Table 2. *Circadian statistics and values for light and dark phases*

Parameter	Wild-Type Mice	Knockout Mice
Blood pressure, mmHg		
MESOR	125 ± 6	109 ± 2*
Amplitude	9 ± 2	6 ± 1
Acrophase (h:min)	0:03 ± 0:56	1:20 ± 0:46
Light phase mean	122 ± 6	107 ± 2*
Dark phase mean	128 ± 6	113 ± 2*
Heart rate, min^{-1}		
MESOR	529 ± 41	565 ± 22
Amplitude	35 ± 8	34 ± 4
Acrophase (h:min)	0:48 ± 0:42	2:50 ± 0:49
Light phase mean	527 ± 28	553 ± 23
Dark phase mean	554 ± 38	575 ± 21
Activity, counts/30 min		
MESOR	135 ± 24	84 ± 17
Amplitude	79 ± 20	25 ± 4*
Acrophase (h:min)	2:09 ± 0:43	2:48 ± 0:13
Light phase mean	98 ± 17	78 ± 17
Dark phase mean	162 ± 26	91 ± 17*

Values are means ± SE of 6 mice in each genotype. MESOR, midline estimating statistic of rhythm. *$P < 0.05$ vs. wild-type mice (t-test).

Perhaps glutamate may be one of such transmitters, since bilateral microinjection into the RVLM of an ionotropic excitatory amino acid receptor antagonist kynurenic acid attenuated an increases in AP and renal sympathetic nerve activity evoked by air jet stress (37) or by direct hypothalamic stimulation (52).

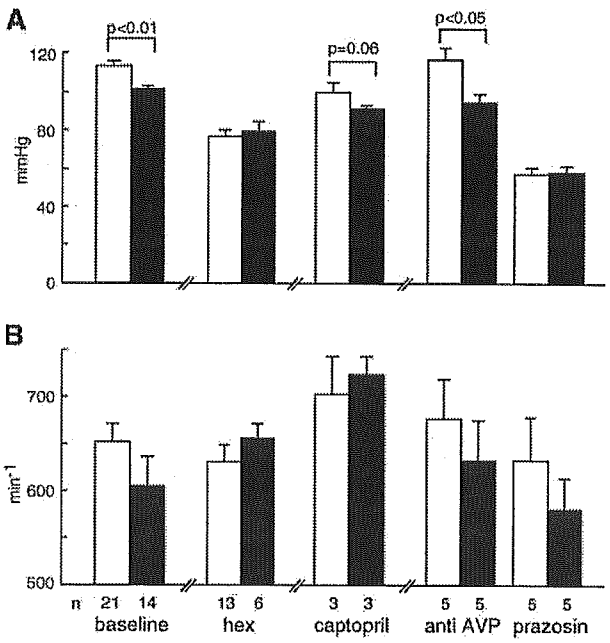

Fig. 7. Cardiovascular effects of intra-arterial injection of hexamethonium (hex), captopril, vasopressin V1 receptor antagonist (anti-AVP), and prazosin. MAP (*A*) and HR (*B*) were measured through indwelling catheter during pre- and postinjection periods in orexin null mutated mice (filled bars) and in WT mice (open bars). Numbers below horizontal axis indicate numbers of animals used in the measurement.

Table 3. *Echocardiography parameters*

Parameter	Wild-Type Mice	Knockout Mice
Body weight, g	30.3 ± 0.7	29.0 ± 1.1
LV diastolic diameter, mm	3.54 ± 0.06	3.45 ± 0.13
LV systolic diameter, mm	1.99 ± 0.06	1.99 ± 0.08
LV end-diastolic volume, μl	46.6 ± 2.2	43.7 ± 4.9
LV end-systolic volume, μl	8.29 ± 0.69	8.42 ± 1.14
Stroke volume, μl	38.3 ± 1.7	35.3 ± 3.9
Fractional shortening, %	43.9 ± 1.1	42.4 ± 0.8
Ejection fraction, %	82.3 ± 1.0	80.8 ± 0.8

Values are means ± SE of 6 mice in each genotype. No parameters were significantly different between orexin-knockout and wild-type mice. LV, left ventricular.

Orexin was able to increase pre- and postsynaptic activity of the glutamate-releasing neurons (54). Thus orexin may act as a modulator of glutamatergic input into the RVLM. A toxin-induced orexin-neuron specific degenerative animal model (21) may help to study other factors, especially cotransmitters contained in the orexin neuron, involved in defense response. On the other hand, above-cited reports dealt with only the cardiovascular components of the defense response. There is little information about neurotransmitter(s) that convey respiratory or behavioral component of the defense response. In this respect, this is the first report suggesting a possible neurotransmitter/modulator that contributes to the simultaneous and coordinated changes in cardiovascular, respiratory, EEG, and behavioral components of the defense response.

The present study did not clarify the site at which orexin acted in the central nervous system and the mechanisms of cardiovascular and respiratory responses induced by disinhibition of GABAergic neurotransmission in the perifornical area. However, previous studies enabled us to suppose key sites where orexin acted as a mediator of defense response. The most apparent site seems to be the RVLM, since inhibition of RVLM or subjacent ventral surface of the medulla (glycine-sensitive area) attenuated sympathetic and defense responses evoked by stimulation to the lateral hypothalamic area (12, 24, 52). A double-virus transneuronal labeling technique revealed that not only perifornical neurons but also RVLM neurons provided a dual input to the sympathetic outflow systems that regulate cardiac and adrenal medullary functions, indicating these neurons were the "central command neurons" of defense response (28). Actually, orexin immunoreactive fibers were seen in the RVLM (44), and exogenous applied orexin into the RVLM elicited AP and sympathetic excitation (7, 36). Present results with hexamethonium and prazosin also support the view since RVLM is the major source of sympathetic outflow (8). Nevertheless, we cannot exclude the possible involvement of sites other than RVLM, such as periaqueductal gray, nucleus tractus solitarius, or intermediolateral cell column of the spinal cord (2, 26), as active sites of orexin in the defense response. This subject needs further experimentation.

In clear contrast to attenuated cardiovascular responses to socioemotional stress by resident-intruder

test, AP and HR responses to noxious stimuli by tail pinch were not different between orexin knockout mice and wild-type mice (Fig. 5). This may be because stimulus duration was shorter in tail pinch (10 s) than in resident-intruder test (5 min). Another explanation may be that cardiovascular responses to noxious stimuli by tail pinch do not always require supraspinal structures (48) and hence the responses are independent from hypothalamic orexin. On the other hand, intrathecal administration of orexin induced analgesia in rats (56). Analgesia but not exaggerated pain response may be advantageous to defense response. Thus orexin may also participate in stress-induced analgesia. Although this hypothesis should be clarified in future experiments, we can say, at present, that the orexin system may be activated by some but not all kinds of stressors. We cannot exclude the possibility that absence of orexin resulted in some deficit in sensory system for socioemotional stress but not for noxious stressor. Nevertheless, results from urethane-anesthetized mice with direct hypothalamic stimulation point to a probable deficit in effecter pathway.

Circadian rhythm of AP and HR, calculated as phase and amplitude, was not significantly different between orexin knockout mice and wild-type mice although activity during dark phase was less in the mutant mice (Fig. 6, Table 2). The latter observation was consistent with previous studies showing reduced awake time and increased sleep time during dark phase in orexin knockout mice (5) and reduced activity during dark phase in orexin neuron-ablated mice (21). It is not clear from the present experiment why amplitudes of circadian fluctuations of AP and HR were not different between the two strains while that of activity was smaller in orexin knockout mice. Activity may not be the sole determinant of AP and HR. Whatever may be the reason, an important point is that AP in orexin knockout mice was smaller than wild-type mice even during the light phase when both animals were at rest and activities were not different between the two. The difference in AP was also independent of HR or cardiac contractility (Table 3). The cause of the difference in AP seemed to be attenuated sympathetic outflow that regulates peripheral vascular resistance in orexin knockout mice (Fig. 7). Basal cardiac sympathetic nerve activity seems not influenced by the absence of orexin. Contribution of the renin-angiotensin system and vasopressin V1 receptor seemed minimal, although small numbers of the animals prevented us from making conclusive remarks about the former. Our conclusion is in line with sympathetic excitation by exogenously administered orexin (3, 15, 50) and extended possible contribution of orexin to basal determination of AP through tonic excitation of sympathetic nervous system.

