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Overproduction of cardiac *S*-adenosylmethionine decarboxylase in transgenic mice

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The present study was designed to provide a better understanding of the role played by AdoMetDC (*S*-adenosylmethionine decarboxylase), the key rate-controlling enzyme in the synthesis of spermidine and spermine, in controlling polyamine levels and the importance of polyamines in cardiac physiology. The αMHC (α-myosin heavy chain) promoter was used to generate transgenic mice with cardiac-specific expression of AdoMetDC. A founder line (αMHC/AdoMetDC) was established with a > 100-fold increase in AdoMetDC activity in the heart. Transgene expression was maximal by 1 week of age and remained constant into adulthood. However, the changes in polyamine levels were most pronounced during the first week of age, with a 2-fold decrease in putrescine and spermidine and a 2-fold increase in spermine. At later times, spermine returned to near control levels, whereas putrescine and spermidine levels remained lower, suggesting that compensatory mechanisms exist to limit spermine accumulation. The αMHC/AdoMetDC mice did not display an overt cardiac phenotype, but there was an increased cardiac hypertrophy after β-adrenergic stimulation with isoprenaline ('isoproterenol'), as well as a small increase in spermine content. Crosses of the αMHC/AdoMetDC with αMHC/ornithine decarboxylase mice that have a > 1000-fold increase in cardiac ornithine decarboxylase were lethal *in utero*, presumably due to increase in spermine to toxic levels. These findings suggest that cardiac spermine levels are highly regulated to avoid polyamine-induced toxicity and that homoeostatic mechanisms can maintain non-toxic levels even when one enzyme of the biosynthetic pathway is greatly elevated but are unable to do so when two biosynthetic enzymes are increased.

Key words: heart, isoprenaline (isoproterenol), *S*-adenosylmethionine decarboxylase, spermidine, spermine, transgenic mice.

INTRODUCTION

The polyamines putrescine, spermidine and spermine have been shown to be essential for mammalian cell growth and differentiation [1,2]. A number of studies suggest that the growth responses of cardiac tissue are accompanied by increases in *de novo* polyamine synthesis, while other investigations confirm a prominent role for polyamines in compensatory cardiovascular hypertrophy associated with hypertension [3–10]. Polyamines are needed for the inward rectification of potassium channels [11,12] and have been shown to regulate the excitability threshold and duration of action potentials in cardiomyocytes, with spermine being responsible for most of the effect [13,14]. These properties may make polyamines a useful target for the development of new ventricular anti-arrhythmic agents.

AdoMetDC (*S*-adenosylmethionine decarboxylase) is a critical enzyme in the synthesis of the polyamines spermidine and spermine [1,15]. The dcAdoMet (decarboxylated *S*-adenosylmethionine) produced by AdoMetDC is used as the aminopropyl donor for the synthesis of these polyamines from putrescine. The activity of AdoMetDC is therefore thought to be an important factor in the control of polyamine levels. In accord with this concept, AdoMetDC is very highly regulated at a number of levels including transcription, translation, proenzyme processing, catalytic activity and degradation [16]. Only a few attempts to increase AdoMetDC content have been reported. A CHO (Chinese-hamster ovary) cell line with a stable amplification of the *AdoMetDC* gene was obtained by growth in the presence of the inhibitor CGP-48664 and had a 10-fold increase in activity when the inhibitor was removed [17]. Increased activity (up to 30–40-fold) was reported in MCF-7 cells [18] and in NIH 3T3 cells [19] transfected with plasmids containing AdoMetDC, as well as in FM3A cells selected for resistance to the inhibitor EGBG [ethylglyoxal bis-(guanylhydrazone)] [20]. These alterations increased the cellular spermine content. A more limited (4-fold or less) increase was seen in transgenic mice in which the *AdoMetDC* gene copy number was increased by transgenic insertion of a 19.5 kb fragment containing the entire rat gene and adjacent sequences [21]. These mice showed no significant alteration in their spermidine and spermine even when bred with mice overexpressing ODC (ornithine decarboxylase), the other enzyme that has been described as rate limiting for polyamine synthesis [21].

AdoMetDC expression from the construct used in these experiments [21] is likely to be subject to all the physiological regulations described above that tend to maintain polyamine homoeostasis, and the AdoMetDC promoter is not particularly active in cardiac tissue. In order to study the effects of polyamines in cardiac physiology, we were interested in transgenic manipulations that might alter polyamine content significantly. The content of dcAdoMet in cardiac tissue is known to be low [22]. We have therefore attempted to overexpress AdoMetDC in the heart using the αMHC (α-myosin heavy chain) promoter in order to study the role played by AdoMetDC in controlling polyamine levels and the importance of polyamines in cardiac physiology. The αMHC promoter is a very strong and highly specific promoter that has been used extensively for cardiac expression studies. Cardiac ODC was increased by > 1000-fold in αMHC/ODC transgenic mice [10]. The αMHC/AdoMetDC mice that were

Abbreviations used: (h)AdoMetDC, (human) *S*-adenosylmethionine decarboxylase; dcAdoMet, decarboxylated *S*-adenosylmethionine; DTT, dithiothreitol; H/E, haematoxylin and eosin; αMHC, α-myosin heavy chain; ODC, ornithine decarboxylase; SSAT, spermidine/spermine-N^1-acetyltransferase; TGF-β, transforming growth factor β; UTR, untranslated region.

