Activation of Histone Deacetylase 2 by Inducible Heat Shock Protein 70 in Cardiac Hypertrophy

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Abstract—Diverse cardiac diseases induce cardiac hypertrophy, which leads to dilatation and heart failure. We previously reported that hypertrophy can be blocked by class I histone deacetylase (HDAC) inhibitor, which prompted us to investigate the regulatory mechanism of class I HDACs. Cardiac hypertrophy was introduced by aortic banding, by infusion of isoproterenol or angiotensin II, or by swimming. Hypertrophic stimuli transiently elevated the activity of histone deacetylase-2 (Hdac2), a class I HDAC. In cardiomyocytes, forced expression of Hdac2 simulated hypertrophy in an Akt-dependent manner, whereas enzymatically inert Hdac2 H141A failed to do so. Hypertrophic stimuli induced the expression of heat shock protein (Hsp)70. The induced Hsp70 physically associated with and activated Hdac2. Hsp70 overexpression produced a hypertrophic phenotype, which was blocked either by siHdac2 or by a dominant negative Hsp70ΔABD. In Hsp70.1−/− mice, cardiac hypertrophy and Hdac2 activation were significantly blunted. Heat shock either to cardiomyocytes or to mice activated Hdac2 and induced hypertrophy. However, heat shock-induced Hdac2 activation was blunted in the cardiomyocytes isolated from Hsp70.1−/− mice. These results suggest that the induction of Hsp70 in response to diverse hypertrophic stresses and the ensuing activation of HDAC2 trigger cardiac hypertrophy, emphasizing HSP70/HDAC2 as a novel mechanism regulating hypertrophy. (Circ Res. 2008;103:1259-1269.)

Key Words: cardiac hypertrophy ■ class I histone deacetylases ■ histone deacetylase 2 ■ heat shock protein 70 ■ Hsp70.1−/− mice

Cardiac hypertrophy is a response, either adaptive or maladaptive, to pressure or volume overload, mutations, or loss of contractile mass. Hypertrophic growth accompanies many forms of heart disease, including ischemic diseases, myocardial infarction, hypertension, aortic stenosis, and valvular dysfunctions. Although the initial hypertrophic responses seem to be an adaptation to those stimuli, the sustained stress may lead to cardiomyopathy and heart failure, a major cause of human morbidity and mortality. However, few interventions have proven effective in blocking the hypertrophy or in preventing the transition to congestive heart failure.

Cardiomyocyte hypertrophy is characterized by an increase in individual myocyte size, enhanced protein synthesis, and heightened organization of the sarcomere,1 which are regulated by activation of heart-specific transcription factors such as GATA4, MEF2, and immediate early genes like c-jun and c-fos.2 The subsequent reactivation of the fetal gene program and repression of adult cardiac genes are closely related to the deterioration of heart function in hypertrophy.

Recently, modulation of gene transcription by altering chromatin structure, especially by adding or removing acetyl groups to histone tails, has been implicated in diverse human pathologies, including cardiac hypertrophy.3 Histone deacetylases (HDACs), which remove the acetyl group, repress downstream gene expression. Although HDACs are divided into 4 families, a major focus has been on class II HDACs (HDAC4, HDAC5, HDAC7, and HDAC9), which antagonize hypertrophy by repressing the fetal gene program4 by phosphorylation-dependent relocation of those proteins from the cytoplasm to the nucleus.5 However, we and others showed that HDAC inhibitors can attenuate cardiac hypertrophy,6–8 which suggests that class I HDACs (HDAC1, HDAC2, HDAC3, and HDAC8), which are readily blocked by those inhibitors, are prohypertrophic.9 In a recent study using Hdac2−/− mice, Trivedi et al10 clearly showed that Hdac2 mediates hypertrophy by modulating inositol polyphosphate-5-phosphatase f (Inpp5f)/glycogen synthase kinase (GSK)ββ signals, further implicating the opposite roles of both classes of HDACs.

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Because class I and class II HDACs share the deacetylase domain with similar enzymatic action, these opposite actions of the two classes of HDACs raise critical questions as to the fundamental roles of HDAC activity. Previous studies of their actions were done either by means of chemical HDAC inhibitors or by utilizing a knockout strategy. Chemical inhibitors raise questions of selectivity, however. In addition, with the knockout, one has to consider the two factors of ‘existence’ and ‘function’ of the enzyme. Indeed, the enzymatic activity of the HDAC is not required in antihypertrophic processes, because MITR, a splice variant of HDAC9 that lacks a deacetylase domain, is still effective. Therefore, the functional role in hypertrophy of HDACs as enzymes, and not merely their presence, and how they are regulated should be investigated. We herewith report that diverse hypertrophic signals induce the enzymatic activation of HDAC2 by inducing heat shock protein (HSP) 70 in commencing cardiac hypertrophy.

Materials and Methods

Animal Model
The experimental protocols were approved by the Chonnam National University Medical School Research Institutional Animal Care and Use Committee. Details regarding animal models and drug administrations are described in the online data supplement, available at http://circres.aha.journals.org.

Cell Cultures, Constructs, siHdac2, and Transfection Study
Protocols for cell cultures of rat neonatal cardiomyocytes, H9c2 cells, and 293T cells, siHdac2, and constructs as well as the antibodies used are described in the online data supplement.

In Vivo Visualization of the Interaction of Hsp70 and Hdac2
To visualize the interaction, we utilized fluorescence protein fragment complementation methods as described (MBL International Corporation, Woburn, Mass.). Protocols for HDAC assay, immunoprecipitation, Western blot analysis, fluorescent immunocytochemistry, cell size measurements, in vitro translation, GST pull-down assay, RT-PCR, and Northern blot are described in the online data supplement.

