Caveolae are flask shaped, 50-100 nm vesicular invaginations of the plasma membrane characterized by high contents of cholesterol and glycosphingolipids [1]. Caveolin, an approximately 20-kDa integral membrane protein, is a principal component of the caveolae membranes. There have been at least three subtypes of caveolin; caveolin-1, -2 are found in many tissues including adipocytes and vascular endothelial cells while caveolin-3 is found exclusively in muscle tissues such as cardiac myocytes [2]. Caveolin is known to compartmentate multiple molecules involved in various signaling pathways and modify their function. Recent studies from mul-

Caveolin-3 inhibits growth signal in cardiac myoblasts in a Ca$^{2+}$-dependent manner

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Received: January 6, 2006; Accepted: February 1, 2006

Abstract

Caveolin, a major protein component of caveolae, directly interacts with multiple signaling molecules, such as Ras and growth factor receptors, and inhibits their function. However, the role of the second messenger system in mediating this inhibition by caveolin remains poorly understood. We examined the role of Ca$^{2+}$-dependent signal in caveolin-mediated growth inhibition using a rat cardiac myoblast cell line (H9C2), in which the expression of caveolin-3, the muscle specific subtype, can be induced using the LacSwitch system. Upon induction with IPTG and serum-starvation, the expression of caveolin-3 was increased by 3.3-fold relative to that of mock-induced cells. The recombinant caveolin-3 was localized to the same subcellular fraction as endogenous caveolin-3 after sucrose gradient purification. Angiotensin II enhanced ERK phosphorylation, but this enhancement was significantly decreased in caveolin-3-induced cells in comparison to that in mock-induced cells. Similarly, when cells were stimulated with fetal calf serum, DNA synthesis, as determined by $[^{3}H]$-thymidine incorporation, was significantly decreased in caveolin-3-induced cells when cells were treated with Ca$^{2+}$-chelator (BAPTA and EGTA), however, this attenuation was blunted. Calphostin (PKC inhibitor), but not cyclosporine A treatment (calcineurin inhibitor), blunted this attenuation in caveolin-3 induced cells. Our findings suggest that caveolin exhibits growth inhibition in a Ca$^{2+}$-dependent manner, most likely through PKC, in cardiac myoblasts.

Keywords: protein kinase C • calcineurin • H9C2 cells • caveolin-3

Introduction

Caveolae are flask shaped, 50-100 nm vesicular invaginations of the plasma membrane characterized by high contents of cholesterol and glycosphingolipids [1]. Caveolin, an approximately 20-kDa integral membrane protein, is a principal component of the caveolae membranes. There have been at least three subtypes of caveolin; caveolin-1, -2 are found in many tissues including adipocytes and vascular endothelial cells while caveolin-3 is found exclusively in muscle tissues such as cardiac myocytes [2]. Caveolin is known to compartmentate multiple molecules involved in various signaling pathways and modify their function. Recent studies from mul-

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tiple laboratories have demonstrated that caveolin directly inhibits the function of molecules involved in cell growth, such as G proteins [3], src tyrosine kinases [4], mitogen-activated protein kinase cascades [5], adenylyl cyclase [6], protein kinase A [7], protein kinase C [8], epidermal or platelet derived growth factor receptor [9, 10], or transforming growth factor receptor pathways [7]. Accordingly, it is now widely believed that caveolin is an inhibitor of cellular growth and proliferation [11].

Despite the above findings, the molecular mechanism of caveolin-mediated inhibition of cardiac growth or hypertrophy has not been well established. The role of each caveolin subtype and molecules that potentially interact with each subtype may also be different, as reviewed recently [12]. A recent study suggested an inhibitory effect of caveolin on the development of cardiac hypertrophy, presumably through regulating ERK activity [13]. As to the exact target of caveolin, however, it is very difficult to determine specific molecules that play a major role(s), simply because caveolin inhibits numerous molecules involved in those pathways. Further, most, if not all, of such studies have been conducted in in vitro assays using partially purified enzymes or caveolin peptides and thus the exact target of caveolin in intact cells has not always been well understood. In this study, we have focused on the second messenger system, in particular, that with Ca^{2+}. We have examined the role of caveolin-3 in the growth of cardiac myoblasts by the use of the caveolin-3 induction system, in which caveolin-3 expression was inducible within the clonal cell line. We thereby demonstrate the involvement of protein kinase C (PKC) in caveolin-mediated growth inhibition.