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generated in the present study had a large increase in enzyme activity. Polyamine distribution was altered towards spermine by this elevation in AdoMetDC but compensatory mechanisms appear to exist that limit spermine accumulation. The overexpression of AdoMetDC did not produce an overt cardiac phenotype, but there was an increased cardiac hypertrophy after β-adrenergic stimulation, and crosses of αMHC/AdoMetDC mice with αMHC/ODC mice were lethal at an early embryonic stage.

MATERIALS AND METHODS

Materials

Isoprenaline ('isoproterenol') and all other chemicals were obtained from Sigma unless indicated otherwise. The protease inhibitor cocktail [500 μM AEBSF (4-(2-aminoethyl)benzenesulphonyl fluoride)/HCl, 150 nM aprotinin, 1 μM of the protease inhibitor E-64 (*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane), 0.5 mM EDTA and 1 μM leupeptin] was from Calbiochem–Novabiochem. The restriction enzymes and Taq polymerase were from Promega and New England Biolabs. Deoxynucleotide triphosphates were from Amersham Biosciences. Plasmid purification columns and genomic DNA isolation kits were purchased from Qiagen. *S*-adenosyl-L-[carboxy-^{14}C]methionine (58 mCi/mmol) was obtained from Amersham Biosciences. [1-^{14}C]Ornithine (47.7 mCi/mmol) was purchased from NEN. [^{14}C]Acetyl-CoA was obtained from ICN Biochemicals. [^{35}S]-dcAdoMet was synthesized from L-[^{35}S]methionine (PerkinElmer Life Sciences, Boston, MA, U.S.A.) as described previously [23].

Generation and identification of αMHC/AdoMetDC mice

Transgenic mice were generated by DNA microinjection of fertilized B6D2F2 oocytes using standard techniques in the Transgenic Core Facility at Pennsylvania State University College of Medicine. The transgene was constructed essentially as described previously for αMHC/ODC mice [10]. A 7.3 kb fragment derived from NotI digestion of a pBR322-based vector containing a murine αMHC promoter (provided by J. Robbins, University of Cincinnati Medical Center, Cincinnati, OH, U.S.A.) [24] was used for insertion of a human AdoMetDC cDNA with greatly abbreviated 5′-UTR (5′-untranslated region) and 3′-UTR. The AdoMetDC cDNA contained 52 bp of 5′-UTR and 66 bp of 3′-UTR. The NotI digestion fragment to be used for microinjection was first purified using the Elutrap system and Elutip columns (Schleicher and Schuell) and then precipitated and resuspended in microinjection buffer (10 mM Tris/HCl, pH 7.4, and 0.25 mM EDTA).

Genomic DNA was isolated from the tails of potential transgenic mice and subjected to PCR analysis to identify mice bearing the transgene. For PCR analysis, the 5′-sense primer was an αMHC promoter sequence (5′-GTAAAAGAGGCAGGGAA-GTGGTG-3′) and the 3′-antisense primer was an oligonucleotide that bound nucleotides coding for amino acids 34–41 of the hAdoMetDC (human AdoMetDC)-coding sequence (5′-GTAT-GTCCCACTCAGATCTTGGG-3′). The amplified 297 bp product spanned the junction between the αMHC promoter and the hAdoMetDC sequence and was only detected in mice bearing the transgene. The transgene was maintained in the hemizygous state by breeding of hemizygous males with B6D2 females (Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.).

Enzyme assays and polyamine analyses

In order to determine AdoMetDC activity, heart or other tissue was placed in 0.5 ml of ice-cold 25 mM Tris/HCl, pH 7.5, 2.5 mM putrescine, 2.5 mM DTT (dithiothreitol), 0.1 mM EDTA, 0.01 % Tween 80 (Fisher Scientific) and 1× protease inhibitor cocktail, homogenized on ice using a Polytron for 15 s on/15 s off/15 s on and centrifuged at 20000 ***g*** for 30 min at 4 °C. The cytosolic fraction was subsequently used to determine AdoMetDC activity and was stored at − 80 °C before use. For measurement of other enzymes [ODC, SSAT (spermine/spermine-N^1-acetyltransferase), spermine synthase and spermidine synthase], tissue was treated in the same manner, except that a different harvesting buffer appropriate for the enzyme of interest was used. The composition of ODC harvesting buffer was 25 mM Tris/HCl (pH 7.5), 2.5 mM DTT, 0.1 mM EDTA, 0.01 % Tween 80 and 1× protease inhibitor cocktail. The composition of SSAT harvesting buffer was the same, except that 50 mM Tris/HCl was used. Spermidine or spermine synthase harvesting buffer was composed of 50 mM sodium phosphate (pH 7.2), 0.3 mM EDTA, 10 mM 2-mercaptoethanol and 1× protease inhibitor cocktail.

All enzyme assays were carried out as reported previously. AdoMetDC and ODC were assayed by measuring the release of $^{14}CO_2$ from *S*-adenosyl-L-[carboxy-^{14}C]methionine [25] and [1-^{14}C]ornithine [26] respectively. Spermidine synthase and spermine synthase assays were carried out by measuring the production of [^{35}S]methylthioadenosine from [^{35}S]dcAdoMet in the presence of the appropriate amine acceptor [23]. SSAT was measured by following the transfer of [^{14}C]acetyl groups from [^{14}C]acetyl CoA to spermidine [27].

Immunoblotting of AdoMetDC was carried out essentially as described previously [28,29]. Cytosolic protein (10 μg) was separated by SDS/PAGE (12.5 % polyacrylamide) and transferred on to PVDF membranes. AdoMetDC protein was detected using a rabbit polyclonal antibody raised against hAdoMetDC and a chemiluminescence detection system. The protein band intensity was quantified using the Gene Genome system, with Gene Snap and Gene Tools software (Syngene Bio Imaging, Cambridge, U.K.).