Perspectives

The difference in AP between two genotypes was maintained after urethane anesthesia and attenuated defense response was observed in both awake and anesthetized conditions. This suggests that the difference in basal AP cannot be explained by possible attenuation of defense response in orexin knockout mice because defense response is not always activated in daily life or under anesthesia. It is possible that there may be two subgroups of orexin-containing neurons: one group contributes to determination of basal AP and another participates in defense response, since orexin-containing neurons widely distribute in lateral hypothalamus and dorsomedial hypothalamus (39) and subpopulations of orexin neurons have been proposed in respect to responses to psychotic drugs (18). However, orexin neurons may be activated only by arousal without any particular stress (17). Therefore, it is also possible that the same orexin system contributes to both basal AP determination by mild activation and to defense response when further activated by stress. We do not have the answer at present on this issue.

There are only a few reports describing autonomic regulation in narcolepsy patients. Sachs and Kaijser (45) reported that never-medicated narcoleptic patients showed attenuated autonomic reflexes (changes in AP and HR) in handgrip test and Valsalva's maneuver, but not in face immersion test or orthostatic standing. Because some but not all reflexes had been disturbed, they proposed intact peripheral nerves and a localization of the defect to the central nervous system. Our findings of attenuated defense response and preserved pain-induced response in orexin-deficient mice are in accordance with their findings in human narcolepsy. Basal AP in narcolepsy patients is rather controversial. The same authors reported normal AP and HR at rest before the autonomic testing (45). However, Guilleminault (20) reported that withdrawal of medication with amphetamine for 4 wk significantly decreased AP in narcoleptic patients, indicating low AP, otherwise taking a central stimulant (20). To the best of our knowledge, 24-h AP and HR have not been reported in narcoleptic patients. We feel that systematic reinvestigation about autonomic regulation in narcoleptic patients is needed, since these reports cited here appeared before 1999 when deficiency of orexin had been revealed as the cause of narcolepsy.

In summary, we found that defense response could be elicited in mice as in the other experimental animals by stimulation to the perifornical region of the posterior hypothalamus. Anesthetized prepro-orexin knockout mice showed lower blood pressure and faster respiratory frequency than those in the wild-type mice and attenuated defense response evoked by microinjection of bicuculline. Attenuated defense response and hypotension was also reproduced in unanesthetized freely behaving mutant mice. The hypotension observed might be due to an attenuated sympathetic outflow. The present study suggests that orexin-containing neurons in the perifornical area play a role for one of the efferent pathways of defense response. Moreover, intrinsic orexin contributes to the maintenance of basal blood pressure.

We thank C. Kumagai and W. Nakamura for excellent technical assistance. We also thank Dr. M. Shimoyama for valuable discussion.

DISCLOSURES

Part of this work was supported by a Grant-in Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (14570037, 14770270) and by grants from the Shimadzu Science Foundation and the Yamanouchi Foundation for Research on Metabolic Disorders.

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This Review is part of a thematic series on **Gene Expression in Hypertrophy and Stress,** which includes the following articles:

Gene Expression in Fibroblasts and Fibrosis: Involvement in Cardiac Hypertrophy

Roles of Cardiac Transcription Factors in Cardiac Hypertrophy

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G Protein–Coupled Signaling and Gene Expression

Genetic Models and Mechanisms of Transcription in Cardiac Hypertrophy

Ryozo Nagai, Guest Editor

Roles of Cardiac Transcription Factors in Cardiac Hypertrophy

Hiroshi Akazawa, Issei Komuro

Abstract—Different cell types, equipped with unique structure and function, synthesize different sets of proteins on the basis of different patterns of gene expression, even though their genomes are identical. Cardiac transcription factors have been reported to control a cardiac gene program and thus to play a crucial role in transcriptional regulation during embryogenesis. Recently, postnatal roles of cardiac transcription factors have been extensively investigated. Consistent with the direct transactivation of numerous cardiac genes reactivated in response to hypertrophic stimulation, cardiac transcription factors are profoundly involved in the generation of cardiac hypertrophy or in cardioprotection from cytotoxic stress in the adult heart. In this review, the regulation of a cardiac gene program by cardiac transcription factors is summarized, with an emphasis on their potential role in the generation of cardiac hypertrophy. **(*Circ Res*. 2003;92: 1079-1088.)**

Key Words: cardiac transcription factors ■ gene expression ■ cardiac hypertrophy ■ cardiogenesis

Cardiomyocytes are terminally differentiated and lose their ability to proliferate soon after birth. Thereafter, cardiomyocytes grow in cell size without cell division to adapt to a demand for an increased workload. In a number of pathological conditions (eg, hypertension, valvular disease, myocardial infarction, and cardiomyopathy) that impose overwork on the heart, postnatal cardiomyocytes undergo cardiac hypertrophy. Although cardiac hypertrophy is initially compensatory for an increased workload, prolongation of this process leads to congestive heart failure, arrhythmia, and sudden death.[1,2] At the cellular level, cardiac hypertrophy is characterized by an increase in cell size and protein synthesis and reactivation of the fetal gene program.[3–5] In addition, recent large-scale expression analyses have identified numerous genes other than fetal genes or immediate-early genes that were upregulated in hypertrophied hearts, including genes encoding proteins involved in signaling pathways and energy metabolism.[6,7] The points at issue are how extracellular hypertrophic stimulation is perceived and converted into intracellular signals and how these signals change the transcriptional program that eventually leads to cardiac hypertrophy. With regard to the differential gene expression induced by hypertrophic stimulation, it is reasonable to assume that cardiac transcription factors play the leading part, because they directly regulate a number of cardiac genes that are upregulated in hypertrophied myocardium.

Cardiac transcription factors are defined, in this context, as essential transcriptional activators that are expressed predominantly in the myocardium and that regulate the expression of the

Original received July 23, 2002; resubmission received October 23, 2002; revised resubmission received April 9, 2003; accepted April 10, 2003.
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Circulation Research **is available at http://www.circresaha.org** **DOI: 10.1161/01.RES.0000072977.86706.23**

TABLE 1. Transgenic Overexpression of Wild-Types or Dominant-Negative Mutants

Cardiac Transcription Factor	Effects	References
GATA4		
Wild-type	Hypertrophic myocardial cell growth with hypertrophic gene expression	20
MEF2		
Dominant-negative	Attenuated postnatal growth of the myocardium	67
Csx/Nkx2-5		
Wild-type	Normal cell size of myocardium with hypertrophic gene expression	133
Dominant-negative	Impaired cardiac function	139

cardiac genes encoding structural proteins or regulatory proteins characteristic of cardiomyocytes. Recent studies have established the notion that cardiac transcription factors govern the intricate process of cardiogenesis by regulating cardiac-specific gene expression.[8] Cardiac transcription factors are represented by the GATA family transcription factors, myocyte enhancer factor 2 (MEF2) transcription factors, and the homeobox transcription factor Csx/Nkx2-5. Accumulating data have suggested the significant role of these transcription factors in postnatal myocardium as well. In the present review, transcriptional regulation by cardiac transcription factors is summarized, with an emphasis on the potential mechanisms involved in cardiac hypertrophy.

GATA Transcription Factors

GATA transcription factors are characterized by the conserved double zinc fingers that are required for binding to the specific consensus DNA sequence (A/T)GATA(A/G).[9] Among 6 GATA transcription factors in vertebrates, GATA4, GATA5, and GATA6 are expressed in the heart.[10] Especially, targeting disruption of *GATA4* in mice resulted in embryonic lethality that was due to failure in the formation of a ventrally fused heart tube, indicating an important role of GATA4 in transcriptional regulation during cardiac morphogenesis.[11,12] Indeed, functional analysis of the *cis*-regulatory elements has revealed that GATA4 directly regulates basal expression of a spectrum of cardiac-specific genes, such as *α-myosin heavy chain* (*α-MHC*), *myosin light chain 1/3* (*MLC1/3*), *cardiac troponin C*, *cardiac troponin I*, *atrial natriuretic peptide* (*ANP*), *brain natriuretic peptide* (*BNP*), *cardiac-restricted ankyrin repeat protein* (*CARP*), *cardiac sodium-calcium exchanger* (*NCX1*), *cardiac m2 muscarinic acetylcholine receptor*, A_1 *adenosine receptor*, and *carnitine palmitoyl transferase I β*.[10,13]

