac remodelling and dysfunction. In this regard, the role of angiotensin II has been well established, and can be exerted through a variety of actions comprising direct effects on cardiomyocytes and other cells of the cardiovascular system and increased aldosterone production. Recent studies have shown that aldosterone promotes heart hypertrophy and fibrosis in rat models, while mineralcorticoid receptor (MR) antagonists significantly reduce morbidity and mortality in patients with heart failure (Hilfinker-Kleiner et al. 2006; Kobayashi et al. 2006; and references within). These findings could not be explained only on the basis of the well-known mineralcorticoid effects on kidney and blood pressure. Instead, several lines of research indicate that heart itself may be an important site of aldosterone action in pathological conditions. Actually aldosterone can provoke hypertrophy and apoptosis of isolated cardiomyocytes (Mano et al. 2004; Okoshi et al. 2004) and also increase in cultured cardiac cells the expression of genes that promote tissue fibrosis (Chun and Pratt 2005). In addition to genomic- and MR-mediated actions that occur after hours, rapid (occurring within minutes), 'non-genomic effects' of aldosterone have been described in cardiac cells, as previously shown for other target cells of this steroid hormone (Chai and Danser 2006; Karmazyn et al. 2003; Mano et al. 2004).

Pioneer studies from this and other laboratories in the seventies of the past century have established a link between polyamines and heart hypertrophy, as it turned out that ODC was rapidly induced and polyamines accumulated in the heart in a number of animal models of cardiac hypertrophy (Flamigni et al. 1986). These observations were then extended by further studies, even employing transgenic mice models (Nisenberg et al. 2006; Shantz et al. 2001). However, polyamines might be involved in allowing and potentially modulating various phenomena of cardiac biology and pathophysiology, including the remodeling occurring in cardiac diseases. Increasing evidence indicates that polyamines are not merely essential factors for cell growth, but play a role even in other cell responses, including apoptosis (Pignatti et al. 2004; Seiler and Raul 2005). In particular we have showed that ODC activity was transiently induced, and polyamine levels increased, in H9c2 cardiomyoblasts exposed to a condition of simulated ischemia that leads to apoptosis (Tantini et al. 2006). In the same model, pre-treatment of the cells with (D,L)-2-(difluoromethyl)ornithine (DFMO), a specific ODC inhibitor, reduced polyamine levels and protected against cell death, inhibiting molecular events related to the mitochondrial pathway of apoptosis. In the present paper, we have extended these studies, by evaluating the ability of DFMO to inhibit adverse actions of aldosterone on cardiac cells, such as its hypertrophic, pro-fibrotic and pro-apoptotic effects.

Materials and methods

Materials

DFMO and N^1,N^4-di(buta-2,3-dienyl)butane-1,4-diamine (MDL 72527) were generous gifts of Patrick M. Woster, Wayne State University, Detroit, MI. Aldosterone and all other biochemical reagents were obtained from Sigma Chemical Company. Polyclonal anti-survivin and monoclonal anti-β actin antibodies were from Santa Cruz and BD, respectively.

Cell cultures and treatments

Cardiomyocyte cultures were prepared from 1- to 3-day-old neonatal Wistar rat hearts. After imbibition of tissue overnight and digestion for 30 min at 37°C, cells were preplated twice, for 30 min and 1 h, to remove non-myocytes and then plated on gelatinized cell culture dishes in Dulbecco's modified Eagle's medium (DMEM, Celbio) DMEM containing 10% heat inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, and 0.1 μg/ml streptomycin. To inhibit non-cardiomyocyte cell proliferation, cytosine arabinose 10 μM was added to culture medium for

48 h. The medium was changed every 2 days. These methods resulted in preparations containing cardiomyocytes, spontaneously beating and showing immunofluorescence staining with antibodies against α-actinin. For experiments, cells were serum-starved for 20 h and then treated with aldosterone.

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 aldosterone treatment (for more details see Tantini et al. 2006). Polyamine depletion was obtained by culturing the cells in the presence of 0.1 mM DFMO, which reduces particularly putrescine and spermidine levels (Tantini et al. 2006). Viable cells were counted following the trypan blue exclusion test. Dead cells (including the dye) were also counted and reported as a percentage of the total number of cells.

