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Research Article

Gene expression profile of cardiomyocytes in hypertrophic heart induced by continuous norepinephrine infusion in the rats

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Abstract. Catecholamines play an important role in the development of cardiac hypertrophy. To observe cardiomyocyte-specific gene expression changes induced by catecholamines in vivo, left ventricular cardiomyocytes were isolated from male Sprague-Dawley rats after continuous infusion of norepinephrine (NE; 0.2 mg/kg per hour intravenously) for 0.5, 1, 2, 3 and 7 days. The gene expression profiles of these cells during different NE infusion stages were assessed by using a cDNA microarray, and the microarray data were further analyzed by a clustering method. Cardiac hypertrophy was induced upon continuous NE infusion, with the peak at 3 days. Meanwhile, manifest changes in gene expression profile within cardiomyocytes over the time course were revealed, most of the genes never having been reported to be involved in cardiac hypertrophy. The number of genes displaying differential expression also peaked at the middle stage of infusion (2–3 days), and the majority of the signaling molecules were found differentially expressed mainly at this stage, including phosphatidylinositol 3-kinase, calcium/calmodulin-dependent protein kinase II and non-receptor tyrosine kinases, etc. The tumor suppressor p53 was found up-regulated at very early (0.5 days) and late stages (7 days) of NE infusion. Self-organization clustering analysis revealed subsets of coordinate regulated genes. One set consisted of several enzymes involved in energy metabolism, including carnitine octanoyltransferase, ATP synthase subunit c, pancreatic lipase and glycogen phosphorylase, possessing a similar expression pattern with a rapidly elevated expression level at the early stage of NE infusion. This is the first study to provide transcriptional information for cardiomyocytes, a single cell type, in the heart during the development of cardiac hypertrophy in vivo, and may provide accurate clues to elaborate hypotheses about the evolution of this pathology.

Key words. Myocyte; cardiac; hypertrophy; norepinephrine; gene expression profile.

Catecholamines have long been considered to be an important cofactor in the development of both physiologic and pathologic cardiac hypertrophy. Numerous investigations on humans and experimental animals have demonstrated that elevated plasma levels of catecholamines are associated with cardiac overload and congestive heart failure. Increased plasma catecholamines and sympathetic neural traffic have also been considered to contribute to exercise-induced myocardial hypertrophy [1, 2]. Although several events, such as the activation of mitogen-activation protein kinases [3] and the re-expression of embryonic genes or expression of proto-oncogenes [4], are well known, we still lack comprehensive understanding for the molecular changes involved in the developmental process of cardiac hypertrophy induced by catecholamines. The

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cDNA microarray, a high-throughput technology, is very useful for investigating gene expression changes on a large scale. Analysis of a gene expression profile with microarray technology during the induction of cardiac hypertrophy has enabled a better understanding of the pathology of this process in the last 4 years [5].

Many models have been employed to demonstrate catecholamine-induced protein synthesis and cell or organ growth [1], including in vitro models such as cultured cardiac cells isolated from neonatal or adult rats as well as isolated perfused hearts, and in vivo models such as intact hypertrophic hearts from experimental animal models or transgenic mice. Compared with intact hearts, the cell cultures have advantages for investigation such as controlability, cell type focus and easy for further analysis or study. However, heart cells are affected by hormonal substances and circulating growth factors in vivo, and their changes often differ from those found in vitro [6]. In addition, different types of cells in the heart can influence each other by paracrine signaling [7, 8]. We recently reported that activation of STAT3 in mice cardiomyocytes with isoproterenol administration in vivo was mediated by the interleukin (IL)-6 family of cytokines secreted by cardiac fibroblasts [9]. There might be great differences in effects of catecholamines on cardiaomyocytes in vivo and in vitro. Given the difficulty to mimic the real conditions in vivo by primary cultured cells, it is valuable to investigate the effects of catecholamines by isolating cells from already hypertrophic hearts induced by catecholamines in vivo.

In this study, we attempted to explore the changes occurring in a single cell type, mainly the cardiomyocyte, during cardiac hypertrophy in vivo. The changes in gene expression profile in cardiomyocytes isolated from hypertrophied left ventricles induced by norepinephrine (NE)-infusion in the rat were determined using a cDNA microarray at different developmental stages of heart hypertrophy. Manifest changes in gene expression profile took place in cardiomyocytes when cardiac hypertrophy was induced by continuous administration of NE and different patterns of changes in gene expression were identified.

Materials and methods

Preparation of cardiac hypertrophy model

Male Sprague-Dawley rats (10 weeks old, 280–290 g) were used in this study, and were maintained in accordance with the 'Ethical Principles and Guidelines for Scientific Experiments on Animals' of the Swiss Academy of Medical Science. The animal model with NE infusion was performed according to a previously described protocol [10] with minor modifications. Osmotic minipumps (model 2002; Alzet, Palo Alto, Calif.) for continuous NE infusion were implanted subcutaneously with a catheter connected to the left jugular vein. NE was dissolved in 0.01% (w/v) ascorbic acid solution and infused at a rate of 0.2 mg/kg per hour. The concentration of NE in serum was confirmed by high-performance liquid chromatography. After 0.5, 1, 2, 3 and 7 days of continuous infusion, the plasma concentrations of NE were significantly elevated compared with the control group (66.1 ± 14.1, 55.6 ± 0.6, 65.0 ± 8.0, 87.9 ± 5.3, 65.1 ± 17.5 µg/l, respectively, vs 5.2 ± 1.3 µg/l; $p < 0.05$, $n = 3$ at each time point). With the study design of a 1-week treatment, animals receiving vehicle for 3 days were used as the putative control group ($n = 3$).

