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Caveolin and β_1-integrin Coordinate Angiotensinogen Expression in Cardiac Myocytes

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Abstract

Background—The cardiac renin-angiotensin system (RAS) has been implicated in mediating myocyte hypertrophy and remodeling, although the biochemical mechanisms responsible for regulating the local RAS are poorly understood. Caveolin-1 (Cav-1)/Cav-3 double-knockout mice display cardiac hypertrophy, and *in vitro* disruption of lipid rafts/caveolae using methyl-β-cyclodextrin (MβCD) abolishes cardiac protection.

Methods—In this study, neonatal rat ventricular myocytes (NRVM) were used to determine whether lipid rafts/caveolae may be involved in the regulation of angiotensinogen (Ao) gene expression, a substrate of the RAS system.

Results—Treatment with MβCD caused a time-dependent upregulation of Ao gene expression, which was associated with differential regulation of mitogen-activated protein (MAP) kinases ERK1/2, p38 and JNK phosphorylation. JNK was highly phosphorylated shortly after MβCD treatment (2 – 30 min), whereas marked activation of ERK1/2 and p38 occurred much later (2 – 4 h). β_{1D}-integrin was required for MβCD-induced activation of the MAP kinases. Pharmacologic inhibition of ERK1/2 and JNK enhanced MβCD-induced Ao gene expression, whereas p38 blockade inhibited this response. Adenovirus-mediated expression of wild-type p38α enhanced MβCD-induced Ao gene expression; conversely expression of dominant negative p38α blocked the stimulatory effects of MβCD. Expression of Cav-3 siRNA stimulated Ao gene expression, whereas overexpression of Cav-3 was inhibitory. Cav-1 and Cav-3 expression levels were found to be positively regulated by p38, but unaffected by ERK1/2 and JNK.

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Conclusion—Collectively, these studies indicate that lipid rafts/caveolae couple to Ao gene expression through a mechanism that involves β_1-integrin and the differential actions of MAP kinase family members.

Keywords

Caveolae; Angiotensinogen; MAP kinases; Cardiac myocytes

1. Introduction

The renin-angiotensin-system (RAS) plays a key role in mediating ventricular hypertrophy and remodeling [1, 2]. Treatment with angiotensin converting enzyme (ACE) inhibitors or angiotensin II (Ang II) type-I receptor (AT_1) blockers significantly improve cardiac function, as well as reverse ventricular remodeling and reduce morbidity and mortality in patients with heart failure [3]. Angiotensinogen (Ao), a substrate of the RAS system, has been implicated in the pathogenesis of hypertension and congestive heart failure. Angiotensin II, the most biologically active peptide of the RAS, affects several aspects of cardiac function including contractility, cell metabolism, cellular growth, differentiation, apoptosis and gene expression [4]. Progression of heart failure is associated with a steady increase of Ang II formation by the myocardium, regardless of the underlying etiology [1, 2]. We have recently reported that β_1-integrin reciprocally regulates mechanical stretch-induced MAP kinase activation [5], and stress activated kinases p38 and JNK play important roles in mechanical stretch-induced regulation of Ao gene expression [6]. However, the specific components coupling β_1 integrin to p38 and JNK activation remain to be determined.