Materials and methods

Materials

Dulbecco’s modification of Eagle’s medium (DMEM), fetal calf serum (FCS), penicillin, and streptomycin were obtained from GIBCO BRL (Rockville, MD). Anti-caveolin-3 monoclonal antibody was obtained from Transduction Laboratories (Lexington, KY). Anti-phospho ERK polyclonal antibodies were obtained from Promega Corporation (Madison, WI). Horseradish peroxidase-linked goat anti-rabbit and goat anti-mouse IgG were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). [3H] Methyl-thymidine was purchased from NEN Life Science Products, Inc. (Boston, MA). Most other reagents were purchased from Sigma (St. Louis, MO) unless specified.

Cell culture

H9C2 cells were cultured in DMEM supplemented with 10% FCS, penicillin, streptomycin and equilibrated with 95% air 5% CO2 at 37°C. When indicated, transfected cells were treated with 10mM isopropyl-D-thiogalactopyranoside (IPTG) various time periods to induce caveolin-3 expression.

Transfection and selection of stable cell lines

Using the LacSwitch Inducible Mammalian Expression system (Stratagene, Inc. La Jolla, CA), we established the stably transfected H9C2 subclones for inducible expression of caveolin-3. Briefly, cDNA of caveolin-3 with myc epitope tag was subcloned into the multiple cloning site (MCS) of the operator vector (pOPRSVI/MCS) of the inducible Lac-Switch system. The vector contains G-418 resistance and the Rous sarcoma virus (RSV) promoter, which drives expression of the gene inserted into the MCS in the control of Lac repressor. H9C2 cells were co-transfected with pOPRSVI-caveolin-3 operator vector and pCMVLacl repressor vector which encoding lac-repressor and hygromycin resistance. After the incubation in medium with 1mg/ml G-418 and 100 μg/ml hygromycin, resistant colonies were isolated. Subclones expressing caveolin-3 were obtained by screening cells that exhibited high caveolin-3 levels by immunoblotting. Induction was carried out for 48 h by incubation in medium containing 10 mM IPTG.

Electron microscopy

The cultured cells in each experimental group were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 24 h. After rinsing, the specimens were postfixed in 1% osmium tetroxide in 0.1% phosphate buffer with 0.5% potassium ferrocyanide for 1 h at 4°C, and dehy-
drated with graded ethanol, and embedded in epon-araldite mixture. Vertical thin sections of the cultured cells were made, and were examined without staining in a Hitachi H-600 transmission electron microscope.

**[3H]-thymidine incorporation**

H9C2 cells were plated onto 24-well culture plates. After 48 h in serum-free media, the cells were incubated in media containing 2% FCS for 20 h. When indicated, various inhibitors were added 1 h before exposure to FCS. [3H]-thymidine (1μCi/ml) was added for the last 4 h of incubation. After incubation, the medium was aspirated, and the cells were washed three times with cold PBS, 10% trichloroacetic acid. DNA was extracted with 0.1% NaOH. The precipitants were filtrated through Whatman GF/B filters and washed three times with cold ethanol and dried, followed by counting in vials containing 5 ml of scintillant. The radioactivity was determined in a liquid scintillation counter (LS 5801, Beckman Instruments Inc. Fullerton, CA).

**Cell fractionation**

Caveolin-enriched membrane fractions were prepared. Briefly, cardiac tissues or cells were homogenated in 2 ml of 500 mM sodium carbonate (pH 11.0) with protease inhibitors (1 μg/ml leupeptin, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 50 U/ml egg white trypsin inhibitor) and lysed by sonication (three 10-s bursts with minimal output power) using a Branson sonicator 250 (Branson Ultrasonic Corp.). The lysate was then adjusted to 45% sucrose by mixing with 2 ml of 90% sucrose prepared in MBS (25 mM Mes, pH 6.5, 0.15 mM NaCl) and placed at the bottom of 5% and 35% discontinuous sucrose gradient (in MBS containing 100 mM sodium carbonate) for an overnight ultracentrifugation (260,000 g). Fractions were removed sequentially from the top and designated as fractions 1 through 13.

**Immunoblot analysis**

Subconfluent cells were serum–starved for 48 h before treatment. 100 nM angiotensin were added for 15 min, and cells were washed twice with ice-cold phosphate buffered saline and lysed in lysis buffer containing 50 mM Tris/HCl, 1mM EDTA, 1mM DTT, 200mM sucrose, 1 μg/ml leupeptin, 0.1 mM PMSF, 50 U/ml egg white trypsin inhibitor, 1mM sodium pyrophosphate, 1mM sodium orthovanadate, and 20 mM NaF. Equal amounts of total protein from each lysate were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to PVDF membranes. Non-specific binding was blocked by the incubation for 1 h with 5% skim milk, 5% BSA in phosphate buffered saline containing 0.1% Tween 20. Immunoblot analysis was carried out with each specific antibody. The blots were then washed extensively and incubated with horseradish peroxidase-linked goat anti-rabbit IgG. Bands were visualized by the Western blotting detection system from PIERCE (Rockford, IL).

