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Central role for ornithine decarboxylase in β-adrenoceptor mediated hypertrophy

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Abstract

Objective: TGF-β stimulation of cardiac myocytes induces a hypertrophic responsiveness to β-adrenoceptor stimulation. This study investigates whether this β-adrenoceptor mediated effect depends on induction of ornithine decarboxylase (ODC). **Methods:** Isolated adult ventricular cardiomyocytes from rats were used as an experimental model. Cells were either cultured in 20% (v/v) FCS to activate autocrine released TGF-β or used without pre-treatment. The hypertrophic response was characterized by an increased ^{14}C-phenylalanine incorporation, RNA and protein mass or by an increased expression of atrionatriurectic factor and ODC. The results on cell cultures were compared to those achieved by isoprenaline perfused mice hearts from transgenic mice overexpressing TGF-β_1. **Results:** ODC activity and expression increased within 2 h in TGF-β_1 pre-treated cells under isoprenaline. In the presence of ODC inhibitors (α-methylornithine or difluoromethylornithine) this increase remained absent and the increases in ^{14}C-phenylalanine incorporation, protein and RNA mass under isoprenaline were abolished. In cells not exposed to TGF-β no induction of ODC was observed. Isoprenaline also induced ODC in isolated perfused ventricles from transgenic mice overexpressing TGF-β_1, but not in ventricles from their nontransgenic counterparts. **Conclusions:** This study shows first, a pivotal role for ODC induction in the hypertrophic response of cardiomyocytes to β-adrenoceptor stimulation and second, that ODC induction in vivo and in vitro requires pre-treatment of cardiomyocytes with TGF-β. It is concluded that TGF-β induces a hypertrophic responsiveness to β-adrenoceptor stimulation that is characterized by ODC induction.

Keywords: Adrenergic (ant)agonist; Cytokines; Hypertrophy; Myocytes; Signal transduction

1. Introduction

Hyperactivation of the sympathetic nerve system plays an important role in the pathogenesis of myocardial hypertrophy and heart failure [1]. It is generally accepted that excess of catecholamines causes myocardial hypertrophy in vivo and in vitro [2–4]. When adult ventricular cardiomyocytes are investigated in vitro, the hypertrophic effect of catecholamines depends on α-adrenoceptor stimulation. Selective α-adrenoceptor stimulation causes an increase in protein synthesis, but selective β-adrenoceptor stimulation does not [2,4]. This is contrasted by the in vivo situation, in which selective β-adrenoceptor stimulation can also cause myocardial hypertrophy [5]. On adult cardiomyocytes in vitro, specific culture procedures restore the in-vivo-like behavior in regard to the hypertrophic responsiveness to β-adrenoceptor stimulation, i.e., pre-cultivation of cardiomyocytes in the presence of low concentrations of isoprenaline [6] or pre-cultivation in the presence of fetal calf serum [7] induces hypertrophic responsiveness of adult ventricular cardiomyocytes to β-

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adrenoceptor stimulation. These culture systems, therefore, permit to analyze the cellular mechanisms by which β-adrenoceptor stimulation induces myocardial hypertrophy.

In cells with hypertrophic responsiveness to β-adrenoceptor stimulation, the hypertrophic effects of α- and β-adrenoceptor stimulation are both characterized by an elevation of total RNA. Approximately 85% of total RNA mass represents ribosomal RNA and thereby the protein synthesis capacity of the cell [8]. Therefore, elevation of total RNA is a key factor in myocardial hypertrophy, that enables the cell to maintain a high rate of protein synthesis. In case of α-adrenoceptor stimulation, RNA elevation is accompanied by an increased ^{14}C-uridine incorporation. This indicates de novo synthesis of RNA. In case of β-adrenoceptor stimulation, however, RNA elevation is not accompanied by an increased ^{14}C-uridine incorporation [7]. That let us conclude that the mechanism by which β-adrenoceptor stimulation elevates RNA mass seems to be due to a decrease of RNA degradation. Since polyamines are known to stabilize RNA [9] this may explain the observed elevation of RNA in the absence of elevated RNA synthesis.

Ornithine decarboxylase (ODC) represents the rate limiting enzyme of the polyamine metabolism. An induction of ODC is causally involved in the mechanism by which β-adrenoceptor stimulation elevates cellular RNA mass [10]. β-Adrenoceptor mediated hypertrophy in vivo is, indeed, accompanied by induction of ODC [5]. The present study investigates on the cellular level, whether ODC is induced under β-adrenoceptor stimulation in cardiomyocytes with hypertrophic responsiveness to β-adrenoceptor stimulation and if this induction is responsible for the hypertrophic effect of β-adrenoceptor stimulation in this cell system.

