Overexpression of antizyme in the hearts of transgenic mice prevents the isoprenaline-induced increase in cardiac ornithine decarboxylase activity and polyamines, but does not prevent cardiac hypertrophy

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Two lines of transgenic mice were produced with constitutive expression of antizyme-1 in the heart, driven from the cardiac α -myosin heavy chain promoter. The use of engineered antizyme cDNA in which nucleotide 205 had been deleted eliminated the need for polyamine-mediated frameshifting, normally necessary for translation of antizyme mRNA, and thus ensured the constitutive expression of antizyme. Antizyme-1 is thought to be a major factor in regulating cellular polyamine content, acting both to inhibit ornithine decarboxylase (ODC) activity and to target it for degradation, as well as preventing polyamine uptake. The two transgenic lines had substantial, but different, levels of antizyme in the heart, as detected by Western blotting and by the ability of heart extracts to inhibit exogenous purified ODC. Despite the high levels of antizyme, endogenous ODC activity was not completely abolished, with 10–39% remaining, depending on the transgenic line. Additionally, a relatively small decrease (30–32%) in cardiac spermidine content was observed, with levels of putrescine and spermine unaffected. Interestingly,

although the two lines of transgenic mice had different antizyme expression levels, they had almost identical cardiac polyamine content. When treated with a single acute dose of isoprenaline (isoproterenol), cardiac ODC activity and putrescine content were substantially increased (by 14-fold and 4.7-fold respectively) in non-transgenic littermate mice, but these increases were completely prevented in the transgenic mice from both founder lines. Prolonged exposure to isoprenaline also caused increases in cardiac ODC activity and polyamine content, as well as an increase in cardiac growth, in non-transgenic mice. Although the increases in cardiac ODC activity and polyamine content were prevented in the transgenic mice from both founder lines, the increase in cardiac growth was unaffected. These transgenic mice thus provide a valuable model system in which to study the importance of polyamine levels in cardiac growth and electrophysiology in response to stress.

Key words: putrescine, spermidine, spermine.

INTRODUCTION

Putrescine, spermidine and spermine, known collectively as polyamines, have long been known to be essential for cell growth, with many studies having shown these compounds to be associated with the synthesis of DNA, RNA and protein (reviewed in [1–3]). Recent studies have indicated that polyamines also have a potentially important role in higher organisms by regulating ion channel function [4–8]. Conversion of the amino acid ornithine into the diamine putrescine in mammalian cells is catalysed by the enzyme ornithine decarboxylase (ODC). The higher polyamines, i.e. spermidine and spermine, are formed subsequently by addition of aminopropyl groups in reactions catalysed by spermidine synthase and spermine synthase respectively. Decarboxylated *S*-adenosylmethionine is formed in a reaction catalysed by *S*-adenosylmethionine decarboxylase, and provides the aminopropyl groups used in the syntheses of the higher polyamines. Apart from dietary uptake, ODC provides the only route for *de novo* polyamine synthesis in mammalian cells [9].

ODC is, therefore, a key polyamine biosynthetic enzyme, and its activity in mammalian cells is highly regulated. Control at the transcriptional, translational and post-translational levels has been well documented (reviewed in [3,10–12]). Recently much attention has been given to the regulation of ODC activity in particular, and to polyamine levels in general, by a protein

termed antizyme. The properties of antizyme, more accurately referred to as antizyme-1, have been widely reviewed [12–14]. Elevated levels of intracellular polyamines induce the synthesis of antizyme-1 by promoting a +1 frameshift that allows translation of the antizyme mRNA. Antizyme-1 binds to the monomeric form of ODC, preventing formation of the enzymically active homodimer, and then targets ODC to the 26 S proteasome, where it undergoes ubiquitin-independent degradation. Antizyme-1 is also known to inhibit the membrane polyamine transporter and to prevent uptake of extracellular polyamines, although the exact mechanism of this effect remains unclear. There are two potential start sites for antizyme-1 translation and both may be used, possibly leading to different subcellular distributions of the protein, the significance of which is not yet understood [14]. Recently a second antizyme protein, antizyme-2, has been described and further antizyme genes reported [15–18]. Antizyme-2 also inhibits ODC activity and polyamine transport, but the ability of the protein to promote the degradation of ODC is unclear [16].

The studies with antizyme-1 described above provide strong evidence that it can regulate ODC activity and cellular polyamine levels, but the extent to which antizyme is a dominant factor in such regulation is unknown. As one approach to understanding the role played by antizyme, we have generated transgenic mice in which a very high level of constitutive antizyme expression is directed specifically to the heart by utilizing a cardiac-specific

Abbreviations used: ODC, ornithine decarboxylase; MHC, myosin heavy chain; DFMO, α -difluoromethylornithine.

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promoter. The mouse heart is a valuable model system for the investigation of cardiac physiology [19], and polyamines might be expected to play a major role in cardiac growth and electrophysiology.