Lipid rafts, plasma membrane domains formed through the association of sphingolipid and cholesterol, have become recognized as essential for many types of cellular signal transduction processes. Caveolins, 22–24 kDa scaffolding proteins which associate with lipid rafts to form caveolae, have been shown to be important structural elements involved in myocyte signal-transduction [7]. Three caveolin isoforms, Cav-1, Cav-2 and Cav-3 have been identified. Cav-1 and Cav-2 are expressed in most cell types, whereas Cav-3 is primarily expressed in striated muscle [8]. In addition to concentrating signal transducers within a distinct region of the plasma membrane [9, 10], caveolins functionally regulate the activation states of several caveolae-associated signaling molecules by sequestering them in an inactive state [11]. Caveolae have also been shown to be required for β_1-integrin-mediated signaling [12], indicating that a functional link exists between β_1-integrins and caveolae. Stretch-induced translocation of caveolin from caveolae to non-caveolar membrane sites has been shown to be critical for coupling integrin signaling to ERK activation in vascular smooth muscle cells [13]. Additionally, caveolae and caveolins have been implicated in the wide range of cardiac protection [14]. It has been suggested that caveolin in caveolae may sequester ERK and prevent its activation, but translocation of caveolin to noncaveolar sites, in response to stretch, induces caveolin-mediated ERK activation through an association with β_1-integrin [13]. Although caveolae and caveolins are important for regulating β_1-integrin-mediated ERK activation, much less is known regarding the importance of caveolins in coupling β_1-integrin to p38 and JNK activation. In this study, we tested the hypothesis that caveolae, together with β_1 integrin and MAP kinases, modulate Ao gene expression in neonatal rat cardiac myocytes.

2. Materials and methods

2.1. Antibodies and reagents

Phospho-ERK-p44/42-Thr^{202}/Tyr^{204} antibody (9101), ERK polyclonal antibody (9102), phospho-p38-Thr^{180}/Tyr^{182} antibody (9211), p38 antibody (5F11), phospho-SAPK/JNK-Thr^{183}/Tyr^{185} antibody (9251), JNK antibody (9252) and horseradish peroxidase-conjugated secondary antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (MAB1501) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Bovine serum albumin (BSA, diagnostic grade K) was obtained from Celluliance (Kanakee, IL). Enhanced chemiluminescence (ECL) reagent (Western Lightning™) was obtained from Perkin Elmer Life Science (Boston, MA). MβCD, U0126, SB202190 and SP600125 were purchased from Calbiochem (San Diego, CA).

2.2. Isolation of neonatal rat ventricular myocytes

Primary cultures of NRVMs were prepared from 1 to 2-day-old Sprague Dawley rats as previously described [5]. Dispersed cardiac cells were separated using a discontinuous Percoll gradient, containing a density of 1.060 gm/L (nonmyocyte layer) and 1.086 gm/L (myocyte layer). NRVM were plated on Bioflex plates (Flexcell International Corporation, Hillsborough, NC), coated with collagen-IV [1 μg/cm^2]), at a density of 0.75×10^6 cells/well in DMEM/M199 medium and maintained at 37°C in humid air with 5% CO_2. Cytosine arabinoside (100 μM) was added to prevent cell division of nonmyocytes and culture media was changed to serum-free 24 h prior to initiation of experiments. NRVM were >95% pure, as revealed by microscopic observation of contractile characteristics and by flow cytometry, after staining with anti-desmin antibody (Sigma Chemical Co., St. Louis, MO). This study conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Experimental protocols were approved by the Institutional Animal Care and Use Committee of Texas A&M Health Science Center.

2.3. Adenovirus expansion and infection of cells

Recombinant adenoviruses expressing Tac-β_{1D} and *lacZ* were gifts from Robert Ross (University of San Diego, San Diego, CA), whereas wild-type p38α (Ad-p38α-WT), and p38α dominant-negative (Ad-p38α-DN) were gifts from Dr. Yibin Wang (University of California, Los Angeles). Amplification of adenovirus was performed in transformed 293 human embryonic kidney (HEK) cells (CRL-1573, ATCC, Manassas, VA), followed by purification on CsCl gradients. Viral MOIs were determined by dilution assay in transformed 293 human embryonic kidney (HEK) cells CRL-1573 (American Type Culture Collection, Manassas, VA) cultured in 6-well clusters, as advised by the supplier. Titration assays were used to determine the lowest MOI which would results a significant increase in expressed protein and/or block endogenous target protein phosphorylation in NRVM. After 24 h of plating, NRVM were infected with following concentrations of adenovirus diluted in DMEM/medium 199: Tac-β_1 (50 MOI), Ad-p38α-WT (50 MOI), or Ad-p38α-DN (100 MOI). Corresponding MOI of adenovirus expressing *lacZ*/green fluorescent protein (GFP) were used to control for viral effects. After 24 h of transfection, the medium was replaced with virus-free DMEM/medium 199, and cells were cultured for an additional 12 h prior to experiments. At optimized virus concentrations, there were no obvious signs of cell toxicity (cell detachment, formation of intracellular vacuoles, cell rounding).