This study was performed in a well-described in vitro system in which ventricular cardiomyocytes isolated from adult rats were cultured for 6 days in serum supplemented culture media. These cells exhibit hypertrophic responsiveness to β_2-adrenoceptor stimulation [11]. It was previously shown that induction of hypertrophic responsiveness depends on the autocrine stimulation with TGF-β of the cultured cells [12]. In accordance with the aforementioned aims, we have analyzed whether pre-stimulation with TGF-β also promotes the β-adrenergic inducibility of ODC in cardiomyocytes. The cell cultures system used in this study exhibits resemblance to the situation of the myocardium in vivo at the transition from compensated to non-compensated cardiac hypertrophy where an increased expression of TGF-β_1 indicates an activation of the local TGF-β system [13]. We were interested to compare the cell culture data to the in vivo situation. We chose to compare hearts from transgenic mice over-expressing TGF-β_1 with hearts from normal mice. The hearts were perfused and responsiveness to β_2-adrenoceptor stimulation was studied in respect to ODC activity and expression. As a molecular marker for myocardial hypertrophy the expression of ANF was also analyzed in these hearts.

2. Methods

All animal studies were performed in accordance with guidelines described in the NIH *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH, publication no. 85-23, revised 1996).

2.1. Cell culture

Ventricular heart muscle cells were isolated from 200- to 250-g male Wistar rats as previously described [4]. Isolated cells were suspended in FCS-free culture medium and plated at a density of 1.4×10^5 elongated cells/35-mm culture dish (Falcon type 3001). The culture dishes had been pre-incubated overnight with 4% fetal calf serum (FCS) in medium 199. The basic culture medium consisted of medium 199 with Earle's salts, 5 mmol/l creatine, 2 mmol/l L-carnitine, 5 mmol/l taurine, 100 I.U./ml penicillin, and 100 μg/ml streptomycin. To prevent growth of nonmyocytes, media were also supplemented with 10 μmol/l cytosine-β-D-arabinofuranoside.

Four hours after plating, cultures were washed twice with culture medium to remove round and nonattached cells and either supplied with FCS-free experimental media, in which cells were incubated for up to 24 h at 37°C or with basic culture medium supplemented with 20% fetal calf serum, in which cells were incubated for 6 days at 37°C. In both cases, the experiments were carried out in basic culture medium under serum free conditions (control), with addition of phenylephrine, isoprenaline or dibutyryl-cyclo-AMP, at concentrations indicated. Ascorbic acid (100 μmol/l) was added to all cultures as an antioxidant.

2.2. Treatment protocol for cardiomyocytes (TGF-β exposure)

Adult ventricular cardiomyocytes from rat were isolated, plated on culture dishes, and cultured either for 24 h under serum-free conditions or for 6 days in presence of 20% (v/v) fetal calf serum (FCS) and thereafter for 24 h under serum-free conditions. TGF-β activity was determined in both culture systems. After 24 h, the media of cardiomyocytes cultured under serum-free conditions had low TGF-β activities (0.3 ± 0.3 ng active TGF-β/ml) and those cultured in presence of high FCS had high TGF-β activities (1.5 ± 0.2 ng active TGF-β/ml; $n=4$, $P<0.05$ vs. serum-free cultures).

2.3. Incorporation of ^{14}C-phenylalanine and changes in cellular protein and RNA mass

Incorporation of phenylalanine into cells was determined by exposing cultures to L-^{14}C-phenylalanine (0.1 μCi/ml) for 24 h and determination of the incorporation of radioactivity into acid-insoluble cell mass as described before [7].

As shown previously ^{14}C-phenylalanine incorporation increases constantly for 36 h under these conditions [7]. Nonradioactive phenylalanine (0.3 mmol/l) was added to the medium to minimize variations in the specific activity of the precursor pool responsible for protein synthesis. In incorporation studies, experiments were terminated by removal of the supernatant medium from the cultures and washed three times with ice-cold phosphate-buffered saline (PBS; composition in mmol/l: 1.5 KH_2PO_4, 137 NaCl, 2.7 KCl, and 1.0 Na_2HPO_4, pH 7.4). Subsequently, ice-cold 10% (w/v) trichloroacetic acid was added. After storage overnight at 4°C, the acid was removed from the dishes. Radioactivity contained in this acid fraction was taken to present the intracellular precursor pool. The dishes were then washed twice with ice-cold PBS. The remaining precipitate on the culture dishes was dissolved in 1 M NaOH–0.01% (w/v) sodium dodecylsulphate (S.D.S) by an incubation for 2 h at 37°C. In these samples protein contents [14] and DNA contents [15] were determined, and the radioactivity was counted. RNA was determined from an aliquot of these samples after precipitation with an equal volume of 10% (w/v) perchloric acid in the remaining supernatant [8]. The RNA content was also expressed relative to the DNA content of the samples.