Results

Hypertrophic Stimuli Activate Hdac2, a Class I HDAC
We first observed changes in Hdac2 activity in the mouse hearts treated with isoproterenol (ISP). Hdac2 activation was observed as early as the 3rd day of administration, when a substantial increase in the heart weight/body weight ratio (HW/BW) was not evident. This activation was blunted after 14 days, whereas the increase in HW/BW was well sustained even after 14 days (Figure 1A). Partial constriction of the ascending aorta by aortic banding (AB) increased the HW/BW. In this case as well, Hdac2 activation preceded the increase in HW/BW (Figure 1B). However, Hdac1, an alternate class I Hdac, was not activated by either ISP or AB (Figure 1A and 1B in the online data supplement). Chronic angiotensin (Ang) II infusion increased HW/BW and Hdac2 activity (Figure 1C and supplemental Figure IC). Interestingly, even 1 day after bolus injection, phenylephrine (PE) activated Hdac2 (Figure 1D and supplemental Figure ID). Forced swimming of mice induced physiological cardiac hypertrophy through AKT pathways (supplemental Figure IE). Swimming of BL6 female mice for 3 days was enough to activate Hdac2 (Figure 1E), although HW/BW was not increased (supplemental Figure IF). We examined the individual enzyme activities of class I HDACs (Figure 1F). ISP activated Hdac2 only.

Activation of Hdac2 in response to hypertrophic stimuli was further investigated in rat neonatal cardiomyocytes. PE significantly increased Hdac2 activity as early as 3 hours (Figure 2B). Significant induction of Natriuretic peptide precursor type A (Nppa) (encoding atrial natriuretic factor, ANF), a marker of hypertrophy, was observed at day 2 (Figure 2A). Transient transfection of Hdac2 activated −3003 Nppa promoter–luciferase (Figure 2C) but not Hdac1 or Hdac3 (Figure 2D).

The Hdac2-induced activation of Nppa promoter (Figure 2E) and Myh7 (myosin heavy polypeptide 7), known as β-myosin heavy chain (supplemental Figure IIIB), was completely blocked by AKT dominant negative (supplemental Figure IIA) and Akti-1/2 (AKT inhibitor VIII, isozyme-selective Akti-1/2, Calbiochem, Figure 2E and supplemental Figure IIB and IIC). Replacement of His141 in Hdac2 with alanine caused a significant reduction in its activity (supplemental Figure III) without altering its binding capacities to other cofactors (data not shown). The enzymatically inert Hdac2 H141A could not activate the promoter–luciferase of either Myh7 (Figure 2F) or Nppa (data not shown). Transfection of wild type Hdac2 caused an increase in cell size and stress fiber formation (Figure 2G and 2H), whereas transfection of the mutant did not.

Hdac2 Regulation Mechanisms
The enzymatic activities of HDACs are regulated by several different mechanisms: (1) HDAC protein amounts, (2) intracellular localization of HDAC protein, (3) phosphorylation status, and (4) association with other cofactors. Changes in protein amounts were ruled out (supplemental Figure IVA through IVC). Substantial Hdac2 activation took place mainly in the nucleus (supplemental Figure VA) because most of the Hdac2 was present in the nucleus (supplemental Figure VB). However, hypertrophy did not induce translocation of Hdac2 (supplemental Figure VB through VE). We also did not observe a significant increase in the phosphorylation of the endogenous Hdac2 (supplemental Figure VF).

Hsp70 Interacts With Hdac2
HDAC activity may be regulated by interacting with other proteins, and HSPs have been reported as one of the regulators of class I HDACs. In heart biology, HSPs, as molecular chaperons, are induced by various stresses to protect cardiomyocytes. Thus, we postulated that hypertrophic stimuli-induced HSPs may work as regulators of HDAC2.

First, we investigated whether HspS physically interact with Hdac2 and found that Hdac2 successfully pulled down Hsp70, but not Hsp56 or Hsc70 (heat shock cognate) (constitutive form; Figure 3A) in 293T cells. Inversely, Hsp70
also recruited Hdac2 (Figure 3B) in H9c2 cells. Likewise, in the cell-free system, recombinant GST-Hsp70 protein successfully pulled down the eluted His-Hdac2 (Figure 3C).

We next sought the Hdac2-interacting domain of Hsp70. Hsp70 has two conserved domains: ATP-binding domain (ABD) and peptide-binding domain (PBD) (Figure 3D).16 His-tagged chimeric proteins of full or truncated Hsp70s were induced from bacteria and in vitro translated Hdac2 was applied. Hdac2 failed to associate with the mutants lacking either part of the PBD (Hsp70ΔC) or the NH2-terminal region (Hsp70ΔN) (Figure 3E).

Hsp70 and Hdac2 were colocalized in the nucleus of H9c2 cells (Figure 3F). Direct in vivo association of both proteins was visualized with fluorescent protein fragment complementation methods using the CoralHue Fluochase system. In this system, fluorescence can be emitted only when divided fluorophore fragments closely approach by the interaction of two target molecules of interest.11 Mammalian expression vectors to make chimeric proteins of both mKGN-Hdac2 and mKGC-Hsp70 were cloned (online data supplement). Green fluorescence was detected in the nucleus of H9c2 cells (Figure 3G) or HeLa cells (data not shown), when both constructs were simultaneously transfected.