2.4. Lipid raft/caveolae disruption using MβCD

NRVM were incubated for various times at 37 °C in serum-free media without (control) or serum-free medium with 5 mM MβCD. MβCD is a chemical that does not enter cells, but disassembles lipid rafts and caveolae by depleting cholesterol from the plasma membrane. This technique has been validated in several cell types, including cardiac myocytes [15, 16].

2.5. siRNA preparation and transfection

In order to selectively "knock down" the expression of Cav-3 in NRVM, we designed a siRNA duplex targeted to the rat Cav-3 (Gene ID: NM_019155.2), sequence sense 5′-GGGCACUUACAGCUUCGAU-3 and antisense 5-AUCGAAGCUGUAAGUGCCC-3,starting at 282 from the open reading frame (136–591) of rat Cav-3 mRNA, by using Invitrogen BLOCK-iT RNAi Designer software. The RNA sequence used as a negative control for siRNA activity was: 5-GGGATTCCGACCTTACGAT-3 designed by BLOCK-iT™ RNAi Designer software. Small interfering RNA duplex oligonucleotides were purchased from Invitrogen. In preliminary experiments, we optimized conditions for the efficient transfection of NRVM using siRNA. Fresh medium was added 6 h post-transfection, and experiments were conducted 48 h after transfection. To assess the specific effect of Cav-3 siRNA on silencing Cav-3 expression, the protein levels of Cav-3 were detected by immunoblot analysis 48 h post-transfection. We found that optimal conditions for siRNA knockdown involved transfecting cardiac myocytes with siRNA (150 nM) and Lipofectamine 2000 (0.15% v/v), following protocols provided by the manufacturer (Invitrogen Life Science, Carlsbad, CA).

2.6. Immunofluorescent staining

Indirect Immunofluorescence staining was performed on cardiac cells cultured on Lab-Tek^{R} chamber slides™ (Nalge Nunc International, Rochester, NY) coated with collagen IV. Briefly, cells were washed with PBS and fixed with 4% paraformaldehyde for 20 minutes at 22°C. Following three extensive washings with PBS, cells were permeabilized with 0.05% Triton-X 100 and blocked with 5% bovine serum albumin in PBS buffer for 30 min at 22 °C. Cells were then incubated with the specific primary antibodies for Cav-1 (1:150, Sc-894 rabbit polyclonal antibody, Santa Cruz Biotechnology), Cav-3 (1:150, BD Transduction Laboratories) and/or mouse desmin (1:200, mouse monoclonal antibody [IgG_{2b}], RD301, Abcam) for 1 h at 37°C. Cells were co-stained for 45 min at 37°C with Alexa-Fluor^{R}-488-labeled chicken anti-rabbit (1:150), Alexa Fluor^{R}-594 labeled goat anti-mouse (1:150) antibodies, goat anti-mouse Alexa-Fluor^{R}-594 IgG_{2b} (1:150). The nucleus was stained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI). Stained cells were mounted with Prolong Gold^{R} antifade mounting medium (Invitrogen Corporation, Carlsbad, CA, USA) and viewed with a FluoView™ FV1000 Olympus epifluorescence microscope (model IX81).

2.7. Lipid rafts/caveolae labeling

Lipid rafts/caveolae were stained in cardiac cells cultured on collagen IV coated Lab-Tek® II chamber slides (Thermo Scientific, Rochester, NY). After treatment with MβCD (5 mM) and cholesterol (1 mM), cells were incubated in the presence of 1 μg/ml of cholera toxin subunit B (CT-B) (Invitrogen Corporation, Carlsbad, CA) for 10 minutes at 4°C. Cells were gently washed with chilled PBS and incubated with rabbit anti-CT-B antibody (Invitrogen Corporation, Carlsbad, CA) for 15 minutes at 4°C to crosslink the CT-B-labeled lipid rafts. Cells were then fixed, permeabilized, immunostained with Alexa 488^{R}-conjugated secondary antibody and visualized as described in section 2.6.

