Adenovirus-Mediated Overexpression of Caveolin-3 Inhibits Rat Cardiomyocyte Hypertrophy

Akimasa Koga, Naoki Oka, Toshio Kikuchi, Hiroshi Miyazaki, Seiya Kato, Tsutomu Imaizumi

Abstract—Caveolae are omega-shaped organelles of the cell surface. The protein caveolin-3, a structural component of cardiac caveolae, is associated with cellular signaling. To investigate the effect of adenovirus-mediated overexpression of caveolin-3 on hypertrophic responses in cardiomyocytes, we constructed an adenovirus that encoded human wild-type caveolin-3 (Ad.Cav-3), mutant caveolin-3 (Ad.Cav-3Δ), or bacterial β-galactosidase (Ad.LacZ). This mutant has been reported to cause human limb-girdle muscular dystrophy. It lacks 9 nucleotides in the caveolin scaffolding domain and behaves in a dominant-negative fashion. Rat neonatal cardiomyocytes were infected with the virus and then harvested 36 hours after infection. In noninfected cells, phenylephrine (PE) and endothelin-1 (ET) increased cell size and [3H]leucine incorporation, along with the induction of sarcomeric reorganization and the reexpression of β-myosin heavy chain, indicating myocyte hypertrophy. Infection with Ad.LacZ had no effect on those parameters. Ad.Cav-3 prevented the PE- and ET-induced increases in cell size, leucine incorporation, sarcomeric reorganization, and reexpression of β-myosin heavy chain. Ad.Cav-3 also blocked the PE- and ET-induced phosphorylations of extracellular signal–regulated kinases (ERKs) but did not affect c-Jun amino-terminal kinase and p38 mitogen-activated protein kinase activities. In contrast, Ad.Cav-3Δ significantly augmented hypertrophic responses to ET, which were associated with increased ET-induced phosphorylation of ERK1/2. These results suggest that caveolin-3 behaves as a negative regulator of hypertrophic responses, probably through suppression of ERK1/2 activity. (Hypertension. 2003;42:213-219.)

Key Words: hypertrophy • receptors, adrenergic • endothelin • myocytes • gene expression

Cardiomyocytes, caveolin-3 might be involved in the regulation of channel functions. The cardiac sodium–calcium exchanger is likely to be associated with caveolin-3. Moreover, a recent report has suggested that caveolin-3 plays a role in the increase in sodium current amplitude in cardiomyocytes. Some reports imply roles for caveolin-3 in pathophysiology of the heart. In the dog with pacing-induced heart failure, expression of caveolin-3 protein is increased. This increase is associated with agonist-stimulated contractile augmentation by inhibition of nitric oxide synthase, suggesting that caveolin-3 is involved in nitric oxide influences on contractility in failing myocardium.

It has been reported that molecules involved in cardiac hypertrophic responses are concentrated in caveolae, eg, G proteins, extracellular signal–regulated kinases (ERKs), Src family kinases, Ras, and protein kinase C. We have reported that caveolin-3 is upregulated in hypertrophied cardiomyocytes induced by phenylephrine (PE). However, the role of caveolin-3 in cardiomyocyte hypertrophy still remains unknown: it might promote hypertrophy, might inhibit hypertrophy, or might simply be a consequence of hypertrophy. We investigated the effect of adenovirus-mediated overexpression of wild-type and dominant-negative caveolin-3 on hypertrophic responses to Gα-dependent agonists in cardiomyocytes.
Methods

Antibodies
An anti–caveolin-3 monoclonal antibody (mAb) was purchased from Transduction Laboratories; an anti–α-c-myc mAb, from Santa Cruz; an anti-ERK1/2 polyclonal antibody (pAb), an anti–phospho-ERK1/2 pAb, an anti–protein kinase (MAP) kinase pAb, an anti–phospho-p38 pAb, an anti–c-Jun amino-terminal kinase (JNK) pAb, and an anti–phospho JNK pAb, from Cell Signaling; biotinylated and horseradish peroxidase–conjugated secondary antibodies, from Amersham; and Alexa Fluor 594–labeled anti-mouse immunoglobulin G from Molecular Probes.