Besides supporting the basal transcription levels of these cardiac genes and thus conferring tissue specificity on cardiomyocytes, GATA4 is critically involved in inducible gene expression evoked by a variety of hypertrophic stimulations. For example, GATA-binding elements are required for the upregulation of *β-MHC* or *angiotensin II type 1a receptor* in response to aortic constriction.[14,15] In addition, GATA-binding elements are responsible for inducible gene expression of *BNP* in the hearts of bilaterally nephrectomized rats.[16] Furthermore, in cultured cardiomyocytes, upregulation of *NCX1* or *BNP* by adrenergic stimulation is mediated by GATA-binding elements within the regulatory regions of the individual genes.[17,18]

Consistent with the essential role of GATA4 in activating the gene program in response to hypertrophic stimulation, the overexpression of GATA4 generated cardiac hypertrophy both in cultured cardiomyocytes[19,20] and in the hearts of mice[20] (Table 1). These results suggest that GATA4 is a sufficient transcriptional regulator for the generation of cardiac hypertrophy. Moreover, the overexpression of a dominant-negative GATA4 by adenoviral gene transfer inhibited an agonist-induced increase in protein synthesis and hypertrophic gene expression in cultured cardiomyocytes.[20] Although electrical stimulation upregulates *GATA4* expression,[21] the expression levels of *GATA4* are not affected by

TABLE 2. Expression Levels or Activities of Cardiac Transcription Factors in Hypertrophied Hearts

Cardiac Transcription Factor	State	Stimulation	References
GATA4	Enhanced DNA binding	Pressure overload	14, 15, 22
		α-Adrenergic agonist	20, 23
		β-Adrenergic agonist	18
		ET-1	25, 26
		Ang II	27
MEF2	Enhanced DNA binding	Pressure overload	70
		Volume overload	70
Csx/Nkx2-5	Upregulated expression	Pressure overload	131
		α-Adrenergic agonist	132
		β-Adrenergic agonist	132
HAND	Downregulated expression	Pressure overload	146
		α-Adrenergic agonist	146

TABLE 3. Posttranslational Modification by Kinases During Cardiac Hypertrophy

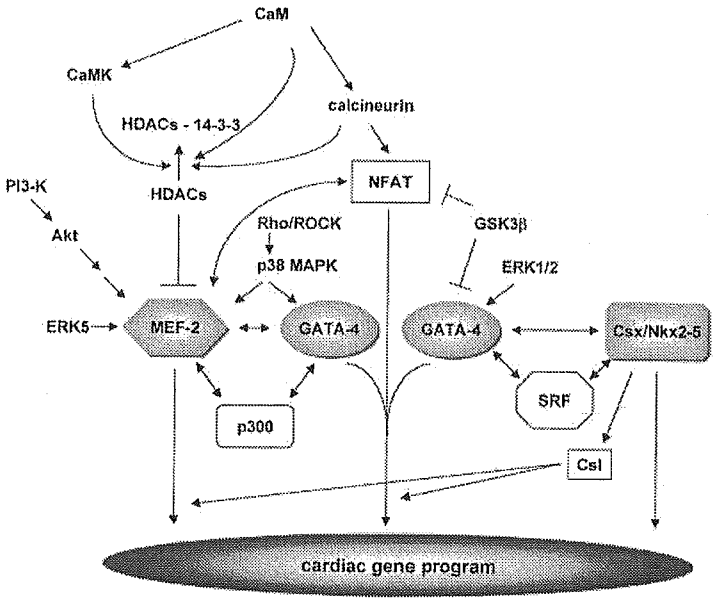
Cardiac Transcription Factor	State	Stimulation	References
GATA4	ERK	Activation	23, 24
	p38 MAPK	Activation	19, 26
	GSK3β	Nuclear export	44
MEF2	p38 MAPK	Activation?	70–73
	ERK5	Activation?	75, 76, 78
Csx/Nkx2-5	Unknown		
HAND	Unknown		

hypertrophic stimulation induced by pressure overload,[22] α-adrenergic agonists,[20,23–25] or endothelin-1 (ET-1).[25] On the basis of an increase in DNA-binding activity of GATA4 in response to pressure overload[14,15,22] or neurohumoral stimulation by α-adrenergic agonists,[20,23] β-adrenergic agonists,[18] ET-1,[25,26] or angiotensin II[27] (Table 2), it is reasonable to postulate that GATA4 is activated through posttranslational modification by hypertrophic stimulation (Table 3).

Indeed, recent studies have demonstrated that GATA4 activation induced by phenylephrine (PE) stimulation is coupled with serine phosphorylation of GATA4.[23,24] Extracellular signal–regulated kinase 2 (ERK2) directly phosphorylates GATA4 in vitro, and PE-induced phosphorylation and activation of GATA4 are inhibited either by incubation with an ERK kinase (MEK1) inhibitor or by adenoviral transfection of dominant-negative MEK1, indicating an essential role of the ERK pathway in GATA4 activation. The ERK pathway, one of the ternary branches of the mitogen-activated protein kinase (MAPK) cascades, is a key biochemical signal that mediates hypertrophic responses.[28,29] In this respect, GATA4 may function as a transcriptional effector acting downstream from the ERK signaling pathway activated by hypertrophic stimulation, because dominant-negative GATA4 inhibited MEK1-induced hypertrophic responses in cultured cardiomyocytes.[24] GATA4 is also activated through direct serine phosphorylation by the p38 MAPK pathway,[19,26] which is another branch of the MAPK cascades and mediates hypertrophic growth in cultured cardiomyocytes.[30–32] Pharmacological inhibition of p38 MAPK attenuated ET-1–induced protein synthesis in addition to DNA binding and phosphorylation of GATA4.[26]

A recent report has suggested that Rho and ROCK, a target of Rho, are linked to PE-induced GATA4 activation through the ERK pathway.[33] Moreover, the potentiation of GATA4 transcriptional activity through p38 MAPK is induced by RhoA,[19] a member of the Rho family of GTPases, which regulate diverse cellular events such as transcriptional regulation, cell growth control, and membrane trafficking as well as cytoskeletal organization.[34] In cardiomyocytes, Rho is critically involved in mediating hypertrophic features[35] induced by mechanical stress[36] and G-protein–coupled receptor agonists such as PE,[37–40] angiotensin II,[41,42] and ET-1.[43] Collectively, these observations highlight the role of GATA4 as an essential transcriptional effector by which divergent protein phosphorylation pathways integrate during the generation of cardiac hypertrophy.

The transcriptional activity of GATA4 is regulated through its nucleocytoplasmic shuttling mechanism. Glycogen synthase kinase 3β (GSK3β) directly phosphorylates GATA4 and thereby decreases basal and β-adrenergic–stimulated GATA4 expression in the nucleus by activating the nuclear export system.[44] Phosphorylation of GATA4 by GSK3β negatively regulates GATA4 transcriptional activity, in con-



Roles of cardiac transcription factors in regulation of cardiac gene program during cardiac hypertrophy. GATA4 transcriptional activity is stimulated through phosphorylation by ERK1/2 and p38 MAPK, although phosphorylation by GSK3β negatively regulates GATA4 activity. In addition, the transcriptional activity of GATA4 is regulated through physical interaction with NFAT, MEF2, SRF, or a coactivator, p300. Most important, in response to hypertrophic stimulation, NFAT is dephosphorylated by calcineurin and translocates into the nucleus, where it activates gene expression partly through forming a complex with GATA4. MEF2 transcriptional activity is enhanced through phosphorylation by p38 MAPK and ERK5 and physical interaction with GATA4, NFAT, and coactivator p300. In addition, MEF2 might be involved in the PI3-K/Akt–mediated hypertrophic signal. Most important, MEF2 factors function as important effectors of Ca^{2+} signaling. MEF2 activity is stimulated by constitutively active calcineurin or CaMK in vivo. Activation of MEF2 is dependent on dissociation from class II HDACs. Signal-mediated phosphorylation of HDACs recruits chaperones 14-3-3 to dissociate the HDAC-MEF2 formation, although the endogenous HDAC kinase has not been determined. Csx/Nkx2-5 might regulate cardiac gene expression (1) directly, (2) via association with GATA4 or SRF, and (3) via upregulation of Csl, which activates transcriptional activities of GATA4 and MEF2. Contribution of Csx/Nkx2-5 transcriptional activity to pathophysiological hypertrophic responses remains undefined.

trast to phosphorylation by ERK or p38 MAPK. Interestingly, recent studies have indicated an inhibitory role of GSK3β in the stimulation of cardiac hypertrophy.[45–47] Therefore, GSK3β is supposed to inhibit cardiac hypertrophy in part by interfering with GATA4 transcriptional activity.