2.8. Preparation of cell lysates and Western blotting

Cell lysates were obtained by lifting NRVM cultured on 6-well pates using lysis buffer (Cell Signaling) containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM 2-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride and 1 mM sodium orthovanadate. Insoluble material was removed by centrifugation for 15 min at 14,000 g and samples were boiled with loading buffer and protein was determined using a kit (Bio-Rad DC Protein Assay) according to the manufacturer's recommendation (Bio-Rad, Hercules, CA). Western blot analysis was performed as previously described [5]. Briefly, equal amounts of protein (30 μg) from cell lysates were separated by SDS-PAGE and blotted onto polyvinylidene fluoride (PVDF) transfer membranes. The membranes were blocked for 2 h using 5% BSA in TBST buffer (10 mM Tris, 100 mM NaCl, 0.1% Tween 20, pH 7.4). Blots were incubated with the primary antibodies in 5% BSA in TBST buffer overnight at 4°C with light agitation. Bound primary antibodies were visualized using horseradish peroxidase-labeled secondary antibodies and detected using ECL. Densities of the protein bands were measured using ImageQuant software. Signals from the phosphoproteins were normalized to total protein, obtained by stripping and reprobing blots with the corresponding total antibody. Blots were again stripped and probed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody to confirm equal loading.

2.8. Quantitative measurement of Ao mRNA using real-time RT-PCR

A commercial kit (Totally RNA™, Ambion, Austin, TX) was used to isolate RNA from NRVM. First strand cDNA was reverse-transcribed (RT) with random hexamer primer using the High Capacity cDNA Archive kit for RT-PCR (Perkin-Elmer-Applied Biosystems Prism, Austin, TX). Real-time RT-PCR was carried out in a MX3005P (Stratagene, Cedar Creek, TX) thermocycler using Taqman Universal PCR Master Mix (Perkin-Elmer-Applied Biosystems). Absolute levels of Ao mRNA were quantified using 21-base sense (5′-AGCACGACTTCCTGACTTGGA-3′) and antisense primers (5′-TTGTAGGATCCCCGATTTCC-3′), which span the second intron of the genomic sequence and produce an 88 base-pair amplicon. Amplification of Ao DNA was performed using oligonucleotide (6FAM-5′-CCGTCTGACCCTGCCGCAGC-3′-TAMRA), as the reporter probe. Parallel reactions, yeast RNA (carrier RNA) spiked with known amounts of synthetic Ao RNA, were used to convert sample determinations to absolute values. Ao primers and reporters were multiplexed with GAPDH, for a "house-keeper" mRNA (proprietary reagents supplied by Perkin-Elmer-Applied Biosystems). The multiplexing was used to control for RNA loading, degradation and PCR efficiency.

3. Statistical analysis

Data are presented as the mean ± the standard error of the mean (SEM). Significant differences among groups were estimated by one-way ANOVA followed by Tukey's multiple comparison test (Instat 3.06, GraphPad Software Inc., La Jolla, CA). A value of $P<0.05$ was considered to denote statistical significance.