Polyamine content was determined by HPLC using an ion-pair reverse-phase HPLC separation method with post-column derivatization using *o*-phthalaldehyde as described previously [30].

Isoprenaline treatment and measurement of cardiac size and function

Adult mice, 7–8 weeks old, were given daily intraperitoneal injections of either saline or 20 mg/kg isoprenaline in 0.9 % sterile saline for 8 days. Mice were killed by CO_2-induced asphyxia 6 h after the last injection and weighed and the hearts were removed immediately. The hearts were then weighed and immediately flash-frozen in liquid N_2. The cardiosomatic index, an indicator of cardiac hypertrophy, was calculated by dividing the heart weight (mg) by the body weight (g). Hearts from 7–8-week-old mice used in the study were sliced first in an apical fashion and the remainder was divided in half with an oblique cut. The sections were weighed and oblique portions were flash-frozen for subsequent enzymatic or polyamine analysis. Apical portions were fixed in 10 % neutral-buffered formalin, embedded in paraffin and cut into 5 μm transverse slices, which were examined after H/E (haematoxylin and eosin) staining or for myocyte size after staining with wheatgerm agglutinin–fluorescein (Sigma) to label cell membranes. Images were taken upon fluorescent microscopy, and the area of cell size was quantified using NIH Image in multiple sections from each heart.

An eight-channel non-invasive blood pressure monitor NIBP-8 with a tail occlusion cuff (Columbus Instruments, Columbus, OH, U.S.A.) was utilized for determination of blood pressure.

Measurements were begun after the mouse was restrained and the tail warmed to 37 °C for 5 min. Left ventricular hypertrophy and systolic function were studied by echocardiography using an Accuson Sequoia instrument paired with a 14 MHz transducer. After light anaesthesia (100 mg/kg ketamine and 10 mg/kg xylazine), the animal was shaved, placed in a supine position and warmed using an isothermal heating pad. M-mode echo was used because it has temporal resolution that can record the rapid heartbeat of the mouse.

Crosses between αMHC/AdoMetDC and αMHC/ODC mice

F2 hemizygous αMHC/AdoMetDC female mice were bred with F5 heterozygous and homozygous males from the previously characterized αMHC/ODC murine line [10]. The females were examined every morning and the appearance of a vaginal mucous plug was considered day 0 of gestation. Pregnant females were killed by cervical dislocation and genomic DNA PCR analysis of the removed embryos and the fetuses was performed as described above. The antisense primer 5′-CTTCTTTAGAATGTCTCCGAGGTCC-3′ used for αMHC/ODC detection was complementary to the amino acids 44–51 of the αMHC/ODC transgene [10].

RESULTS

Generation of transgenic mice with cardiac AdoMetDC expression

Previously, mouse lines transgenic for the full rat genomic sequence of AdoMetDC exhibited only a slight (2–4-fold) increase in AdoMetDC activity [21,31]. Since this might have been due to the weak activity of the promoter or to regulatory sequences in the UTR of AdoMetDC mRNA, which are known to impart cell type-specific expression and regulation by polyamines [32,33], the AdoMetDC cDNA was placed under the regulation of the strong well-characterized, cardiac-specific αMHC promoter [24] and deletions in the 5′- and 3′-UTRs of the AdoMetDC mRNA coding sequence were made to abrogate post-transcriptional regulation. Three potential founder mice were identified by genomic DNA screening. However, only one founder both expressed and transmitted the transgene. All results were obtained using progeny from this one transgenic line, αMHC/AdoMetDC. Southern-blot and dot-blot analyses of DNA from the αMHC/AdoMetDC showed the presence of the transgene. The copy number was estimated to be < 5.

AdoMetDC activity in αMHC/AdoMetDC mice

AdoMetDC activity was assayed in the cytosolic fraction from mouse hearts at 12 developmental time points from 1 day after birth to adulthood (Table 1). The activity in the transgenic mice was maximal by 5 days of age. Over the age range from 5 days to 3 weeks, there was a > 200-fold increase in cardiac AdoMetDC over the controls. At later ages up to 24 weeks, the fold increase was slightly less as the non-transgenic AdoMetDC was slightly higher, but there was still > 100-fold increase in activity.

The tissue specificity of αMHC-driven AdoMetDC expression was tested by assaying tissue panels (brain, spleen, lungs, liver, small intestine and gastrocnemius muscle) of 3-week-old mice. In agreement with previous findings on transgenes expressed from the αMHC promoter [10,24], the only other tissue that showed any expression of the transgene was the lung. The lungs of transgenic animals showed a moderate 5-fold increase in AdoMetDC activity as compared with control littermates [162 ± 34 versus 28 ± 2 pmol · (30 min)$^{-1}$ · (mg of protein)$^{-1}$]. This is explained by the expression of αMHC in pulmonary vasculature [24]. The rest of the tissues tested showed no statistically significant increase in AdoMetDC activity (results not shown).

Table 1 Cardiac AdoMetDC activity in control and αMHC/AdoMetDC mice

Hearts were isolated from mice at the indicated ages and AdoMetDC-specific activity was determined in cytosolic fractions from hearts of transgenic animals and littermate controls. Values are means ± S.E.M. for the number of mice indicated in parentheses for each group.