In addition to phosphorylation, the transcriptional activity of GATA4 is regulated through interaction with cofactors such as p300[48] and other transcription regulators such as serum response factor (SRF),[49] Csx/Nkx2-5,[50–52] MEF2,[53] nuclear factor of activated T cells (NFAT),[54] dHAND/HAND2,[55] FOG-2,[56,57] YY1,[58] and peroxisome proliferator–activated receptor binding protein.[59] p300 interacts with GATA transcription factors to enhance the promoter activation of the *ANP*[48,60] and *β-MHC* genes,[48] which is dependent on the histone acetyltransferase (HAT) activity of p300. With respect to protein-protein interaction with other transcription factors, the interaction between GATA4 and NFAT is particularly noteworthy because NFAT plays a critical role in activating the hypertrophic gene program. In response to hypertrophic stimuli, NFAT is dephosphorylated by calcineurin and translocates into the nucleus, where it activates gene expression partly through forming a complex with GATA4.[54,61] SRF is a transcriptional regulator of a wide variety of cardiac-specific genes, and cardiac overexpression of SRF induces hypertrophic features in mice.[62] MEF2 is another important transcription factor regulating the cardiac gene program during myocardial cell hypertrophy. Transcriptional synergy based on protein-protein interaction involving GATA4 and these transcriptional factors may be implicated in the generation of cardiac hypertrophy.

Taken together, GATA4 transcriptional activity is positively regulated by multiple signaling pathways in response to hypertrophic stimulation. GATA4 plays an essential role in transcriptional regulation during the generation of cardiac hypertrophy (Figure).

MEF2 Transcription Factors

MEF2 transcription factors contain a MADS (indicating MCM1, agamous, deficiens, and SRF) domain and an adjacent MEF2-specific domain in the N-terminus, which together direct dimerization and binding to their cognate DNA sequence CAT(A/T)4TAG/A.[63,64] In vertebrates, 4 members (MEF2-A, MEF2-B, MEF2-C, and MEF2-D) have been identified. Although MEF2A through MEF2-D are expressed in many types of cells, their specific functions are assigned to transcriptional regulation in the immune system, neurons, and striated muscle. Especially, targeted disruption of *MEF2C* has been shown to lead to arrested cardiac looping and right ventricular formation during embryogenesis, and several cardiac genes have been shown to be downregulated in *MEF2C*-null embryos,[65,66] indicating an essential role of MEF2 in myocardial cell differentiation. MEF2C is involved in transcriptional regulation in postnatal hearts as well, inasmuch as transgenic mice expressing a dominant-negative MEF2C have displayed attenuated postnatal growth of the myocardium[67] (Table 1). Consistently, the MEF2-binding A/T-rich DNA sequences have been identified within the promoter regions of a number of cardiac genes, (eg, *muscle creatine kinase* gene, *α-MHC*, *MLC1/3*, *MLC2v*, *skeletal α-actin*, *sarcoplasmic reticulum* Ca^{2+}*-ATPase*, *cardiac troponin T*, *cardiac troponin C*, *cardiac troponin I*, *desmin*, and *dystrophin*).[63,68]

In addition, MEF2 transcription factors are critically involved in the regulation of inducible gene expression during myocardial cell hypertrophy, inasmuch as the MEF2-binding site within the *MLC2* promoter is required during PE-mediated and ET-1–mediated hypertrophy,[69] and MEF2 DNA-binding activity is increased in the hearts of rats subjected to pressure overload or volume overload[70] (Table 2). Recent studies have elucidated complex signaling pathways that link hypertrophic stimulation and MEF2 activation (Table 3). First, MEF2 is phosphorylated by p38 MAPK.[67,71–73] Specifically, p38 MAPK-MEF2 signaling is implicated in the regulation of skeletal muscle cell differentiation[74] and immune response.[71] Although activation of p38 MAPK induces hypertrophic growth in cultured cardiomyocytes[30–32] and p38 MAPK phosphorylates MEF2 in hypertrophied heart, the pathophysiological significance of the p38 MAPK–MEF2 pathway during cardiac hypertrophy has not been fully determined. Second, MEF2 is activated through phosphorylation by ERK5, also known as big MAPK 1.[75,76] The ERK5-MEF2 pathway participates in inducible gene expression of an immediate-early gene c-*fos* in response to growth stimulation such as serum[75] or G-protein–coupled receptor agonists.[77] A recent study has demonstrated that ERK5 is activated by hypertrophy-stimulating factors such as PE, leukemia inhibitory factor, and oxidative and osmotic stress in cultured cardiomyocytes.[78] Additionally, dominant-negative MEK5, the MAPK kinase for ERK5, inhibited leukemia inhibitory factor–induced hypertrophic features, and transgenic overexpression of constitutively active MEK5 in the heart resulted in eccentric hypertrophy.[78] Collectively, these results suggest a role of the ERK5-MEF2 pathway in the generation of cardiac hypertrophy, although it is not determined whether MEF2 is an essential downstream effector of ERK5-induced cardiac hypertrophy.

During the skeletal muscle differentiation evoked by insulin-like growth factor-1 (IGF-1), the transcriptional activity of MEF2 is activated through the phosphoinositide 3-kinase (PI3-K)–Akt pathway.[79,80] Interestingly, transgenic mice overexpressing the constitutively active form of either PI3-K or Akt exhibit physiological cardiac hypertrophy characterized by proportional myocardial cell growth without interstitial fibrosis or deterioration of cardiac function.[81,82] Although the transcriptional activity of MEF2 has not been examined in these transgenic mice, it may be possible that MEF2 is involved in PI3-K/Akt–mediated hypertrophic growth of cardiomyocytes.

Most important, the MEF2 factors function as important effectors that converge in the binary downstream pathway of the Ca^{2+} signaling. A growing body of evidence has suggested that Ca^{2+} signaling plays a critical role in the generation of cardiac hypertrophy.[83] Increased intracellular Ca^{2+} binds to and activates Ca^{2+}-binding proteins, including calmodulin (CaM), which regulates several downstream effectors, such as calcineurin and Ca^{2+}/CaM-dependent protein kinases (CaMKs). Activation of either calcineurin[54] or CaMKs[84,85] induces cardiac hypertrophy both in cultured

cardiomyocytes and in murine hearts. The MEF2 activity is stimulated by CaMK, as indicated by LacZ expression in the hearts of double transgenic animals harboring activated CaMKIV and a MEF2-dependent *LacZ* reporter.[85] Although CaMKs directly phosphorylate MEF2D in vitro,[86] the activation of MEF2 by CaMK is mediated mainly through the phosphorylation of transcriptional repressors, the histone deacetylases (HDACs).[87] Especially, class II HDACs (HDAC-4, HDAC-5, HDAC-7, and HDAC-9) associate with MEF2 to repress MEF2-induced gene expression.[88–92] In general, transcriptional activity is controlled by the state of histone acetylation, the balance of which is maintained through opposing activities of HDACs and HATs.[93] HDACs repress gene expression through intrinsic deacetylase activity and recruitment of a transcriptional corepressor COOH-terminal–binding protein.[94] Recent studies have demonstrated that phosphorylation of HDACs by CaMKs results in the recruitment of intracellular chaperones 14-3-3 to dissociate the HDAC-MEF2 formation.[95–97] Consequently, HDACs are sequestered in the cytoplasm by the nucleocytoplasmic shuttling mechanism,[95,98,99] and MEF2 is released from HDACs in the nucleus and transcriptionally activated through binding to coactivators harboring intrinsic HAT activity, such as p300[100–102] and CBP.[103] HDAC4 has a CaM-binding domain that overlaps the MEF2-binding domain, and dissociation of MEF2 from HDACs is also regulated by CaM,[101] indicating that the HDAC-MEF2 complex is controlled by a series of mediators in the Ca^{2+} signaling pathway.

