Targeted overexpression of ornithine decarboxylase enhances β -adrenergic agonist-induced cardiac hypertrophy

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These studies were designed to determine the consequences of constitutive overexpression of ornithine decarboxylase (ODC) in the heart. Induction of ODC is known to occur in response to agents that induce cardiac hypertrophy. However, it is not known whether high ODC levels are sufficient for the development of a hypertrophic phenotype. Transgenic mice were generated with cardiac-specific expression of a stable ODC protein using the α -myosin heavy-chain promoter. Founder lines with > 1000-fold overexpression of ODC in the heart were established, resulting in a 50-fold overaccumulation of putrescine, 4-fold elevation in spermidine, a slight increase in spermine and accumulation of large amounts of cadaverine compared with littermate controls. Despite these significant alterations in polyamines, myocardial hypertrophy, as measured by ratio of heart to body weight, did not develop, although atrial natriuretic factor RNA was slightly elevated in transgenic ventricles. How-

ever, stimulation of β -adrenergic signalling by isoproterenol resulted in severe hypertrophy and even death in ODC-overexpressing mice without further altering polyamine levels, compared with only a mild hypertrophy in littermates. When β 1-adrenergic stimulation was blocked by simultaneous treatment with isoproterenol and the β 1 antagonist atenolol, a significant, although reduced, hypertrophy was still present in the hearts of transgenic mice, suggesting that both β 1 and β 2 adrenergic receptors contribute to the hypertrophic phenotype. Therefore these mice provide a model to study the *in vivo* cooperativity between high ODC activity and activation of other pathways leading to hypertrophy in the heart.

Key words: β -adrenergic-induced hypertrophy, isoproterenol, polyamine biosynthesis, transgenic mice.

INTRODUCTION

The polyamines putrescine, spermidine and spermine have been shown to be essential for cell growth and differentiation. The polyamine-metabolizing enzymes are activated by a variety of growth factors, tumour promoters, viruses and oncogenes, and the cellular activities of these enzymes are very tightly controlled, with extensive alterations in enzyme activities occurring very quickly. The importance of understanding the role of the polyamine pathway in cardiac physiology is underscored by the findings that changes in intracellular polyamines may strongly alter cardiac excitability through interactions with inwardly rectifying K^+ channels [1,2]. In addition, it has been known for some time that the activity of ornithine decarboxylase (ODC), the first and potential rate-limiting enzyme of the polyamine pathway, and levels of the polyamines themselves, are induced by a variety of stimuli which cause cardiac hypertrophy, including thyroxine, the β -adrenergic agonists isoproterenol and clenbuterol and the α 1-adrenergic agonists phenylephrine and methoxamine [3–8]. Indeed, ODC induction has been used as a marker for the development of the hypertrophic phenotype. Administration of α -difluoromethylornithine, a suicide inactivator of ODC, reduced polyamine content in all cases and attenuated isoproterenol- and clenbuterol-induced cardiac hypertrophy, but did not prevent thyroxine-induced hypertrophy [4–6]. These results suggest ODC-dependent and -independent processes leading to the hypertrophic state, depending on the inducer. The experiments described in the present study use a transgenic approach to examine the effects of overexpression of

ODC in the heart, both in the absence and presence of a hypertrophy-inducing stimulus. The mouse has been established as a valid model system in which to study cardiac hypertrophy, since it conserves many features of larger mammalian model systems. Directed overexpression of ODC to the heart is accomplished through the use of the well-characterized α -myosin heavy-chain (α MHC) promoter. The use of this promoter allows expression of ODC in both the atria and ventricles of transgenic animals throughout adult life.

EXPERIMENTAL

Materials

Isoproterenol and biochemical reagents were purchased from Sigma. Restriction enzymes and *Taq* polymerase were from New England Biolabs and Life Technologies, Inc. dNTPs used in PCR reactions were from Amersham Pharmacia. Plasmid purification columns and genomic DNA isolation kits were purchased from Qiagen. L-[1-¹⁴C]Ornithine (52 mCi/mmol) was from DuPont-New England Nuclear.