	AdoMetDC activity (nmol/30 min per mg of protein)		
Age	Control	αMHC/AdoMetDC	Fold increase
1 day	0.034 ± 0.008 (6)	2.4 ± 0.4 (6)	71
3 days	0.068 ± 0.007 (6)	7.7 ± 0.7 (6)	113
5 days	0.060 ± 0.008 (5)	13.8 ± 1.4 (6)	230
1 week	0.057 ± 0.010 (5)	13.7 ± 1.2 (6)	240
2 weeks	0.052 ± 0.003 (5)	13.8 ± 0.7 (5)	265
3 weeks	0.049 ± 0.008 (6)	12.4 ± 0.4 (7)	253
4 weeks	0.055 ± 0.009 (4)	10.2 ± 0.3 (7)	185
5 weeks	0.083 ± 0.004 (5)	12.4 ± 0.9 (5)	149
6 weeks	0.073 ± 0.034 (5)	8.7 ± 0.5 (5)	119
11 weeks	0.055 ± 0.018 (3)	8.8 ± 1.8 (3)	160
16 weeks	0.078 ± 0.005 (3)	11.4 ± 0.7 (4)	146
24 weeks	0.073 ± 0.009 (4)	9.9 ± 1.3 (5)	136

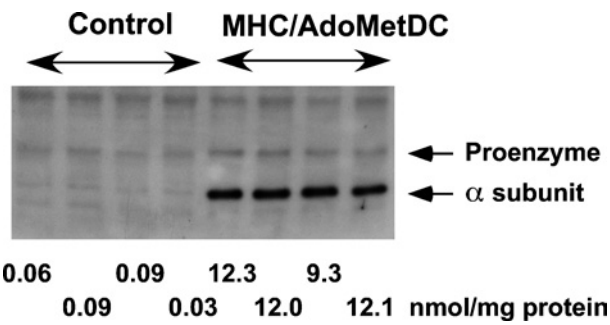

Figure 1 Increase in AdoMetDC protein in αMHC/AdoMetDC mice

Hearts were isolated from four control (left lanes) and four αMyHC/AdoMetDC mice. Aliquots of 10 μg of protein were used in Western blots developed with an antiserum that recognizes both the AdoMetDC proenzyme and the 31 kDa α subunit. The measured AdoMetDC activities of each sample are shown below each lane.

Western blotting of the approx. 31 kDa subunit confirmed that there was a large increase in the amount of AdoMetDC protein (Figure 1). The level of AdoMetDC protein in the control mice was too low for accurate quantification with the antiserum available but, based on these densitometric measurements, there was an approx. 480-fold increase in the amount of protein. AdoMetDC is synthesized as an approx. 38 kDa proenzyme that undergoes a non-hydrolytic serinolysis cleavage reaction to generate two non-identical subunits, with the pyruvate prosthetic group formed from an internal serine residue at the N-terminus of the approx. 31 kDa α-subunit and a much smaller approx. 8 kDa subunit [34]. This processing is believed to occur spontaneously, but a role for other cellular factors in facilitating the reaction is not ruled out. There was a small increase in a protein that was detected in the blots at the appropriate position for the AdoMetDC proenzyme. There was a non-specific background band in the extracts from the control mice at this position, which interferes with accurate quantification, but at least 90 % of the expressed AdoMetDC was processed, indicating that any factors needed for cleavage of the AdoMetDC are not limiting in the formation of the active protein in αMHC/AdoMetDC mice (Figure 1).

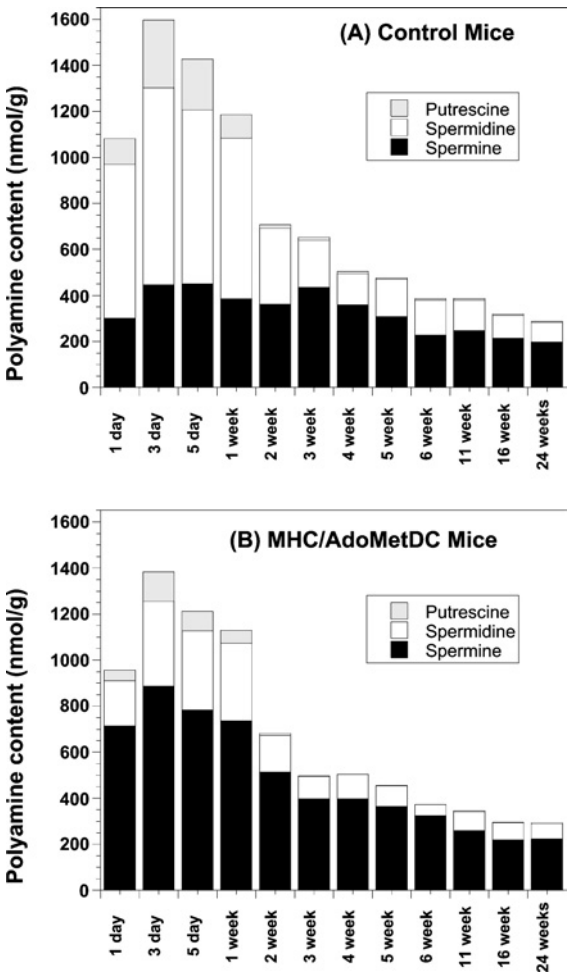

Figure 2 Polyamine content in control and αMHC/AdoMetDC mice

Mice were killed at the indicated ages and polyamines were determined and plotted as a column where the shaded area corresponds to the polyamine indicated and the total polyamine content is represented by the total height of the column. Supplementary Table 1 http://www.BiochemJ.org/bj/393/bj3930295add.htm shows these results in a Table (mean ± S.E.M.). Results for littermate controls (**A**) and αMyHC/AdoMetDC mice (**B**) are shown. Polyamines are expressed as nmol/g of tissue.