The implication of class II HDACs during cardiac hypertrophy is underscored by a recent report demonstrating that *HDAC9*-deficient mice display spontaneous cardiac hypertrophy and are predisposed to more severe hypertrophic growth after banding of the thoracic aorta.[104] In cultured cardiomyocytes, overexpression of class II HDACs with mutations of 2 conserved CaMK phosphorylation sites blocks hypertrophic features, including agonist-induced gene expression of *ANP* and *β-MHC* and histone acetylation of the promoter regions of these genes.[104] These data indicate the repressive role of class II HDACs in the generation of cardiac hypertrophy. Although the HDAC kinase activity is enhanced in cardiac extracts from hypertrophied hearts of mice and although CaMKs are capable of phosphorylating HDACs, it remains unclear whether CaMKs are the functional HDAC kinases that are responsive to hypertrophic stimulation, because HDAC kinase activity in in vitro kinase assays is only partially blocked by CaMK inhibitors.[104] Instead, HDAC kinase activity is enhanced by calcineurin signaling, inasmuch as the hypertrophic features in *HDAC9*-deficient mice harboring the activated calcineurin transgene are more prominent with increased transcriptional activity of MEF2.[104] In T lymphocytes or skeletal muscle, activated calcineurin promotes complex formation between MEF2 and NFAT to synergistically transactivate downstream target genes.[86,105–107] Calcineurin-mediated dephosphorylation of MEF2 is observed in skeletal muscle and neurons.[107–109] The precise role of calcineurin in the activation of MEF2 during cardiac hypertrophy remains to be determined.

The transcriptional activity of MEF2, like that of GATA4, is regulated through protein-protein interaction with other transcription factors, such as MyoD,[110] GATA4,[53] NFAT,[86,105,106,109] thyroid hormone receptor,[111] and Smad proteins.[112] Especially, MEF2 and GATA4 synergistically activate the transcription of several cardiac genes, such as *ANP*, *BNP*, *α-MHC*, and *cardiac α-actin*, indicating a significant cooperative role of MEF2 and GATA4 in the transcriptional regulation of these cardiac genes. However, it remains to be determined whether the transcriptional synergy between MEF2 and GATA4 is implicated in the generation of cardiac hypertrophy.

Recent reports have demonstrated that a novel cardiac helicase, CHAMP, is activated by MEF2 protein[66] and acts as a suppressor of cardiac hypertrophy.[113] *CHAMP* was originally identified by differential array analysis as a cardiac-specific gene downregulated in *MEF2C*-deficient embryos.[66] Overexpression of CHAMP in cultured cardiomyocytes impairs PE- and serum-induced hypertrophic gene expression.[113] These data appear contradictory to the notion that the MEF2 factors are important in regulation of hypertrophic gene expression. However, *CHAMP* expression is downregulated in the hearts of transgenic mice overexpressing activated calcineurin. On the basis of the enhanced activity of MEF2 in these mice,[104] it is plausible that *CHAMP* expression is not dependent on MEF2 in the postnatal heart.

Collectively, MEF2 activity is enhanced in response to hypertrophic stimulation, and MEF2 functions as an essential effector of divergent intracellular signaling pathways mediating hypertrophic features (Figure).

Cardiac Homeobox Transcription Factor Csx/Nkx2-5

Csx/Nkx2-5 is a homeodomain-containing transcriptional activator, originally identified as a potential homologue of *Drosophila tinman*.[114,115] The homeodomain of Csx/Nkx2-5 has a helix-turn-helix motif that binds to the specific consensus DNA sequence T(C/T)AAGTG.[116] Targeted disruption of *Csx/Nkx2-5* in mice caused embryonic lethality due to the arrested looping morphogenesis of the heart tube and growth retardation.[117,118] The expression of several cardiac genes in the heart of *Csx/Nkx2-5*–deficient embryos (including *MLC2v*, *ANP*, *BNP*, *CARP*, *MEF2-C*, *eHAND/HAND1*, *N-myc*, *Iroquois homeobox gene 4*, and *HOP*) was reduced.[117–122] In addition, direct downstream targets for Csx/Nkx2-5 (such as *ANP*,[52,123] *cardiac α-actin*,[124] *A_1 adenosine receptor*,[125] *calreticulin*,[126] *connexin40*,[127] and *NCX1*) have been identified.[128] These results indicate a functional role of *Csx/Nkx2-5* in the transcriptional regulation of a cardiac gene program.

In contrast to the essential role of Csx/Nkx2-5 during embryogenesis, its functional role in the postnatal heart has not been fully determined. *Csx/Nkx2-5* is expressed in the adult heart,[114,129,130] and notably, its expression is upregulated in hypertrophied hearts (Table 2). Banding of the feline pulmonary artery induces right ventricular hypertrophy with increased expression of *Csx/Nkx2-5* and its downstream target genes, *ANP* and *cardiac α-actin*.[131] In PE- or isoproterenol-mediated hypertrophic hearts, expression of *Csx/Nkx2-5* is stimulated as well as the expression of fetal genes, such as *ANP* and *β-MHC*, and immediate-early genes,

such as c-*fos*, c-*jun*, and *Egr-1*.[132] The upregulation of *Csx/Nkx2-5* expression in pressure overload–induced and agonist-induced hypertrophic hearts indicates a potential role of *Csx/Nkx2-5* in the process of cardiac hypertrophy in general.

However, transgenic mice overexpressing Csx/Nkx2-5 under the control of the cytomegalovirus enhancer/chicken *β-actin* promoter exhibit normal-sized hearts[133] (Table 1). The expression levels of cardiac genes such as *ANP*, *BNP*, *CARP*, and *MLC2v* are upregulated in the hearts of *Csx/Nkx2-5* transgenic mice. These gain-of-function studies suggest that Csx/Nkx2-5 is not sufficient for the generation of cardiac hypertrophy but that Csx/Nkx2-5 functions to control cardiac gene program in adult hearts as well as in embryonic hearts. Csx/Nkx2-5 interacts with other cardiac transcription factors. Transcriptional activity of Csx/Nkx2-5 is modulated through physical interaction with other transcription factors such as GATA4,[50–52,134] SRF,[124] T-box–containing transcription factor Tbx5,[127,135] Tbx2,[136] and eHAND/HAND1.[137] We have identified several factors that interact with Csx/Nkx2-5 and modulate Csx/Nkx2-5–induced gene expression. One of the coactivating factors potentiates Csx/Nkx2-5–induced promoter activation in response to a signal evoked by hypertrophic stimulation (authors' unpublished data, 2003). Therefore, combinatorial regulation involving Csx/Nkx2-5 and its coactivators might be necessary for the generation of cardiac hypertrophy, although it is still speculative.

A novel muscle-specific gene, *Chisel* (*Csl*), was identified by a differential screening as a target gene downregulated in *Csx/Nkx2-5*–null embryonic hearts.[138] Overexpression of Csl in C2C12 myoblasts induced lamellipodia formation and differentiation into large myosacs in the presence of IGF-1 as a result of enhanced cell fusion. Interestingly, Csl augmented transcriptional activities of MEF2 and NFAT in an IGF-1 signaling-dependent manner. Both MEF2 and NFAT are important in the differentiation and hypertrophy of cardiac muscle as well as skeletal muscle. Although the activation of NFAT by Csl in the presence of IGF-1 is not dependent on the calcineurin pathway, it is intriguing that the downstream target of Csx/Nkx2-5 might operate in connection with the NFAT and MEF-2 transcription factors, which are involved in the generation of cardiac hypertrophy.

Recently, transgenic mice overexpressing dominant-negative mutant of Csx/Nkx2-5 under control of *α-MHC* promoter have been generated[139] (Table 1). These mice show impaired cardiac function with the degeneration of cardiomyocytes. Furthermore, in response to doxorubicin, dominant-negative Csx/Nkx2-5 transgenic mice show more severe cardiac dysfunction accompanied by a larger number of apoptotic myocardial cells, although doxorubicin-induced myocardial damage is mild in transgenic mice overexpressing the wild-type of Csx/Nkx2-5. These results indicate a cardioprotective role of Csx/Nkx2-5 in postnatal hearts.

Taken together, Csx/Nkx2-5 is upregulated in response to hypertrophic stimulation and may have implications in the transcriptional regulation of the cardiac gene program in hypertrophied hearts (Figure). In the adult heart, Csx/Nkx2- 5 also plays an important role in protecting the myocardium against cytotoxic damage.