2.4. TGF-β activity

TGF-β activity in the media was determined as described [16] by the growth inhibitory effect of TGF-β on the proliferation of microvascular endothelial cells. Isolation and cultivation of microvascular endothelial cells was described earlier [17]. Supernatants of the cell culture media were diluted and added to subconfluent monolayers of microvascular endothelial cells plated on 96-well dishes. After 48-h protein contents of the wells were determined. The cultures were fixed by addition of 3% (v/v) paraformaldehyde, stained by replacement of the paraformaldehyde by 1 (% v/v) methylene blue dissolved in TBE buffer (composition: in mM 89 tris(hydroxymethyl)aminomethane (Tris), 89 borate, 2 EDTA). Finally, the dishes were washed twice and incubated with ethanol–HCl (1:1, v/v). The density of the cultures was determined at 630 nm. The growth inhibitory effect was compared to a standard curve using activated TGF-β isolated from porcine platelets (British Biotechnology Products, Oxon, UK). The results are expressed as ng active TGF-β/ml. Specificity of the inhibitory effect of media supernatants to TGF-β was further demonstrated by the use of a neutralizing antibody to TGF-β_1 (British Biotechnology Products), which abolished the growth inhibitory effect.

2.5. ODC activity

The activity of ODC was determined as described by Meilhoc et al. [18]. Cardiomyocyte cultures were washed twice with ice-cold phosphate buffered saline, scrapped off, and centrifuged for 2-min at 3000 *g*. The pellet was resuspended in lysis buffer (composition in mM: 10 tris(hydroxymethyl)aminomethane (Tris)·HCl, 250 sucrose, 5 dithiothreitol, 1 EDTA, 0.5 pyridoxalphosphate, pH 7.3) and homogenized by sonification. The homogenate was centrifuged again for 2 min at 3000 *g*. This supernatant was used to determine ODC activity. ODC activity from ventricles of mice hearts was determined by homogenization of ventricle samples in lysis buffer (composition as above) using a ultravox homogenizer, and the suspension was used as described for cardiomyocyte cultures. ODC activity in the supernatants was determined by the release of $^{14}CO_2$ from 1-^{14}C-ornithine. A 50-μl volume of the supernatant and 250 μl of lysis buffer including 0.1 μCi/ml 1-^{14}C-ornithine and 3 mM ornithine was incubated for 30 min in a closed tube equipped with filter-paper wetted in 1 M KOH to trap released CO_2. The incubation was terminated by incubation over night at 4°C to adsorb released $^{14}CO_2$. To estimate non-specific CO_2 release during the incubation, blank tubes were set up and the nonspecific release was subtracted from sample release. The filter papers were removed, and trapped $^{14}CO_2$ was counted by liquid scintillation.

2.6. RT-PCR

Total RNA from cardiomyocytes and ventricular tissue was extracted with RNA-Clean (AGS, Heidelberg, Germany) as described by the manufacturers. Reverse transcription reactions were performed for 1 h at 37°C in a final volume of 10 μl RNA, 100 ng oligo(dT)$_{15}$ (Boehringer Mannheim, Germany), 1 mM dNTPs (Gibco-BRL,) 8 U RNAse Block (Promega, Mannheim, Germany) and 60 U M-MLV reverse transcriptase (Gibco-BRL). Aliquots (1.5 μl) of the synthesized cDNA were used for polymerase chain reaction (PCR) in a final volume of 10 μl 1.5 μM of primer pairs, 0.4 mM dNTPs, 1.5 mM $MgCl_2$, and 1 U Taq-polymerase (Gibco-BRL). Amplification was performed under the following cycle conditions: 1 min 93°C, 1 min 57°C, 3 min 72°C. For each assayed gene the number of cycles resulting in a linear amplification range was tested. Oligonucleotide primers were synthesized by Gibco-BRL and had the following sequences: β-actin sense 5′-GAAGTGTGACGTTGACATCCG-3′ and antisense 5′-TGCTGATCCACATCTGCTGGA-3′, for amplification between bp 2731 and 3081 of rat β-actin gene [19]; ODC sense 5′-GAAGATGAGTCAAACGAGCA-3′ and antisense 5′-AGTAGATGTTTGGCCTCTGG-3′, for amplification between bp 5777 and 6352 [20]; ANF sense 5′-ATGGGCTCCTTCTCCATCAC-3′ and antisense TCTTCGGTACCGGAAGCT-3′, for amplification between bp 64 and 520 [21]; c-fos sense 5′-TGCCAGATGTGGACCTGTCTG-3′ and antisense 5′-CCACAGCTTGGTGTGTTTCAC-3′, for amplification between bp 999 and 1390 [22].

After amplification reaction products were separated on

5% polyacrylaminde gels, stained with ethidium bromide and photographed under UV illumination. For quantification density of the DNA fragments were determined by IMAGE QUANT (Molecular Dynamics, Krefeld, Germany). The results for c-fos, ODC, and ANF expression were normalized for equal loading by β-actin amplification.