Our results show that a high level of constitutive antizyme expression can be achieved, but that this did not totally abolish endogenous ODC activity, and was not incompatible with apparently normal cardiac development. However, the induction of cardiac ODC activity, and the subsequent polyamine accumulation caused by both a single acute dose of, and prolonged exposure to, isoprenaline (isoproterenol) was prevented. Interestingly, the expression of antizyme had no effect on the increase in cardiac growth caused by prolonged exposure to isoprenaline. Such mice may thus provide a better understanding of the association of ODC activity, polyamines and cardiac hypertrophy.

MATERIALS AND METHODS

Materials

All chemicals, unless otherwise noted, were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Production of transgenic mice

Transgenic mice were generated by DNA microinjection of fertilized B6D2F2 oocytes using standard techniques [20] in the Transgenic Core Facility, Pennsylvania State University College of Medicine. The transgene was a 6.8 kb fragment derived from *NotI* digestion (New England Biolabs, Beverly, MA, U.S.A.) of a pBR322-based vector containing a murine α -myosin heavy chain (α -MHC) promoter [21] upstream of a full-length rat antizyme-1 cDNA which had been engineered to contain a single nucleotide deletion at position 205 [22]. This deletion eliminates the need for translational frameshifting, as is normally required in the production of wild-type antizyme, and thus permitted constitutive expression of antizyme in the heart.

Genomic DNA was isolated from the tails of potential founder mice (DNeasy tissue kit; Qiagen, Valencia, CA, U.S.A.) and subjected to two different PCR analyses to identify mice bearing the transgene. The 5' sense primers were α -MHC promoter sequences and the 3' antisense primers were oligonucleotides that corresponded to antizyme coding sequences. Each amplified product therefore spanned the junction between the α -MHC promoter and the antizyme sequence, and was only detected in mice bearing the transgene. Primers were synthesized in the Macromolecular Core Facility, Pennsylvania State University College of Medicine. PCR reagents used and *Taq* DNA polymerase were purchased from Promega (Madison, WI, U.S.A.), and an Ultrapure dNTP Set was from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.).

Two transgenic founders (α MHCAZ34 and α MHCAZ35) were identified, and thereafter the transgene was maintained in the hemizygous state by breeding male transgenic animals with B6D2F1 females (The Jackson Laboratory, Bar Harbor, ME, U.S.A.), resulting in approximately equal numbers of transgenic pups and normal littermates. Transgenic progeny were identified using the same PCR procedures as detailed above, and all experiments were carried out using F2 generation male and female mice. Animals were killed for all experiments using an inhalation anaesthetic, methoxyflurane (Metofane; Schering-Plough Animal Health, Union, NJ, U.S.A.), followed by cervical dislocation.

Transgene copy number was estimated using a slot-blotting procedure as detailed by Hogan and co-workers [20]. Briefly,

genomic DNA was blotted on to a nylon transfer membrane (Hybond-N⁺; Amersham Pharmacia Biotech) and hybridized with a fluorescein-labelled probe (ECF Random Prime Labeling Module; Amersham Pharmacia Biotech) designed to bind to the transgene in genomic DNA. Detection was achieved using a fluorescent detection system (ECF Signal Amplification Module; Amersham Pharmacia Biotech) and a FluorImager 595 (Molecular Dynamics, Sunnyvale, CA, U.S.A.). Copy number was estimated by quantitative analysis (ImageQuant application software; Molecular Dynamics) of the intensity of binding of the fluorescent probe to genomic DNA of transgenic animals, compared with a standard curve.

Characterization of cardiac antizyme expression and polyamine metabolism in transgenic mice

Transgenic mice and their non-transgenic littermate controls were killed at 6 weeks of age and the hearts removed immediately. The hearts were divided in half by a mid-sagittal cut, flash-frozen in liquid nitrogen and stored at -80°C until required for analysis. Half of the heart was placed in 1 ml of ice-cold ODC assay buffer [25 mM Tris/HCl, pH 7.5 (Boehringer Mannheim Corp., Indianapolis, IN, U.S.A.), 2.5 mM dithiothreitol, 0.1 mM EDTA; $1\times$ protease inhibitors (Protease Inhibitor Cocktail Set I; Calbiochem-Novabiochem Corp., La Jolla, CA, U.S.A.) and 0.01% Tween 80 (Fisher Biotech, Fairlawn, NJ, U.S.A.)] and homogenized on ice using a Polytron for 15 s on/15 s off/15 s on. Centrifugation was carried out at 4°C for 30 min at 20000 *g*, and the cytosolic fraction was used to assay ODC activity, antizyme activity and antizyme expression. Protein measurements were carried out using Bio-Rad reagent (Bio-Rad, Hercules, CA, U.S.A.) with BSA as a standard [23].

The presence of free antizyme in the extracts was assayed by measuring the ability of heart extract to inhibit the activity of purified recombinant mouse ODC. The activity of the purified ODC was much greater than the activity present in the heart extracts alone. ODC assay buffer was placed in an assay tube and an assay mix {50 μl , containing 12.5 μl of 1 M Tris/HCl, pH 7.5, 5 μl of 2 mM pyridoxal 5'-phosphate, 2.5 μl of 250 mM dithiothreitol, 5 μl of 20 mM L-ornithine, 2.5 μl of L-[1- ^{14}C]ornithine (specific radioactivity 47.70 mCi/mmol; NEN, Boston, MA, U.S.A.) and 22.5 μl of deionized water} was added. Heart extract from non-transgenic littermates and transgenic animals, containing a known amount of protein in the range 5–150 μg , depending on the transgenic line, was then added (ODC assay buffer and heart extract to total a volume of 190 μl), followed by, finally, 10 μl of a solution containing mouse ODC (500 ng/ml), which had been purified as described by Poulin and co-workers [24]. ODC activity was then determined by measuring the release of $^{14}\text{CO}_2$ over a 30 min incubation period at 37°C [25]. The amount of protein in heart extracts required to inhibit the purified ODC activity by 50% was calculated from the best-fit line for the data from all the transgenic progeny from the relevant founder.