HAND Transcription Factors

dHAND/HAND2 and eHAND/HAND1 are basic helix-loop-helix transcription factors that have distinctive roles in cardiac and extraembryonic development.[140] The expression of *eHAND* is predominant in the left ventricle and is excluded from the right ventricle. Analysis of *eHAND*-null mice defined an essential role of *eHAND* in myocardial differentiation of the left ventricle.[141–143] In contrast, the expression of *dHAND* is restricted to the right ventricle, and development of the right ventricle has been shown to be selectively compromised in *dHAND*-null embryos.[144]

Initial insight into postnatal HAND function was provided by a report showing that the expression of *dHAND* and *eHAND* is detectable in human adult hearts and that the cardiac expression of *eHAND* is significantly downregulated in patients with cardiomyopathies.[145] Likewise, in a PE-induced hypertrophic mouse model, a chamber-specific downregulation of *eHAND* in the left ventricle and *dHAND* in the right ventricle was observed[146] (Table 2). In addition, in abdominal aorta–banded rats, the expression of *dHAND* and of *eHAND* was shown to be downregulated in both the ventricles. The reduced expression of *HAND* genes may indicate a role in the inhibition of myocardial cell growth. At present, a limited number of direct downstream target genes of the HAND transcription factors have been identified. Through binding to p300, dHAND interacts with GATA4 to induce synergistic transactivation of the promoters of *ANP*, *BNP*, and *α-MHC*.[55] Similarly, eHAND interacts with Csx/Nkx2-5 to synergistically transactivate the *ANP* promoter.[137] Elucidation of the molecular basis of the HAND transcription factors will be required to understand the postnatal roles relevant to their reduced expression in hypertrophied hearts.

Future Issues

Functional roles of the cardiac transcription factors during cardiogenesis have been considerably deciphered. Many downstream target genes are identified, and transcriptional regulatory mechanisms whereby protein-protein interactions with other cardiac transcription factors allow fine-tuned gene expression have been clarified. Recent studies have demonstrated that GATA4 and MEF2 are involved in reactivation of the fetal gene program in response to a variety of hypertrophic stimulation and that these factors function as important effectors during the generation of cardiac hypertrophy. Furthermore, mechanistic insights have been provided into the signaling pathways that enhance the transcriptional activities of these transcription factors. In contrast to GATA4 and MEF2, Csx/Nkx2-5 participates in the activation of the hypertrophic gene program but does not have the ability to induce hypertrophic myocardial cell growth. In this respect, a challenging problem (ie, how the cardiac transcription factors influence an increase in protein synthesis and myocardial cell size) remains unsolved. An increased capacity of protein synthesis underlying hypertrophic growth is facilitated not by increased translational efficiency but by ribosome accumulation resulting from increased transcription of ribosomal DNA by the nucleolar factor UBF.[147,148] Interestingly, adenoviral introduction of UBF antisense RNA into cultured cardiomyocytes abolished an increase in general protein synthesis and

hypertrophic cell growth in response to α-adrenergic and contraction stimulation but had little effect on fetal gene expression.[148] It has not been clarified whether the UBF activity is influenced by cardiac transcription factors. Comprehensive analyses of target genes regulated by cardiac transcription factors during cardiac hypertrophy will provide a clue toward solving this problem.

Transcriptional regulation by multiple cardiac transcription factors such as GATA4, MEF2, and Csx/Nkx2-5 is interrelated. It is conceivable that combinations of the ubiquitous and tissue-specific transcription factors execute regulatory decisions under a spectrum of hypertrophic conditions as well as during embryogenesis. Although transcriptional synergy has been reported to be significant in controlling the expression of several cardiac genes, an important issue (ie, how much the cooperative transcriptional regulation weighs with the generation and progression of cardiac hypertrophy) remains unsolved. It is also undetermined how the mutual interaction is regulated in response to hypertrophic stimulation. Functional analysis of the individual cardiac transcription factors and clarification of their interactive roles will be required.

Finally, whether the cardiac transcription factors may be potential therapeutic targets in cardiovascular diseases is a challenging problem. Although compensatory cardiac hypertrophy is beneficial in some pathological conditions, evidence-based studies have suggested that the regression of cardiac hypertrophy in patients leads to better prognosis.[2] It is an ideal adaptation to excessive workload to enhance myocardial contractility without a pathological increase in left ventricular mass, which may be feasible, as exemplified by a rat model of N^G-nitro-L-arginine methyl ester–induced hypertension.[149] Cardiac transcription factors are the potential candidates, because it is now clear that they orchestrate inducible gene expression in postnatal cardiomyocytes. Further investigation will be required to understand the molecular basis of the gene expression program directing cardiac hypertrophy and to target this for therapeutic purposes.

Acknowledgments

This review was supported in part by grants from the Japanese Ministry of Education, Science, Sports, and Culture; Japan Health Sciences Foundation; Takeda Medical Research Foundation; Takeda Science Foundation; Uehara Memorial Foundation; Kato Memorial Trust for Nambyo Research; Japan Medical Association (to Dr Komuro); Japanese Heart Foundation; and Kanae Foundation for Life and Sociomedical Science (to Dr Akazawa). Dr Akazawa is a Research Resident for Research on Human Genome, Tissue Engineering Food Biotechnology of the Japan Health Sciences Foundation.

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Cdc42 plays a critical role in assembly of sarcomere units in series of cardiac myocytes

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Received 21 April 2003

Abstract

Cardiomyocyte hypertrophy is observed in various cardiovascular diseases and causes heart failure. We here examined the role of small GTP-binding proteins of Rho family in phenylephrine (PE)-or leukocyte inhibitory factor (LIF)-induced hypertrophic morphogenesis of cultured neonatal rat cardiomyocytes. Both LIF and PE increased cell size of cardiomyocytes. LIF induced an increase in the length/width ratio of cardiomyocytes, while PE did not change the ratio. Adenoviral gene transfer of constitutively active mutants of Cdc42 increased the length/width ratio of cardiomyocytes and dominant negative mutants of Cdc42 conversely inhibited LIF-induced cell-elongation, while mutants of RhoA and Rac1 did not affect the length/width ratio of cardiomyocytes. These results suggest that Cdc42, but not RhoA and Rac1, is involved in LIF-induced sarcomere assembly in series in cardiomyocytes.

Keywords: RhoA; Rac1; Cdc42; Leukocyte inhibitory factor; Cardiac hypertrophy

One of the distinct features of cardiomyocyte hypertrophy is the assembly of contractile proteins into organized sarcomeric units. Immunocytochemical studies have revealed the process of sarcomere assembly (myofibrillogenesis) in cultured myocytes and developing chicken heart [1]. The morphological phenotypes of ventricular myocytes are different among physiological, concentric, and eccentric hypertrophy [2]. Physiological hypertrophy is characterized by proportional increases in width and length of cardiomyocytes. Concentric hypertrophy is induced by pressure overload and characterized by more increase in width than length while eccentric hypertrophy is induced by volume overload and characterized by more increase in length of cardiomyocytes. Many growth factors induce cardiomyocyte hypertrophy in vitro with distinctive features through various intracellular signaling pathways [3–6]. Concentric hypertrophy is induced by mechanical stretch and agonists of Gq protein-coupled receptors such as phenylephrine (PE), angiotensinII (AngII), and endothelin-1 [4]. In contrast, gp130 ligands such as cardiotrophin-1 (CT-1) or leukocyte inhibitory factor (LIF) induced cardiomyocyte hypertrophy with an increase in rather cell length than width and this feature of cardiomyocytes resembles that of eccentric hypertrophy [5].

Small molecular weight GTP-binding proteins (small G-proteins) of Rho family including RhoA, Rac1, and Cdc42 play critical roles in cell shape, adhesion, and motility in various types of cells [7–10]. In cardiomyocytes, RhoA, and Rac1 are involved in AngII- and PE-induced cardiac hypertrophy [11,12]. In this study, we examined the roles of small G-proteins of Rho family in PE- or LIF-induced sarcomere assembly of cardiac myocytes using adenovirus-mediated gene expression system. We here report that among RhoA, Rac1, and Cdc42, only Cdc42 is involved in LIF-induced myofibril elongation.

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doi:10.1016/S0006-291X(03)00838-6

Materials and methods

Antibodies and reagents. Monoclonal anti-myc antibody (9E10) and monoclonal anti-cardiac myosin heavy chain antibody (MF20) were prepared from American Type Culture Collection. TRITC-labeled phalloidin and phenylephrine were purchased form Sigma–Aldrich Japan, rhodamine conjugated anti-mouse IgG was from Chemicon, and FITC conjugated goat anti-mouse IgG was from American Qualex. LIF was purchased from Chemicon.

Cell culture. Primary cultures of cardiac ventricular myocytes from 1-day-old Wistar rats were obtained as described previously [13]. Cells were plated at a field density of 1×10^5cells/cm^2 on either collagen-coated 35-mm culture dishes or coverslips with 2 ml of Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Culture media were changed to serum-free media after 24 h. Myocytes were further cultured under serum-free conditions for 24 h and then treated with either 10^{-9} M LIF or 10^{-5} M PE for 48 h.