2.7. Transgenic animals

The trangenic animals used in this study have been described in detail by Sanderson et al. [23]. They overexpress TGF-β_1 in which Cys^{223} and Cys^{225} codons were replaced with serine codons, resulting in preferential secretion of the mature form of TGF-β_1 [24]. Their TGF-β_1 plasma concentrations have been documented from the second week on [23]. All animals used in this study were used at an age of 8 weeks and compared to non-transgenic control animals from the same strain.

2.8. Heart perfusion

Hearts from mice were isolated as described above for rat hearts to prepare cardiomyocytes preparations and quickly connected to a Langendorff perfusion system. Hearts were perfused at 37°C and at a flow-rate of 0.5 ml/min. The composition of the perfusion buffer was as follows (mM): 145 NaCl, 25 $NaHCO_3$, 5.9 KCl, 1.2 mM KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 0.5 EDTA, 0.1 ascorbic acid, 5.5 glucose, 0.001 isoprenaline, 0.01 atenolol, gassed with 95% O_2–5% CO_2. Hearts were allowed to beat spontaneously. After an initial equilibration period (5 min) the first heart was removed and taken as time 0. The perfusion was prolonged for up to 2 h. Release of lactate dehydrogenase was monitored continuously as described before [25] to control sarcolemmal integrity throughout the whole perfusion time.

2.9. Statistics

Data are given as means±S.E.M. from *n* different culture preparations. Statistical comparisons were performed by one-way analysis of variance and use of the Student–Newman–Keuls test for post hoc analysis [26]. Differences with $P<0.05$ were regarded as statistical significant. All data analyses were computed using SAS software, version 6.11 (SAS Institute, Cary, NC, USA).

2.10. Materials

Falcon tissue culture dishes were obtained from Becton-Dickinson (Heidelberg, Germany). Boehringer Mannheim was the source for glutamine-free medium 199 and fetal calf serum. Cytosine-β-D-arabinofuranoside, L-carnitine, creatine, taurine, L-phenylephrine hydrochloride, ICI 118,551, and DL-isoproterenol hydrochloride were obtained from Sigma (Deisenhofen, Germany). All other chemicals were of analytical grade.

3. Results

3.1. Effect of active TGF-β on hypertrophic responsiveness and ODC induction under isoprenaline

On cardiomyocytes cultured with low TGF-β activity, isoprenaline did not increase ^{14}C-phenylalanine incorporation, indicating hypertrophic growth of cardiomyocytes, and did not induce ANF expression, a molecular marker of hypertrophy (Fig. 1). In contrast, isoprenaline increased ^{14}C-phenylalanine incorporation and ANF expression in cardiomyocytes pre-exposed to high TGF-β activity (Fig. 1).

ODC induction under isoprenaline was investigated next. On cells cultured under low TGF-β activity, isoprenaline did not increase ODC activity or expression (Fig. 2). On cells pre-cultivated with high TGF-β_1-activity,

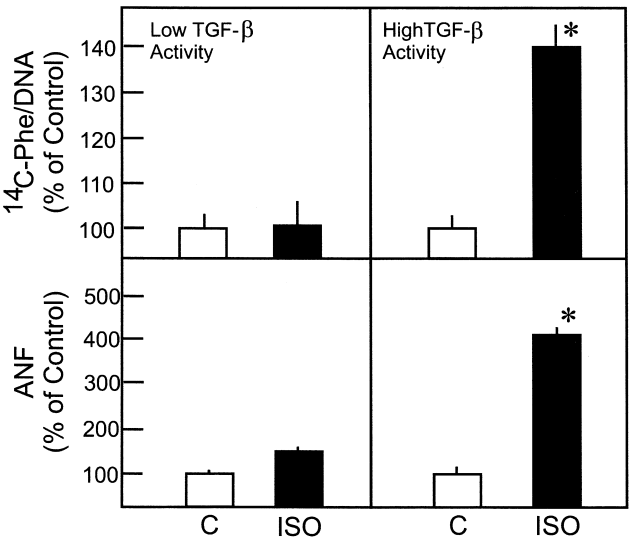

Fig. 1. Incorporation of ^{14}C-phenylalanine and expression of atrionatriuretic factor (ANF) in cardiomyocytes cultured either for 24 h under serum free conditions with low TGF-β activity in absence (C) and presence of isoprenaline (ISO, 1 μM) or in cardiomyocytes cultured for 6 days in high TGF-β activity and subsequently for 24 h under serum free conditions in the absence or presence of ISO. In case of ^{14}C-phenylalanine incorporation, ^{14}C-phenylalanine was included in the medium and its incorporation into the protein fraction was calculated as ^{14}C-phenylalanine per μg DNA and expressed as percentage of the controls. S.E.M. bars of the controls represent variations between four different culture preparations. In case of ANF expression, cells were harvested at the end of the incubation time and the mRNA content of ANF was determined by RT-PCR (23 cycles). In each case, data are given as percent values relative to the controls and the S.E.M. bars of the controls represent the variations between four different culture preparations. *, $P<0.05$ vs. C; $n=4$ cultures.