In order to estimate the amount of antizyme present in the hearts of transgenic mice, recombinant antizyme was produced and its ability to inhibit purified mouse ODC activity was also measured. Using the same plasmid as was used to produce the transgenic mice, the antizyme-1 cDNA was amplified by PCR and ligated into a pQE30 vector (Qiagen). The protein expressed from the resulting pQE30-AZ plasmid had the N-terminal sequence MRGSHHHHHHGSAS in place of the MP (Met-Pro) that is present when the second potential start codon of the antizyme mRNA is utilized. Transformed *Escherichia coli* JM109 cells containing the pQE30-AZ plasmid were induced to express

the poly-histidine-tagged antizyme (6 × His-AZ) by the addition of isopropyl β -D-thiogalactopyranoside (150 μ M). Cells were lysed by sonication and the affinity-tagged antizyme was purified from the bacterial extract using a TALON Metal Affinity Column (Clontech, Palo Alto, CA, U.S.A.) according to the manufacturer's instructions. The antizyme was further purified by gel filtration (Superose 12, HR10/30; Amersham Pharmacia Biotech).

Endogenous ODC activity was assayed using extracts prepared from whole mouse hearts, as described above. For this assay, ODC assay mix {200 μ l, containing 50 μ l of 1 M Tris/HCl, pH 7.5, 20 μ l of 2 mM pyridoxal 5'-phosphate, 10 μ l of 250 mM dithiothreitol, 4 μ l of L-[1- 14 C]ornithine (specific radioactivity 47.70 mCi/mmol) and 116 μ l of deionized water} was added to 800 μ l of heart extract, and the assay reaction was carried out at 37 °C for 1 h.

To ensure that measured enzymic activity was indeed ODC activity, heart extracts from both non-transgenic mice and transgenic mice from both founder lines were incubated with 5 mM α -difluoromethylornithine (DFMO), an irreversible inhibitor of ODC, for 30 min at 37 °C prior to an ODC assay. Controls were incubated with the same volume of deionized water.

Heart extracts containing 100 μ g of protein were used to investigate the amount of antizyme protein present by Western blotting. Samples were subjected to SDS/PAGE using 15 % gels, transferred to PVDF-PLUS transfer membrane (Micron Separations Inc., Westborough, MA, U.S.A.) and hybridized with an affinity-purified anti-antizyme polyclonal antibody (1:400 dilution of supplied material) [26,27]. The antibody was kindly provided by Dr J. L. A. Mitchell (Department of Biological Sciences, Northern Illinois University, DeKalb, IL, U.S.A.). Protein was visualized using an ECF Western Blotting Reagent Pack (rabbit) (Amersham Pharmacia Biotech), fluorescence detection was performed using a FluorImager 595 (Molecular Dynamics) and quantitative analysis was carried out using ImageQuant application software (Molecular Dynamics).

Cardiac polyamine content was determined using samples corresponding to half of the heart. Polyamines were acid-extracted using 10 % trichloroacetic acid (Fisher Scientific) and aliquots were analysed using an ion-pair reverse-phase HPLC separation method with post-column derivatization using o-phthalaldehyde, as described previously [28].

Effects of acute and chronic treatment of mice with isoprenaline

Transgenic mice and non-transgenic littermates from each founder were taken at 8 weeks of age and injected subcutaneously with 20 mg/kg isoprenaline in 0.9 % sterile saline (total volume 100 μ l). Controls for each group received 0.9 % sterile saline only. Mice were killed 6 h after treatment, and the hearts were removed and analysed for endogenous ODC activity and polyamine content as well as Western blot analyses for antizyme content, as described above. For chronic isoprenaline treatment, mice were injected daily with 20 mg/kg isoprenaline for 10 days, and were killed 6 h after the final injection and weighed. The heart was then removed and weighed also. The cardiosomatic index, an indicator of cardiac hypertrophy, was calculated by dividing the heart weight by the body weight.

Statistical analysis

Analysis of all results was carried out using two-tailed Student's *t*-tests.

RESULTS

Generation of transgenic mice with cardiac antizyme expression

Two distinct founder lines, termed α MHCAZ34 and α MHCAZ35, in which the antizyme transgene DNA was clearly present were established and propagated. The viable progeny of matings between transgenic founder males and normal females had the expected 50:50 distribution between transgenic and non-transgenic mice, indicating that antizyme expression did not significantly affect survival. The transgenic animals appeared healthy and their breeding, raising of young and length of life up to 1 year were unaffected.