Enzymatic assays

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

Immunoblotting

The proteins survivin and β-actin 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″, followed by amplification (45 cycles: 94°C for 15″, appropriate annealing temperature for each target gene as detailed below kept for 20″ and then 72°C for 10″). 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 plasminogen activator inhibitor-1 (PAI-1); 58°C for atrial natriuretic factor (ANF), GATA-4 and β-myosin heavy chain (β-MHC). 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. The comparative C_t method is also known as the $2^{-[\mathrm{delta}][\mathrm{delta}]Ct}$ 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 a reference sample, also normalized to the endogenous housekeeping gene.

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

Gene	Primers	Sequence
GAPDH	Forward	GAC_CTC_AAC_TAC_ATG_GTC_TAC_A
	Reverse	ACT_CCA_CGA_CAT_ACT_CAG_CAC
PAI-1	Forward	GAA_CGC_CCT_CTA_TTT_CAA_CGG
	Reverse	CTC_TGT_TGG_ATT_GTG_CCG_AAC
ANF	Forward	TGG_GCT_CCT_TCT_CCA_TCA_CC
	Reverse	GCC_AAA_AGG_CCA_GGA_AGA_GG
β-MHC	Forward	GCC_TAC_CTC_ATG_GGA_CTG_AA
	Reverse	ACA_TTC_TGC_CCT_TTG_GTG_AC
GATA-4	Forward	AGA_AGG_CAG_AGA_GTG_TGT_CA
	Reverse	CAG_TGT_GGT_GGT_GGT_AGT_CT

Results and discussion

DFMO inhibits the expression of genes related to heart hypertrophy and fibrosis in cardiac cells stimulated by aldosterone

Aldosterone is directly implicated in cardiac hypertrophy and fibrosis. In particular, the addition of aldosterone to cultured neonatal rat cardiomyocytes can induce cellular hypertrophy as judged by both morphological and

biochemical criteria (Karmazyn et al. 2003; Okoshi et al. 2004), including the up-regulation of the hypertrophy marker genes ANF, α-MHC and β-MHC. These hypertrophic responses were generally suppressed by MR antagonists. The transcription factor GATA-4, a master regulator of cardiac genes, has been implicated in the transcriptional activation of genes encoding ANF and contractile proteins and in sarcomeric reorganization in response to hypertrophic stimulation (Pikkarainen et al. 2004). GATA-4 itself can be regulated by various hypertrophic stimuli at gene expression and/or post-translational level, but a possible modulation by aldosterone has not yet been investigated. On the other hand, aldosterone has been reported to increase the expression of PAI-1, a factor that inhibits extracellular matrix-degrading metalloproteinases and promote fibrosis, in neonatal rat cardiomyocytes and in H9c2 rat cardiomyoblasts (Chun and Pratt 2005; Tanaka et al. 2007). Moreover, this effect was prevented by a MR antagonist in both cardiac cell types. Since the occurrence of MR has been demonstrated in H9c2 cardiac cells (Chun and Pratt 2005), this cell line was used for gene expression studies. H9c2 cells were treated with DFMO and/or aldosterone and then analyzed for their relative levels of mRNA for PAI-1, ANF, β-MHC and GATA-4 by real-time PCR, employing the specific primers reported in Table 1. Figure 1 shows that the expression of all these genes was markedly stimulated by the treatment with 100 nM aldosterone for 4 h. Moreover, DFMO pre-treatment largely prevented the aldosterone-induced increase in mRNA levels. A marked inhibitory effect was exerted by DFMO even if aldosterone concentration was elevated to 10 μM, but at this high dosage the hormone appeared somewhat less effective in stimulating the expression of the hypertrophy-related genes ANF, β-MHC and GATA-4 (not shown). The correlation between polyamines and cardiac hypertrophy has been known for long time, however, information about the molecular targets of the action of polyamines and polyamine inhibitors is lacking. Early work showed that DFMO decreased synthesis, levels and secretion of ANF by rat atria and neonatal rat cardiomyocytes (Tipnis et al. 1993, 1994). Although to our knowledge no information is available in the literature about the effect of DFMO on cardiac fibrosis, a correlation has been shown between polyamines levels in the heart of isoproterenol and/or arginine-treated rats and collagen fibers, assessed by Van Gieson staining, as well as ANF mRNA levels and other indexes of cardiac hypertrophy (Lin et al. 2008).