Preparation of Recombinant Adenovirus Vector
We constructed a recombinant adenovirus that encoded human wild-type caveolin-3 (Ad.Cav-3), mutant caveolin-3 (Ad.Cav-3Δ), or bacterial β-galactosidase (Ad.LacZ). This mutant has been reported to cause human limb-girdle muscular dystrophy. It lacks 9 nucleotides encoding threonine, phenylalanine, and threonine in the caveolin scaffolding domain and behaves in a dominant-negative fashion.5,12 The human caveolin-3 cDNA was cloned from autopsied heart by reverse transcription–polymerase chain reaction (PCR) with a primer set containing 5′-ATGGCAGAAGAGCACACAG-3′ and 5′-ATGGGGTATGGGACGTC-3′. To construct Ad.Cav-3, a c-myc-epitope tag was added to the cDNA at the 5′ end, and KpnI sites were added to both ends by PCR. Primers used in this procedure were 5′-GGGTACCCCATGGGACGTTAATCTGGATTGACG4-3′ and 5′-GGGTACCCCATGGGACGTTAATCTGGATTGACG3′. To construct Ad.Cav-3Δ, we deleted the 9 nucleotides (bp 186 to 194) from the wild-type cDNA with use of a site-directed mutagenesis kit (Stratagene). Primers used in this procedure were 5′-GTGGAAAGGT-GAGGTACCCCATGGGACGTTAATCTGGATTGACGTC-3′. To confirm the adenovirus-mediated expression of caveolin-3 protein, we performed immunoblot analysis of cardiomyocytes with the anti–caveolin-3 and the anti–c-myc mAbs (Figure 1). In noninfected cardiomyocytes, a single band of ≈20 kDa was detected in caveolin-3 blots. A band of ≈25 kDa could be detected 12 hours or more after infection with Ad.Cav-3, in association with an increased expression of 20-kDa proteins (Figure 1A). To verify that Ad.Cav-3 produces 20-kDa protein as well as 25-kDa protein that were myc-tagged caveolin-3, human embryonic kidney (HEK) 293 cells, which do not express caveolin-3, were infected with Ad.Cav-3. Infection of HEK293 cells with Ad.Cav-3 induced both 20- and 25-kDa proteins in a time- and dose-dependent manner. Thus, Ad.Cav-3 infection might produce both 20-kDa protein of the native form and 25-kDa myc-tagged caveolin-3. In Figure 1B, dose dependencies of the immunoblots of these proteins are shown at 36 hours after viral infection. Recombinant caveolin-3 protein was dose-dependently upregulated, as shown in blots of cardiomyocytes infected with Ad.Cav-3 at 10 MOI or more. On the basis of these results, cardiomyocytes that were infected with 100 MOI of the virus and at 36 hours after infection were used for the following experiments.

Effect of Ad.Cav-3 Infection on Hypertrophic Responses to Agonists
For an in vitro model of cardiac hypertrophy, we used PE (α1-adrenergic agonist) and ET. α1-Adrenergic receptors share common intracellular signaling pathways with ET.15,16 These agonists bind to specific receptors that belong to the G protein–linked superfamily of heptahelical transmembrane proteins and chiefly activate Gαs. Activation of Gαs might be both necessary and sufficient to cause hypertrophy in cardiomyocytes.15,16 In noninfected cells, the cell area was signifi-
nonstimulated cells did not induce sarcomeric reorganization (data not shown). Infection with Ad.LacZ of those cells (Figure 3) and significantly (P<0.01) augmented phosphorylation of ERK1/2 and JNK in cardiomyocytes infected with Ad.LacZ (Figure 6). p38 MAP kinase was not phosphorylated by those agonists. The agonist-induced phosphorylation of ERK1/2 was significantly (P<0.01) inhibited by infection with Ad.Cav-3; however, that of JNK was not altered.