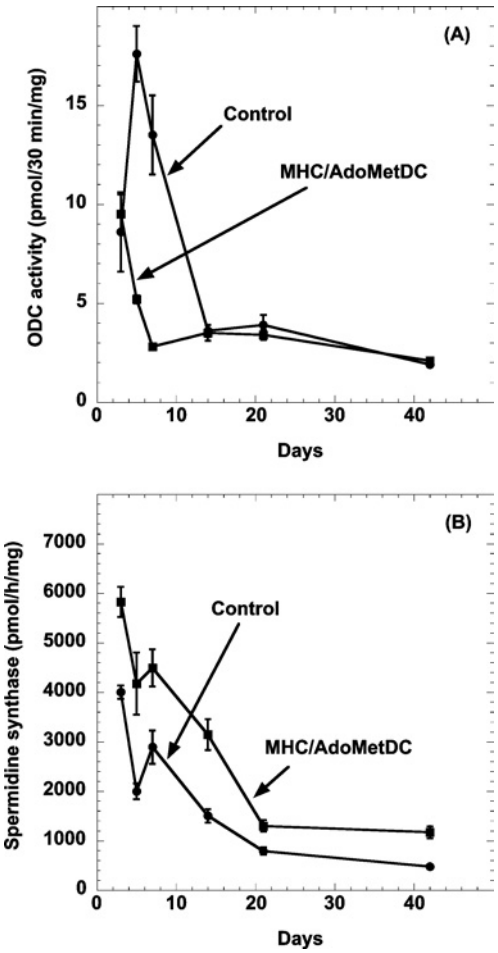

Figure 3 Changes in ODC and spermidine synthase in αMHC/AdoMetDC mice

ODC (**A**) and spermidine synthase (**B**) activities were determined in extracts from littermate controls and αMyHC/AdoMetDC mice at the ages shown. Ten mice per group were used for 3 and 5 day time points and five mice per group for later time points. Values are expressed as means ± S.E.M. Differences in spermidine synthase are statistically significant at all time points ($P < 0.01$) and differences in ODC are statistically significant only at 5 and 7 days.

Changes in polyamine levels due to αMHC/AdoMetDC

Heart polyamine levels were measured over the same time course from 1 day to 24 weeks (see Figure 2; see Supplementary Table 1 at http://www.BiochemJ.org/bj/393/bj3930295add.htm). Younger animals were shown to have the greatest concentration of the polyamines and polyamine levels declined with age. Transgenic expression of AdoMetDC did not increase total polyamine levels and actually decreased them slightly during the first week. The overexpression of AdoMetDC altered the distribution of polyamines, particularly at early ages. Putrescine levels (which were much higher in the first week of life) were reduced to about one-half of the controls at all time points studied. The ratio of spermine to spermidine was increased by the high AdoMetDC activity. At ages up to approx. 3 weeks, this alteration in ratio was brought about by a decrease in spermidine and an increase in spermine. Spermidine levels were approx. 50 % of the controls and spermine levels were elevated by approx. 2-fold until week 3. However, at later times, spermidine levels reached 80 % of the control levels and spermine content was near control levels (5–20 % higher, but not statistically significant). These results suggest that the polyamine regulatory mechanisms are better able to normalize polyamine levels as time progresses. Equal numbers of males and females were used in these experiments, and no gender differences were observed in the levels of cardiac polyamines at any age tested.

Alterations in other enzymes of polyamine metabolism

Cardiac ODC activity was much higher over the first week of life in control mice, reaching a peak at 5 days of age. On day 2, the ODC activity was not different in controls and αMHC/AdoMetDC mice; however, on days 5 and 7, the activity in the αMHC/AdoMetDC mice was highly repressed towards adult levels (Figure 3A). After day 14, there was no difference in ODC between the control and αMyHC/AdoMetDC mice.

Spermidine-synthase activity was also altered in the αMHC/AdoMetDC mice (Figure 3B). This activity (which also decreases with age over the first 3 weeks of life) was increased by approx. 50 % at all ages in the transgenic mice. In contrast, spermine synthase, which, as previously reported [23], is much lower than spermidine synthase, was not altered significantly (results not shown). SSAT was below the limit of detection in the cardiac tissue from both control and αMHC/AdoMetDC mice.

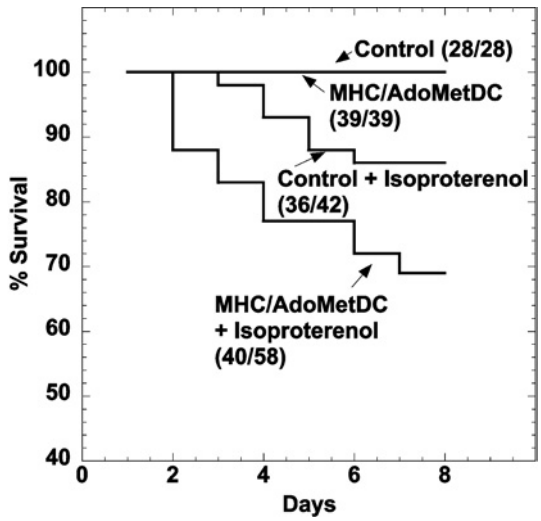

Figure 4 Survival of αMHC/AdoMetDC mice and control mice treated with isoprenaline ('Isoproterenol')

Transgenic mice and littermate controls (7–8 weeks old) were treated with daily intraperitoneal injections of either saline or 20 mg/kg isoprenaline for up to 8 days. Three separate experiments with approx. 10–20 mice and approximately equal numbers of each sex in each group were carried out with similar results and the results were combined to give the survival curves shown. The number of animals remaining after 8 days and the total number of animals are indicated in parentheses.