Assessment of cardiac function

To evaluate left ventricular thickness and contraction function in vivo, the rats were anesthetized with an intraperitoneal injection of 1 mg/kg urethane after 3 days of continuous NE infusion, and transthoracic echocardiography was performed with an ultrasonic machine (Sequoia C256; Acuson Co., Mountain View, Calif.) and 8-MHz sector scan probe. Briefly, once the short-axis two-dimensional (2D) image of the left ventricle was obtained at the papillary muscle level, 2D guided M-mode images crossing the anterior and posterior walls were recorded (sweep speed 100 mm/s). Parameters measured digitally on the M-mode trace were the posterior wall thickness and inner dimension of diastolic or systolic left ventricles (PWT_d, PWT_s, $LVID_d$ and $LVID_s$), and fractional shortening [$FS=(LVID_{d-} LVID_s) /LVID_d$].

Left ventricular weight assessment and histologic examination

Parallel series of rats were sacrificed after 0.5, 1, 2, 3 and 7 days of continuous NE infusion, the heart was cut and the left ventricle was quickly separated from the atria and right ventricular free wall and weighed. Then, left ventricles were fixed overnight in 4% paraformaldehyde before embedding in paraffin. Sections of 7.5 µm were prepared, and stained with hematoxylin-eosin or Sirius red for evaluation of myocyte hypertrophy and collagen content, respectively.

Data analysis

Results are expressed as mean ± SE. One-way ANOVA was performed to assess differences in parameters between groups. Significant differences were then subjected to post hoc analysis using the Newman-Keuls method. A value of $p < 0.05$ was considered significant.

Isolation of cardiomyocytes

After 0.5, 1, 2, 3 and 7 days of continuous NE infusion, left ventricular cardiomyocytes were isolated with a modified protocol described by Claycomb and Palazzo [11]. Animals were anesthetized with 1 mg/kg urethane and

heparinized with 100 units/kg heparin intraperitoneally. The heart was excised and perfused retrogradely with a Ca^{2+}-free perfusion buffer (PB) containing (in mmol/L) NaCl 118, KCl 4.7, $MgSO_4$, 1.2, KH_2PO_4 0.93, $NaHCO_3$ 25 and glucose 10 (at 37 °C, pH 7.4, gassed with 95% O_2/5% CO_2). Perfusion was then switched for 10 min to 0.05% collagenase (type II; Worthington Biochemical Co., Lakewood, N. J.) bovine serum albumin and 0.1% (BSA) in PB. The left ventricle was separated and digestion continued in the collagenase solution in a 37 °C water bath. After the first 10 min of digestion, the solution was discarded. Fresh collagenase solution was added for another 20 min of digestion. Then, the digestion solution was filtered through a nylon mesh with 250-μm pore size, and centrifuged at 25 g for 1 min. The precipitate was suspended in PB followed by 15 min sedimentation. Then the loose pellet containing 80–85% rod-shaped cardiomyocytes was obtained. Finally, these cells were further collected with a centrifugation step at 250 g for 1 min.

Gene expression profile

For microarray assays, triplicates of cardiomyocyte RNA samples from three separate left ventricles were analyzed independently for each time point.

RNA extraction, labeling of cDNA probes and hybridization to microarray

RNA was extracted from isolated cardiomyocytes using TRIzol reagent according to the manufacturer's instructions. Total RNA (8 μg) wase reverse-transcribed in the presence of 300 units of SuperScript II RNase H Reverse Transcriptase (100 μCi; 1 Ci = 37 GBq) of [α-^{33}P]dCTP (10 mCi/ml, 3,000 Ci/mmol; ICN, Irvine, Calif.) and 2 μg of oligo(dT). Each reaction was carried out at 37 °C for 90 min in 30 μl of buffer consisting of 50 mM Tris·HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 3.3 mM tithiothreitol (DTT), and 1 mM each of dATP, dGTP and dTTP. The resulting ^{33}P-cDNA probes were purified with Bio-Spin 6 Chromatography Columns (Bio-Rad, Hercules, Calif.). Rat GeneFilters (GF300 from Invitrogen. Carlsbad, Calif., representing 5300 rat cDNA clones) were used for differential-expression screening. The membranes were first pretreated with boiled 0.5% SDS for 10 min. Prehybridizations were performed for 2 h at 42 °C in MicroHyb hybridization solution with poly(dA) and denatured Cot-1 as blocking reagents. The column-purified and denatured probes were then added and hybridized at 42 °C for 16 h. After hybridization, the membranes were washed twice in a solution containing 2×standard saline citrate (SSC) and 1% SDS for 20 minutes at 50 °C followed by two additional washes performed at the same temperature in a washing solution consisting of 0.5×SSC and 1% SDS for 15 min each. The membranes were then exposed to phosphor image screens for 48 h. Images were acquired using a Cyclone Phosphor System (Packard, Meriden, Conn.), and analyzed using the PATHWAYS 3.0 software (Invitrogen, USA) and exported as EXCEL documents (Microsoft, Redmond, Wa.).

Data extraction and quantification

The spots with raw signal intensity greater than sixfold of background signal intensity were selected for further analysis. Empirically, these clones represented the subset of clones with detectable expression in heart. To ensure sufficient data for analysis of each likely involved gene, those genes fitting the above criteria at any experimental time point were collected. This yielded a set of 731 genes from all arrays applied across different time points.