**Hsp70 Activates Hdac2**

Next, we investigated whether Hsp70 can activate Hdac2. Transfection of Hsp70 successfully activated Hdac2 in cardiomyocytes (Figure 4A) and in H9c2 cells, whereas other Hsps failed (Figure 4B), although those proteins were correctly expressed (data not shown). Hsp70 did not activate Hdac1 (supplemental Figure VI). The recombinant GST-Hdac2 protein did not elicit the intrinsic Hdac2 activity (Figure 4C). Previously, it was reported that all HDACs except HDAC8 generated from bacteria are enzymatically inactive and that cellular components are required in the Hdac assay.17 We also measured the enzymatic activity of the recombinant proteins when H9c2 cell lysates were applied. Interestingly, Hdac2 activity was further increased when recombinant Hsp70 was added (Figure 4C).

The domain mapping study with Hsp70 gave us the idea that the Hsp70ΔABD that can bind to Hdac2 may work in a
dominant negative fashion. When pCMV-Hsp70ΔABD was cotransfected, the Hdac2 recruited by Hsp70 full was significantly reduced (Figure 4D). Next, Hdac2 activity was measured in the presence of Hsp70ΔABD in cardiomyocytes. Hsp70ΔABD itself did not activate Hdac2 (Figure 3E). However, transfection of Hsp70ΔABD dose-dependently blocked Hsp70 full-induced activation of Hdac2 (Figure 4F). Hsp70ΔN or Hsp70ΔC, both of which failed to interact with Hdac2 (Figure 3E), could not activate Hdac2 (Figure 4E), further suggesting that binding of intact Hsp70 is required for the activation of Hdac2.

**Hsp70 Induces Cardiac Hypertrophy**

Hsps are induced by hypertrophic stimuli. Thus, we postulated that induction of Hsp in response to hypertrophic stresses and subsequent activation of Hdac2 may induce cardiac hypertrophy. First, we studied which HSPs are upregulated by hypertrophic signals in mice. At the 5th day of ISP-infusion, Hsp70 transcript was dramatically increased, whereas Hsp27 (also known as Hspb2), Hsp40 (Dnajb1), Hsp56 (Fkbp4), Hsp90α/β, and Hsc70 (Hspa8) were not changed (Figure 5A). The increase in Hsp70 protein was also observed in AB or swimming models (supplemental Figure VIIA and VIIB). Hypertrophic stresses such as pressure overload have been reported to elevate Hsp70 expression within 1 hour after the stimuli. Likewise, in our experiments, administration of ISP or PE to mice dramatically increased the expression of Hsp70 as early as 3 hours, implying that Hsp70 precedes eventual heart weight increase (Figure 5B and supplemental Figure VIIIC). The increase in Hsp70 was attenuated at 14 days (Figure 5A).

Phosphorylation of AKT and the following inactivation by phosphorylating antihypertrophic GSK3β are key markers of the physiological hypertrophy resulting from increased oxygen demand, such as exercise (supplemental Figure IIE). Activation of AKT/GSK3β signal cascades are also involved in pathological hypertrophy, especially at the early phase. In our experimental model using ISP or AB, both of which are the typical inducers of pathological hypertrophy, Akt/Gsk3β phosphorylation was transiently upregulated after ISP infusion (Fig-
We next studied whether hypertrophy is associated with the increase in binding of Hsp70 and Hdac2. AB induced Hsp70, which results in its increased binding to Hdac2, compared with sham-operated mice (Figure 5D). Likewise, in PE-treated cardiomyocytes, greater amounts of Hsp70 were recruited by Hdac2 than in those without PE treatment (Figure 5E).

On the basis of the activation of Hdac2 by direct association with Hsp70 and the increase in Hsp70 in the early phase of cardiac hypertrophy, we postulated that HSP70 may play a role as a prohypertrophic mediator. We tested this by checking whether Hsp70 induces hypertrophic markers. Transfection of Hsp70 dose-dependently increased promoter activity of Nppa (Figure 5F) and Myh7 (Figure 5G). Interestingly, this Hsp70-induced activation of the Nppa promoter was completely abolished by transfection of siHdac2, suggesting that Hsp70-induced events are Hdac2-dependent. We also studied whether Hsp70ΔABD, a dominant negative Hsp70 mutant that failed to activate Hdac2, blocks Hsp70-induced activa-

**Figure 3.** Hsp70 physically interacted with Hdac2. A, Hsp70, but not Hsp56 or Hsc70, was immunoprecipitated by Hdac2 in 293T cells. B, Hsp70 immunoprecipitated Hdac2 in H9c2 cells. C, Recombinant glutathione S-transferase–Hsp70 (GST-Hsp70) pulled down recombinant H$_{6}$-tagged Hdac2 (His-Hdac2) in complete cell-free condition. D, A schematic diagram of Hsp70 and its mutants. ABD indicate ATP-binding domain; PBD, peptide-binding domain. E, Hdac2-interacting domain mapping of Hsp70. In vitro–translated Hdac2 was applied to recombinant proteins of His-tagged Hsp70 mutants. Both the NH$_{2}$-terminal region and the COOH-terminal region including PBD are important for the interaction with Hdac2. F, Confocal image showing colocalization of Hdac2 and Hsp70 in the nucleus. G, In vivo interaction of Hsp70 and Hdac2 in the nucleus of H9c2 cells was visualized with the fluorophore protein fragment complementation technique after transfection of phmKGN-MC-Hdac2 and phmKGC-MC-Hsp70.
tion of Nppa promoter. First, the effect of Hsp70/H9004 ABD itself on the promoter activity was examined. Like Hsp70/H9004 N and Hsp70/H9004 C, both of which failed to interact with Hdac2 (Figure 3E), Hsp70/H9004 ABD did not induce promoter activation (supplemental Figure VIII). Addition of dominant negative Hsp70ΔABD completely abolished Hsp70 full-induced −3003 Nppa promoter activity (Figure 5H). Electroporation of Hsp70 caused stress fiber formation (Figure 5I) and an increase in cell size (Figure 5J), clearly demonstrating that Hsp70 induces hypertrophic phenotypes.