Presence of free antizyme protein in hearts from transgenic mice expressing antizyme

In order to test whether free antizyme was present in the hearts of the transgenic mice, extracts were prepared and tested for their ability to inactivate exogenous purified recombinant mouse ODC (Figure 1). Extracts from the non-transgenic littermates had no inhibitory activity in this assay (Figure 1). (The small increase in activity was probably due to stabilization of the added ODC which, in the very dilute solution used, is relatively unstable.) In

Figure 1 Effect of heart extracts from progeny of founder α MHCAZ34 (upper panel) and progeny of founder α MHCAZ35 (lower panel) on the activity of purified ODC

The activity of mouse ODC protein (5 ng) was determined in the presence of the indicated amount of heart extract protein. ODC activity measured in the absence of heart extract was defined as 100%. Each point represents the average of two independent assay measurements using the same heart. Filled symbols represent non-transgenic mice, and open symbols represent transgenic mice.

Figure 2 Effect of poly-histidine-tagged antizyme (6 × His-AZ) on the activity of purified ODC

The activity of mouse ODC protein (10 ng) was determined in the presence of the indicated amount of 6 × His-AZ. ODC activity measured in the absence of 6 × His-AZ was defined as 100%. Each point represents the average of two independent measurements.

Figure 3 Antizyme Western blot of heart extracts from non-transgenic mice and transgenic progeny from founders α MHCAZ34 and α MHCAZ35

Portions of 100 μ g of protein were used in Western blots. The positions of molecular mass standards are indicated at the left. AZ, antizyme.

contrast, heart extracts from transgenic progeny of both founder lines reduced ODC activity. The extracts from transgenic progeny of founder α MHCAZ34 (Figure 1, upper panel) were clearly more active than those from founder α MHCAZ35 (Figure 1, lower panel). In order to obtain 50% inhibition of the activity of 5 ng of purified ODC, approx. 7 μ g of protein was needed from transgenic progeny of founder α MHCAZ34 and approx. 35 μ g of protein was required from transgenic progeny of founder α MHCAZ35.

A standard curve in which purified recombinant antizyme was added to recombinant ODC showed that approx. 2.5 ng of purified antizyme gives 50% inhibition of 10 ng of purified ODC (Figure 2). This is consistent with stoichiometric (1:1) binding of antizyme to the ODC monomer [12]. Assuming that additional proteins in the heart extracts do not enhance the inactivation of exogenous ODC, these results suggest that antizyme represents approx. 1 part in 5600 of the soluble cardiac protein in transgenic progeny from founder α MHCAZ34; the equivalent value for transgenic progeny from founder α MHCAZ35 is 1 part in 28000.

Both in the assays of heart extracts (shown in Figure 1) and in the inactivation of purified ODC upon addition of purified antizyme (Figure 2), ODC was not inactivated completely even when a considerable excess of extract or antizyme was added. Residual activity of approx. 10% of the added ODC was

Table 1 Cardiac ODC activity in transgenic mice expressing antizyme and non-transgenic littermates

Values are means $\pm$ S.E.M. Significant differences, comparing transgenic groups with non-transgenic littermates, are shown as: **0.001 < P $\leq$ 0.01; *** P $\leq$ 0.001. Numbers of mice are shown in parentheses.

Mice	Mean ODC activity (pmol of CO ₂ /h per mg of protein)
α MHCAZ34 non-transgenic (21)	1.32 $\pm$ 0.19
α MHCAZ34 transgenic (18)	0.51 $\pm$ 0.13**
α MHCAZ35 non-transgenic (19)	1.65 $\pm$ 0.88
α MHCAZ35 transgenic (23)	0.17 $\pm$ 0.02***

observed. A similar lack of complete inactivation of ODC by excess antizyme has been observed by others [29–32].

Western blot analysis of the cardiac extracts with an antibody against antizyme also indicated that antizyme was present in extracts from the transgenic mice at much higher levels than in those from the non-transgenic controls, and that transgenic progeny of founder α MHCAZ34 had more cardiac antizyme than transgenic progeny of founder α MHCAZ35 (Figure 3). There was a clear band with a molecular mass suggestive of antizyme-1A (29.5 kDa) [15], expressed from the first possible initiation codon of the antizyme mRNA [33], in the extracts from transgenic mice. This band was very faint in the controls and could not be quantified accurately. Quantitative analysis of this upper band for the transgenics showed that the expression of antizyme in the heart extracts from transgenic progeny of founder α MHCAZ34 was 2.1-fold greater than the expression of antizyme in hearts from transgenic progeny of founder α MHCAZ35, which is in reasonable agreement with the estimates of 5-fold greater expression derived from the inhibition studies with purified ODC activity. Additionally, there was a clear band suggestive of antizyme-1B, expressed from the second possible initiation codon of antizyme mRNA, which has a molecular mass of 24 kDa (Figure 3) [15], in the transgenics, but only very weak staining in the controls. Quantification of this band indicated that expression of antizyme in hearts from transgenic progeny of founder α MHCAZ34 was 1.7-fold greater than the expression of antizyme in hearts from transgenic progeny of founder α MHCAZ35. More diffuse bands centred around the 21.5 kDa marker were also seen only in the extracts from transgenic mice (Figure 3). These may correspond to minor bands of antizyme reported previously (22 and 18 kDa) [14,34], but their origin and ability to interact with ODC is unclear.