Normalized intensity values for genes in each array were generated using the overall mean intensity of the array as the normalization standard. The three separate hybridizations for the time course of NE infusion were compared to control cardiomyocytes within the same hybridization experiment, and the gene expression ratios were calculated by dividing the normalized intensities from treatment samples by the normalized ones from control samples. Results are expressed as mean±SE. The expression ratios reported were the average of three experiments. To make the extents between up-regulation and down-regulation comparable, a changed expression ratio is shown in this study. This was obtained by subtracting 1 from expression ratios if expression ratios were more than 1, or by subtracting the reciprocal of expression ratios from 1 if expression ratios were less than 1. A paired Students t test was performed to assess the changes in the expression ratios. A value of $p < 0.05$ was considered significant, and the spot images of all these genes were visually inspected to ensure that the signal density was not the result of an artifact or high background signal.

Self-organization clustering

The genes with changed expression ratios were analyzed with the software of self-organization clustering, which was obtained from Stanford University (see http://rana.stanford.edu/software/.

Compounds

All reagents for microarray experiments were purchased from Invitrogen. Other drugs and chemicals were purchased from Sigma (St. Louis, Mo.).

Results

Effects of NE infusion on left ventricular weight, wall thickness and function

Upon continuous infusion of NE, the ratio of left ventricular weight (LVW) to body weight (BW) increased in a time-dependent manner, and peaked at 3 days of NE infusion (3.183 ± 0.101 vs 2.193 ± 0.083; $p < 0.05$, $n = 3$).

Echocardiography showed that at this time point, the thickness of the diastolic and systolic left ventricle also increased significantly (2.53 ± 0.24 vs. 1.95 ± 0.12 mm, 4.00 ± 0.21 vs. 3.20 ± 0.14 mm; $p < 0.05$, respectively, n = 3 in each group). FS was elevated from 60.5 ± 3.8 to 70.8 ± 2.6%; ($p < 0.05$, n = 3) (table 1, fig. 1) .

The histologic examination showed abundant hematoxylin-stained nuclei indicating inflammatory cell infiltration in endocardium and increased Sirius-stained collagens in cardiac interstitium at 3 and 7 days of NE infusion. Compared with the control group, the averaged cross-sectional area of cardiomyocytes increased by 83% and 88% after 3 and 7 days NE infusion, respectively (fig. 2).

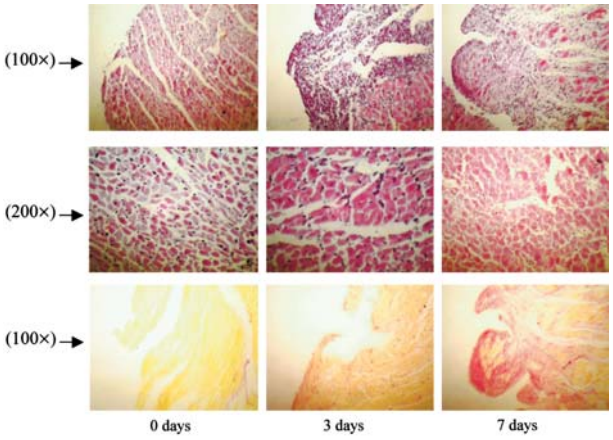


Figure 2. Histochemistry examination of hyperthrophic heart induced by NE infusion for 0, 3 and 7 days. The images in the upper and middle rows show hematoxylin-eosin staining at endo- and intersections of heart issue slices, respectively. Images in the lower row show Sirius Red-stained collagens in cardiac interstitium.

NE infusion induced gene expression profile changes in cardiomyocytes

To identify the cardiomyocyte-specific changes in gene expression during NE-induced cardiac hypertrophy, the cardiomyocytes were isolated from left ventricles by collagenase digestion after 0.5, 1, 2, 3 and 7 days of continuous NE infusion in vivo, and total RNA from each time point along with that from the vehicle control was simultaneously labeled and hybridized to rat cDNA GeneFilters.

Table 1. Effects of NE infusion (0.2 mg/kg per hour for 3 days) on left ventricular weights and thickness.

	Vehicle	Norepinephrine
BW (g)	250 ± 10	248 ± 3
LVW (g)	0.57 ± 0.02	0.75 ± 0.02*
LVW/BW	2.30 ± 0.09	3.02 ± 0.10*
PWT_d (mm)	1.95 ± 0.12	2.53 ± 0.24*
PWT_s (mm)	3.20 ± 0.14	4.00 ± 0.21*
$LVID_d$ (mm)	5.39 ± 0.28	4.83 ± 0.48
FS (%)	60.5 ± 3.8	70.8 ± 2.6*

BW, body weight one week after treatment; LVW, left ventricular weight; PWT_d and PWT_s, thickness of diastolic and systolic left ventricular; $LVID_d$, inner dimension of diastolic left ventricular; FS, fractional shortening; Data are means ± SE (n = 5 in each group). *$p < 0.05$ vs vehicle group.

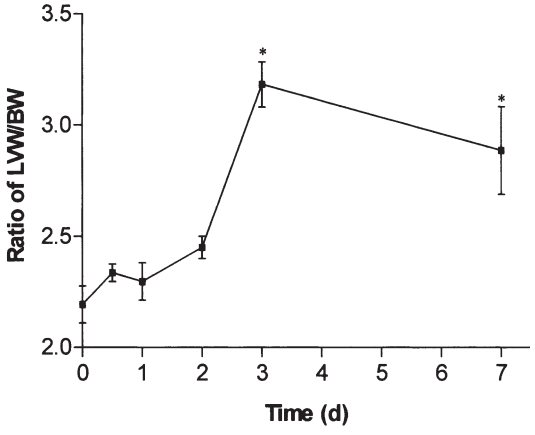


Figure 1. Time course of left ventricular weight (LVW) to body weight (BW) ratios following NE infusion. * $p<0.05$ vs. control (n=3).