**Data analysis and statistics**

All data were expressed as means ± S.D. A value of p<0.05 was considered statistically significant.

**Results**

**Induction of recombinant caveolin-3 expression in H9C2 cells**

To examine the effect of overexpressing caveolin-3 on the growth of cardiac myoblasts, we established H9C2 subclones for inducible expression of caveolin-3. H9C2 cells were co-transfected with the Lac operator vector harboring a full-length caveolin-3 cDNA and the Lac repressor vector, and were screened for subclones with high caveolin-3 inducibility by IPTG. The time course of changes in the expression of recombinant caveolin-3 following IPTG treatment was examined by immunoblot (Fig. 1). The amount of recombinant caveolin-3 was increased gradually after IPTG treatment and reached the maximum level at 48 h. The peak expression level of total caveolin-3 was 3.3-fold greater than that of total caveolin-3 in mock-induced cells, and was maintained for at least another 48 h. As recombinant caveolin-3 was expressed with a myc epitope tag, the band appeared larger in size compared with the endogenous caveolin-3 (Fig. 1).

We then examined the intracellular localization of recombinant caveolin-3 by immunoblotting
after cell protein fractionation. As shown in Fig. 2, recombinant caveolin-3 was detected only in the high buoyancy fractions in the same manner as the endogenous caveolin-3, i.e., fractions 5 and 6 at the boundary between 5 and 30% sucrose layers.

We also examined whether recombinant overexpression of caveolin-3 increased the formation of caveolae in H9C2 cells. As shown in Fig. 3, electron microscopic examination detected an increased number of caveolae in caveolin-3 overexpressed cells. Thus, the overexpression of caveolin-3 lead to increased caveolin protein expression and caveolar formation.

**Effect of recombinant caveolin-3 overexpression on angiotensin-induced ERK activation in H9C2 cells**

We next examined the functional consequence of overexpressing caveolin-3. We examined changes in angiotensin-induced ERK activation, major growth stimulation in cardiac cells, between caveolin-3- and mock-induced cells from the same subclone. As shown in Fig. 4, angiotensin stimulation (100 nM) led to increased ERK phosphorylation in H9C2 cells in both caveolin-3- and mock-induced cells. However, the magnitude of ERK
activation was significantly decreased in caveolin-3-induced cells (by 35.7 ± 6.6 %, $P<0.05$, $n=4$), suggesting that the overexpression of caveolin-3 inhibited angiotensin-induced ERK signal. Similar, but somewhat less changes were observed when 2% FCS was used instead of angiotensin.
Effect of recombinant caveolin-3 expression on growth of H9C2 cells and the role of Ca\(^{2+}\) signal

To examine the effect of caveolin-3 overexpression on the growth of H9C2 cells, we measured \[^{3}H\]-thymidine incorporation. In the presence of 2% FCS, \[^{3}H\]-thymidine incorporation was increased dramatically in both caveolin-3- and mock-induced cells. However, this increase was significantly smaller in caveolin-3-induced cells (by 20.4 ± 7.6 %, * P<0.05, n=4) than in mock-induced cells. Ca\(^{2+}\) chelation was performed by incubating cells with 5μM BAPTA and 1 mM EGTA for 1 h, followed by 2% FCS treatment. Note that the attenuation of DNA synthesis by caveolin-3-induction was abolished by Ca\(^{2+}\) chelation.

Accordingly, we examined potential involvement of molecules that are regulated by Ca\(^{2+}\) within the growth pathway. \[^{3}H\]-thymidine incorporation was examined in the presence of calphostin C, a PKC inhibitor, or cyclosporine A, a calcineurin inhibitor; both are known to be activated by Ca\(^{2+}\) and stimulate growth signal. As shown in Fig. 6, cyclosporine A treatment significantly inhibited \[^{3}H\]-thymidine incorporation in both caveolin-3- and mock-induced cells. However, the difference in \[^{3}H\]-thymidine incorporation between caveolin-3- and mock-induced cells remained unchanged (by 41.2 ± 4.7 %, * P<0.05, n=4), suggesting that calcineurin is not responsible for caveolin-3-mediated inhibition. In contrast, calphostin C treatment decreased \[^{3}H\]-thymidine incorporation in both caveolin-3- and mock-induced cells, and the difference between the two cells disappeared. These findings suggest that caveolin-3 most likely inhibited growth signal through the inhibition of PKC, but not calcineurin.
In this study, we have demonstrated that caveolin-3 overexpression in cardiac myoblasts resulted in the inhibition of growth signal as demonstrated by significant attenuation of ERK signal and thymidine uptake activity. This inhibition appeared involving Ca\(^{2+}\) signal because the effect of caveolin-3 overexpression was negated in the presence of Ca\(^{2+}\) chelation. We found that it was most likely through inhibiting PKC, but not calcineurin, among various Ca\(^{2+}\)-dependent signal pathways.