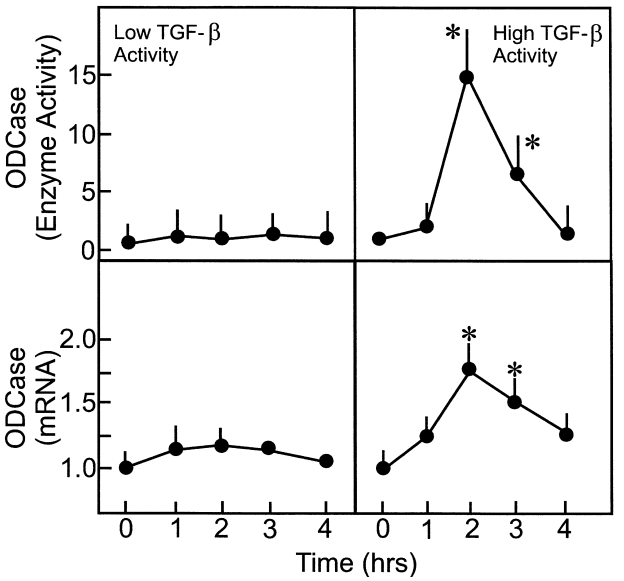

Fig. 2. Expression and activity of ornithine decarboxylase (ODC) in cardiomyoytes following treatment with isoprenaline (1 μM). Cells were cultured as described for Fig. 1. ODC enzyme activity was determined in cell extracts from the respective cultures as the release of $^{14}CO_2$ from 1-^{14}C-ornithine and expressed as mU/min. ODC mRNA expression was determined by RT-PCR (25 cycles). Data are means±S.E.M. from $n=4$ cultures. *, $P<0.05$ vs. 0 h; $n=4$ cultures.

however, enzyme activity and expression increased within 2 h after addition of isoprenaline (Fig. 2). In these cells, actinomycin D, a transcriptional inhibitor, attenuated ODC induction under isoprenaline (Table 1), indicating that the increase in ODC mRNA under isoprenaline requires transcriptional activation. The effect of isoprenaline on ODC induction could be mimicked by dibutyryl-cAMP and antagonized by the β_2-adrenoceptor antagonist ICI 118,551 (Table 1). Two different ODC inhibitors, namely α-methylornithine and difluoromethylornithine (DFMO), abolished ODC induction in these cells (Table 1). In contrast, stimulation of α-adrenoceptors by phenylephrine did not induce ODC activity (Table 1).

Table 1
ODC activity in cardiomyocytes pre-treated with TGF-β for 6 days[a]

	ODC activity (mU/min)
Control	0.6±0.8
Isoprenaline (1 μM)	13.5±2.4*
Isoprenaline (1 μM)+ICI 118,551 (1 μM)	0.9±1.1
Isoprenaline (1 μM)+actinomycin D (5 μM)	0.6±0.7
Dibutyryl-cyclo-AMP (1 mM)	7.5±1.3*
Isoprenaline (1 μM)+α-methylornithine (10 mM)	0.5±0.8
Isoprenaline (1 μM)+difluoromethylornithine (1 mM)	0.4±0.3
Phenylephrine (10 μM)	0.6±0.4

[a] ODC enzyme activity was determined in cell extracts from the respective cultures as the release of $^{14}CO_2$ from 1-^{14}C-ornithine and expressed as mU/min. Cells were treated with isoprenaline, dibutyryl-cyclo-AMP, or phenylephrine for 2 h. Data are means±S.E.M. from $n=4$ cultures. *, $P=0.05$ vs. control.

3.2. Causal relationship between ODC induction and hypertrophy

The above reported results show that β-adrenoceptor stimulation is accompanied by ODC induction. Whether ODC induction and induction of hypertrophy are causally linked was investigated by pharmacological inhibition of ODC. In this set of experiments, cardiomyocytes with hypertrophic responsiveness to β-adrenoceptor stimulation were used, namely 6-day-old cultures with high TGF-β activity. α-Methylornithine dose-dependently attenuated the increase in protein and RNA mass in response to isoprenaline (Fig. 3). The inhibitory effect of α-methylornithine on enlargement of protein and RNA mass was accompanied by an inhibition of ^{14}C-phenylalanine incorporation (Table 2). A similar result was obtained by use of DFMO that attenuated ^{14}C-phenylalanine incorporation in response to isoprenaline. In contrast, under α-adrenoceptor stimulation by phenylephrine, the presence of α-methylornithine did not attenuate this hypertrophic effect (Table 2).