Recombinant adenovirus vector and adenoviral gene transfer. Six types of E1-deleted, replication deficient adenovirus vectors expressing myc-epitope tagged constitutively active or dominant negative recombinant cDNA of Rho family were used. Adenoviruses encoding C.A.RhoA (RhoA V14), D.N.RhoA (RhoA N19), C.A.Rac1 (Rac1 V12), and D.N.Rac1 (Rac1 N17), C.A.Cdc42 (Cdc42 V12), and D.N.cdc42 (Cdc42 N17) were prepared. Adenovirus vector containing the LacZ gene was used as a control vector. Cardiomyocytes were infected with recombinant adenoviruses at a multiplicity of infection (MOI) of 50 U in 0.5 ml DMEM for 1 h at 37 °C in humidified 5% CO_2 incubator and further cultured in serum-free DMEM for 48 h. The expression of RhoA, Rac1, and Cdc42 was confirmed by Western blotting using antibodies specific for Rho, Rac1, and Cdc42 (data not shown). Efficiency of gene transfer into cardiomyocytes was assessed by X-gal staining [14]. More than 95% of cardiomyocytes exhibited LacZ-positive staining (data not shown).

Immunofluorescent cytochemistry. Immunostaining to examine the morphology of cardiomyocytes was performed as described previously [15]. Briefly, cells were fixed in 3% paraformaldehyde for 10 min, treated with 50 mM NH_4Cl, and permeabilized with 0.1% Triton X-100 and 5% fetal bovine serum for 30 min at 37 °C. The samples were incubated with either anti-myc antibody (9E10) to assess adenoviral mediated gene expression or anti-cardiac myosin heavy chain antibody (MF20) to examine myofibrils followed with FITC-conjugated anti-mouse IgG second antibody and TRITC-labeled phalloidin.

To assess the hypertrophic phenotype of cardiomyocytes, cell length and width were measured and length/width ratio was calculated. Definitions of morphometric parameters were as previously described [5]. Cell length was defined as the maximum longitudinal extension of individual cells. Maximum cell width was measured perpendicular to the axis defining cell length. These parameters were measured with graphic analyzing software, IP lab on the digital images taken by CCD camera on the fluorescent microscope. More than 100 cells on every dish were measured.

Statistics. All results (means ± SE) were obtained from three independent experiments. Statistical comparison of the control group with treated groups was carried out with 1-way ANOVA and Dunnett's t test. The accepted level of significance was $p < 0.05$. More than 100 cardiomyocytes were examined in each experiment.

Results and discussion

Role of small G-proteins of Rho family in sarcomeric organization

To investigate the role of small G-proteins RhoA, Rac1, and Cdc42 in sarcomeric organization in cultured

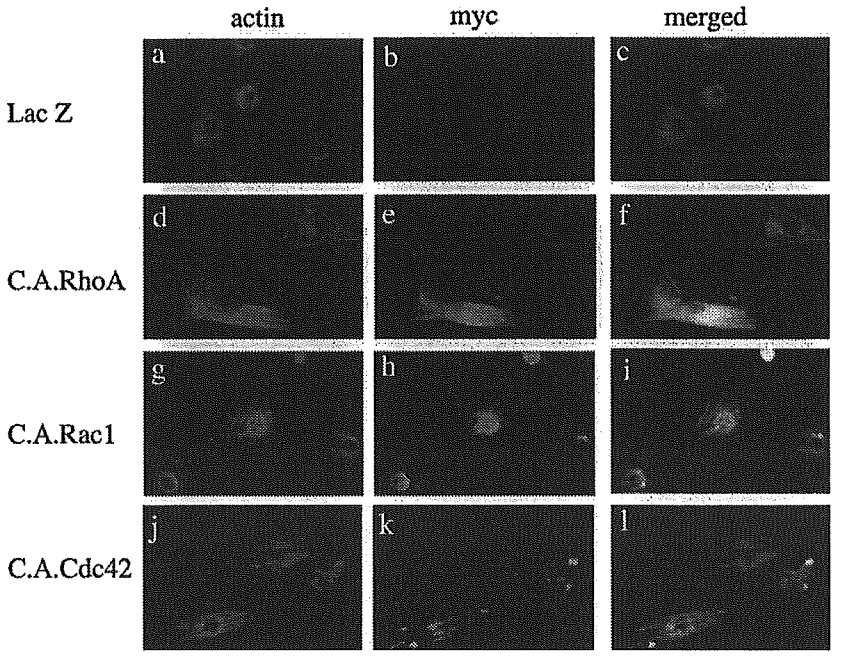

Fig. 1. Constitutively active mutants of Rho family proteins induce sarcomeric organization in cardiomyocytes. Cardiomyocytes were infected with adenovirus constructs containing LacZ without myc tag (a–c), constitutively active mutants of RhoA (d–f), Rac1 (g–i), and Cdc42 (j–l) for 48 h and then stained with anti-myc monoclonal antibody (9E10) followed by FITC-conjugated anti-mouse immunoglobulin (green) and TRITC-labeled phalloidin for actin (red).

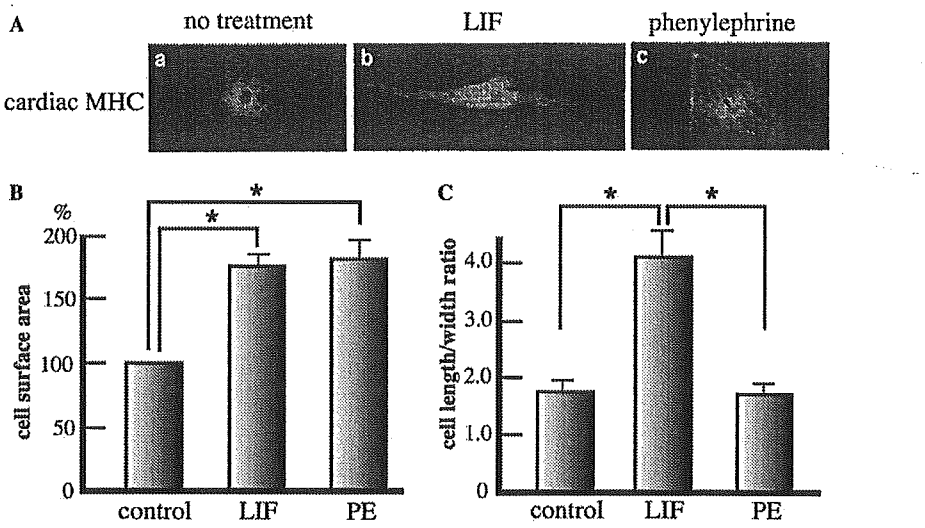

Fig. 2. Effects of LIF and PE on cell-length/width ratio and cell surface area. (A) Cardiomocytes were untreated (a) or treated with 10^{-9} M LIF (b) or 10^{-5} M PE (c) for 48 h and stained with anti-cardiac myosin heavy chain antibody (MF20) followed by FITC-conjugated anti-mouse immunoglobulin. (B) In each experiment, the cell surface area of over 100 cardiomyocytes was measured. The data represent the mean percentage of controls (100%) from three independent experiments (means ± SE). $*p < 0.01$ vs control. (C) Cell-length/width ratio of over 100 cardiomyocytes was quantitatively examined in each experiment. The results are from three independent experiments (means ± SE). $*p < 0.05$ vs control and $**p < 0.05$ vs control treated with LIF.

cardiac myocytes, cells were infected with adenovirus encoding C.A. mutant of RhoA, Rac1, or Cdc42. Immunocytochemical staining revealed that each of the three types of mutant induced sarcomeric organization in infected cells (Fig. 1). When cardiomyocytes were incubated with either LIF or PE for 48 h, clear striated bands reflecting sarcomere were observed (Figs. 2A b,c) and the cell size was increased (control 100% vs LIF 175%, $p < 0.01$; control 100% vs PE 180%, $p < 0.01$, Fig. 2B). However, there was considerable difference in cell shape between LIF- and PE-treated cardiomyocytes. LIF induced an increase in the length/width ratio, leading to the elongated cell shape, while PE did not change the ratio (Figs. 2A b, c, and C).