Production and characterization of transgenic mice

Transgenic mice were generated by DNA microinjection of fertilized C57BL/6xSJL oocytes using standard techniques [9]. The transgene was a 7.5 kb fragment derived from *NotI* digestion of a pBR322-based vector containing a murine α MHC promoter (provided by J. Robbins, University of Cincinnati Medical

Abbreviations used: ODC, ornithine decarboxylase; α MHC, α -myosin heavy-chain; ANF, atrial natriuretic factor; AdoMetDC, S-adenosylmethionine decarboxylase; ERK, extracellular signal-regulated kinase.

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Figure 1 Generation and identification of mice transgenic for the truncated ODC protein

Transgenic mice were generated as described in the Experimental section. **(A)** Transgenic vector. The α MHC promoter was ligated 5' to the coding region of a mouse ODC cDNA engineered to produce a protein truncated to 425 amino acids. At the 3'-end of the ODC cDNA, the intron and polyadenylate-containing sequences of the human growth hormone were added. The positions of the PCR primers used to detect the transgene are indicated. **(B)** Detection of the transgene in animals from transgenic line MHC-ODC21 by PCR with the primers indicated in **(A)**. 1 kb = DNA ladder. **(C)** Southern-blot analysis of DNA from hemizygous transgenic mice (Tg) and nontransgenic controls (C). *Bam*HI-digested genomic tail DNA (10 μ g) was hybridized with an ODC protein coding region probe. Size of the transgene band is 7.5 kb, as indicated by the positive plasmid control (M).

Center, Cincinnati, OH, U.S.A.) [10] upstream of a murine ODC cDNA which had been engineered to contain an introduced stop codon at position 425 (see Figure 1A).

The 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 α MHC promoter sequence (5'-GTAAAAGAGGCAGGGAA-GTGGTG-3') and the 3' antisense primer was an oligonucleotide which corresponded to an ODC-coding sequence (5'-CTT-CTTTAGAATGTCTCCGAGGTCC-3'). The amplified 267 bp product spans the junction between the α MHC promoter and the ODC sequence, and is therefore detected only in mice bearing the transgene. Control PCR reactions were performed by using two other primers designed to amplify exon 11 of the endogenous *ODC* gene in both normal and transgenic mice (results not shown). The transgene is maintained in the hemizygous state by breeding of hemizygous males with B6D2 females (Jackson Laboratories, West Grove, PA, U.S.A.). This results in approximately equal numbers of transgenic pups and normal littermates for experiments.

Southern-blot analysis

For Southern-blot analysis, genomic DNA from potential transgenic mice was digested with *Bam*HI and electrophoresed on 0.8% agarose gels. The DNA was then transferred to nylon membranes (Amersham Pharmacia), and probed using a 1500 bp ODC cDNA fragment corresponding to the ODC open reading frame. The probe was labelled with fluorescein by random prime

labelling and the blot was processed using the enhanced chemifluorescence ('ECF') detection technique (Amersham Pharmacia). Bands were visualized on a fluorimager 595 (Molecular Dynamics) and compared with purified plasmid digested with *Bam*HI.

Immunohistochemical analysis

Hearts from 2-, 3- and 7-week-old mice were fixed in 10% neutral-buffered formalin, embedded in paraffin and cut into 5 μ m sections. Haematoxylin and eosin staining was used for histological examination of hearts from normal and α MHC-ODC21 transgenic mice. For immunohistochemical analysis of ODC expression in the heart, sections were deparaffinized, endogenous peroxidase activity was quenched by incubation in 0.3% H_2O_2 , and sections were blocked in 1.5% normal goat serum. Sections were then incubated overnight at 4 $^{\circ}$ C in 0.1 ng/ μ l affinity-purified ODC polyclonal antibody [11], 1% normal goat serum and 0.1% Triton X-100. ODC expression was visualized using a Vectastain Elite ABC Kit with 3,3'-diaminobenzidine as the substrate (Vector Laboratories, Inc., Burlingame, CA, U.S.A.). Sections were counterstained with haematoxylin and pictures taken on a Nikon TMS microscope with a 10 $\times$ objective.