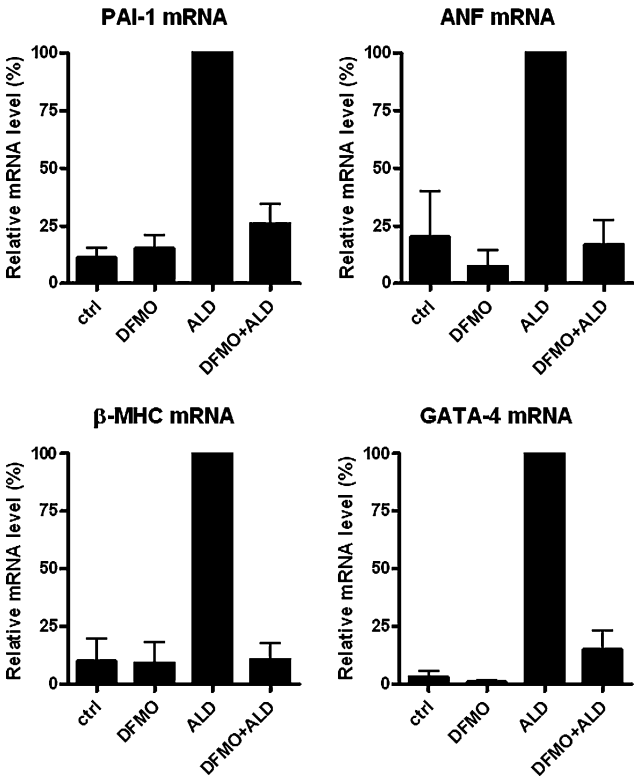

Fig. 1 Involvement of polyamines in the expression of hypertrophy- and fibrosis-related genes in cardiac cells stimulated by aldosterone. H9c2 cells were pre-incubated after seeding in the absence or in the presence of 0.1 mM DFMO for 24 h. Then cells were further treated with or without 100 nM aldosterone (ALD) for 4 h. Control cells received no treatment. Cells were harvested and assayed for mRNA levels by RT-PCR as detailed in "Materials and methods". Data are expressed as % of aldosterone-stimulated cells and represent mean ± SEM. The relative values of mRNA content after DFMO plus aldosterone treatment were significantly different with respect to cells treated with aldosterone only ($p < 0.05$ for ANF and $p < 0.01$ for PAI-1, β-MHC and GATA-4, by paired t test; $N = 5$ for PAI-1 and $N = 3$ for ANF, β-MHC and GATA-4)

DFMO reduces cardiomyocyte apoptosis induced by aldosterone

It has been reported that aldosterone can induce apoptosis of neonatal rat cardiomyocytes, most likely through a rapidly activated, non-genomic pathway involving mitochondria (Mano et al. 2004). The apoptotic response was time- and dose-dependent, with a maximum effect at a 10 μM concentration. Therefore, neonatal rat cardiomyocytes were utilized and exposed to this high dose of aldosterone for 24 h before measuring caspase activity. Caspases are a family of proteases that execute the death program and represent a well-known biochemical marker of apoptosis. Aldosterone caused an increase in caspase activity that was largely prevented by pre-treatment with the ODC inhibitor DFMO (Fig. 2a). Interestingly DFMO effectively abolished a transient increase in ODC activity that was noted after a 3 h treatment with aldosterone (Fig. 2b), thus preceding caspase activation. Furthermore, survivin, an anti-apoptotic member of the inhibitor of apoptosis protein (IAP) family, was down-regulated after aldosterone treatment and inhibition of polyamine