Characteristics of αMHC/AdoMetDC mice

Despite the great increase in AdoMetDC activity and a significant alteration in cardiac polyamine levels, no obvious physiological impairments were apparent in αMHC/AdoMetDC transgenics. There may be a very small difference in survival; at 3 weeks of age, there were 199/359 (55 %) control mice and 160/359 (45 %) transgenics from the matings of control and αMHC/AdoMetDC mice. Both male and female transgenics exhibited a normal adult lifespan and were fertile. Measurements of heart rate and blood pressure in adult, 7-week-old mice showed no significant differences between transgenics and controls (results not shown). As described below, the αMHC/AdoMetDC mice consistently showed a slight increase in the heart weight/body weight ratio, suggesting a slight cardiac hypertrophy, but this difference was not statistically significant.

Effects of treatment of αMHC/AdoMetDC mice with isoprenaline

The development of cardiac hypertrophy in response to treatment with β-adrenergic agonists such as isoprenaline is well established [4,5,10,35]. Such treatment induces cardiac ODC activity, causing increases in putrescine and spermidine and producing a marked cardiac hypertrophy. This hypertrophy was substantially enhanced in αMHC/ODC transgenic mice [10].

The effect of isoprenaline on the αMHC/AdoMetDC mice was examined by giving daily injections of 20 mg/kg of isoprenaline for 8 days. There was an increased mortality in the αMHC/AdoMetDC mice treated with isoprenaline (Figure 4) with 31 % of the treated αMHC/AdoMetDC mice dying compared with 14 % of the control mice.

Isoprenaline treatment had no effect on AdoMetDC activity in either αMHC/AdoMetDC or non-transgenic mice (Table 2). ODC activity was increased by isoprenaline in both groups but to a lesser extent in the non-transgenic mice. The increase in ODC was reflected in a clear increase in putrescine and spermidine content and in total polyamines in the control mice (Table 3).

Table 2 Effect of isoprenaline on cardiac AdoMetDC and ODC activities in control and αMHC/AdoMetDC mice

αMHC/AdoMetDC mice and control littermates (7–8 weeks old) were treated with saline or isoprenaline (20 mg/kg) daily for 8 days. Hearts were removed from surviving mice 6 h after the final injection, and ODC and AdoMetDC activities were determined. Values are means ± S.E.M. for the number of mice indicated in parentheses for each group.

Treatment group	ODC activity (pmol/ 30 min per mg of protein)	AdoMetDC activity (nmol/ 30 min per mg of protein)
Control saline	2.7 ± 0.4 (6)	0.04 ± 0.01 (11)
Control isoprenaline	8.0 ± 1.0* (6)	0.02 ± 0.005 (10)
αMHC/AdoMetDC saline	1.9 ± 0.2 (6)	8.6 ± 0.64 (17)
αMHC/AdoMetDC isoprenaline	8.3 ± 0.9* (6)	6.8 ± 0.16 (12)

* Significantly different from the group not treated with isoprenaline ($P < 0.0001$).

Table 3 Effect of isoprenaline on cardiac polyamine content in control and αMHC/AdoMetDC mice

αMHC/AdoMetDC mice and control littermates (7–8 weeks old) were treated with saline or isoprenaline (20 mg/kg) daily for 8 days. Hearts were removed from surviving mice 6 h after the final injection, and polyamine content was determined. Values are expressed as means ± S.E.M. for the number of mice indicated in parentheses for each group.

	Polyamine content (nmol/g of tissue)			
Treatment group	Putrescine	Spermidine	Spermine	Total
Control saline (19)	7.7 ± 0.3	120 ± 4	319 ± 16	447
Control isoprenaline (23)	35.5 ± 0.4*	247 ± 4*	313 ± 15	596
αMHC/AdoMetDC saline (18)	4.8 ± 0.4	74 ± 4	362 ± 18	441
αMHC/AdoMetDC isoprenaline (19)	10.9 ± 1.1*	93 ± 5*	434 ± 37*	538

* Significantly different from the group not treated with isoprenaline ($P < 0.01$).

Table 4 Effect of isoprenaline on cardiac hypertrophy in control and αMHC/AdoMetDC mice

αMHC/AdoMetDC mice and control littermates (7–8 weeks old) were treated with saline or isoprenaline (20 mg/kg) daily for 8 days. Hearts were removed from surviving mice 6 h after the final injection, and heart weight/body weight ratio was determined. Values are means ± S.E.M. for the number of mice indicated in parentheses for each group.

Treatment group	Heart weight/body weight ratio
Control saline (21)	5.79 ± 0.17
Control isoprenaline (24)	6.68 ± 0.17*
αMHC/AdoMetDC saline (19)	5.96 ± 0.16
αMHC/AdoMetDC isoprenaline (20)	7.76 ± 0.22†‡

* Significantly different from control saline ($P < 0.001$).
† Significantly different from αMHC/AdoMetDC saline ($P < 0.001$).
‡ Significantly different from control isoprenaline ($P < 0.001$).

These increases were less in the αMHC/AdoMetDC mice but the latter had a significant increase in spermine (Table 3).