Biochemical analyses

When hearts were assayed for ODC activity, the sample was resuspended in ODC assay buffer [50 mM Tris/HCl, pH 7.5, 2.5 mM dithiothreitol, 0.1 mM EDTA and protease inhibitor cocktail (Calbiochem)], subjected to three 15 s pulses using a polytron on ice, and centrifuged for 30 min at 30 000 *g* and 4 $^{\circ}$ C. ODC was assayed at 37 $^{\circ}$ C by measuring the release of $^{14}CO_2$

from L-[1-¹⁴C] ornithine using saturating concentrations (800 μ M) of ornithine as substrate and 40 μ M pyridoxal 5-phosphate [12]. Cytosolic protein (10 μ g) was subjected to SDS/PAGE on 10% gels and transferred to PVDF membrane (Micron Separations, Inc., Westborough, MA, U.S.A.). Western-blot analysis was performed using a fluorescent detection system (Amersham Pharmacia), and wild-type and truncated ODC proteins were detected using a purified rabbit polyclonal antibody against mouse ODC [11]. For polyamine analysis, samples were acid-extracted using 10% trichloroacetic acid and analysed for polyamines using reverse-phase HPLC analysis, as described previously [13].

Morphometric and genetic analyses of hypertrophy

Adult mice, 6–8 weeks old, were given 10 daily intraperitoneal injections of either saline or 20 mg/kg isoproterenol in saline. Mice were sacrificed 6 h after the tenth injection, weighed, and the hearts removed immediately. Hearts were then weighed and immediately frozen in liquid N₂. When it was necessary to sacrifice transgenic mice before the end of the experiment, these mice were treated identically. For Northern blots of atrial natriuretic factor (ANF) mRNA, frozen hearts were powdered with a mortar and pestle, and RNA was isolated using the Totally RNA kit (Ambion, Austin, TX, U.S.A.) according to the manufacturer's instructions. Total RNA (20 μ g) was separated on a 1.2% agarose/formaldehyde gel, transferred to nylon membrane (Amersham), and UV-crosslinked using a Stratalinker (Stratagene). cRNA probes were synthesized from pGEM-ANF (provided by K. R. Chien, University of California, San Diego, CA, U.S.A.) using T7 RNA polymerase and fluorescein-12-UTP (Roche Molecular Biochemicals). Blots were hybridized overnight and analysed by fluorimager. RNA loading differences were normalized using ethidium bromide staining of 18 S and 28 S bands.

Statistical analysis

Statistical analysis of results was carried out using a two-sample equal variance Student's *t* test with two-tailed distribution.

RESULTS

Identification of transgenic mice overexpressing ODC in the heart

The mice described in these studies were engineered to express a truncated form of the wild-type ODC. It has been shown previously that this truncated ODC is more stable than wild-type ODC (half-life of approx. 3 h compared with 20 min), and the activity of this truncated mutant is equivalent to that of the wild-type [14,15]. In addition, the construct used contained only 70 bp from the ODC 5'-untranslated region, in order to avoid the well-established translational regulation of ODC synthesis, which is mediated through the long (320 bp), structured 5'-leader sequence (reviewed in [16]). The promoter driving expression of the transgene is the α MHC promoter. This promoter has been used previously to express various proteins to high levels within the heart, as described below. During the fetal stage of development of mouse cardiac muscle, the α MHC gene is expressed in the atrium, whereas the β MHC gene is expressed in the ventricle. After birth, the β MHC is downregulated and the α MHC becomes the predominant MHC transcript in both the atria and ventricles and remains so in the adult animal [10,17].

Results of PCR analysis on the progeny of one founder are shown in Figure 1(B). Only transgenic animals showed the 267 bp product generated by the α MHC-ODC junction primers.

Figure 2 Expression of ODC protein in MHC-ODC transgenic mice

Hearts were isolated from 6-week-old mice and processed as described in the Experimental section. (A) Western-blot analysis of cytosolic protein (10 μ g) from transgenic mice (T) and littermates (N) resulting from F1 crosses of two different MHC-ODC founders with B6D2 females. A band cross-reacting with the ODC antibody is visible only in transgenic mice. (B) ODC specific activity measurements in cytosolic fractions from hearts of transgenic animals and littermate controls from two different founder lines. Values are expressed as means $\pm$ S.E.M. for the number of mice indicated in each group.

Using this PCR screening procedure, nine founder animals have been identified and two founders (MHC-ODC6 and MHC-ODC21) have been further characterized. All the results shown here were obtained using progeny of founder 21. Similar results have been obtained using progeny of founder 6.