4. Results

4.1. Caveolae disruption by MβCD or knockdown of Cav-3 by siRNA activates Ao gene expression

MβCD has been consistently used as a pharmacological tool to study the role of lipid rafts/caveolae in cardiac myocytes and other cellular models [15–17]. In NRVM with lipid rafts/caveolae labeled with cholera toxin-B (CT-B), MβCD treatment induced detectable disruption of these plasma membrane structures within 10 min (Fig. 1A). To test whether caveolae may couple to regulation of Ao gene expression, NRVM were treated with 5 mM

of MβCD for different times (0 – 8 h). MβCD treatment increased Ao gene expression in a time-dependent manner, with significant changes in Ao gene expression initially observed at 4 h (2.16 ± 0.17 fold, P<0.01) (Fig. 1B). To determine the role of caveolae in regulation of Ao gene expression, we selectively knocked down the expression of Cav-3 protein (dominant isoform in cardiac myocyte) by transfecting NRVM with Cav-3 siRNA duplex. Fig 1C shows an immunoblot probed for Cav-3 protein in NRVM cell lysates harvested after 48 h transfection with Cav-3 specific siRNA. Cav-3 expression was efficiently knocked-down by transfection with Cav-3 siRNA, however, transfection with a control random sequence siRNA (con siRNA) did not affect the expression of Cav-3 (Fig 1C). siRNA-mediated knockdown of Cav-3 protein significantly increased Ao gene expression (2.31 ± 0.18 fold, P<0.01) (Fig. 1D). In contrast, adenovirus-mediated overexpression of Cav-3 (Fig. 1E) decreased Ao gene expression, compared to cells overexpressing control vector (GFP) (Fig. 1F).

4.2. Caveolae disruption by MβCD or knockdown of Cav-3 by siRNA activates ERK, JNK and p38

We compared the time-course of p38 and JNK activation with that of ERK1/2 in NRVM treated with MβCD (Fig. 2). Within 2 min of MβCD treatment, there were significant increases in phosphorylation of ERK (1.32 ± 0.039 fold, $P < 0.05$), p38 (1.12 ± 0.039 fold, $P<0.05$) and JNK (2.41 ± 0.047 fold, $P < 0.001$) ($N = 4$), suggesting that caveolae/lipid rafts serve to maintain these MAP kinases in an inactive state. However, continued treatment with MβCD resulted in different activation patterns among the MAP kinases. MβCD-mediated ERK1/2 and p38 activation peaked at 2 h, (5.23 ± 0.48 fold, $P < 0.001$ and 4.98 ± 0.23 fold, $P < 0.001$, respectively) and persisted up to 8 h (final time point). In contrast to ERK and p38, JNK phosphorylation peaked early, within 10 min of treatment (4.55 ± 0.13, $P < 0.001$) and declined. We also observed that prolonged treatment of MβCD down-regulated total ERK1/2 expression (Fig. 2A). However, further studies are required to establish the role of cholesterol depletion on ERK1/2 expression and to identify the underlying mechanism. To further confirm the role of caveolae in the differential regulation of MAP kinase phosphorylation, NRVM were transfected with Cav-3 siRNA or control siRNA. siRNA-mediated knockdown of Cav-3 significantly increased ERK (1.51 ± 0.085 fold, $P < 0.01$) and p38 (1.82 ± 0.21 fold, $P < 0.01$) phosphorylation (Fig. 2G and 2H). However, no significant difference was observed in JNK phosphorylation (control siRNA vs. Cav-3 siRNA group). Thus, the pattern of Cav-3 siRNA-mediated JNK activation was similar to that observed with chronic MβCD treatment. These data suggest that individual MAP kinase members have different roles in mediating caveolae regulated cellular events.

4.3. β_1-integrin is required for MβCD-mediated MAP kinase activation

We previously demonstrated that mechanical stretch-induced MAP kinase activation is β_1-integrin-dependent [5]. To test whether β_1-integrin may also be required for mediating MβCD-induced activation of MAP kinases, NRVM infected for 24 h with matched titers of recombinant adenoviruses that express either Tac-β_{1D} or control (*lacZ*) transgenes, were treated with MβCD for 1 h. NRVM transfected with Tac-β_1 expressing adenovirus showed high cell surface expression (fluorescence intensity of Tac-β_1), compared to cells transfected with *lacZ* as determined by flow cytometry (data not shown) [5]. Tac-β_{1D} functions as a dominant-negative for β_{1A} subunit, as well as the predominantly expressed β_{1D} subunit in myocytes. Disruption of β_1-integrin signaling significantly inhibited MβCD-mediated activation of ERK1/2 (0.728 ± 0.05 fold, $P < 0.01$), p38 (1.147 ± 0.06 fold, $P < 0.001$) and JNK (1.80 ± 0.04 fold, $P < 0.001$) (Fig. 3). These results indicate that β_1-integrin plays an integral role in the activation of ERK1/2, p38 and JNK following MβCD treatment.