**ISP-Induced Hypertrophy Is Blunted in Hsp70.1−/− Mice**

We further confirmed that Hsp70 activates Hdac2 in response to hypertrophic stimuli by utilizing Hsp70.1−/− mice.21 In contrast with Hsp70.1+/− mice, the increase in Hdac2 activity was completely blocked in Hsp70.1−/− mice (Figure 6A). In addition, although ISP could still elevate HW/BW, the increase in HW/BW was significantly blunted in Hsp70.1 mutant mice (Figure 6B and supplemental Table I). Ang II–induced cardiac hypertrophy was also significantly attenuated in the null mice (data not shown). Echocardiogram analysis showed that ISP-induced thickening of ventricular walls was significantly blunted (Figure 6C and see supplemental Table II) in the null mice. We next investigated the responses of hypertrophy markers in Hsp70.1 mutant mice. The increase in either ANF or α-tubulin, an alternate hypertrophy marker,22 was significantly blunted in Hsp70.1−/− mice (Figure 6D).

Our observations that HSP70/HDAC2 induces activation of AKT signaling pathways led us to investigate if Akt/Gsk3β signal activation is impaired in Hsp70.1−/− mice as was previously reported in Hdac2−/− mice.10 Our results...
showed that the phosphorylation of Akt (Figure 6E) and Gsk3β (Figure 6F) in the ISP-treated heart was significantly blunted.

**Heat Shock Induces Cardiac Hypertrophy In Vivo and In Vitro**

Given that Hsp70 is induced by heat shock, the question arises whether heat shock can activate Hdac2 and induce hypertrophy. One-hour heat shock to cardiomyocytes, which induces Hsp70, was sufficient to activate Hdac2 (Figure 7A). To rule out the possible involvement of Hsps other than Hsp70, heat shock was introduced to cardiomyocytes isolated from Hsp70.1−/− mice. Heat shock failed to activate Hdac2 in Hsp70.1-null cardiomyocytes, indicating that heat shock-induced activation of Hdac2 is mediated by Hsp70 (Figure 7B). Heat shock for 1 hour elevated Nppa promoter-luciferase activity (Figure 7C). Likewise, heat shock significantly increased the individual cell size (Figure 7D).

Next, we extended our hypothesis to the animal model by examining whether heat shock to live mice can induce cardiac hypertrophy as well as activation of Hdac2. First, we treated CD1 male mice with single heat shock (30 minutes at 42°C) and measured Hdac2 activity as well as HW/BW; we did not observe any significant changes (data not shown). On the contrary, repeated heat shock for 5 consecutive days dramatically activated Hdac2 (Figure 7E), accompanied by an increase in HW/BW (Figure 7F). The inductions of ANF and Hsp70 were confirmed by Western blot (Figure 7G).

**Discussion**

A model for the HSP70/HDAC2-mediated cardiac hypertrophy signaling pathway is shown in Figure 8. In summary, we found that diverse hypertrophic stimuli activate HDAC2, a class I HDAC, in the early phase of cardiac hypertrophy, and the induction of HSP70 in response to exogenous hypertrophic stimuli is a key regulatory mechanism to activate HDAC2. Our present observations highlight new directions. First, we clearly showed that HDAC2 activation mediates cardiac hypertrophy in response to exogenous stresses. Our work extends prior studies by identifying new aspects of HDAC2 function initiating cardiac hypertrophy. Second, we elucidated a key mechanism bridging HSPs to cardiac hypertrophy. Because HSPs possess a protective function against exogenous stresses including heat or ischemia by inhibiting apoptotic signaling pathways, it is plausible that the heart may react to protect itself from those stresses, especially in the early adaptive phase, by increasing the antiapoptotic cascades such as AKT signaling, possibly via an HSP70/HDAC2 pathway.

**Enzymatic Activation of Hdac2 Is Required for Cardiac Hypertrophy**

Although Trivedi et al observed that Hdac2 knockout mice are resistant to exogenous hypertrophic stresses, the implications of HDAC2 in the development of cardiac hypertrophy are in debate, because other recent work showed that Hdac2 knockout mice are still vulnerable to hypertrophy. However,
these studies with knockout mice may reflect the presence of the enzymes rather than their enzymatic activities. Moreover, a sustained, lifetime loss of the enzyme may not account for the acute enzymatic responses to the exogenous hypertrophic stimuli. Indeed, deletion of one HDAC could cause compensatory activation of redundant HDACs, which may affect the function of individual enzymes.26 In this study, we measured the enzymatic activities of HDACs induced by hypertrophic stimuli, which circumvents either long-term effects of loss of enzyme or compensatory mechanisms of other HDACs in knockout mice, and clearly demonstrated that diverse hypertrophic stimuli induce enzymatic activation of HDAC2. It is noteworthy that activation of HDAC2 preceded substantial hypertrophic events, implying that activation of HDAC2 is not a result but a cause of hypertrophy.

**Hsp70 Regulates Hdac2 Enzymatic Activity**

We found no evidence that alterations in either protein amounts or localization are involved in the mechanism of Hdac2 regulation of cardiac hypertrophy. Likewise, in our experimental model, we did not observe a dramatic increase in the phosphorylation level of the endogenous Hdac2. We next investigated HSP as a regulator of HDAC2 in response to cardiac hypertrophic stresses. We showed that Hsp70 activated Hdac2 by direct association. In addition, we showed that binding capacity of Hsp70 to Hdac2 as well as a functioning domain is required for the activation.
Hsp70 Triggers Cardiac Hypertrophy

In the present study, Hsp70 expression and its association with Hdac2 was transiently increased in the early phase of cardiac hypertrophy, which coincided well with the activation of Hdac2. In addition, we showed that forced expression of Hsp70 exaggerated hypertrophy in cardiomyocytes. This finding was further supported by the in vivo animal model: impairment of Hdac2 activation accompanied the attenuation of the ISP-induced increase in HW/BW in \textit{Hsp70.1}\textsuperscript{−/−} mice.