Continuous NE infusion induced manifest changes in the gene expression profile within cardiomyocytes, and those genes with differential expression over the time points were classified into five groups according to the functions of their corresponding proteins (table 2). As expected, several genes showing changes in expression had been reported in previous studies of hypertrophy, such as ANF, collagen and genes participating in energy metabolism. The manner of ANF expression change was very similar to the ratio of LVW to BW, with an acme at 3 days of NE infusion.

At the early stage of NE infusion (0.5–1 days), the genes involved were mainly related to metabolism and structure. Most of the genes scanned in our study, covering all the functional groups, showed differential expression at the middle stage of infusion (2–3 days). The majority of the signaling molecules were found differentially expressed mainly at this stage, with phosphatidylinositol 3-kinase, calcium/calmodulin-dependent protein kinase II and two non-receptor tyrosine kinases, syk and fgr, up-regulated, whereas p21-activated kinase was down-regulated. The tumor suppressor protein, p53, took a different expression course increasing only at the early and late stage of NE infusion. The gene expression changes at the late stage of infusion (7 days) were minor, and no stage-specific gene was found.

Analysis of gene expression data with self-organization clustering

With the method of self-organization clustering, those genes changing their expression levels in cardiomyocytes clustered into seven groups (fig. 3). The results showed

Table 2. Up- or down-regulated genes in rat cardiomyocytes during the development of cardiac hypertrophy upon the continuous infusion of NE.