Southern-blot analysis of tail DNA showed that multiple copies of the transgene were integrated into the genomic DNA of offspring from founder 21. All mice transgenic by PCR exhibited the same 7.5 kb fragment following *Bam*HI digestion of tail DNA, as did the purified plasmid DNA (Figure 1C). Quantitative slot-blot analysis of genomic DNA from founder 21 estimated the copy number to be between 5 and 10 (results not shown). Northern-blot analysis of RNA from the hearts of MHC-ODC21 mice revealed > 1000-fold overexpression of ODC RNA compared with levels in littermate controls (results not shown).

Expression of ODC in the hearts of transgenic mice

For initial expression studies, progeny of the MHC-ODC founders were sacrificed at 6 weeks of age, and the hearts were immediately removed, cut in half along a midsagittal plane, and snap-frozen in liquid N₂. In the experiment shown, hearts from four F1 progeny from founder 21 and four F1 progeny from founder 6 were analysed by Western blotting. In each group, two were identified as transgenic by PCR screening and Southern-blot analysis. These mice all showed a dramatic accumulation of transgene-derived ODC protein (Figure 2A). The ODC derived from the transgene is truncated to 425 amino acids and has a molecular mass of approx. 47 kDa, whereas endogenous ODC has a molecular mass of 51 kDa. Therefore transgene-derived ODC can easily be distinguished from the endogenous protein on a Western blot. However, the ODC levels in normal hearts, although measurable by ODC activity assay (see below), are below the limits of detection by the ODC antibody used in these Western analyses (Figure 2A).

Hearts from 6-week-old mice were also assayed for ODC activity using our standard assay procedure [12]. Figure 2(B) shows that in hearts from MHC-ODC21 transgenic mice the ODC activity is increased approx. 5000-fold compared with their littermate controls. The ODC activity in MHC-ODC6 transgenic

Figure 3 Immunohistochemical analysis of ODC expression in hearts of MHC-ODC21 mice and littermate controls

Hearts were sectioned and immunohistochemical analysis performed as described in the Experimental section. (A) Uniform staining is seen in myocytes, but not blood vessels, in the hearts of transgenic mice. (B) Control hearts show virtually no staining. Magnification $\times 200$.

hearts was increased by approx. 1500-fold, reflecting the slightly lower amount of ODC protein also seen in the Western blot of these hearts (Figure 2A).

As expected, immunohistochemical analysis of 7-week-old hearts indicates a uniform expression of ODC protein throughout the transgenic heart (Figure 3A). Unlike the Western blot, the ODC antibody does not distinguish between endogenous and transgenic ODC in immunohistochemical analysis. However, virtually no ODC protein can be detected in the control hearts (Figure 3B), confirming that the ODC protein seen in transgenic hearts is derived from the *ODC* transgene. Histological analysis of the hearts revealed no obvious abnormalities in the MHC-ODC21 mice at this age. Hearts were also histologically normal at 2 and 3 weeks of age (results not shown).

ODC protein expression in transgenic hearts as a function of age

In order to determine the time course of expression of transgene-derived ODC activity in the heart as a function of age, mice between the ages of 2 days and 6 months were sacrificed and the whole hearts analysed for ODC activity. Levels of the α MHC cardiac isoform have been reported to increase from 30 % in the ventricles at birth to 70 % by day 6 [17]. By 2 days of age, levels of ODC activity were increased 150-fold in MHC-ODC21 mice

Figure 4 ODC specific activity as a function of age in MHC-ODC21 mice

Hearts were isolated from mice at the indicated ages and ODC activity was determined in the cytosolic fraction as described in the Experimental section. Values are expressed as means $\pm$ S.E.M. of 3–8 mice in each group. Control values were 2.6 nmol/30 min per mg of protein, and were unchanged with age.

compared with littermate controls (Figure 4). Levels of ODC activity reached a maximum by 3 weeks of age, and remained relatively constant at approx. 6000-fold induction between 3 weeks and 6 months (Figure 4).