Estimates of transgene copy number in the two lines were in agreement with the higher expression of antizyme in line α MHCAZ34. DNA from transgenic progeny of founder α MHCAZ34 had many more transgene copies than DNA from transgenic progeny of founder α MHCAZ35, which appeared to have approx. 20 copies of the transgene (results not shown).

Effects of transgenic antizyme expression on cardiac ODC activity and polyamine content

Cardiac ODC activity was reduced in transgenic mice from both founders compared with that in non-transgenic littermates, but there was still significant residual ODC activity (Table 1). Transgenic progeny from founder α MHCAZ35 were observed to have lower ODC activity (approx. 10% of controls) than

Table 2 Cardiac polyamines in transgenic mice expressing antizyme and non-transgenic littermates

Values are means $\pm$ S.E.M. Significant differences, comparing transgenic groups with non-transgenic littermates, are shown as: **0.001 < $P \leq$ 0.01; *** $P \leq$ 0.001. Numbers of mice are shown in parentheses.

Mice	Mean polyamine content (nmol/g of tissue)		
	Putrescine	Spermidine	Spermine
α MHCAZ34 non-transgenic (11)	3.8 $\pm$ 0.6	74 $\pm$ 2	174 $\pm$ 10
α MHCAZ34 transgenic (14)	3.4 $\pm$ 0.3	52 $\pm$ 2***	185 $\pm$ 9
α MHCAZ35 non-transgenic (22)	3.8 $\pm$ 0.3	76 $\pm$ 3	197 $\pm$ 6
α MHCAZ35 transgenic (16)	3.0 $\pm$ 0.2	52 $\pm$ 3***	170 $\pm$ 6**

transgenic progeny from founder α MHCAZ34 (approx. 39% of controls). Thus, despite the presence of free antizyme levels that are greatly in excess of those normally present and that are able to inactivate exogenous ODC (Figure 1), endogenous ODC activity was not fully inhibited. Preincubation of heart extracts with 5 mM DFMO showed that the enzymic activity measured was indeed ODC activity, with the activity in extracts from non-transgenic mice, transgenics from founder α MHCAZ34 and transgenics from founder α MHCAZ35 being inhibited by 82%, 79% and 76% respectively after incubation for 30 min (results not shown).

Measurements of cardiac polyamine content in transgenic mice from both founders showed only a slight effect of antizyme expression on polyamine content (Table 2). Putrescine levels, which are very low, were unaffected by expression of the transgene. Spermidine levels were also largely unaffected, although the level in the transgenic progeny of founder α MHCAZ35 was reduced slightly. Spermidine levels were, however, significantly reduced in both lines of transgenic mice by 30–32%. Therefore the reduction in cardiac spermidine was approximately the same for both lines of transgenic mice, despite the different degrees of antizyme expression.

No significant differences were observed in the endogenous ODC activity measurements or in the cardiac polyamine content of non-transgenic mice from the two founder lines (Tables 1 and 2). This provides evidence that the mixed DBA/2 and C57BL/6 genetic background of these mice did not influence cardiac ODC activity or polyamine levels. Therefore data for the non-transgenic mice from each founder line are pooled together as 'non-transgenic mice' for all other results presented.

Effects of transgenic antizyme expression on the responses of cardiac ODC and polyamine content to a single acute dose of isoprenaline

Cardiac ODC activity was measured in transgenic mice from both founder lines and in non-transgenic littermates 6 h after administration of 20 mg/kg isoprenaline. This treatment induced ODC activity in non-transgenic littermates by 14-fold (Table 3). This result is in excellent agreement with previous findings that showed a 16-fold peak induction of cardiac ODC activity in adult Sprague–Dawley rats treated in the same manner [35]. However, in both lines of transgenic mice the expression of antizyme almost completely prevented the induction of cardiac ODC activity by isoprenaline (Table 3). The 6 h treatment with isoprenaline increased putrescine content substantially (4.7-fold) and spermidine content slightly (1.3-fold) in the hearts of non-

Table 3 Cardiac ODC activity in transgenic mice expressing antizyme and non-transgenic littermates treated with a single acute dose of isoprenaline

Values are means $\pm$ S.E.M. A significant difference, comparing mice treated with isoprenaline with mice treated with saline, is shown as: *0.01 < $P \leq$ 0.05. Numbers of mice are shown in parentheses.

Mice	Mean ODC activity (pmol of CO ₂ /h per mg of protein)
Treated with 0.9% NaCl	
Non-transgenic (4)	1.17 $\pm$ 0.37
α MHCAZ34 transgenic (4)	0.60 $\pm$ 0.24
α MHCAZ35 transgenic (4)	0.19 $\pm$ 0.05
Treated with 20 mg/kg isoprenaline	
Non-transgenic (4)	16.33 $\pm$ 4.15*
α MHCAZ34 transgenic (4)	1.38 $\pm$ 0.24
α MHCAZ35 transgenic (4)	0.32 $\pm$ 0.03

transgenic mice (Table 4). Similarly, these increases were completely prevented in transgenic mice from both founder lines.