Acc. No.	Gene name	NE infusion				
		0.5 day	1 day	2 days	3 days	7 days
Cell signaling/communication						
AA819343	natriuretic peptide precursor, Anf	0.52 ± 0.24	0.31 ± 0.25	0.58 ± 0.23	**1.83 ± 0.30**	**1.49 ± 0.18**
AI045921	glypican 3	–0.03 ± 0.13	**6.17 ± 1.36**	0.03 ± 0.27	–0.13 ± 0.54	–0.51 ± 0.34
AI028944	connexin 43	0.39 ± 0.14	**1.43 ± 0.36**	–0.17 ± 0.17	–0.03 ± 0.13	–0.25 ± 0.28
AA996434	PI3-kinase	0.02 ± 0.06	–0.01 ± 0.11	0.13 ± 0.13	**0.91 ± 0.02**	0.05 ± 0.11
AI045851	CaM kinase II β	0.20 ± 0.15	0.67 ± 0.31	–0.17 ± 0.21	**8.08 ± 2.36**	0.33 ± 0.24
AA997367	p21-activated kinase 3	–0.01 ± 0.05	–0.04 ± 0.03	–0.27 ± 0.27	**–0.63 ± 0.15**	–0.96 ± 0.64
AA956939	spleen tyrosine kinase (syk)	0.02 ± 0.15	–0.01 ± 0.03	0.04 ± 0.12	**1.88 ± 0.47**	–0.01 ± 0.06
AA901385	Fgr (tyrosine kinase)	–0.22 ± 0.12	–0.92 ± 0.50	**1.02 ± 0.24**	–0.25 ± 0.33	–0.42 ± 0.19
AA875052	p53	**2.57 ± 0.74**	–0.11 ± 0.13	0.16 ± 0.08	0.49 ± 0.28	**0.71 ± 0.07**
Gene expression						
AI071579	NF-E2-related factor 2	**1.82 ± 0.31**	0.08 ± 0.15	0.02 ± 0.16	0.13 ± 0.29	–0.06 ± 0.12
AI029791	scaffold attachment factor B	0.43 ± 0.12	0.02 ± 0.07	**0.44 ± 0.07**	**0.67 ± 0.10**	0.16 ± 0.18
AA900875	transcription factor MRG2	–0.37 ± 0.13	–0.35 ± 0.19	**–0.57 ± 0.15**	–0.40 ± 0.20	**–0.22 ± 0.05**
Protein expression						
AA818442	Ribosomal protein S11	**–0.41 ± 0.03**	**–0.52 ± 0.07**	**–0.35 ± 0.13**	0.20 ± 0.14	**–0.45 ± 0.14**
AA819818	glucose – regulated protein (58KD)	0.23 ± 0.20	–2.16 ± 0.73	**–1.92 ± 0.60**	**–1.58 ± 0.37**	–0.16 ± 0.12
Cell/organism defense						
AA924772	metallothionein-III	0.22 ± 0.16	–0.2 ± 0.24	–0.25 ± 0.12	**1.20 ± 0.17**	–0.18 ± 0.20
AI028945	RT1.D beta chain	0.34 ± 0.15	**3.77 ± 0.43**	0.21 ± 0.07	–0.27 ± 0.35	–0.14 ± 0.18
Cell structure/motility						
AI045419	cardiac myosin heavy chain 11	0.06 ± 0.02	–0.09 ± 0.04	**0.51 ± 0.04**	–0.04 ± 0.10	0.10 ± 0.09
AA818610	cardiac myosin light chain 3	**–0.31 ± 0.02**	–0.09 ± 0.16	**–0.89 ± 0.12**	–0.62 ± 0.22	**–0.40 ± 0.05**
AA926237	cardiac troponin I	–0.03 ± 0.28	0.49 ± 0.26	**–1.62 ± 0.24**	–1.32 ± 0.33	**–1.70 ± 0.56**
AA998487	dynamin 2	**–0.54 ± 0.02**	**–0.36 ± 0.09**	**–0.84 ± 0.17**	**–1.12 ± 0.23**	**–0.39 ± 0.07**
AA925301	coronin	0.27 ± 0.13	**2.82 ± 0.55**	0.03 ± 0.08	–0.14 ± 0.30	–0.17 ± 0.23
AA875497	α2 procollagen I	0.52 ± 0.26	**0.65 ± 0.19**	**0.78 ± 0.21**	0.30 ± 0.18	**0.60 ± 0.23**
AA925675	α1 procollagen III	0.88 ± 0.44	**12.19 ± 4.17**	**0.84 ± 0.28**	1.25 ± 1.00	1.13 ± 0.37
Metabolism						
AA817808	steroidogenic acute regulatory protein	–0.14 ± 0.08	0 ± 0.13	0.03 ± 0.08	**0.60 ± 0.10**	0.17 ± 0.09
AA900776	ATP synthase subunit c	**0.82 ± 0.33**	**0.86 ± 0.43**	–0.05 ± 0.53	0.12 ± 0.52	0.24 ± 0.29
AA925568	carnitine octanoyltransferase	**2.88 ± 0.87**	1.33 ± 0.61	0.70 ± 0.42	**0.60 ± 0.17**	0.78 ± 0.45
AI059021	pancreatic lipase	0.91 ± 0.52	**0.78 ± 0.15**	0.24 ± 0.54	0.48 ± 0.06	–0.22 ± 0.28
AA899469	glycogen phosphorylase	**0.95 ± 0.15**	0.08 ± 0.18	**–0.56 ± 0.12**	–0.45 ± 0.14	–0.02 ± 0.09
AI030101	arachidonate 5-lipoxygenase activating protein	**0.45 ± 0.11**	–0.02 ± 0.10	**0.53 ± 0.03**	0.49 ± 0.20	0.22 ± 0.10
AA924534	lysophospholipase	0.48 ± 0.17	0.13 ± 0.05	0.34 ± 0.16	**0.61 ± 0.15**	**0.37 ± 0.09**
AA818217	small zinc finger-like protein (TIM13)	**0.29 ± 0.09**	0.32 ± 0.12	**0.71 ± 0.04**	0.56 ± 0.17	0.25 ± 0.14
AA859955	mitochondrial processing peptidase β	0.16 ± 0.07	–0.72 ± 0.16	–0.32 ± 0.12	**–1.02 ± 0.20**	–0.3 ± 0.29
AA817825	Peptidylglycine α-amidating monooxygenase	–0.05 ± 0.06	0.07 ± 0.16	0.13 ± 0.10	**0.61 ± 0.14**	0.42 ± 0.17
AI059788	NAAG-peptidase	**–0.57 ± 0.07**	**–0.41 ± 0.11**	**–0.37 ± 0.03**	**–0.70 ± 0.16**	–0.3 ± 0.11
AA924540	xanthine dehydrogenase	0.63 ± 0.10	**0.35 ± 0.07**	**0.71 ± 0.13**	0.48 ± 0.12	**0.49 ± 0.04**
AI043796	solute carrier family 18 A2	0.86 ± 0.38	**0.89 ± 0.11**	0.22 ± 0.12	0.64 ± 0.30	**1.01 ± 0.13**
AA866330	Hemoglobin, beta	0.07 ± 0.14	**0.59 ± 0.17**	0.79 ± 0.59	0.44 ± 0.23	0.33 ± 0.27
AI043801	peroxisomal membrane protein 3	**0.27 ± 0.04**	0.23 ± 0.03	**0.35 ± 0.12**	**0.63 ± 0.04**	0.35 ± 0.13
EST						
AA875448	EST	0.17 ± 0.05	–0.01 ± 0.04	0.14 ± 0.05	**–0.61 ± 0.04**	0.02 ± 0.04
AA819572	EST	**0.60 ± 0.23**	0.26 ± 0.21	0.38 ± 0.33	0.12 ± 0.16	0.07 ± 0.15
AA900845	EST	**0.34 ± 0.04**	0.22 ± 0.08	**0.68 ± 0.15**	–0.12 ± 0.65	0.12 ± 0.23
AA817878	EST	0.2 ± 0.08	**0.40 ± 0.08**	0.10 ± 0.06	**0.67 ± 0.18**	0.32 ± 0.15
AA858846	EST	0.17 ± 0.15	**0.21 ± 0.02**	–0.22 ± 0.29	**0.73 ± 0.21**	0.12 ± 0.24
AA866221	EST	0.18 ± 0.11	0.28 ± 0.07	0.51 ± 0.22	**1.90 ± 0.33**	0.03 ± 0.13
AA818614	EST	0.24 ± 0.23	0.05 ± 0.18	**2.73 ± 0.45**	**4.92 ± 1.42**	0.96 ± 0.49
AA899608	EST	–1.23 ± 0.75	–1.33 ± 0.68	1.18 ± 0.40	**1.75 ± 0.38**	–1.43 ± 0.89

Table 2 (continued).