ODC activity in other organs

Most investigators have found cardiac-specific expression of α MHC-driven transgenes [18–20], whereas one study [10] also reported low levels of expression in the lung. In order to examine the specificity of ODC expression in MHC-ODC mice, 6-week-old F1 progeny of the MHC-ODC21 founder were sacrificed and ODC activity was measured in the liver, small intestine, spleen and brain, in addition to the heart and lung. As expected, ODC activity in most organs of both transgenic mice and their littermate controls was almost immeasurably low. In contrast, ODC activity in the lungs of transgenic mice was induced (245 ± 9 nmol/30 min per mg of protein). While the transgenic lung ODC activity is only 2 % of the activity in the transgenic heart, it is more than 1000-fold higher than the activity found in the lungs of control mice (0.13 ± 0.06 nmol/30 min per mg of protein). This difference in ODC activity was not, however, sufficient to change the levels of any of the polyamines in the total transgenic lungs compared with control lungs, perhaps due to expression in limited cell populations (results not shown). Future experiments will determine whether this activity is found primarily in the vasculature of the lung, or if this represents ODC expression in specific cell types of the lungs of transgenic mice. Previous studies [10] have reported localization of an α MHC-driven chloramphenicol acetyltransferase reporter gene to the pulmonary vasculature.

Polyamine measurements in transgenic animals and littermate controls

Table 1 shows that 6-week-old transgenic mice have almost a 50-fold over-accumulation of putrescine compared with their control littermates, as well as an approx. 4-fold induction in spermidine and a slight increase in spermine. In addition, transgenic mice accumulate large amounts of cadaverine, which is formed by the decarboxylation of lysine [21]. Lysine is a poor substrate for ODC, with a K_m approx. 100-fold higher compared with that of ornithine. It seemed possible that the ODC activity in transgenic

Table 1 Polyamine levels in the hearts of MHC-ODC mice and their littermate controls

Mice were sacrificed at the indicated ages and polyamines were determined as described in the Experimental section. The values are means $\pm$ S.D. from 5–11 mice.

Mouse	Age	Polyamines nmol/g of tissue			
		Putrescine	Spermidine	Spermine	Cadaverine
MHC-ODC21	6 weeks	189 $\pm$ 52	168 $\pm$ 35	109 $\pm$ 28	223 $\pm$ 54
Littermates	6 weeks	4.2 $\pm$ 1.8	42 $\pm$ 9	86 $\pm$ 17	< 1
MHC-ODC21	6 months	380 $\pm$ 38	159 $\pm$ 13	190 $\pm$ 20	421 $\pm$ 98
Littermates	6 months	6.3 $\pm$ 2.2	56 $\pm$ 9	198 $\pm$ 39	< 1

Figure 5 Genetic measurement of hypertrophy in MHC-ODC21 mice and littermate controls

Transgenic mice and littermate controls (6–8 weeks old) were treated as described in the Experimental section, with intraperitoneal injections of either saline or 20 mg/kg isoproterenol for up to 10 days. Total RNA was isolated from the hearts of two transgenic and two control mice from each treatment group and 20 μ g was analysed for ANF expression.

animals is such that all of the available ornithine was converted into putrescine, leaving lysine as the only substrate for ODC. However, neither intraperitoneal injection nor infusion, using an osmotic pump of 500 mM ornithine, significantly raised levels of polyamines in the hearts of transgenic mice. Total ornithine levels in the heart were also measured after injection, and were not different in transgenics and controls, with an average value of 6.9 nmol/g of tissue in saline-treated mice, 10.3 nmol/g of tissue in ornithine-treated nontransgenics and 10.9 nmol/g of tissue in ornithine-treated transgenics. These results suggest that the production of cadaverine might be related to a large excess of lysine compared with ornithine in the heart cytosol, and also to the inability of spermidine synthase to further metabolize cadaverine effectively.

Interestingly, putrescine as well as cadaverine appears to increase in the hearts of transgenic mice as they age. In 6-month-old mice, both putrescine and cadaverine are almost doubled in MHC-ODC21 mice compared with 6-week-old mice (Table 1). Spermine also increases dramatically in these mice as they age, whereas spermidine levels remain unchanged. However, spermine levels in littermate control mice also increase to the same degree,

Table 2 Morphometric measurement of hypertrophy in MHC-ODC21 mice and littermate controls

Transgenic mice and littermate controls (6–8 weeks old) were treated as described in the Experimental section with intraperitoneal injections of either saline or 20 mg/kg isoproterenol for up to 10 days. Hearts were removed from mice immediately after sacrifice, washed with PBS, emptied of blood and weighed. Ratio of total heart weight/body weight (mg/g) is shown. Values are expressed as means $\pm$ S.D. of 5–8 mice in each group. *Significantly different from non-transgenic saline ($P = 0.0004$); **significantly different from non-transgenic isoproterenol ($P = 0.0014$) and MHC-ODC21 saline ($P = 0.0002$).