Effect of Dominant-Negative Caveolin-3 on Hypertrophic Responses to ET

Finally, we investigated the effect of dominant-negative caveolin-3 on hypertrophic responses to ET. The viability of cells infected with Ad.Cav-3Δ was comparable to those infected with Ad.Cav-3 or Ad.LacZ (data not shown). In contrast to the effect of the overexpression of wild-type caveolin-3, Ad.Cav-3Δ significantly augmented the ET-induced [3H]leucine incorporation into cardiomyocytes (Figure 7A). This augmentation was associated with increased phosphorylation of ERKs (Figure 7B).

Discussion

We have previously reported that the expression of caveolin-3 was upregulated in α1-adrenergic agonist–induced hypertrophied cardiomyocytes.11 However, it had not yet been clarified whether the upregulation of caveolin-3 in cardiomyocyte hypertrophy was simply a consequence of
hypertrophy, promoted hypertrophy, or inhibited hypertrophy. In this study, we overexpressed caveolin-3 in cardiomyocytes and investigated the effect of that overexpression on hypertrophic responses to Gq-dependent agonists. We demonstrated that the overexpression of caveolin-3 inhibited hypertrophic responses. In addition, dominant-negative caveolin-3 augmented the hypertrophic responses. Thus, our results might suggest that endogenous caveolin-3 act as an inhibitor of myocyte hypertrophy.

Methodologic Considerations
To modulate the level of intracellular caveolin-3 protein expression and to explore the function of caveolin-3, we constructed an adenovirus harboring human caveolin-3 cDNA and infected myocytes with the virus. As shown in the Results, the virus produced caveolin-3 protein in time- and dose-dependent manners. Although construction of the virus

Figure 2. Overexpression of caveolin-3 suppresses development of cardiomyocyte hypertrophy. A, Light microscopic observations. Cells were infected with or without the indicated virus and then treated with agonists. B, Relative myocyte area. Graph shows cell size relative to vehicle-treated, noninfected cells. *P<0.001 vs vehicle without infection; †P<0.001 vs Ad.LacZ treated with the indicated agonist. C, [3H]leucine incorporation into myocytes. Shown is [3H]leucine incorporation relative to vehicle-treated, noninfected cells. *P<0.001 vs vehicle without infection; †P<0.001 vs Ad.LacZ treated with the indicated agonist. In Ad.LacZ-infected cells, both cell area and leucine incorporation into myocytes were significantly increased by both PE and ET. Infection with Ad.Cav-3 reduced those responses.

Figure 3. Caveolin-3 overexpression inhibits agonist-induced sarcomeric reorganization. Cells were stained with phalloidin-FITC to assess sarcomeric reorganization. In Ad.LacZ-infected cardiomyocytes, both agonists markedly induced sarcomeric reorganization. Ad.Cav-3 prevented agonist-induced sarcomeric reorganization.

Figure 4. Overexpression of caveolin-3 suppresses reexpression of $\beta$MHC in hypertrophied cardiomyocytes. Immunoblotting was performed with anti-polyclonal $\beta$MHC. In Ad.LacZ-infected cardiomyocytes, expression of $\beta$MHC was significantly increased by PE and ET. Ad.Cav-3 attenuated those responses. *P<0.001 vs vehicle; †P<0.001 vs Ad.LacZ treated with the indicated agonist.
vector encoding caveolin-1 has been reported, to our knowledge, the present study is the first report of construction of the adenovirus harboring caveolin-3 cDNA. Viral infection not only produced the band of 25 kDa (recombinant caveolin-3 with myc-epitope tag) but also increased the band of 20 kDa. The increased band of 20 kDa would be a splicing variant that was produced by use of the start codon of a native caveolin-3 sequence. Although viral infection increased the protein level of caveolin-3, the cellular localization of the recombinant, wild-type caveolin-3 protein was similar to that of endogenous caveolin-3. Thus, our results indicate the overexpression of recombinant, wild-type caveolin-3 as a useful tool for investigation of the function of caveolin-3.