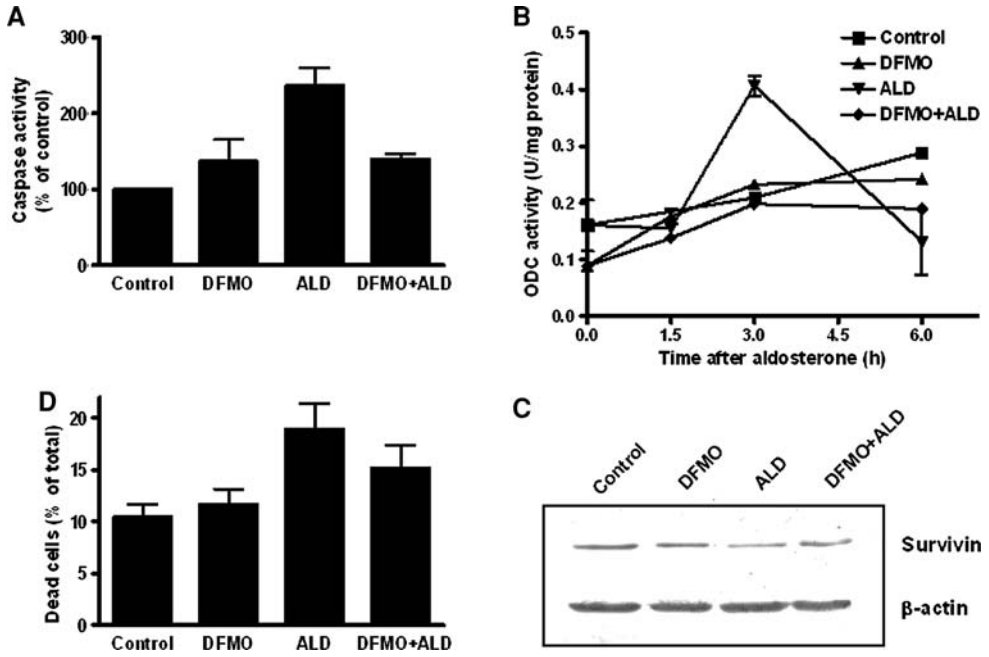

Fig. 2 Involvement of polyamines in aldosterone-promoted apoptosis of cardiac muscle cells. Neonatal rat cardiomyocytes or H9c2 cardiomyoblats were cultured for 3 days in the absence or presence of 0.1 mM DFMO, and then treated with 10 μM aldosterone (ALD). **a** DFMO pre-treatment inhibits effector caspase activation by aldosterone in neonatal rat cardiomyocytes. Cells were collected after treatment with aldosterone for 24 h and analyzed for caspase activity (DEVDase). **b** Time course of ODC activity following aldosterone treatment of neonatal rat cardiomyocytes pre-incubated with or without DFMO. Cells were analyzed for ODC activity at the indicated time after aldosterone. **c** DFMO pre-treatment contrasts the reduction in survivin level following aldosterone. Neonatal rat cardiomyocytes were collected after treatment with aldosterone for 24 h and analyzed for the content of survivin or β-actin (as a control) by Western blot. Densitometric analysis showed that survivin content was 90, 38, and 68% of control cells after treatment with DFMO, aldosterone or DFMO plus aldosterone, respectively. **d** DFMO pre-treatment reduces the death of H9c2 cells enhanced by aldosterone. H9c2 cells were collected after treatment with aldosterone for 48 h and counted to assess cell viability by trypan blue exclusion test. The percentage of dead cells after aldosterone treatment was significantly different with respect to control cells ($p < 0.05$ by paired t test, $N = 5$) or to cells treated with DFMO plus aldosterone ($p < 0.05$ by paired t test, $N = 5$). Instead no significant difference was found between control cells and DFMO-treated cells or DFMO plus aldosterone treated cells by paired t test, $N = 5$. Data shown in graphs are mean ± SEM

biosynthesis by DFMO partially opposed survivin decrease (Fig. 2c). Altogether, these results indicate that DFMO can interfere with caspase activation, suggesting that polyamines are involved in the execution of the death program activated by aldosterone in cardiomyocytes. Accordingly, Fig. 2d shows that DFMO pretreatment significantly reduced the increase in the percentage of dead cells observed after 48 h incubation of H9c2 cardiomyoblasts with 10 μM aldosterone.