Genotype	Treatment	Heart weight/body weight (mg/g)
Non-transgenic	Saline	5.5 $\pm$ 0.3
Non-transgenic	Isoproterenol	6.7 $\pm$ 0.3*
MHC-ODC21	Saline	5.9 $\pm$ 0.4
MHC-ODC21	Isoproterenol	9.6 $\pm$ 1.4**

suggesting that the increase in spermine is not related to transgene expression.

Morphometric and genetic measurements of cardiac hypertrophy in MHC-ODC mice

Despite the dramatic overexpression of ODC activity and accompanying alterations in cardiac polyamines in MHC-ODC21 mice, no obvious physiological consequences are apparent. Both male and female MHC-ODC21 mice are fertile and have a normal life span. Since ODC overexpression has been implicated in the hypertrophic response to a variety of chemical stimuli, we examined both morphometric and genetic measurements of hypertrophy in MHC-ODC21 mice and their littermate controls in the presence and absence of a hypertrophy-inducing stimulus. The markers used in these studies were the ratio of heart weight/body weight and ventricular expression of ANF.

For these experiments, 6–8-week-old mice were injected with either saline or a solution of 20 mg/kg of the β -adrenergic agonist isoproterenol for 10 days [22]. Mice were sacrificed 6 h after the last injection. In saline-treated mice, there was no significant difference in the ratio of heart weight/body weight in MHC-ODC21 mice compared with littermate controls (Table 2). In addition, no difference in ratio of heart weight/body weight between untreated transgenics and littermate controls was observed at 2, 3 or 4 weeks, or at 6 months of age (results not shown). When non-transgenic mice were treated with isoproterenol for 10 days, the ratio of heart weight/body weight increased by approx. 18%. In contrast, treated MHC-ODC21 mice showed an increase of almost 40% (Table 2). Indeed, of eight transgenic mice treated, five had to be sacrificed before the

end of the experiment. Thus this dramatic hypertrophic response occurred in as little as 5 days after starting injections in several MHC-ODC21 mice. Both male and female transgenic mice were included in this experiment, and there was no difference in the response (results not shown).

Ventricular ANF message was also measured in saline- and isoproterenol-treated mice. A distinctive feature of cardiac hypertrophy in both human heart failure and physiologically relevant animal models is the increased ventricular expression of fetal genes, particularly ANF [20,23]. Figure 5 shows that in non-transgenic controls, very low levels of ANF message are present in the ventricles of saline-treated mice, and these levels increase approx. 2-fold upon treatment with isoproterenol for 10 days. Interestingly, ANF levels in MHC-ODC21 saline-treated mice are similar to isoproterenol-treated nontransgenic mice, suggesting a mild hypertrophy in the transgenic mice. This is in agreement with our other studies, in which we have measured a 13% increase in membrane capacitance in isolated myocytes from MHC-ODC21 mice compared with littermate controls, which generally correlates well with cell size [24]. Treatment of MHC-ODC21 mice with isoproterenol further increases ANF message by another 1.6-fold (Figure 5).

Polyamine levels and ODC activity were measured in MHC-ODC21 mice treated with isoproterenol. Cardiac ODC activity did not change significantly compared with saline-treated MHC-ODC21 mice, and putrescine and spermidine pools were each increased by only approx. 1.5-fold as a result of isoproterenol treatment. It is unlikely that this small elevation in putrescine and spermidine is important to the development of the severe hypertrophic phenotype seen in the treated transgenic mice, since the levels achieved were not higher than those seen in older untreated mice as described in Table 1. Non-transgenic mice treated with isoproterenol also exhibited an approx. 2-fold increase in putrescine and a 1.5-fold increase in spermidine. These data suggest that the massive overexpression of ODC and overaccumulation of polyamines already present in the hearts of the MHC-ODC21 mice co-operate with other factors induced by the β -adrenergic agonist to result in an enhanced response to isoproterenol treatment.