Inhibition of Myocyte Hypertrophy by Caveolin-3

In this study, we examined cardiomyocyte hypertrophy by several methods. PE and ET increased leucine incorporation and cell area and changed the immunostaining pattern of phalloidin from a dense staining without agonists to fiberlike staining, indicating that myocyte hypertrophy did occur at the protein and structural levels. Moreover, βMHC expression was markedly augmented by PE and ET, suggesting the transformation of myosin. These results indicate that PE and ET induced pathologic hypertrophy in cardiomyocytes. Infection with Ad.Cav-3 prevented all of these changes induced by PE and ET. Thus, caveolin-3 might act as an inhibitor of myocyte hypertrophy. The effects of overexpression of wild-type caveolin-3 were not nonspecific, because infection with Ad.LacZ did not affect myocyte hypertrophy. Overexpression of caveolin-3 in Ad.Cav-3–infected cells was associated with the prevention of both agonist-induced hypertrophy and sarcomeric disorganization seen in Ad.LacZ-infected and control cells with the same stimulus. It has been reported that caveolin-3–null mice have degeneration of muscle fibers in skeletal muscle but not in cardiac muscle.17,18 These results might appear to contradict ours; however, that study was done without stress. Caveolin-3 in the heart might not be an essential protein under normal conditions but might play a pivotal role under pathologic conditions.

Hypertrophic responses to Gq-dependent agonists are mediated by several intracellular signaling cascades. Because the MAP kinase family is 1 of the critical components of the Gq-dependent signaling pathway, we examined the involvement of the MAP kinase family in the attenuation of hypertrophy by Ad.Cav-3. As shown in Figure 6, PE and ET phosphorylated ERK and JNK, as reported previously, but not p38. It is not clear in our study why p38 was not phosphorylated by Gq-dependent agonists. Only ERK phosphorylation was prevented by the overexpression of wild-type caveolin-3. Previously, an interaction between caveolins and ERKs has been reported by several investigators.20–23 ERKs are concentrated in caveolae at least partially, and ERK2 activity is regulated by the scaffolding domain of caveolins in vitro. Park and coworkers21 have reported that all caveolin isotypes are upregulated in senescent rat cells and organs, including the heart, in association with a reduction in epidermal growth factor–induced phosphorylation of ERKs. Their report suggests that upregulation of caveolin attenuates epidermal growth factor signaling including ERKs in vivo. Moreover, Furuchi and Anderson22 have shown that choles-
Overexpression of dominant-negative caveolin-3 augments ET-induced hypertrophic responses of cardiomyocytes through hyperactivation of ERKs. A, [3H]leucine incorporation into cardiomyocytes infected with Ad.Cav-3Δ or Ad.LacZ. Adenovirus encoding dominant-negative caveolin-3 cDNA (Ad.Cav-3Δ) was used to infect cardiomyocytes. Hypertrophic response to ET was assessed by [3H]leucine incorporation into cardiomyocytes. Leucine incorporation was augmented by Ad.Cav-3Δ compared with the infection with Ad.LacZ. B, Phosphorylation of ERKs in cells infected with Ad.Cav-3Δ or Ad.LacZ. Ad.Cav-3Δ augmented phosphorylation of ERKs. *P<0.001 vs Ad.LacZ treated with the indicated agonist.

Perspective

The current study provides insight into the role of caveolin-3 in the development of cardiomyocyte hypertrophy. Our results suggest that caveolin-3 behaves as an endogenous, negative regulator of hypertrophic response, probably via suppression of ERK1/2 activity. Given this crucial role in cardiomyocyte hypertrophy, caveolin-3 might represent an important target for treatment of cardiomyocyte hypertrophy.

Acknowledgments

This study was supported by grants from the Kimura Memorial Heart Foundation (to N.O.), the Kaibara Morikazu Medical Foundation (to N.O.), a grant-in-aid for encouragement of young scientists from the Ministry of Education, Science, Sports and Culture of Japan (to A.K. and N.O.), and a grant for science frontier research promotion centers from the Ministry of Education, Science, Sports and Culture, Japan.


Adenovirus-Mediated Overexpression of Caveolin-3 Inhibits Rat Cardiomyocyte Hypertrophy

Akimasa Koga, Naoki Oka, Toshio Kikuchi, Hiroshi Miyazaki, Seiya Kato and Tsutomu Imaizumi

Hypertension. 2003;42:213-219; originally published online July 7, 2003; doi: 10.1161/01.HYP.0000082926.08268.5D

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/42/2/213

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/