Cardiac hypertrophy was induced by isoprenaline treatment in both control and αMHC/AdoMetDC mice (Table 4). The increase in heart weight was greater in the αMHC/AdoMetDC mice; the difference between the isoprenaline-treated control and isoprenaline-treated αMHC/AdoMetDC mice was highly significant ($P < 0.001$). Microscopic analysis of H/E sections also revealed an increase in myocyte size and myofibrillar disorganization in isoprenaline-treated αMHC/AdoMetDC mice that is consistent with hypertrophic phenotype (results not shown).

In the results shown in Table 4 and in all other comparisons of αMHC/AdoMetDC and control littermates, there was a small increase in the heart weight/body weight ratio of transgenic mice,

but this difference did not achieve statistical significance and it does not account for the much greater difference between the two groups when treated with the β-agonist. Echocardiography of mice treated with either saline or isoprenaline for 8 days showed that left ventricular function, as measured by shortening fraction, ejection fraction and cardiac output, was not different in transgenics compared with identically treated controls (results not shown).

Studies of crosses between αMHC/AdoMetDC and αMHC/ODC mice

The results described above with αMHC/AdoMetDC mice and those published previously with αMHC/ODC mice [10] indicate that a large increase in the level of either enzyme activity in the heart can be tolerated without major deleterious effects unless subjected to additional stimuli such as β-adrenergic stimulation. This may be due to compensatory mechanisms that act to resist alterations in cellular polyamine content and/or downstream alterations resulting from changed polyamine content. These factor(s) may have a limited capacity to respond to further increases in polyamine biosynthesis. The αMHC/ODC mice have a 50-fold increase in putrescine and 3.6-fold increase in spermidine [10], but the absence of an increase in spermine may be due to a lack of dcAdoMet. We therefore bred both homozygous and heterozygous αMHC/AdoMetDC mice with αMHC/ODC mice. No double transgenic offspring were born. Examination of the fetuses at gestation days 7, 9, 10, 11 and 12 indicated that embryonic lethality occurred before day 11 of gestation. Of more than 100 potential offspring of ages greater than E11 (embryonic day 11) examined, no viable double transgenics were found.

DISCUSSION

The results presented in this paper are the first report of a significant alteration in the content of polyamines in an AdoMetDC-overexpressing transgenic mouse line. In previous work with mice transgenic for full rat genomic sequence of AdoMetDC, AdoMetDC activity was increased only 2–4-fold relative to their syngenic littermates and spermine levels were unchanged, while putrescine and spermidine were only slightly decreased [21,31]. Polyamine levels in a cross of these mice with those overexpressing ODC were also normal [21]. There was a much larger increase in cardiac AdoMetDC activity in our αMHC/AdoMetDC mice and, particularly at early ages, this was reflected in a significant alteration in the relative polyamine content. From birth until 3 weeks of age, transgenic cardiac tissue showed a 50 % reduction in putrescine and in spermidine and a 2-fold increase in spermine content. From 3 weeks on, spermine levels returned to near control levels, while spermidine was decreased 20–40 % and putrescine was decreased 50 % compared with controls. The decrease in putrescine and spermidine levels is most likely due to their rapid conversion into spermine in the presence of excess dcAdoMet, the aminopropyl donor produced by increased activity of the transgenic AdoMetDC. The alteration in polyamine levels leading to increased spermine is consistent with that reported in tissue culture studies with transiently or stably overexpressed AdoMetDC [17,19,20,36], although spermine accumulation is less marked in the mice despite a higher level of AdoMetDC activity.

These results are consistent with the concept that the supply of dcAdoMet limits the production of spermine. When this supply is increased, putrescine and spermidine decrease and spermine increases. However, it is noteworthy that there was no increase in the total polyamine content (putrescine + spermidine + spermine) in the αMHC/AdoMetDC mice. In fact, this total decreased slightly at early ages. This suggests that the total polyamine content is controlled by the ODC level and its ability to supply the 1,4-diaminobutane moiety. The decrease in total polyamines in the αMHC/AdoMetDC mice could be due to the repression of ODC activity shown in Figure 3(A). This reduction is likely to be due to the increase in spermine, which is the most active polyamine in causing the induction of antizyme and the subsequent degradation of ODC [37].

There was a significant increase in spermidine synthase in the αMHC/AdoMetDC mice. Most of the work on the regulation of polyamine synthesis has focused on the two decarboxylases, ODC and AdoMetDC, which have been found to be altered in many physiological and pathological states, but spermidine synthase has previously been reported to be increased during liver regeneration, hormonal stimulation and lectin-induced lymphocyte activation and to be down-regulated in response to TGF-β (transforming growth factor β) (see [38] and references therein). Since the former conditions increase AdoMetDC activity and TGF-β decreases it, it is possible that there is a link between spermidine synthase and AdoMetDC and the possibility that spermidine synthase is regulated by dcAdoMet should be investigated.

The alterations in spermine and spermidine content in the αMHC/AdoMetDC mice were less pronounced at ages above 3 weeks, suggesting that compensatory mechanisms come into play to limit spermine accumulation despite the presence of non-physiological levels of AdoMetDC activity in cardiac tissue. Such mechanisms may contribute to the absence of a phenotype in the αMHC/AdoMetDC mice since over-accumulation of spermine may be toxic [1,39,40]. The observed normalization of spermine levels cannot be explained by regulatory changes in polyamine biosynthetic enzymes that were measured and other mechanisms must be considered. One possibility is an increase in a polyamine export system, but this seems unlikely since the only known export systems are most active on putrescine [1,2,41]. Although a limitation in methionine or *S*-adenosylmethionine that prevents further production in spermine cannot be ruled out, a more attractive possibility is induction of the recently discovered spermine oxidase that converts spermine into spermidine [42,43]. Such an up-regulation would create a futile cycle with spermidine being continuously shuffled into creation of spermine and the spermine being converted back into spermidine at the same rate. At present, there are no established assay methods for this spermine oxidase activity in crude tissue samples.