Western blot analysis of isoprenaline-treated and control heart extracts from transgenic progeny of founders α MHCAZ34 and α MHCAZ35 showed that isoprenaline treatment led to a decrease in the amount of antizyme present in the mouse heart (Figure 4). Quantitative analysis of the well defined upper band thought to correspond to antizyme-1A indicated that the antizyme content was reduced by approx. 50% in transgenic progeny from founder α MHCAZ34 and by 59% in transgenic progeny from founder α MHCAZ35. Similarly, quantification of the lower band, thought to correspond to antizyme-1B, indicated decreases in antizyme content of 46% and 66% in transgenic progeny from founders α MHCAZ34 and α MHCAZ35 respectively. The isoprenaline-mediated decrease in antizyme levels is consistent with the hypothesis that antizyme degradation is enhanced when it is complexed with ODC. Although it is well reported that antizyme acts to target ODC for proteasomal degradation in a recyclable manner [36], there are other suggestions that some fraction of the antizyme population may itself be degraded during this process [12].

Table 4 Cardiac polyamines in transgenic mice expressing antizyme and non-transgenic littermates treated with a single acute dose of isoprenaline

Values are means $\pm$ S.E.M., except where indicated ($\dagger$, values for two mice given). Significant differences, comparing mice treated with isoprenaline with mice treated with saline, are shown as: **0.001 < $P \leq$ 0.01; * $P =$ 0.053. Numbers of mice are shown in parentheses.

Mice	Mean polyamine content (nmol/g of tissue)		
	Putrescine	Spermidine	Spermine
Treated with 0.9% NaCl			
Non-transgenic (3)	6.2 $\pm$ 0.4	90 $\pm$ 2	142 $\pm$ 9
α MHCAZ34 transgenic (2) $\dagger$	7.0, 7.1	49, 62	162, 180
α MHCAZ35 transgenic (2) $\dagger$	4.1, 5.2	46, 58	155, 194
Treated with 20 mg/kg isoprenaline			
Non-transgenic (3)	29.2 $\pm$ 3.6**	115 $\pm$ 9*	154 $\pm$ 16
α MHCAZ34 transgenic (3)	6.1 $\pm$ 0.3	65 $\pm$ 3	198 $\pm$ 19
α MHCAZ35 transgenic (3)	5.2 $\pm$ 0.5	51 $\pm$ 4	170 $\pm$ 25

Figure 4 Antizyme Western blot of heart extracts from transgenic progeny of founders α MHCAZ34 and α MHCAZ35 treated with a single acute dose of 0.9% saline or 20 mg/kg isoprenaline

The left-hand lane of each pair is heart extract from a mouse treated with saline; the right-hand lane of each pair is heart extract from a mouse treated with isoprenaline. Portions of 50 μ g of protein were used in Western blots. The positions of molecular mass standards are indicated at the left. AZ, antizyme.

Table 5 Cardiac ODC activity in transgenic mice expressing antizyme and non-transgenic littermates treated with isoprenaline for 10 days

Values are means $\pm$ S.E.M. A significant difference, comparing mice treated with isoprenaline with mice treated with saline, is shown as: *** $P \leq 0.001$. Numbers of mice are shown in parentheses.

Mice	Mean ODC activity (pmol CO ₂ /h per mg of protein)
Treated with 0.9% NaCl	
Non-transgenic (8)	1.6 $\pm$ 0.37
α MHCAZ34 transgenic (9)	1.3 $\pm$ 0.22
α MHCAZ35 transgenic (7)	1.7 $\pm$ 0.62
Treated with 20 mg/kg isoprenaline	
Non-transgenic (10)	7.4 $\pm$ 1.6***
α MHCAZ34 transgenic (9)	1.8 $\pm$ 0.4
α MHCAZ35 transgenic (6)	3.1 $\pm$ 1.3

Effects of transgenic antizyme expression on the response of cardiac ODC, polyamine content and cardiac growth to chronic treatment with isoprenaline

When mice were treated with 20 mg/kg isoprenaline for 10 days, the final cardiac ODC activity was induced in non-transgenic littermates by 4.6-fold (Table 5). This induction of ODC activity was less than that observed after a single acute dose of isoprenaline (Table 3) and may indicate some compensatory down-regulation, since this prolonged treatment with isoprenaline caused a highly significant 5.1-fold accumulation of putrescine, while spermidine levels were also increased by 1.7-fold (Table 6). Such increases in polyamine content were similar to those observed following the single acute dose of isoprenaline (Table 4). In both lines of transgenic mice the expression of antizyme prevented both the induction of cardiac ODC activity (Table 5) and the increases in polyamine levels (Table 6).

The cardiosomatic index of non-transgenic mice was increased by 19.8% following 10 days of treatment with isoprenaline (Table 7). Transgenic progeny from both founder α MHCAZ34 and founder α MHCAZ35 were also observed to have significant hypertrophy in response to chronic isoprenaline treatment, with increases in their cardiosomatic indices of 20.2% and 17.1%

Table 6 Cardiac polyamines in transgenic mice expressing antizyme and non-transgenic littermates treated with isoprenaline for 10 days

Values are means $\pm$ S.E.M. Significant differences, comparing mice treated with isoprenaline with mice treated with saline, are shown as: ** $0.001 < P \leq 0.01$; *** $P \leq 0.001$. Numbers of mice are shown in parentheses.