These results are in contrast to a previous report that hearts from Hsp70 knockout mice are enlarged,\textsuperscript{27} which could be interpreted as showing that Hsp70 may function as an antihypertrophic regulator. Indeed, we also observed that basal levels of some hypertrophic indicators, such as HW/BW or ANF expression, were slightly increased in our 5- to 6-week-old null-mice, although not significantly so. It is noteworthy, however, that in the previous report,\textsuperscript{27} hypertrophy was apparent when the mutant mice grew older than 20 weeks. Considering that heat shock factor (HSF)/HSP70 maintains physiological hypertrophy and that declines of HSF/HSP70 trigger transition to pathological hypertrophy when the stresses are sustained for a long time,\textsuperscript{28} it is quite likely that HSP70 may work to prevent maladaptive hypertrophy by maintaining physiological hypertrophy. Thus, the cardiac hypertrophy induced by the sustained loss of HSP70 in the older knockout mice might be maladaptive and pathological. On the contrary, our experiments focused on seeking the initial triggering signals in the early phase. In agreement with previous reports,\textsuperscript{19,29} we observed that HSP70 is dramatically increased even before the hypertrophic phenotype appears. Moreover, we clearly showed that forced expression of Hsp70 in cardiomyocytes provoked hypertrophy and that

\begin{figure}[h]
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\caption{Heat shock–activated Hdac2 and induced cardiac hypertrophy. A, Heat shock (1 hour at 42°C) significantly increased Hdac2 activity in rat neonatal cardiomyocytes (n=4). B, Heat shock could not activate Hdac2 in \textit{Hsp70.1}\textsuperscript{−/−} cardiomyocytes. C, Heat shock increased −3003 \textit{Nppa} promoter-luciferase activity in cardiomyocytes (n=6). D, Heat shock enlarged cardiomyocytes. E, Repeated heat shock to mice (15 minutes at 42°C/d for 5 consecutive days, n=7) dramatically activates cardiac Hdac2. F and G, Repeated heat shock to CD1 male mice significantly increased HW/BW (F) (n=23) and ANF-expression (G).}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure8.png}
\caption{The HSP70/HDAC2 signal cascade in the development of cardiac hypertrophy. Stress signals such as hypertrophic stimuli or even heat to cardiomyocytes can induce Hsp70 expression, followed by activation of cardiac HDAC2, a class I HDAC. The increase in HDAC2 activity is then expected to induce cardiac hypertrophy by inhibiting antihypertrophic gene expression.}
\end{figure}
response to hypertrophic stimuli was significantly blunted in young Hsp70 knockout mice.

Hsp70 protein is produced by the duplicated genes Hsp70.1 (Hspa1b) and Hsp70.3 (Hspa1a), which differ in only one amino acid. Thus, one may argue that half of the protein is Hsp70.1 (data not shown). Thus, the amount of Hsp70 partially induce the transcription of Hsp70.1 rather than that of Hsp70.3 (data not shown). Thus, the amount of Hsp70 proteins induced by hypertrophic stimuli in Hsp70.1−/− mice must be much less than 50%, which may explain why Hdac2 activation and hypertrophy was significantly blunted in Hsp70.1−/− mice in our study.

The stresses used to induce pathological hypertrophy, such as adrenergic stimuli or AB, can cause the phosphorylation of AKT and GSK3β, both of which are key markers of physiological hypertrophy. In our experimental models, phosphorylation of Akt and Gsk3β was transiently increased in the early phase of hypertrophy induced by ISP and by AB, as well as by swimming. In addition, Hdac2-induced activation of Nppa or Myh7 promoter was blocked by either Akt-1/2 or dominant negative Akt. These results suggest the possible involvement of the AKT signal in the relaying of HDAC2 to hypertrophic phenotypes, consistent with the previous report that Akt-phosphorylation was decreased in Hdac2-knockout mice but was increased in Hdac2-transgenics.

**Heat Shock Induces Cardiac Hypertrophy**

One striking finding of this study is the induction of cardiac hypertrophy by heat shock. First, in the cardiomyocyte model, heat shock induced Hdac2 activation, ANF-expression, and enlargement of the cells. Interestingly, the heat-shock-induced cardiac hypertrophy was reproduced in the animal model; repeated exposure to heat shock activated Hdac2 and increased HW/BW, suggesting that heat shock is a prohypertrophic stimulus.

In summary, we herewith propose that induction of HSP70 and subsequent activation of HDAC2 are important triggering signals of cardiac hypertrophy. It is noteworthy, however, that the HSP70/HDAC2 axis is not the only pathway in hypertrophy signaling. For example, the importance of neurohormonal signals mediated by G-protein and calcium-dependent signaling pathways in cardiac hypertrophy is well established. Nevertheless, the signal cascades proposed in the present work are worthy of further scrutiny, because they may provide novel targets for overcoming or averting cardiac disorders.

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**Disclosures**

None.