Acc. No.	Gene name	NE infusion				
		0.5 day	1 day	2 days	3 days	7 days
AA818174	EST	–0.03 ± 0.39	–0.01 ± 0.33	–0.11 ± 0.39	**5.85 ± 2.18**	0.07 ± 0.28
AA859341	ESTs	0.26 ± 0.12	–0.72 ± 0.23	**–1.77 ± 0.25**	**–1.50 ± 0.30**	**–1.33 ± 0.32**
AA858894	ESTs	–0.51 ± 0.14	–0.21 ± 0.06	–0.53 ± 0.18	**–0.54 ± 0.05**	–0.06 ± 0.13
AA818533	ESTs	**1.75 ± 0.28**	0.14 ± 0.23	0.01 ± 0.04	–0.18 ± 0.18	**0.84 ± 0.06**
AA819259	ESTs	0.38 ± 0.16	**0.76 ± 0.21**	–0.05 ± 0.27	0.52 ± 0.32	**0.38 ± 0.05**
AA900594	ESTs	0.22 ± 0.23	**0.63 ± 0.14**	**0.89 ± 0.21**	–0.49 ± 0.83	0.40 ± 0.13
AA817763	ESTs	0.35 ± 0.12	0.45 ± 0.26	0.23 ± 0.19	**0.85 ± 0.07**	–0.06 ± 0.08
AA819278	ESTs	0.17 ± 0.06	**0.38 ± 0.04**	0.24 ± 0.13	**0.66 ± 0.12**	0.18 ± 0.09
AA818861	ESTs	0.07 ± 0.17	0.14 ± 0.10	0.30 ± 0.14	**0.69 ± 0.20**	0.35 ± 0.08
AA866228	ESTs	0.36 ± 0.14	0.34 ± 0.19	**0.61 ± 0.03**	**1.40 ± 0.17**	0.08 ± 0.46
AA819303	ESTs	0.02 ± 0.16	0.07 ± 0.05	**0.40 ± 0.11**	**0.76 ± 0.26**	0.23 ± 0.20
AA818433	ESTs	0.07 ± 0.05	0.25 ± 0.09	0.06 ± 0.03	**0.68 ± 0.11**	–0.03 ± 0.08
AA818966	ESTs	–0.15 ± 0.27	0.22 ± 0.18	0.11 ± 0.26	**4.45 ± 1.82**	**1.33 ± 0.48**
AA899898	ESTs	–0.64 ± 0.22	–0.36 ± 0.19	**0.42 ± 0.13**	–0.12 ± 0.18	**–0.70 ± 0.06**

The numerical data in the table were changed expression ratios ± SE (n=3). Values > 0 are up-regulations; values < 0 are down-regulations. Changed expression ratios corresponding to those genes with a significant difference in expression levels between the treatment and control group are shown in bold.

that several enzymes involved in energy metabolism, including carnitine octanoyltransferase, ATP synthase subunit c, pancreatic lipase and glycogen phosphorylase, gathered in one cluster. They possessed similar expression patterns, with a characteristic of rapidly elevated expression levels during the early stage of NE infusion.

The gene expression changes in clusters 1, 2 and 3 seemed similar, in that they increased their expression to a maximal extent at 3 days of NE infusion. However, they really exhibit diversities, which were revealed through clustering analysis. For example, expression changes in genes in cluster 3 persisted through the whole experimental course, which was not observed for genes in clusters 1 and 2. ANF was included in this cluster, which indicated a change in expression similar to the development of cardiac hypertrophy.

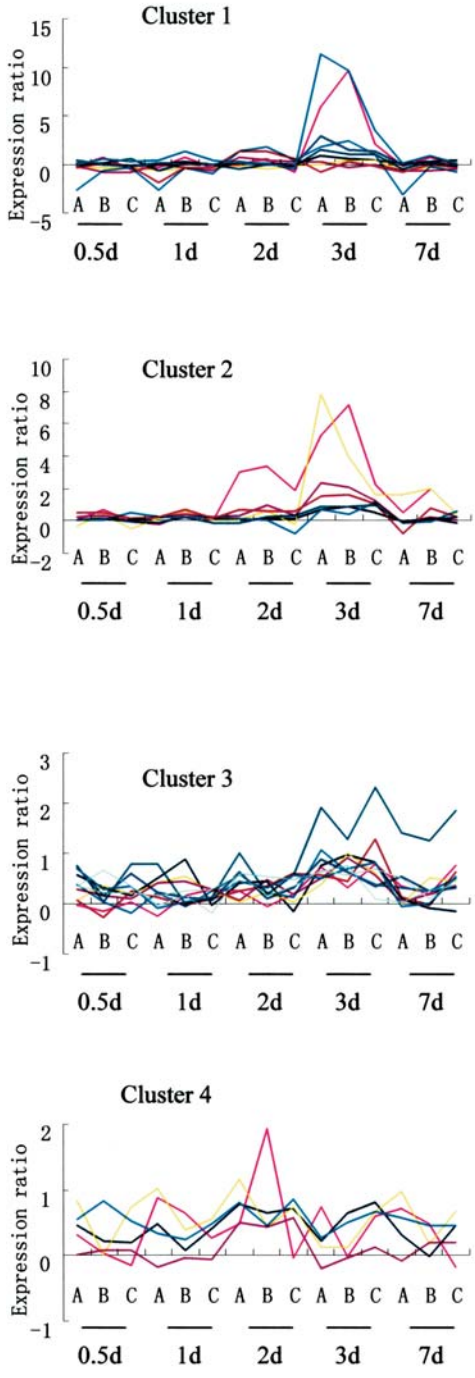
Except for cardiac myosin heavy chain 11, the other three structural genes, dynamin 2, cardiac myosin light chain 3 and troponin I, were clustered into one group, in which most genes were persistently down-regulated through the course of heart hypertrophy. In general, distinct groups of genes played the predominant role at different developmental stages of heart hypertrophy.

Discussion

Analysis of gene expression profiles with microarray technology during the induction of cardiac hypertrophy has enabled a better understanding of the pathology of this process. However, few studies have focused on changes occurring within a single cell type in heart in vivo, which comprises multiple cell types interacting with each other. In the present study, left ventricular cardiomyocytes were isolated from hypertrophic heart in adult rats with collagenase digestion, and cardiomyocyte-specific gene expression profiles during cardiac hypertrophy induced by NE infusion in vivo were assessed for the first time.