Mice	Mean polyamine content (nmol/g of tissue)		
	Putrescine	Spermidine	Spermine
Treated with 0.9% NaCl			
Non-transgenic (4)	6.7 $\pm$ 1.21	83 $\pm$ 3.9	228 $\pm$ 10.4
α MHCAZ34 Transgenic (4)	5.8 $\pm$ 1.33	50 $\pm$ 4.8	216 $\pm$ 12.7
α MHCAZ35 Transgenic (4)	4.4 $\pm$ 0.33	40 $\pm$ 5.6	177 $\pm$ 13.0
Treated with 20 mg/kg isoprenaline			
Non-transgenic (4)	34.0 $\pm$ 2.26***	141 $\pm$ 10.9**	220 $\pm$ 10.7
α MHCAZ34 Transgenic (4)	8.9 $\pm$ 2.12	56 $\pm$ 5.0	188 $\pm$ 12.6
α MHCAZ35 Transgenic (4)	7.4 $\pm$ 1.23	58 $\pm$ 6.2	177 $\pm$ 11.0

Table 7 Cardiosomatic index in transgenic mice expressing antizyme and non-transgenic littermates treated with isoprenaline for 10 days

Cardiosomatic index is defined as: [heart weight (g)/body weight (g)] $\times$ 1000. Values are means $\pm$ S.E.M. Significant differences, comparing mice treated with isoprenaline with mice treated with saline, are shown as: *** $P \leq 0.001$. Numbers of mice are shown in parentheses.

Mice	Cardiosomatic index
Treated with 0.9% NaCl	
Non-transgenic (8)	4.99 $\pm$ 0.09
α MHCAZ34 transgenic (9)	5.09 $\pm$ 0.18
α MHCAZ35 transgenic (7)	6.19 $\pm$ 0.15
Treated with 20 mg/kg isoprenaline	
Non-transgenic (10)	5.98 $\pm$ 0.12***
α MHCAZ34 transgenic (9)	6.12 $\pm$ 0.10***
α MHCAZ35 transgenic (6)	7.25 $\pm$ 0.15***

respectively. These results indicate that hypertrophy was induced in all three groups of mice. The increase in the cardiosomatic index of all three groups was significant for both males and females, with no sex-related difference being observed in the response to the drug treatment (results not shown). It should also be noted that the cardiosomatic index of untreated transgenic mice from founder α MHCAZ35, but not from founder α MHCAZ34, was higher than that of the untreated non-transgenic mice (Table 7), but that both transgenic lines showed a significant increase in heart weight in response to isoprenaline.

DISCUSSION

Although antizyme is clearly a major factor in the regulation of ODC activity and polyamine levels in mammalian cells, it is normally present in only very small amounts, even after induction by exposure to high levels of polyamines [33,37]. It is therefore quite surprising that viable transgenic mice expressing antizyme from the very strong α -MHC promoter could be obtained with no obvious signs of abnormal cardiac function. The levels of antizyme detected in these mice at 6 weeks of age were vastly greater than those normally present. Based on the titration of free antizyme in heart extracts from transgenic mice with purified ODC shown in Figure 1, the extracts contain 36–180 ng of free antizyme protein per mg of cytosolic protein. This level of expression is consistent with the amounts of ODC produced

from the α -MHC promoter in transgenic mice expressing ODC using a similar construct (L. M. Shantz, D. J. Feith and A. E. Pegg, unpublished work). Antizyme, even when induced, has only been reported to be present at levels of 3–5 ng/mg of HTC cell protein [33] and less than 1 ng/mg of cytosolic protein in crude liver extracts [37]. We did not examine mice at earlier stages of development, and it is possible that some major compensatory changes occur to maintain polyamine homeostasis during this period. It is known that the α -MHC gene is expressed in the atrium during the fetal stage of development of mouse cardiac muscle, while the β -MHC gene is expressed in the ventricle. After birth, the β -MHC gene is down-regulated and the α -MHC gene becomes the predominant MHC transcript in both the atria and ventricles [21]. Thus the use of the α -MHC promoter allows overexpression of antizyme in both the atria and ventricles of transgenic animals from approximately 2 weeks of age through-out adult life.

The lack of physiological effects of antizyme expression is clearly explained by the observation that ODC activity levels, although reduced, are maintained at 10–39 % of normal values. Similarly, the only polyamine substantially affected in the heart is spermidine, with a 30–32 % decrease in content. Some previous studies in which cardiac ODC activity was reduced substantially by exposure of rats to DFMO have also indicated that decreased ODC activity does not have profound effects on cardiac function [38,39]. However, these experiments lasted only for a short time period, and a compensatory increase in polyamine transport can occur following DFMO treatment, whereas polyamine transport is expected to be blocked by constitutive expression of antizyme.