Role of small G-proteins of Rho family in morphological change

Overexpression of D.N.RhoA, D.N.Rac1, or D.N.Cdc42 inhibited LIF- and PE-induced sarcomere assembly (Fig. 3). Although overexpression of D.N. RhoA or D.N. Rac1 had no effect on LIF-induced cell elongation, D.N.Cdc42 inhibited sarcomere organization in series (Fig. 3). D.N.Cdc42 significantly reduced the length/width ratio of LIF-treated cardiomyocytes but neither D.N.RhoA nor D.N.Rac1 had the effect on the length/width ratio (Fig. 4). Although all three types of C.A.Rho family proteins induced sarcomere organization and cellular hypertrophy, C.A. Cdc42-overexpressed cardiomyocytes showed significant increase in the length/width ratio that mimics the LIF-treated cardiomyocytes, while neither C.A.RhoA nor C.A.Rac1 affected the length/width ratio (Figs. 1 and 4).

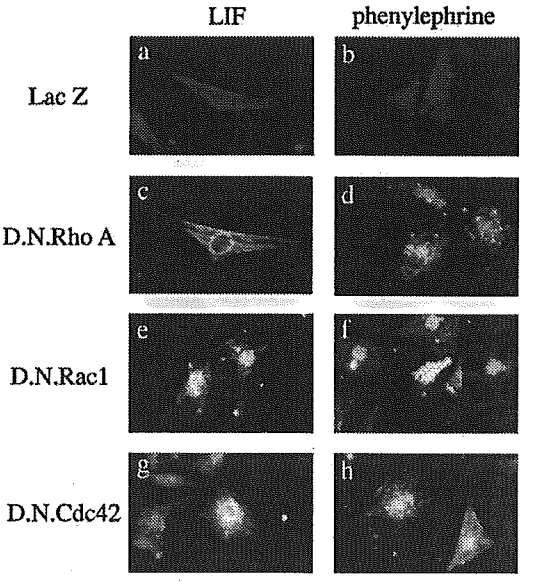

Fig. 3. Effects of dominant negative Rho family proteins on LIF- or PE-induced sarcomeric organization in cardiomyocytes. Cardiomyocytes were infected with adenovirus construct encoding LacZ (a, b), D.N.RhoA (c, d), D.N.Rac1 (e, f), or D.N.Cdc42 (g, h) for 1 h followed by treating with either 10^{-9} M LIF (a, c, e, g) or 10^{-5} M PE (b, d, f, h) for 48 h. Cardiomyocytes were stained with anti-myc monoclonal antibody (9E10) followed by FITC-conjugated anti-mouse immunoglobulin (green) and TRITC-labeled phalloidin for actin (red).

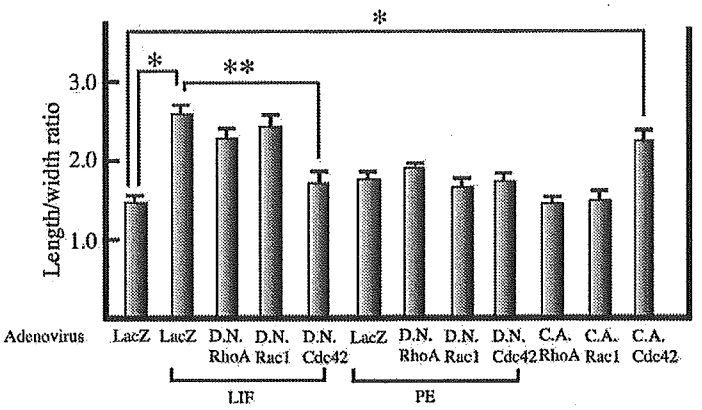

Fig. 4. Quantitative analysis of effects of mutant forms of Rho family proteins on the cell-length/width ratio in cardiomyocytes. Cardiomyocytes were infected with adenovirus construct and treated with either 10^{-9} M LIF or 10^{-5} M PE as described in the legend of Fig. 2. The effect of D.N.RhoA, D.N.Rac1, or D.N.Cdc42 on LIF or PE-induced cardiac hypertrophy and the effect of C.A.RhoA, C.A.Rac1, and C.A.Cdc42 were quantitatively analyzed by measuring cell-length/width ratio. In each experiment, over 100 cardiomyocytes were estimated and results are from three independent experiments (means ± SE). $*p < 0.05$ vs control and $**p < 0.05$ vs control treated with LIF.

The small GTP-binding proteins of Rho family have been demonstrated to play pivotal roles in various cytoskeletal functions such as cell shape, adhesion, and motility in various types of cells [16–19]. In cultured cardiomyocytes, we have reported that RhoA and Rac1 play critical roles in mechanical stress-induced hypertrophic responses [20]. Several groups have reported that RhoA and Rac1 are required for AngII- and PE-induced myofibrillogenesis [11,12], but the role of Cdc42 in sarcomere assembly was unclear. Overexpression of C.A. forms of RhoA, Rac1, and Cdc42 induced sarcomeric organization in cultured neonatal rat ventricular myocytes, and D.N.RhoA, Rac1, and Cdc42 inhibited LIF- and PE-induced myofibril organization. These results suggest that Cdc42 as well as RhoA and Rac1 play an important role in sarcomere organization of cardiac myocytes.

LIF, a member of IL-6 superfamily, activates the JAK-STAT signaling pathway through a common receptor gp130. Although LIF induces cardiomyocyte hypertrophy as well as PE and AngII, the shape of LIF-treated cells was quite different from that of PE-treated cells [4,5]. LIF induced assembly of sarcomeric units in series, rather than in parallel as seen after activation of G-protein-coupled receptors by PE, AngII, or endothelin-1 [7]. Myocytes from the heart with eccentric hypertrophy that is often induced by volume overload display assembly of sarcomeric units in series, while myocytes from pressure overload-induced concentric hypertrophy show parallel assembly of sarcomeric units [18–20]. Although RhoA or Rac1 has been reported to be required for AngII- or PE-induced parallel sarcomeric organization [11,12], the signaling pathway leading to this morphological difference was unknown. In the present study, C.A.Cdc42 promoted myofibril assembly in series rather than in parallel. However, neither C.A.RhoA nor C.A.Rac1 induced cell elongation. Furthermore although D.N.RhoA or D.N.Rac1 had no effect on LIF-induced cell elongation, D.N.Cdc42 inhibited LIF-induced sarcomere organization in series. These results suggest that only Cdc42 is critically involved in LIF-induced sarcomere assembly in series, although it remains to be determined whether LIF activates Cdc42 in cardiomyocytes. It has been reported that Cdc42 plays an important role in cell–cell adhesion with cadherin and β-catenin system at intercalated discs of cardiomyocytes [21,22], and that sarcomeric organization begins at intercalated discs in vitro and in vivo [23,24]. Therefore, Cdc42 may induce myofibril assembly in series by regulating the cell–cell adhesion system. Further studies are necessary to elucidate the molecular mechanism of how Cdc42 induces sarcomere assembly in series.

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Myocardial Fibrosis in Fabry Disease Demonstrated by Multislice Computed Tomography

Comparison With Biopsy Findings

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A 54-year-old man presented with dyspnea on effort. Echocardiogram revealed reduced apical wall motion of the left ventricle (LV) with extreme hypertrophy of the interventricular septum (IVS). Conventional coronary angiogram showed normal coronary arteries. Endomyocardial-biopsy specimens obtained from the IVS revealed extensive vacuolization of cardiac myocytes and mild fibrosis on light microscopy, and typical lysosomal inclusions with a concentric lamellar configuration were seen with electron microscopy (Figure 1). With these findings and low plasma α-galactosidase activity, he was diagnosed as having Fabry disease. To evaluate the characteristics of the LV, ECG-gated enhanced multislice computed tomography (CT) (Light Speed Ultra, General Electric) was performed with a 1.25-mm slice thickness, helical pitch 3.25. After intravenous injection of 100 mL of iodinated contrast material (350 mgI/mL), CT scanning was performed with retrospective ECG-gated reconstruction at 30 seconds and 8 minutes after injection. In the axial source images, extreme hypertrophy of the IVS and the posterior wall of the LV compared with the apical and lateral walls of the LV could be observed (Figure 2). The apical and lateral portions of the LV revealed lower CT intensity than the IVS in the early phase (arrows), and in the late phase they were abnormally enhanced compared with the IVS, suggesting fibrotic changes in the apical and lateral myocardium. Therefore, we concluded that despite the IVS biopsy results, more fibrotic changes occurred in the apical and lateral portions of the LV rather than in the IVS.

Acknowledgments

This work was supported by Grant from Takeda Science Foundation.

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(*Circulation.* **2003;107:2519-2520.**)

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