In order to determine the contribution of β 1 versus β 2 adrenergic receptors to the development of cardiac hypertrophy in MHC-ODC21 mice, transgenic and control mice were treated for 14 days with the β 2 adrenergic agonist clenbuterol (intraperitoneal injections twice per day at a dose of 1.5 mg/kg). However, unlike previously reported results [6], no evidence of induction in ODC activity or hypertrophy was seen in the hearts of either transgenic or control mice treated with clenbuterol. Similar results were obtained when clenbuterol was delivered by osmotic pumps over a 14 day period. This lack of effect may be due to strain differences between the two studies (B6D2 mice in the present study compared with Balb/c mice in previously reported studies [6]). An alternate strategy was therefore used to selectively activate β 2 receptors in which MHC-ODC21 mice were treated using osmotic pumps (Alza Corp., Palo Alto, CA, U.S.A.) for 10 days with either saline, isoproterenol (20 mg/kg per day) or isoproterenol and the β 1 adrenergic antagonist atenolol (5 mg/kg per day). Isoproterenol treatment resulted in a 36% increase in ratio of heart weight/body weight compared with saline-treated MHC-ODC21 mice, with a ratio of 6.31 ± 0.08 in saline-treated mice compared with 9.80 ± 1.06 in isoproterenol-treated mice ($P = 0.01$ with respect to saline-treated mice). These numbers are in good agreement with the results reported in Table 2. When mice were treated with isoproterenol and atenolol, the heart weight/body weight ratio was still increased by 19% over saline-treated mice (7.85 ± 0.57 in Iso/AT-treated

mice; $P = 0.02$ with respect to saline-treated mice), suggesting that both β 1 and β 2 receptors contribute to the development of the hypertrophic phenotype in MHC-ODC21 mice treated with isoproterenol.

DISCUSSION

Both pressure-overload hypertrophy and agonist-mediated hypertrophy are known to induce a pattern of immediate-early gene expression in the heart, including *c-fos*, *c-jun*, *c-myc* and *ODC* [5,25,26]. The mechanism of induction of ODC during hypertrophy is unknown. However, the promoter of the *ODC* gene contains a cAMP-response element, and the β -adrenergic receptor pathway activated by agonists such as isoproterenol involves the stimulatory GTP regulatory protein (G_s) activation of adenylate cyclase and accumulation of cAMP [27,28]. A recent study [29] has shown that ODC activity can be induced in myocyte culture by dibutyryl cAMP. ODC has also been identified as a transcriptional target of c-Myc [30,31]. The present study examines the effects of lifelong ODC overexpression in the heart, both in the resting state and in the presence of β -adrenergic receptor activation.

The data show clearly that transgenic mice can withstand a massive lifelong overexpression of ODC in the heart (up to 5000-fold induction of ODC activity) with no obvious physiological consequences in the absence of external stress. Total heart weight/body weight ratios are not significantly different from littermate controls, although ANF message and membrane capacitance are up-regulated in the ventricles of untreated MHC-ODC21 mice. These observations indicate that overexpression of ODC in the heart is not in itself sufficient to cause an obvious hypertrophic phenotype. These data are in agreement with recent observations using mice overexpressing antizyme in the heart. Antizyme is an inhibitor of ODC, which binds to the ODC monomer and stimulates its degradation [32]. Overexpression of antizyme driven from the α MHC promoter (MHC-ATZ mice) to similar protein levels as those reported in the present study for ODC also caused no abnormal phenotype in untreated mice. When MHC-AZ mice were treated with isoproterenol for 10 days, ODC activity was depressed compared with control mice, but MHC-AZ mice exhibited the same hypertrophic response as their littermates [33].