**References**


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Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2008/10/10/01.RES.0000338570.27156.84.DC1
**Supplemental Material**

**Online Table I.** Heart weight and body weight in wild type and \textit{Hsp70} knockout mice treated with ISP for 5-7 days

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* p<0.05 with vehicle, ** p<0.01 with vehicle
† p<0.05 with \textit{Hsp70}\textsuperscript{+/+}

NS not significant with vehicle, NS not significant with \textit{Hsp70}\textsuperscript{+-}

Data are mean ± SEM of 15 to 27 mice.
**Online Table II.** Echocardiographic parameters and heart weight in wild type and Hsp70 knockout mice hearts treated with ISP for 2 weeks

<table>
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<tr>
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<th>Hsp70+/-</th>
<th>Hsp70-/-</th>
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<tr>
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<td>Vehicle (n=7)</td>
<td>ISP (n=7)</td>
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<tr>
<td>LVWT, mm</td>
<td>0.81 ± 0.03</td>
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<td>LVDd, mm</td>
<td>3.94 ± 0.09</td>
<td>3.37 ± 0.28</td>
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<td>PWT, mm</td>
<td>0.96 ± 0.06</td>
<td>1.50 ± 0.12 *</td>
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<tr>
<td>LVSd, mm</td>
<td>3.01 ± 0.17</td>
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<tr>
<td>FS, %</td>
<td>24.2 ± 3.40</td>
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<tr>
<td>HW, g</td>
<td>0.14 ± 0.01</td>
<td>0.18 ± 0.01 *</td>
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* p<0.01 in HSP70+/- group  
† p<0.01 in HSP70-/- group

Data are mean ± SEM of 6 to 7 mice. LVWT indicates end-diastolic left ventricular wall thickness; LVDd, LV end-diastolic dimension; PWT, posterior wall thickness; LVSd, LV end-systolic dimension; FS, Fractional shortening; and HW, heart weight.
Online Figure III
Online Figure IV

A

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Online Figure VIII

-3003 Nppa promoter activity (fold change)

mock  Hsp70 full  Hsp70 ΔN  Hsp70 ΔABD  Hsp70 ΔC

**  *
Online Figure Legends

Online Figure I. ISP (A) and AB (B) did not activate Hdac1 (n=4-6). (C) AngII infusion for 5 days increased HW/BW (n=8). (D) PE injection did not increase HW/BW at 1 day (n=6). (E) Swimming induced phosphorylation of Akt at 3 days. (F) Swimming induced increases in HW/BW only at 14 days but not at 3 days (n=12).

Online Figure II. Dominant negative (dn) Akt blocked the increase in the activities of Nppa or Myh7 promoters. (A) Transfection of either Akt wild type (wt) or constitutively active (ca) forms induced the activation of -3003 Nppa promoter, whereas Akt dn did not (n=6). (B) Hdac2-induced activation of -3003 Nppa promoter was significantly reduced by transfection of Akt dn in cardiomyocytes (n=12). (C) Hdac2-induced activation of -299 Myh7 promoter was also abolished by Akt dn (n=6).

Online Figure III. Substitution of H141 in Hdac2 with alanine (H141A) results in a loss of enzymatic activation (n=4-8).

Online Figure IV. Hdac2 regulation mechanism. (A-B) Hdac2 expression was not altered in ISP-treated (A) or AB-heart (B). (C) PE treatment of cardiomyocytes did not alter the expression of Hdac2. Numbers above the pictures represent the harvesting time after the treatment with ISP (A), AB (B), or PE (C).

Online Figure V. Localization and phosphorylation of endogenous Hdac2 were not altered in hypertrophied heart. (A) Hdac2 activity in nuclear but not cytosolic fractions was increased in ISP-treated mouse hearts. Hdac2 enzymatic activities in both nucleus and cytoplasm were measured after the fractionation of the ISP-treated heart lysates. In contrast to nucleus, the increase in Hdac2 in the
cytoplasm was not significant (n=4). (B) ISP did not change the distribution of Hdac2 between the
cytosol and nucleus. Most Hdac2 was localized in the nucleus. However, no shift in the amounts was
observed in the ISP-treated hearts. (C-D) Hdac2 was still localized in the nucleus in the hypertrophic
hearts by ISP (C) or AB (D). Sections were obtained from ISP- or AB-treated hearts and subjected to
immunohistochemistry with Hdac2 antibody. (E) Hdac2 signal was detected only in the nucleus of PE-
treated rat neonatal cardiomyocytes. PE was administered to cardiomyocyte and the cells were
immunostained for Hdac2. (F) Endogenous Hdac2 was not phosphorylated by ISP. The phosphorylation
after the hypertrophic signal was investigated with anti-phosphoserine antibody after pulling-down with
Hdac2 antibody. Although Hdac2 is basally phosphorylated, neither an increase in the amount of
phosphorylated proteins, nor shift of the bands upside was seen.

**Online Figure VI.** Transfection of Hsp70 does not activate Hdac1 (n=4). After the transfection of
pcDNA6-myc-Hsp70, H9c2 cell lysates were immunoprecipitated with Hdac1 antibody and the enzymatic
activity of Hdac was measured.

**Online Figure VII.** The expression of Hsp70 and the phosphorylation of Akt were increased in cardiac
hypertrophy. (A) Hsp70, but not Hsc70, a constitutive heat shock cognate 70, was induced in 5 day AB
heart. (B) Forced swimming for 3 days was enough to induce Hsp70. After training for 8 days, the mice
underwent swimming for 90 min twice daily for 3 consecutive days. Swimming, a typical inducer of
physiological hypertrophy, triggered the induction of Hsp70. (C) One bolus injection of PE for 1 day to
mice significantly induced Hsp70 expression. Note that eventual increases in HW/BW were not observed
with swimming for 3 days (B) or in 1 day after PE-bolus injection (C). (D) AB increased phosphorylation
of Akt at day 5. The increase in phosphorylation was blunted 14 days after AB.
Online Figure VIII. Both PBD and ABD domains of Hsp70 were necessary for the activation of \(-3003\) \textit{Nppa} promoter. Only intact full Hsp70 successfully activated the promoter, whereas the truncated Hsp70s did not, suggesting that both binding and activating capacities are required for the function of Hsp70.
Online Supplementary Methods