In this study, the commercial GeneFilters from Invitrogen were used, and the validity of this type of microarray has been confirmed in a number of laboratories [12–14]. However, single hybridization is clearly not sufficient for reliable results. This uncertainty was overcome by three separate hybridizations using independent RNA preparations in our study, which is in agreement with other reported microarray experiments [15]. Confirming the reliability of our results, several genes that show elevated expression were previously reported utilizing traditional methodology. In addition, for many genes scanned in the present study, differential expression was observed at multiple time points, which also indicated reliability in the results.

Reviewing the time-dependent changes in gene expression profiles, an important reason for the alterations in gene expression during NE infusion, especially at the early stage (i.e. 0.5–1 day), is likely due to alterations in cardiac function, for a number of genes coding for metabolic enzymes or transporters of energy substrates altered expression at this stage when cardiac hypertrophy is not significant. During the late stages of NE treatment, these changes due to adrenoceptor-mediated signaling pathways leading to hypertrophic growth become evident: many of the signaling molecule genes selected by our study showed differential expression. The chanes in expression of different genes in cardiomyocytes presented distinct patterns upon continuous administration of NE in vivo. These provide useful clues to gain insight into the underlying molecular basis of cardiac hypertrophy. Most



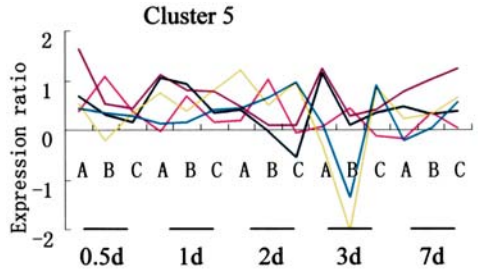
Acc No.	Gene titles
AA818442	Ribosomal protein S11
AA901385	fgr
AA817808	Steroidogenic acute regulatory protein
AA924772	Metallothionein-III
AA956939	Spleen tyrosine kinase(syk)
AI045851	CaM kinase II β
AA818174	EST
AA899608	EST
AA899898	EST

AA996434	Phosphatidylinositol 3-kinase
AA818433	ESTs
AA818614	EST
AA818966	ESTs
AA858846	EST
AA866221	EST
AA866228	ESTs

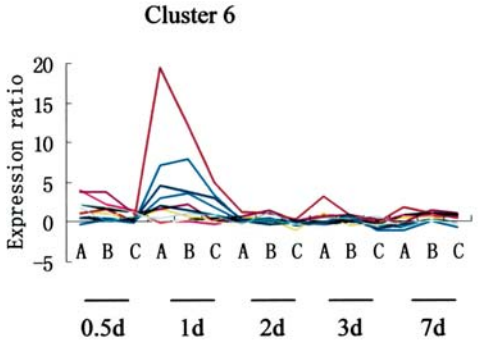
AA819343	Natriuretic peptide precursor A (Anf)
AI029791	Scaffold attachment factor B
AA924534	Lysophospholipase
AI030101	Arachidonate 5-lipoxygenase activating protein
AI043801	Peroxisomal membrane protein 3(35 kDa)
AA817825	Peptidylglycine α-amidating monooxygenase
AA817763	ESTs
AA817878	EST
AA818861	ESTs
AA819278	ESTs
AA819303	ESTs

AI045419	Myosin heavy chain 11
AA875497	Procollagen I, alpha 2
AA818217	Small zinc finger-like protein (TIM13)
AA866330	Hemoglobin β
AA924540	Xanthine dehydrogenase

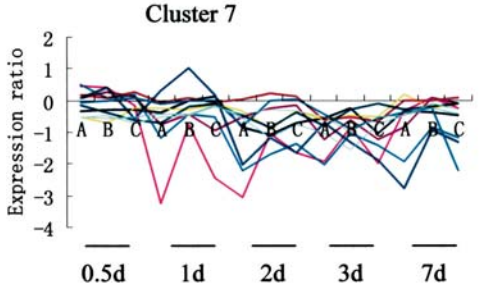
Figure 3. Self-organization clustering analysis of gene expression profile in cardiomyocytes during different development stages of hypertrophy (0.5, 1, 2, 3 and 7 days) induced by continuous infusion of NE. Data from triplicate microarray hybridizations (A, B, C) for each time point were enrolled for clustering analysis.



AI043796	Solute carrier family 18 A2
AA819259	ESTs
AA819572	EST
AA900594	ESTs
AA900845	EST



Acc No.	Gene titles
AA925675	α 1 procollagen III
AA875052	Tumor protein p53
AA925301	Actin binding protein 1B (Coronin)
AI028944	Connexin 43
AI028945	RT1.D β chain
AI045921	Glypican 3
AI071579	NF-E2-related factor 2
AA900776	ATP synthase subunit c
AA925568	Carnitine octanoyltransferase
AI059021	Pancreatic lipase
AA899469	Glycogen phosphorylase
AA818533	ESTs



AA818610	Cardiac myosin light chain 3
AA926237	Cardiac troponin I
AA998487	Dynamin 2
AA859955	Mitochondrial processing peptidase β
AI059788	NAAG-peptidase
AA900875	Transcription factor MRG2
AA819818	Glucose regulated protein, 58KD
AA997367	p21 (CDKN1A)-activated kinase 3
AA858894	ESTs
AA859341	ESTs
AA875448	EST

Figure 3 (continued).