It is also possible that physiological factors relating to polyamine homoeostasis limit the increase in AdoMetDC activity in the αMHC/AdoMetDC mice. This activity reached a peak on day 5, whereas it has been reported that the αMHC promoter increases progressively in activity after birth, becoming 70 % active at 1 week of age and maximally active at 3 weeks of age [10,24,44]. The content of ODC in αMHC/ODC mice was not maximal until day 21 and was 14 % of maximal at 2 days and 36 % of maximal at 14 days [10]. This discrepancy suggests that some post-transcriptional regulatory factors influence the amount of AdoMetDC activity in αMHC/AdoMetDC mice. Sequences influencing translation were removed from the UTR of the transgene but it is possible that post-translational mechanisms influence the content of active AdoMetDC protein. One possibility is that the substrate-mediated transamination of the decarboxylase is increased, which converts the covalently bound pyruvate prosthetic group into alanine, permanently inactivating the enzyme [45]. This is known to occur in the recombinant enzyme expressed to very high levels in bacteria [46] and may be a key step in the rapid degradation of mammalian AdoMetDC [29]. Such transamination would be reflected in a difference between the amount

of protein and its enzymatic activity and could account for the apparent greater increase in protein based on Figure 1 and activity measurements, but the levels of protein in the controls were too low for confidence in this difference. In any event, post-translational mechanisms do not explain the reversion towards normal in the polyamine content in the adult αMHC/AdoMetDC mice since there was still a > 100-fold increase in AdoMetDC activity in these mice.

The αMHC/AdoMetDC mice resemble αMyHC/ODC mice in the pathological consequences of their response to pharmacological doses of the β-agonist, isoprenaline. Both strains of transgenic mice show an increased cardiac hypertrophy after this treatment although the additional alteration in the polyamine content is limited. The αMHC/ODC mice have a much more striking alteration in polyamines (with a 50-fold increase in putrescine and a 3.6-fold increase in spermidine [10]) than the αMyHC/AdoMetDC mice, which have only a small increase in spermine that is increased further by isoprenaline. These results suggest that other factors influenced by the β-agonist are more effective in bringing about hypertrophy when cardiac polyamines are increased and that increased spermine is much more effective than increased spermidine and putrescine.

It is unlikely that the increased mortality in the isoprenaline-treated αMHC/AdoMetDC mice is related to the hypertrophy since (i) most of the mortality occurs in the first 3–4 days of treatment and (ii) the increase in heart size is not accompanied by any other evidence of heart failure as indicated by the echocardiography. A more probable explanation is that the modest increase in spermine (and/or the spermine/spermidine ratio) alters the electrophysiology in the hearts and the changes in Kir currents cause potentially fatal arrhythmias. This hypothesis is supported by several studies. For example: an increase in spermine levels leads to reduction of K^+ currents in neuronal cells [47]; the ablation of inward rectifying K^+ current leads to hypertrophy and destabilization of resting potential of murine cardiomyocytes [48]; and transgenic up-regulation of Kir2.1 channels in the mouse heart leads to multiple abnormalities of cardiac excitability [49].

Both αMHC/AdoMetDC and αMHC/ODC have normal lifespans and the only phenotype seen in the untreated mice is a very modest reduction in the number of transgenic mice at weaning. This suggests that even in cardiac tissue, compensatory mechanisms leading to polyamine homoeostasis can prevent the accumulation of toxic levels of polyamines despite many studies showing that elevated polyamines may cause a variety of toxic effects including apoptosis and perturbation of critical ion channels. However, the very early lethality in the double transgenic mouse line overexpressing both AdoMetDC and ODC enzymes under the control of the αMHC promoter suggests that these homoeostatic mechanisms cannot cope with the co-ordinated increases in polyamine precursors. Although αMHC promoter is not fully active at the time at which the double transgenic mice die, it is expressed from 7.5 days post-conception and is localized mainly to the atria in the developing heart [50]. The very early lethality shows vividly the importance of regulation of polyamine content, but the very small amount of relevant tissue at this time prevents us from measuring the concentration at which cardiac polyamine content becomes toxic. Since the mice died at a time too early for us to measure polyamine content, it is not clear whether the toxic polyamine is spermidine or spermine but we would favour the latter since: (i) αMHC/ODC mice already have a large increase in spermidine with no toxicity; and (i) the crosses between αMHC/AdoMetDC mice and CAG (cytomegalovirus immediate-early enhancer, β-actin promoter, β-globin poly A signal)/spermine synthase mice (which overproduce spermine synthase) are also embryonic lethal [23].

This work was supported by grant CA-18138 to A. E. P. from the National Cancer Institute, National Institutes of Health (Bethesda, MA, U.S.A.) and by grant 004014N from the American Heart Association to L. M. S. The echocardiographic studies were supported by a Commonwealth of Pennsylvania Tobacco Settlement Award and a Penn State University Dean's Feasibility grant (to A. E. P.). We are grateful to Suzanne Sass-Kuhn for technical assistance.

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Received 22 July 2005/7 September 2005; accepted 12 September 2005
Published as BJ Immediate Publication 12 September 2005, doi:10.1042/BJ20051196