Animal model

Animal models for cardiac hypertrophy with ISP, AngII, or AB were confirmed by echocardiogram and pressure gradient as described previously.1,2 Protocols for histology were described previously.1 For PE administration, 6 week-old CD1 mice were injected intraperitoneally with either PE (10 mg/kg at every 12h) or saline. For swimming exercise, 8-week-old BL6 female mice were prepared as described previously.3 Mice were swum in water tanks twice daily for 90 min. Before the swimming exercise, the mice practiced swimming for 8 days. Evaluation of cardiac hypertrophy was done by weighing the heart and body or by measuring the cross-sectional diameter of individual cardiomyocytes after H&E staining in a blind-fashion.2 Hsp70.1−/− mice were described previously.4 Preparation of either the nuclear or the cytosolic fraction from mouse heart was done with a nuclear preparation kit (Panomics Inc., Fremont, CA).

Constructs and transfection study

Preparation of neonatal rat cardiomyocytes was performed as described.5 H9c2 and 293T cells were obtained from the Seoul Korean Cell Line Bank (Seoul, Korea) and were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. pcDNA6-Hsp70-‐myc construct was prepared by subcloning from pCMV-‐human HSP70.1 construct. pET30a-Hsp70, pET30a-Hsp70ΔN, pET30a-Hsp70ΔABD, pET30a-Hsp70ΔC, pCMV-Hsp70, pCMV-Hsp70ΔN, pCMV-Hsp70ΔABD, and pCMV-Hsp70ΔC were kindly provided by Dr. Eui-‐Ju Choi (Korea University, Seoul, Korea).

pCMV SPORT6-‐human Hsp27 constructs were purchased from KUGI (Korean Unigene Information).

pFlag CMV2-Hsp40 and pFlag CMV2-Hsc70 were kindly provided by Dr. Ki Sun Kwon (Korea Research
Institute of Bioscience and Biotechnology, Taejon, Korea). pcDNA6-human HSP56-myc was constructed by RT-PCR-based cloning. pcDNA3.1-HDAC2 wild type-V5/His construct was prepared with pcDNA3.1V5/His TOPO TA construction kit (Invitrogen, Carlsbad, CA) after PCR-amplification of the coding region of pME18S-HDAC2 (kindly provided by Dr. Edward Seto, H. Lee Moffitt Cancer Center and Research Institute, Florida). On the basis of a previous report,6 dominant negative mutant H141A was prepared by site-directed mutagenesis kit (Stratagene. La Jolla, CA). All constructs were confirmed by direct sequencing of the entire coding region.

The transfection study was described previously.1 pGL3-3003 and -638 Nppa-promoter-luciferase constructs (kindly provided by Prof. Young Sook Lee, University of Wisconsin Medical School, Madison, WI) and pGL3-3500 and -299 Myh7-promoter luciferase constructs (kindly provided by Dr. Fadia Haddad, University of California, Irvine, CA) were transfected with Lipofectamine Plus (Invitrogen) to cardiomyocyte or H9c2. Twenty-four hours after transfection, the cells underwent heat shock (42 °C) for an hour and were then maintained at 37 °C for the next 6 hours before harvest for luciferase assay (Promega, Madison, WI).

Electroporation of plasmids into cardiomyocytes was performed according to the optimized protocols provided by the manufacturer (Amaxa Biosystem, Cologne, Germany). Briefly, cells were gently resuspended in 100 μl of rat cardiomyocyte-neonatal nucleofector solution, mixed with DNA plasmids, and pulsed with the program G-09. Immediately after, cells were transferred into prewarmed fresh medium in plates. Cells were analyzed 48 hours after electroporation for cell size determination.

siHdac2
A mixture of four siRNAs (10 nmol/L) targeting rat Hdac2 (ON-TARGET plus™, SMARTpool siRNA,
Catalog number: L-094936-00-0010, Dharmacon, Lafayette, CO) was transfected to H9c2 cells by DharmaFECT® 1 reagent as recommended by manufacturer. Reduction of Hdac2 was confirmed by RT-PCR and western blot (data not shown). Non-targeting siRNA (Accell non-targeting negative control, Dharmacon) was used for as negative control.

**Antibodies**

Anti-phosphoserine antibody was purchased from Zymed. Other antibodies used were as follows: Hsp27, Hsp56 (FKBP59), and Hsp70 from Stressgen; Hsp90α/β, actin and myc from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); Hdac1 and Hdac8 from Upstate Biotechnology; Hdac2 and Hdac3 from Zymed; α-tubulin from Abcam; ANF from Biodesign; V5 from Invitrogen; pAkt, Akt, and pGsk3β from Cell Signaling; and Gsk3β from Santa Cruz Biotechnology. Anti-mouse or anti-rabbit IgG peroxidase-conjugated secondary antibody (Sigma, St. Louis, MO) was used for Western blot analysis, and Alexa Fluor 488-conjugated anti-mouse IgG secondary antibody (Molecular Probes) was used for fluorescent immunocytochemistry.

**Immunoprecipitation and Western blot**

After cells were harvested with lysis buffer [50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP40, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na3VO4, and 1 μg/ml each of leupeptin, pepstatin, and aprotinin], immunoprecipitation and Western blot were performed as described previously.