of the genes that displayed differential expression had not been reported to be involved in cardiac hypertrophy. For example, glypican 3, a heparan sulfate proteoglycan, which could regulate the activities of several growth factors [16], and NF-E2-related factor 2 (Nrf2), a transcription factor, which plays a major role in transcriptional activation of antioxidant responsive element-driven genes [17], were up-regulated significantly at the early stage of NE infusion. These molecules might modulate the early responses of cardiomyoctes to stimulations of various growth factors and oxidative stress. Similarly, non-receptor tyrosine kinases such as syk and fgr were mainly reported to participate in the activation of immune cells [18, 19]. But they increased their expression at the middle stage of NE infusion in our experiment. This implies that they might also be involved in cardiac hypertrophy. For down-regulated genes, glucose-regulated protein (grp58), a chaperone in the endoplasmic reticulum lumen [20], was significantly down-regulated at the middle stage of infusion. Interestingly grp58 was recently reported to associate with STAT3 and may regulate signaling by sequestering inactive and activated STAT3 in human hepatoma Hep3B cells [21]. It is tempting to speculate that grp58 might also regulate the Jak-STAT3 pathway in cardiomyocytes, the signaling pathway that has been widely studied during cardiac hypertrophy and failure [22]. Of particular note is the mechanoenzyme dynamin, whose mRNA levels are decreased throughout the time course of adrenergic

stimulation in cardiomyocytes. Dynamin proteins are required for endocytosis, synaptic vesicle recycling and membrane trafficking [23]. This protein was recently demonstrated to play an essential role in maintaining normal heart function in the *Drosophila* [24]. Thus, expression information presented here is very useful in generating testable hypotheses, though firm functional associations among proteins cannot yet be established.

The tumor suppressor p53 was biphasically up-regulated at early and late stages of NE infusion. p53 showed transiently elevated expression at the very early stage of infusion, only half a day, when cardiac hypertrophy had not yet been induced. p53 may be involved in the apoptosis of cardiomyocytes induced by NE stimulation. However, this probability is minor since most of the cardiomyocytes isolated according to the methods described in this study remained in a good condition. Thus, instead of apoptosis, this transiently elevated expression of p53 might participate in the induction of the hypertrophy in cardiomyocytes. Insulin-like growth factor I (IGF I), a factor that can rescue cardiomyocytes from apoptosis, has also been reported to induce p53 expression [25]. The up-regulation of p53 mRNA might retain the cell cycle arrest when cardiomyocytes are stimulated by various pro-growth factors, which would permit cardiomyocte hypertrophy rather than proliferation.

The use of the method of self-organization clustering revealed some important messages hidden beyond the cardiomyocyte gene expression profile. Most representative was the clustering of those enzymes involved in energy metabolism into one group. The expression changes of these genes indicated their functional cooperativity. Expression of carnitine octanoyltransferase, an important enzyme in fatty acid β-oxidation [26], increased rapidly after half a day of NE infusion. It indicated an enhancement in fatty acid utilization in the early stage of induction of cardiac hypertrophy. At the same time, the expression of pancreatic lipase and glycogen phosphorylase was also elevated, which suggested an enhanced supplementation of both fatty acids and glucose. The coordinat expression of these enzymes may enable the heart to adapt to an elevated requirement for energy upon NE stimulation. Hints for the possible functions of some genes could also be gleaned from the clustering results. For example, the gene expression pattern of ANF was very similar to that of the LVW-to-BW ratio. This pattern was also possessed by other genes in cluster 3 including ANF. Although the functions of most genes in this cluster were unclear or unknown in heart their expression changes might be positively correlated to the development of cardiac hypertrophy like that of ANF.

All these gene expression data were from cardiomyocytes of hypertrophic heart. Our previous study had revealed that the gene expression changes in cardiomyocytes differed greatly from those in heart tissue during hypertrophy. For example, the expression level of PI3-kinase rose to 1.91-fold of control levels in cardiomyocytes after 3 days NE infusion. But there was no significant expression change detected in heart tissue. There were many similar examples, including CaM kinase II β, steroidogenic acute regulatory protein, glucose-regulated protein (58 kDa) and dynamin 2 (data not shown). Thus, our present study provided valuable knowledge of gene expression profiles with an accurate cellular location. In addition, some transcriptional information, diluted by the expression signals from other types of cell in the heart, was revealed.

There are limitations to our study. The ventricular myocytes isolated with the method described were always those with the most vigor in hypertrophic heart. Those with poor vigor, which possessed lower gravity, were often discarded, especially in the sedimentation step. Thus, the gene expression data obtained in this study are strictly speaking only relevant to viable cardiomyocytes, and new appproaches must be developed for studying cardiomyocytes undergoing apoptosis of which are in poor condition for other reasons. In addition, the gene coverage of the cDNAs on the Rat GeneFilters is limited and most of them are ill-defined ESTs, and this commercial microarray is not cardiac specific, which also limits what can be extracted from microarray data.

More recently, using an α_{1b} adrenergic receptor-overexpressing mice model, Yun et al. [27] examined the gene expression profile of adrenergic stimulation-induced cardiac hypertrophy. Because of many differences in experiment design and in the arrays used between their and our experiments, few involved genes common to both studies were identified. But similar broad-based genome-wide analysis will enable better understanding of adrenergic signaling in the heart.

In general, the present method provided a time-dependent of gene expression profile with a relatively accurate cellular location in cardiomyocytes during cardiac hypertrophy in vivo, which will be helpful for gaining clearer insight into the molecular changes in the heart during cardiac hypertrophy, especially when microarray technology is employed.

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