3.3. Effect of β-adrenoceptor stimulation on ODC induction in isolated perfused mice hearts

To investigate, whether the observed induction of hypertrophic responsiveness to β-adrenoceptor stimulation by

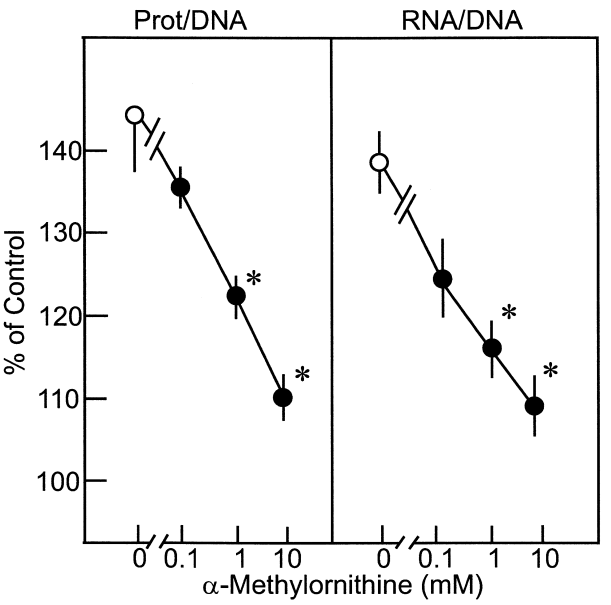

Fig. 3. Influence of α-methylornithine on isoprenaline induced increases of protein and RNA mass. Cardiomyocytes were pre-cultured for 6 days in the presence of high TGF-β activity and subsequently for 24 h under serum free conditions with isoprenaline (1 μM) and addition of α-methylornithine at concentrations as indicated. Protein and RNA mass of the cultures were calculated relative to the DNA content of the dishes and expressed as percentage relative to untreated controls. Data are means±S.E.M. of $n=4$ cultures. *, $P<0.05$ vs. without α-methylornithine.

Table 2
Influence of ODC inhibitors on ^{14}C-phenylalanine incorporation in cardiomyocytes pre-treated with TGF-β for 6 days[a]

	^{14}C-Phenylalanine incorporation (% of control)
Isoprenaline (1 μM)	141±5
Isoprenaline (1 μM)+α-methylornithine (10 mM)	119±4*
Isoprenaline (1 μM)+difluoromethylornithine (1 mM)	103±4*
Phenylephrine (10 μM)	150±9
Phenylephrine (10 μM)+α-methylornithine (10 mM)	147±6
α-Methylornithine (10 mM)	102±9
Difluoromethylornithine (10 mM)	95±12

[a] C-phenylephrine incorporation was determined in cardiomyocytes pre-treated for 6 days with TGF-β during the subsequent 20-h under serum-free conditions in presence of isoprenaline or phenylephrine. The control value (100%) was $4.6 \cdot 10^{-2}$ dpm per μg DNA. Data are expressed as means±S.E.M. from $n=4$ cultures relative to untreated controls. *, $P<0.05$ vs. isoprenaline.

TGF-β on cultured cardiomyocytes bears relevance to the whole heart, we determined the expression of ANF, a molecular marker for the onset of myocardial hypertrophy in hearts from transgenic mice over-expressing TGF-β_1 and non-transgenic control mice from the same strain. Hearts from transgenic animals exhibited already elevated ANF mRNA levels compared to non-transgenic animals (Fig. 4). Hearts were connected to a Langendorff perfusion system and perfused with a constant flow (0.5 ml/min) for 2 h. Isoprenaline (1 μM) and, to abolish β_1-adrenoceptor stimulation, the β_1-adrenoceptor antagonist atenolol (10 μM) were added to the perfusion system. Atenolol was added because on the isolated cell system atenolol did not abolished the hypertrophic response to isoprenaline in responsive cells and the perfusion conditions were aimed to mimic closely those previously used on the cell culture