4.4. β_1-integrin-dependent regulation of MβCD induces Ao gene expression

Because adenovirus-mediated over expression of dominant negative β_1-integrin (Tac-β_1) significantly inhibited MβCD-induced activation of MAP kinases, we tested whether inhibition of β_1-integrin signaling may also inhibit the effects of MβCD on Ao gene expression. This postulate was tested by infecting NRVM with adenoviruses expressing either *lacZ* (control) or dominant-negative β_1-integrin (Tac-β_1) prior to MβCD treatment. Unexpectedly, Tac-β_1 significantly increased MβCD-induced Ao gene expression compared to *lacZ* (control) expressing cells (3.04 ± 0.61 fold, P<0.001) (Fig. 4).

4.5. ERK is a negative regulator of MβCD and Cav-3 siRNA-induced Ao gene expression

To determine whether ERK may play a role in MβCD or Cav-3 siRNA-induced Ao gene expression, cells were pretreated with a specific ERK inhibitor U0126 (10 μM) or PD98059 (20 μM) for 1 h prior to MβCD treatment. Although U0126 did not affect basal Ao gene expression, it significantly enhanced the stimulatory effects of MβCD (1.84 ± 0.50 fold, P<0.001) (Fig. 5A) and Cav-3 siRNA (1.20 ± 0.34 fold, P<0.05) (Fig. 5C) on Ao gene expression, suggesting that ERK negatively regulates MβCD or Cav-3 siRNA-induced Ao gene expression.

4.6. Negative regulation of Ao gene expression by JNK is independent of caveolae

To determine whether JNK may play a role in MβCD-induced Ao gene expression, cells were pretreated with a specific JNK inhibitor (20 μM SP600125) for 1 h before MβCD treatment. Incubation with SP600125 increased basal Ao mRNA expression several fold (10.0 ± 0.77 fold, P<0.001) (Fig. 5B), which was independent of MβCD treatment. Similar results were observed when Cav-3 siRNA transfected cells were treated with JNK inhibitor SP600125 (Fig. 5D). These results suggest that JNK regulates basal Ao gene expression in a caveolae-independent manner.

4.7. p38 mediates MβCD or Cav-3 siRNA-induced Ao gene expression

To test whether p38 may have a role in Ao gene expression in a caveolae-independent manner, cells were pretreated with SB202190 (10 μM) or SB203580 (10 μM), specific inhibitors of p38 MAP kinase for 1 h before MβCD treatment. Both pharmacological p38 inhibitors significantly inhibited MβCD treatment induced Ao gene expression, SB 202190 (2.57 ± 0.34 fold, P<0.001) and SB203580 (2.26 ± 0.42 fold, P<0.001) (Fig. 6A). Furthermore, pharmacological inhibition of p38 by SB202190 significantly inhibited Cav-3 siRNA-induced Ao gene expression (1.09 ± 0.083 fold, P<0.01) (Fig. 6B). To further confirm this observation, NRVM were transfected with adenoviruses expressing wild-type p38 or p38α-DN (dominant p38 isoform in cardiac myocytes). Expression of wild-type p38α significantly increased the MβCD-induced Ao gene expression (2.36 ± 1.13 fold, P<0.001) (Fig. 6C). Conversely, expression of dominant-negative p38α significantly inhibited MβCD-induced Ao gene expression (1.91 ± 0.14 fold, P<0.001) (Fig. 6C), further indicating that p38 is a primary mediator of MβCD treatment or Cav-3 siRNA-induced Ao gene expression in cardiac myocytes.