We also examined the levels of polyamines in the hearts of transgenic mice and found a dramatic elevation of putrescine and cadaverine, a 4-fold elevation in spermidine, and very little effect on spermine pools. While this increase in spermidine is approx. 10 times lower than that seen for putrescine, it is still quite substantial compared with that seen in many studies. It has been shown in a variety of *in vitro* systems that high levels of putrescine do not necessarily lead to an increase in the higher polyamines [34–36]. These findings point to S-adenosylmethionine decarboxylase (AdoMetDC) and not ODC as the rate-limiting factor in spermidine and spermine synthesis. An increase in spermidine, such as that seen in the transgenic hearts, is known to down-regulate the transcription of the *AdoMetDC* gene [13], and although no change in AdoMetDC activity was detected in the hearts of 8-week-old MHC-ODC21 mice compared with littermate controls (results not shown), the enzyme activity measured was very low and represents only a single time point. In addition, it cannot be ruled out that levels of S-adenosylmethionine, the substrate for the AdoMetDC reaction, are limiting in the hearts of these mice.

ODC induction is often used as a marker for the development of cardiac hypertrophy in response to various treatments. However, it is not clear whether high levels of ODC activity contribute

to the development of this phenotype. To address this question, we studied the effect of β -adrenergic stimulation in MHC-ODC21 mice using treatment with isoproterenol for 10 days. These mice responded much more dramatically than their littermate controls, with rapid development of a severe hypertrophic phenotype, as measured by both morphometric and genetic markers. In fact, several transgenic mice died before the scheduled end of the experiment, whereas their littermates developed only a very mild hypertrophy over the course of 10 days of injections. It is not known why the high ODC levels in the hearts of these mice leads to this magnified response. However, recent studies have provided evidence for the involvement of apoptotic pathways during the transition between cardiac hypertrophy and heart failure in animal models (reviewed in [37]), and several *in vitro* studies have indicated that loss of ODC regulation and overaccumulation of putrescine results in apoptosis [36,38–40]. In other systems, apoptosis has been shown to be due to deregulation of polyamine transport and overaccumulation of spermidine and spermine [41,42], and a recent study [43] suggests that polyamines can directly induce the release of cytochrome *c* from heart mitochondria, which triggers caspase activation. These studies raise the possibility that ODC-overexpressing hearts are more susceptible to induction of apoptosis in response to stress than normal hearts.

There are several specific components of the β -adrenergic pathway that may co-operate with the overproduction of polyamines to produce enhanced hypertrophy. For example, it is possible that the positively charged polyamines, particularly the diamine putrescine, affect Ca^{2+} mobilization in response to β -adrenergic receptor activation. Activation of β -adrenergic receptors causes Ca^{2+} mobilization through phosphorylation of the L-type Ca^{2+} channel by the cAMP-dependent protein kinase A, and also through direct coupling of Gs to Ca^{2+} channel activation [27,44]. It has been suggested that polyamines may play a role in stimulation of Ca^{2+} influx in rat myocytes in the presence of isoproterenol [45].

Several studies have suggested that the induction of ODC in response to β -adrenergic stimulation is primarily through the β 2-adrenergic receptors, which are a minor component of the β -adrenergic receptors in the heart, but may become more important in the failing heart (reviewed in [27]). Our studies reported here using isoproterenol and the β 1-receptor-specific antagonist atenolol suggest that both β 1 and β 2 receptors contribute to the hypertrophy seen in the hearts of MHC-ODC21 mice. In addition, it has been shown that cardiac hypertrophy resulting from treatment with the β 2-specific agonist clenbuterol results in an accumulation of polyamines in the heart, which could be reversed by α -difluoromethylornithine. ODC induction by isoproterenol both *in vivo* and in an *in vitro* cardiac myocyte system could also be prevented by β 2-receptor specific antagonists [6,29,46]. A recent study [47] suggests that both G_s and G_i proteins, which are activated by the β 2-adrenergic receptor pathway, are involved in cardiac hypertrophy induced by isoproterenol, and these pathways induce a Ras-dependent activation of extracellular-signal-regulated kinases (ERKs). Activation of the ERK pathway is required for many growth-related processes, and mice overexpressing Ras or Gzq in the heart are known to develop hypertrophy [20,23,48,49]. We and others [11,50] have shown that ODC induction is central to the activation of Ras-dependent pathways in a variety of conditions involving abnormal cell growth. Thus the more rapid development of β -adrenergic mediated hypertrophy seen in the transgenic mice may stem from an enhanced activation of these pathways in ODC-overexpressing hearts. Future experiments will use isolated myocyte cultures to determine whether ODC

overexpression in the hearts of MHC-ODC21 mice activates a pattern of gene expression which includes pathways downstream of Ras.

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