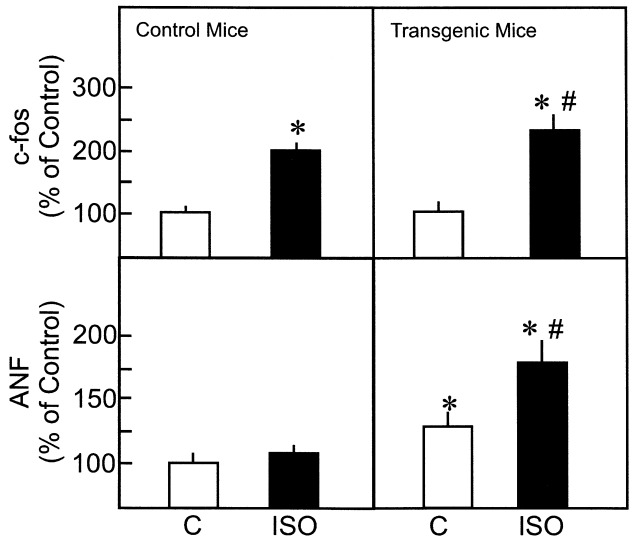

Fig. 4. ANF and c-fos expression in mice ventricles. Hearts from transgenic mice over-expressing TGF-β_1 and their non-transgenic counterparts were perfused for 2 h without additions (C, control), or with isoprenaline (ISO, 1 μM) and atenolol (10 μM). The expression of c-fos and ANF was determined by RT-PCR (23 cycles). Data are means±S.E.M. from $n=4$ hearts. *, $P<0.05$ vs. control hearts from non-transgenic hearts; #, $P<0.05$ vs. control hearts from transgenic hearts.

system [11]. In response to isoprenaline and atenolol ANF mRNA contents increased in hearts from transgenic mice but not in control hearts, indicating hypertrophic responsiveness of hearts from transgenic mice. In hearts from transgenic animals and from non transgenic control mice, isoprenaline induced c-fos mRNA, indicating that even the control hearts were able to respond to isoprenaline with expressional changes. Finally, the induction of ODC in whole hearts isolated from transgenic mice over-expressing TGF-β_1 and control mice was investigated. ODC activity and expression was found significantly increased within 2 h in hearts from transgenic animals. In hearts from control mice, however, ODC activity and expression did not increase (Fig. 5).

4. Discussion

This study investigated whether induction of ODC, the rate limiting enzyme of the polyamine metabolism, is causally involved in the hypertrophic response to β-adrenoceptor stimulation in cardiomyocytes with hypertrophic responsiveness to β-adrenoceptor stimulation. Induction of such a hypertrophic responsiveness depends on TGF-β activity. The main findings are first, that β-adrenoceptor mediated hypertrophy is indeed dependent on the induction of ODC in responsive cardiomyocytes, and, second, that such an induction of ODC is observed only when cells have been pre-exposed to TGF-β. Experiments in whole hearts from normal or TGF-β_1 over-expressing mice confirmed these conclusions.

This study has used a previously well characterized cell system in which hypertrophic responsiveness to β-adrenoceptor stimulation is inducible by addition of serum [7]. The cultured cardiomyocytes release TGF-β into the medium which, in presence of a serum supplement, is activated. Active TGF-β_1 induces the hypertrophic responsiveness to β-adrenoceptor stimulation as characterized by elevation of protein and RNA mass and ^{14}C-phenylalanine incorporation and induction of ODC and ANF expression. We now demonstrate further on transgenic animals over-expressing TGF-β_1, that TGF-β_1 also induces hypertrophic

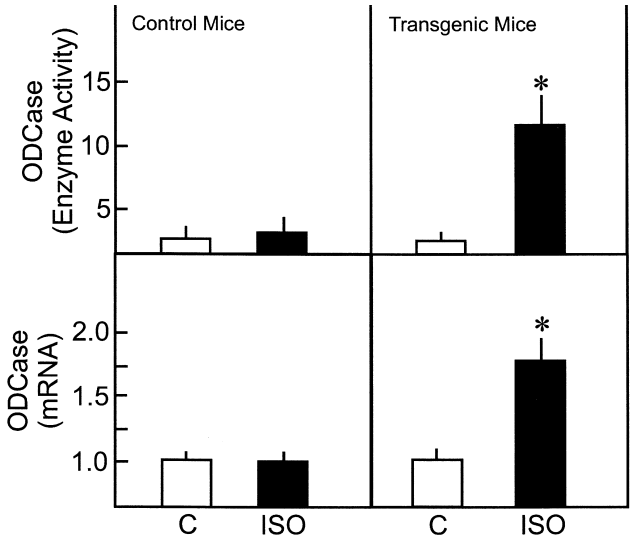

Fig. 5. Expression and activity of ornithine decarboxylase (ODC) in mice ventricles. Hearts were perfused as indicated in Fig. 4. ODC enzyme activity was determined in extracts from the ventricles as the release of $^{14}CO_2$ from 1-^{14}C-ornithine and expressed as mU/min. ODC mRNA expression was determined by RT-PCR (25 cycles). Data are means±S.E.M. from $n=4$ hearts. *, $P=0.05$ vs. control hearts from non-transgenic and transgenic mice.

responsiveness to β-adrenoceptor stimulation in vivo. Induction of ODC and ANF expression in the ventricles of transgenic and non-transgenic animals were used as molecular markers for myocardial hypertrophy