To detect Hsps or Hdacs in mouse hearts, 50 μg of heart lysates was separated on an SDS-PAGE gel and specific antibodies were applied. In the experiment for Hdac2 phosphorylation, for the negative control, heart lysates were treated with calf intestinal phosphatase (Biolabs, 10 units) for 1 hour with appropriate buffer. The experimental groups were treated with buffers without the phosphatase.
Fluorescent immunocytochemistry, confocal microscopy and cell size measurements

Neonatal rat cardiomyocytes or H9c2 cells were plated on 8-well chambers and fluorescent immunocytochemistry was performed as described previously. Hdac2, Hsp70, or actin was visualized with specific antibodies or with Texas-Red-X phalloidin. Under fluorescent microscopy, cell sizes were determined by use of the i solution program (Image & Microscope Technology Inc., Vancouver, Canada). At least 80 cells were measured for each treatment, and each experiment was repeated three times.

For confocal microscopy, the samples were analyzed with a Leica TCS SP5/Tandem laser confocal scanning microscope (Leica Microsystems, Mannheim, Germany) using an immersion oil objective (lambda blue 63×, N.A. 1.40) at Kores Basic Science Institute, Gwangju Branch. To remove cross-talk image, Images were performed by sequential scan acquisition of the Leica TCS SP5 software.

Binding assay in cell-free system, in vitro translation, GST pull-down assay, and domain mapping study

To investigate the direct interaction between Hdac2 and Hsp70, an in vitro binding assay was performed. Glutathione S transferase (GST)-hsp70 fusion proteins were produced in Escherichia coli (E. coli) and purified. Recombinant hexahistidine-tagged Hdac2 (His-Hdac2) was induced in E. coli and immobilized on Ni²⁺-NTA-agarose beads. After the elution, His-Hdac2 was applied to GST-Hsp70 or GST only beads. The bound Hdac2 was detected with Western blot analysis using Hdac2 antibody.

In vitro transcription/translation was performed as described in the protocol (Invitrogen). In vitro transcription-translated Hdac2 proteins were incubated overnight at 4°C with GST or GST-hsp70 proteins
immobilized on Sepharose 4B beads in a solution containing binding buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.5, 1 mmol/L Na3VO4, 1 mmol/L PMSF, 1 mmol/L NaF, 1% Nonidet P-40, 10% glycerol, and protease inhibitors). The beads were washed and analyzed by Western blot after separation on SDS-PAGE gels.

For the domain mapping study, recombinant hexahistidine-tagged Hsp70 full, Hsp70ΔN, Hsp70ΔABD (ATP-binding domain), and Hsp70ΔC, which lack the COOH-terminal part and PBD (protein binding domain), were induced in *E. coli* and immobilized on Ni2+-NTA-agarose beads. The schematic structures of truncated Hsp70 are shown in Figure 3D. After *in vitro* translated Hdac2 was applied to the beads, the bound Hdac2 was detected with Western blot analysis.

**Hdac assay**

Hdac activity was measured by using the HDAC Fluorimetric Assay/Drug Discovery kit (Biomol International, Plymouth Meeting, PA) with the modifications discussed in a previous report.1 After harvesting hearts from mice, 6 mg of lysates were preincubated with 1.25 μg of specific antibodies or unimmunized IgG. After recovering by protein A/G agarose beads, Hdac enzyme activity was measured in the precipitates. The absorbances from IgG-treated controls were subtracted as the basal level, and the values from Hdac2-precipitated controls were calculated as 100% to obtain the relative activity.

Lysates (2 mg) from cardiomyocytes were also used to measure Hdac activity. In each set of experiments, the experimental condition was duplicated, absorbances were measured three times, and the values were averaged. At least three experimental sets were repeated for each condition.

The Hdac assay was performed with recombinant Hdac2 and Hsp70 proteins. Because recombinant
Hdac2 did not have intrinsic activity in the preliminary study, the cell lysates were applied as described.\textsuperscript{7} His-Hdac2 was incubated with H9c2 cell lysates and either GST-Hdac2 or GST only was applied to the beads. Total amounts of the sepharose A/G beads were maintained equally in each experimental condition. The Hdac assay was done with the bead-containing recombinant protein.

**In vivo visualization of the interaction of Hdac2 and Hsp70**

To visualize the *in vivo* interaction, we utilized fluorescence protein fragment complementation methods with CoralHue® Fluo-Chase kit (MBL International Corporation, Woburn MA).\textsuperscript{8-10} According to the protocol, we generated 4 pairs of constructs: *phmKGN-MC-Hdac2, phmKGN-MC-Hdac2, phmKGN-MN-Hdac2, phmKGC-MN-Hdac2, phmKGN-MC-Hsp70, phmKGN-MC-Hsp70, phmKGN-MN-Hsp70, and phmKGC-MN-Hsp70*. As recommended, four combinational pairs to generate mKGN- and mKGC-chimeric proteins were transfected to H9c2 and HeLa cells and the fluorescence were visualized by fluorescent microscopy. Among the pairs, when the combinations of either *phmKGN-MC-Hdac2 + phmKGC-MC-Hsp70* or *phmKGN-MN-Hdac2 + phmKGC-MC-Hsp70* were transfected, the fluorescence was detected. In the following experiments, pairs of *phmKGN-MC-Hdac2 + phmKGC-MC-Hsp70* were investigated for further analysis (Figure 3G lower left panel).

For the negative control, *phmKGN-MC* and *phmKGC-MC* vectors were transfected together with *phmKGN-MC-Hdac2 and phmKGC-MC-Hsp70*, respectively (Figure 3G upper panels). We observed the similar strong fluorescence signals when those two constructs were co-transfected to HeLa cells (data not shown). For the positive control, pCONT-A (p50 partial domain from NK-κB complex) and pCONT-B (p65 partial domain from NF-κB complex) were co-transfected to H9c2 cells (Figure 3G lower right panel).
RT-PCR and Northern blot

RT-PCR and Northern blot analysis were performed as standard methods as described previously.1,2 The primer sequences used for gene amplification are available upon request.

References for Supplementary Methods