Recent studies have demonstrated a major role of calcineurin in developing cardiac hypertrophy. Calcineurin is a calmodulin-binding, broad serine/threonine phosphatase [14]. Treatment of cardiac myocytes with the calcineurin inhibitors such as cyclosporine A successfully blocked the development of growth factor-induced hypertrophy [15]. Transgenic overexpression of active calcineurin led to the development of severe cardiac hypertrophy and failure. Similarly, adenovirus-mediated introduction of calcineurin induced hypertrophy in cultured cardiac myocytes [16, 17]. These findings indicated that calcineurin, and more likely the targets of calcineurin, such as nuclear factor of activated T cells, play a major role in the development of cardiac hypertrophy. However, calcineurin is not likely the major target of caveolin-mediated inhibition of growth signal, at least, in cardiac myoblasts.

Our findings instead suggested that PKC played a major role in caveolin-mediated inhibition of cardiac growth signal. Cardiomyocytes express multiple PKC subtypes that play key roles in a spectrum of adaptive and maladaptive cardiac responses including the development of hypertrophy [18]. In vitro experiments by using caveolin scaffolding domain peptides demonstrated a potent inhibition of PKC kinase activity or the translocation of PKC upon activation by growth factor stimulation [8, 19]. PKC subtypes are located in caveolae and are inhibited by caveolin-3; however, this inhibition occurred in a PKC-subtype specific manner [8]. The scaffolding domain peptide of caveolin-3, but not -2, inhibit-
ed the kinase activity of PKC-α and -ζ, but not of PKC-ε, indicating that the inhibition of PKC by caveolin occurs to specific PKC subtypes. Because PKC-α is a conventional, Ca^{2+}-dependent subtype, and is also abundantly expressed in the heart, it is most likely involved in the caveolin-mediated inhibition of cardiac growth signal in our study as well. More recently, a study from our group has demonstrated that the molecules involved in α1-adrenergic signaling, a major stimulatory signal for cardiac hypertrophy, are accumulated in caveolae; these molecules included the α1-adrenergic receptor, Gq, and PLC-β subtypes (PLC-β1, -β3) [20]. Of note, a decrease in caveolin-3 expression was accompanied by the development of cardiac hypertrophy in rats, suggesting that disinhibition of growth signal by the reduction of caveolin played a role in promoting cardiac hypertrophy [20].

Putting together, PKC may be a major target of caveolin-3 mediated inhibition of cardiac growth even though we do not deny the involvement of calcineurin in cardiac growth and PKC-mediated stimulation of cardiac growth. To further confirm this, targeted disruption of PKC subtypes or calcineurin by the use of siRNA technique, for example, would be required. It has been demonstrated that calcineurin promoted PKC and ERK activation in cardiac myocytes [21]. In T cells, calcineurin crosstalks with PKC and PKA signaling pathways in the regulation of cytokine gene expression [22, 23]. These findings suggest the presence of crosstalk between calcineurin and PKC pathways to regulate hypertrophic responses in coordination with other intracellular signal transduction pathways. Nevertheless, our findings suggest that caveolin-3 is a potent inhibitor of cardiac growth and thus hypertrophy. The hypertrophic growth of the myocardium is induced by load to the heart, initially as a compensatory response. However, sustained cardiac hypertrophy is associated with the development of heart failure, dilated cardiomyopathy, arrhythmia, and sudden death [24]. Thus, inhibition of cardiac hypertrophy is thought to result in improvement of prognosis of patients with cardiovascular disease. In this regard, it might be possible to utilize caveolin as a tool to inhibit or modify the course of the development of cardiac hypertrophy in the future therapy [13].

**Aknowledgements**

Contract grant sponsor: United States Public Health Service; **Contract grant number:** GM067773. **Contract grant sponsors:** the Japanese Ministry of Education, Culture, Sports, Science, and Technology, the Japan Space Forum, and the Kitsuen Kagaku Research Foundation.

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