In TGF-β exposed cells and transgenic animals, β-adrenoceptor stimulation evoked the induction of ODC. It was found in the cell system that the induction of ODC could be abolished by the presence of two chemically distinct ODC inhibitors (α-methylornithine and difluoromethylornithine). With either one of these inhibitors present, the stimulatory action of β-adrenoceptor stimulation on ^{14}C-phenylalanine incorporation was suppressed. For α-methylornithine it was also shown that the increase in cellular protein and RNA mass was dose-dependently reduced. These results suggest, that an activation of the polyamine metabolism, as indicated by ODC induction, is involved in the elevation of RNA that is observed under β-adrenoceptor stimulation. As a control for the specificity of ODC inhibition on the β-adrenoceptor hypertrophic response, the same inhibitors were applied under α-adrenoceptor stimulation. In this case, they had no effect on the increase in RNA mass and protein synthesis. In summary, these results indicate that the hypertrophic effect of β-adrenoceptor stimulation in responsive cardiomyocytes depends specifically on the induction of ODC.

The role of ODC in cellular growth has been investigated in several cell culture systems. Inhibition of ODC by DFMO depletes cellular polyamines and thereby reduces the proliferation rate, e.g. in Ehrlich ascites tumor cells [27]. High levels of polyamines seem to be required for the initiation of the cell cycle. Adult ventricular cardiomyocytes are unable to divide and thus grow by hypertrophy. One common feature of hypertrophic and hyperplastic growth is, that under both conditions an increase in protein synthesis is necessary. This requires an elevation of the translational machinery as indicated by an increase in rRNA and, since this is >80% of all RNA, of total RNA. Our data indicate, that an induction of ODC is causally involved in this process in adult cardiomyocytes. A correlation between ODC induction and increase in tissue content of the polyamines spermidine, spermine, putrescine, and total RNA has been shown before on stimulated bovine lymphocytes as well [10].

The hypertrophic effect of β-adrenoceptor stimulation in this model of cultured cardiomyocytes is due to a stimulation of β_2-adrenoceptors [11]. Likewise the hypertrophic response characterized before, ODC induction by isoprenaline in responsive cells could be antagonized by ICI 118,551, a β_2-adrenoceptor antagonist. Together with the aforementioned results this suggests that ODC induction in these cells is due to β_2-adrenoceptor stimulation. In this context, we also were able to induce ODC in hearts from transgenic mice over-expressing TGF-β_1 by isoprenaline in the presence of a β_1-adrenoceptor antagonist, again suggesting that most likely β_2-adrenoceptors are involved in the observed induction of hypertrophic responsiveness by TGF-β. In agreement with this conclusion, it has been found that clenbuterol, a β_2-adrenoceptor agonist, can induce ODC in vivo [28]. The β_2-adrenoceptor mediated hypertrophic effect is known to be cAMP dependent [11]. Consistent with this finding and the conclusion that hypertrophic growth depends on ODC induction is the present observation, that ODC induction can also be achieved by addition of the cell permeable cAMP analogue db-cAMP.

In the cell culture system investigated here induction of ODC and hypertrophy upon β-adrenoceptor stimulation requires a previous exposure of the cells to TGF-β. We used a whole animal model of cardiac pre-exposure to TGF-β_1 to test the hypothesis that in intact myocardium the induction of ODC by β-adrenoceptor stimulation also depends on a pre-exposure to TGF-β_1. For this purpose hearts from normal animals were compared to those from animals with elevated circulating TGF-β levels. The latter animals were transgenic animals with an increased expression and release of active TGF-β_1. The hearts from transgenic animals showed a similar induction of ANF expression, a molecular marker of myocardial hypertrophy, upon β_2-adrenoceptor stimulation than the isolated cardiomyocytes upon isoprenaline treatment. These hearts, however, had already elevated ANF expression prior to β-adrenoceptor stimulation. They had fibrosis, as expected from the known effect of TGF-β_1 on fibroblasts, and this seems to cause a hypertrophic response caused by increased stiffness of the ventricles. However, stimulation of β-adrenoceptors further induces ANF and ODC expression only in hearts from transgenic animals.

Hearts undergoing myocardial hypertrophy show a transitory increased expression of TGF-β_1 [13]. In these hearts, the expression of TGF-β_1 is increased at the transition from compensatory hypertrophy to heart failure. The results of the present study suggest that in this pathophysiological transition stage, TGF-β_1 stimulation renders cardiomyocytes responsive to a hypertrophic action of β-adrenoceptor stimulation. This adds a further receptor-mediated stimulus to the growth promotion of the myocardial cells, which under normal conditions would not be found. Recently we showed that neuropeptide Y, which under normal conditions does not elevate protein synthesis either, can also increase protein synthesis of cardiomyocytes after pre-exposure to TGF-β [29]. Therefore, a pivotal role for TGF-β_1 for cardiomyocyte hypertrophy seems more important than expected from the present study alone.

In summary, our study demonstrates a causal role for ODC in the hypertrophic response to β-adrenoceptor stimulation.

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