The reason for the lack of complete inhibition of ODC activity, in spite of the high amounts of antizyme protein in the heart, is unclear. Even though antizyme inhibitor is known to bind to antizyme more strongly than antizyme binds to ODC [40,41], this observation is unlikely to be due to a large excess of antizyme inhibitor protein in the heart extracts, since the extracts were capable of inactivating exogenous ODC (Figure 1), and we were unable to detect any difference in the levels of antizyme inhibitor protein in the transgenic lines and non-transgenic mice using Western blotting (results not shown). Several other possible explanations may contribute to the maintenance of residual ODC activity. Firstly, as shown in Figures 1 and 2, despite the addition of an apparent excess of antizyme-containing heart extract or purified antizyme to purified ODC, some ODC activity remained. The reason(s) for this phenomenon is unknown; however, a similar lack of complete inhibition of ODC activity by high amounts of antizyme has been observed by other researchers [29–32]. Secondly, transient compensatory mechanisms leading to increased synthesis of ODC may occur periodically in response to the decreases in ODC activity and polyamine levels caused by antizyme. There is excellent evidence that ODC expression can be regulated at the levels of transcription and translation, and that large changes in ODC synthesis rates may occur in response to appropriate stimuli [3,10,42–45]. Finally, it is also conceivable that a compensatory post-translational modification of cardiac ODC occurs to render it resistant to antizyme binding, thereby resulting in residual endogenous ODC activity.

It is unlikely that there is a large accumulation of ODC protein in an inactive antizyme–ODC complex in the transgenic mice expressing antizyme. Western blots failed to show any difference in ODC content in heart extracts from non-transgenic mice and transgenic mice from both founder lines when up to 100 μ g of protein was analysed. In addition, an extremely low amount of ODC protein was detected, at least 100 times less than that seen in extracts from transgenic mice expressing ODC from the

α -MHC promoter described above (results not shown). It is possible that, *in vivo*, the antizyme present in the heart is in some way (such as compartmentalization or sequestration) rendered incapable of reacting with some fraction of the ODC. Recent work suggests that the larger 29.5 kDa antizyme-1A contains a targeting sequence that could direct it to the mitochondria and result in processing of the protein [14]. The ability of antizyme to block polyamine transport, as well as some localization studies, suggest that a fraction of the protein may be bound to membranes [12]. A compensatory increase in synthesis of the polyamine transporter is also possible, and the inhibition of polyamine transport requires the same antizyme domain that is responsible for ODC binding [46]. We did not specifically investigate the presence of antizyme in mitochondria or in membranes, but it is clear from the experiments shown in Figures 1 and 3 that there is excess cytosolic antizyme, which is capable of interacting with exogenous ODC.

Furthermore, the blockage of the induction of ODC activity by both a single acute dose and chronic doses of isoprenaline indicates that the antizyme can repress increases in ODC activity caused by exogenous stimuli. This inhibition of induction of ODC activity is consistent with the concept described above that compensatory increases in ODC synthesis come into play in the transgenic mice in order to permit polyamine levels to be maintained, and that further up-regulation of ODC synthesis in response to β -adrenergic agonists or other factors would not be possible. Although the level of antizyme protein in the extracts from transgenic progeny of founder line α MHCAZ35 was lower than that in extracts from transgenic progeny of founder α MHCAZ34, the cardiac polyamine content and lack of response to both treatment regimes of isoprenaline was similar in the two groups. It is noteworthy that the higher residual ODC activity was actually seen in transgenic mice from founder α MHCAZ34, which have the greater antizyme content. The reason for this apparent paradox is unknown, but it is possible that increased up-regulation of ODC synthesis occurs to different extents in mice from the two founder lines.

The antizyme-expressing transgenic mice should provide a useful model in which to evaluate the role of enhanced ODC activity and polyamine content in response to various stresses, including aortic and pulmonary artery constriction, hypoxia, exercise, adrenergic agonists and thyroxine, all of which have been shown previously to be associated with increased ODC activity, increased polyamine content and cardiac hypertrophy [47–55]. Several previous studies [38,39,52,56–58] have attempted to examine the importance of such induction by using inhibitors of polyamine synthesis such as DFMO, 1,3-diaminopropanol and 1,3-diaminopropane. Such studies have indicated that preventing the induction of cardiac ODC activity and the accumulation of polyamines, as caused by isoprenaline, leads to an attenuation of the associated cardiac growth. However, preventing the induction of ODC activity and polyamine accumulation associated with thyroxine treatment does not affect the associated cardiac growth. One major problem with such studies is that it is difficult to treat animals with inhibitors of polyamine biosynthesis for prolonged periods and to rule out non-specific effects or influences from other tissues, since the inhibitors alter polyamine metabolism systemically. The results of the present study indicate that increased cardiac ODC activity and the accumulation of cardiac polyamines are not necessary for isoprenaline-induced cardiac hypertrophy, in contrast with the findings of previous work. Only one indicator of cardiac hypertrophy (cardiac mass) was measured in the present study; nevertheless, these mice provide a useful model in which to study further the relationship between ODC activity, polyamines and

cardiac growth in response to a variety of stimuli. The presence of two separate lines of transgenic mice expressing antizyme will ensure that results are due to antizyme expression rather than artifacts related to transgene insertion sites and interference with the expression of other genes.

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