4.8. p38 regulates Cav-1 and Cav-3 expression in cardiac myocytes

MAP kinases regulate caveolin gene expression in various noncardiac cell types [18–20], however, regulatory effects of MAP kinases on Cav-1 and Cav-3 expression in cardiac myocytes have not been studied. We assessed the role of ERK1/2, p38 and JNK in the regulation of Cav-1 and Cav-3 expression in cardiac myocytes. To test the role of MAP kinases in Cav-1/Cav-3 expression, NRVM were incubated for 24 h with specific inhibitors for ERK (10 μM U0126), p38 (10 μM SB202190) and JNK (20 μM SP600125). Western blot and immunofluorescent staining revealed that NRVM express both Cav-1 and Cav-3, in

which Cav-1 expression was much less compared to Cav-3 (Fig. 7A and 7B). Western blot analysis revealed that p38 increased both Cav-1 and Cav-3 gene expression in NRVM (Fig. 7C and 7D). However, Cav-1 and Cav-3 protein expression was independent of ERK and JNK activation (data not shown).

5. Discussion

To our knowledge, this is the first study to demonstrate a role for caveolae in the regulation of Ao gene expression. Numerous studies have unequivocally shown that the RAS plays an important role in the heart, under a variety of pathologic conditions [1, 2]. Cardiac myocyte specific overexpression of Ao results in cellular remodeling and age-dependent cardiac dysfunction and failure [21]. Furthermore, adenovirus-mediated delivery of Ao antisense attenuates hypertension and cardiac hypertrophy [22]. Caveolae and caveolins have been implicated in the wide range of cardiac protection [14, 16, 23]. Given the cardioprotective role of caveolae, we hypothesized that caveolae may suppress Ao gene expression in cardiac myocytes. Results from the present study indicate that chemical disruption of caveolae by MβCD induces Ao gene expression in a time-dependent manner, and requires β_1-integrin and p38 MAP kinase. However, ERK1/2 was identified as a negative regulator of this response. These findings suggest that caveolae-mediated cardiac protection may be due to suppression of Ao production by cardiac myocytes.

Disruption of caveolae/caveolin induces hyperactivation of the ERK pathway [24], which is thought to be responsible for hypertrophic growth. However, experimental evidence confirming such a mechanism is lacking. Although ERK activation has been mostly implicated in response to caveolae or caveolin modulation, other MAP kinase members, such as p38 and JNK, are known to be activated by modulation of caveolae/caveolins in various cell types as well [25].

Caveolae-mediated MAP kinase activation has been implicated in many diseases in various systems; however, the molecular activation mechanisms remain to be completely explored. In the present study, an unexpected finding was the identification of β_1-integrin as a major receptor required for the activation of MAP kinases in response to caveolae disruption. Caveolins and β_1-integrins colocalize within caveolae in the basal condition in cardiac myocytes, and caveolae disruption stimulates translocation of integrins from caveolae to noncaveolar sites within the membrane [12]. Because caveolae are flask-shaped invaginations located at cell surfaces [26], it is unlikely that integrins bind to ECM when located within caveolae. Together, these findings suggest that caveolae sequesters β_1-integrin, effectively preventing β_1-integrin from interacting with the ECM. Thus, disruption of caveolae activates β_1-integrin signaling by translocating β_1-integrin to non-caveolar sites within the membrane, which facilitate β_1-ECM interaction. However, further studies are required to identify the underlying mechanisms of caveolae-mediated sequestration of β_1-integrin.