These results are consistent with a previous paper from our group reporting a similar protective effect of DFMO against apoptosis of rat H9c2 cardiac cells exposed to a condition of simulated ischemia (Tantini et al. 2006). Besides in both cases, a transient rise in ODC activity, abolished by DFMO treatment, was observed. In addition, we observed that cardiomyocytes isolated from transgenic mice over-expressing cardiac ODC, thus with a fourfold increased putrescine level, show a higher caspase activation with respect to cells from control mice (Tantini et al. 2006). Therefore, the present results confirm and extend the view of polyamines as pro-apoptotic factors in cardiac cells. Besides the present paper individuates survivin, which is known to inhibit caspases and other mitochondrial events related to apoptosis, as a possible target of the apoptosis-facilitating action of polyamines. Survivin myocardial expression appears to be important for cardiac function (Levkau et al. 2008) and has been associated with reduced apoptosis and more favorable cardiac remodeling in spontaneously hypertensive rats (Abbate et al. 2006).

Recent studies have focused on the ability of polyamines to affect survival and death of cardiomyocytes subjected to ischemia and reperfusion without employing DFMO (Han et al. 2007; Zhao et al. 2007). In particular, the addition of putrescine to cultures of neonatal rat cardiomyocytes was shown to enhance the rate of apoptosis measured after simulated ischemia/reperfusion (Han et al. 2007), however, the treatment with spermine or spermidine resulted to decrease it. Activation of polyamine catabolism following induction of SSAT and/or SMO has been suggested to favor cell death by perturbation of polyamine levels and/or by generation of H_2O_2 and other potentially toxic by-products (Wang and Casero 2006). Quite recently, the

expression of SSAT has been found to increase in the ischemic myocardium following coronary ligation in rats (Ryu et al. 2008), but SSAT appeared to function as a cardioprotectant in this context. However in our cell models, we were not able to show SSAT or SMO induction, nor any protective effect of the APAO and SMO inhibitor MDL 72527 (not shown). In summary, all these studies report a variety of results, in part related to the different experimental models, which confirm the complex involvement of polyamines and polyamine-related enzymes in the control of cell death and survival. In fact, the relationship between polyamines and apoptosis appears to be dependent on the cell type and death stimulus, as well as on the actual levels of the single polyamines and activated polyamine pathways (Pignatti et al. 2004; Seiler and Raul 2005).

Concluding remarks

In the present paper, we have confirmed that the hormone aldosterone can act on rat cardiac cells directly, favoring the expression of genes related to hypertrophy and fibrosis, such as ANF, β-MHC and PAI-1. GATA-4 can be added to this list and may play a pivotal role by promoting the expression of other genes up-regulated by aldosterone in cardiac cells. We have not investigated the involvement of MR in this study, however, our results may be compatible with a classical, "genomic" mode of action of aldosterone. Moreover, a sustained treatment with aldosterone can lead cardiomyocytes to apoptosis, possibly through non-genomic mechanisms (Mano et al. 2004). The present study shows that selective inhibition of polyamine biosynthesis by DFMO can prevent the hypertrophy- and fibrosis-related genic responses of cardiac cells and also contrast their apoptotic response to aldosterone. The precise mechanisms by which DFMO and consequent lack of polyamines realize these effects remain to define.

Most of the efforts in polyamine field have been driven by the hope to find novel and efficacious ways to treat or prevent cancer. However, we have recently reviewed some papers showing that inhibition of polyamine biosynthesis, catabolism or uptake may prevent or reduce apoptosis triggered by a variety of stimuli in non-tumoral cells, such as cardiac cells, stem cells, chondrocytes or infected-macrophages (Flamigni et al. 2007), and proposed polyamine biosynthesis as a target to reduce deleterious apoptosis in some pathological contexts. Since hypertrophic stimuli are known to activate polyamine biosynthesis and pathological cardiac hypertrophy is associated to tissue fibrosis and apoptotic cell death of cardiomyocytes, our results hint at the possibility to target polyamine metabolism to interfere with these alterations and contrast progression of heart failure. Of course a possible use of these findings would require in vivo investigation to validate that found in cell culture.

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