Several equally robust studies conducted in cultured cardiac myocytes and transgenic animal models reported paradoxical roles for β_1-integrin in the myocardium [27–32]. For example, wild-type (WT) and β_1-integrin heterozygous knockout (hKO) mice studies reveal that a deficiency of β_1-integrin results in increased myocardial dysfunction after myocardial infarction [33], suggesting the protective nature of β_1-integrin in the myocardium. Consistent with these observations, β_1-integrin signaling has been reported to protect cardiac myocytes against β_1-adrenergic-stimulated apoptosis [27]. Moreover, cardiac myocyte-specific excision of the β_1-integrin gene results in myocardial fibrosis and cardiac failure [30]. In contrast, other studies have shown that β_1-integrin mediates cardiac hypertrophy [29, 31, 32]. Thus, taken together these studies suggest that β_1-integrin has dichotomous

regulatory functions in the myocardium. In light of the conflicting reports regarding protective and maladaptive roles of β_1-integrin in the heart, it was not too surprising to observe that expression of Tac-β_1 further increased MβCD-induced Ao gene expression. Taken together, these results indicate that β_1-integrin regulates MβCD-induced Ao gene expression via MAP kinase dependent and independent pathways.

Myocardial ERK1/2 activation is increased during cardiac hypertrophy [34] and hyperactivation of the ERK cascade is implicated in mediating the pathological effects caused by disruption of caveolae/caveolin [24, 35, 36]. Thus, an unexpected finding of the present study was the finding that ERK1/2 is a negative regulator of Ao gene expression in NRVM, as demonstrated by disruption of caveolae using MβCD. Recent studies using genetically-modified mice suggest that ERK1/2 signaling is not required for mediating physiologic or pathologic cardiac hypertrophy, although it may play a protective role in response to pathologic stimuli [37, 38]. The mechanisms by which ERK1/2 negatively regulates Ao gene expression in the myocardium remain to be identified. The observation that JNK negatively regulates Ao gene expression is consistent with studies, which suggest a protective role for the JNK cascade in the myocardium [39–42]. p38 has been implicated in both cardiac protection and injury [42–44]. However, the molecular basis for the divergent functions of p38 activation is unknown and requires further investigation. The finding that p38 mediates MβCD-induced Ao gene expression is consistent with recent reports suggesting that p38 may induce maladaptive effects in the myocardium [42–45]. Studies using pharmacological inhibitors [46–49] and genetic manipulations [42, 44] suggest a detrimental role for p38 in mediating events associated with cardiac hypertrophy, remodeling and contractile dysfunction. The downstream effectors responsible for mediating p38 effects on Ao gene expression remain to be identified.

Results from this study suggest that upregulation of Cav-1 and Cav-3 protein expression by p38 indirectly serves as an important negative feedback system by sequestering p38 to the plasma membrane via caveolin, thereby playing a cardioprotective role. Similar results have been shown in vascular smooth muscle cells, in which p38 activation also upregulates Cav-1 expression [19, 20]. Interestingly, in the present study, ERK and JNK, negative regulators of Ao gene expression in NRVM, had no effect on Cav-1 and Cav-3 protein expression. This is in contrast to H-Ras (G12V)-transformed NIH3T3 cells, in which increased expression of ERK caused down regulation of Cav-1 [50]. However, differential regulation of caveolins among cell types has been reported [51] and may relate to the pathophysiological state of the tissue. The biochemical and molecular mechanisms responsible for regulation of caveolin expression remain to be further explored in the myocardium.

6. Limitations

Currently, MβCD is the primary method to induce acute disruption of both lipid rafts and caveolae. Although complementary molecular approaches were also used to verify specificity of MβCD and Cav-3 on signaling effects and Ao expression, some subtle effects may have been mediated through single and/or clustered lipid rafts as well. A better understanding of signaling events mediated by lipid rafts and caveolae will become possible as new technologies are developed to better determine the composition and function of these membrane structures.

7. Conclusion

In summary, these results suggest that lipid rafts/caveolae have an important role in maintaining basal Ao gene expression in cardiac myocytes and that activation of MAP kinases ERK, p38 and JNK, caused by caveolae disruption, requires β_1-integrin function. These observations provide new insights into the importance of caveolae in controlling

cardiac growth. The finding that only p38 kinase was required for MβCD-induced upregulation of Ao gene expression suggests that targeting the p38 pathway may provide a therapeutic regimen for protecting the injured myocardium.

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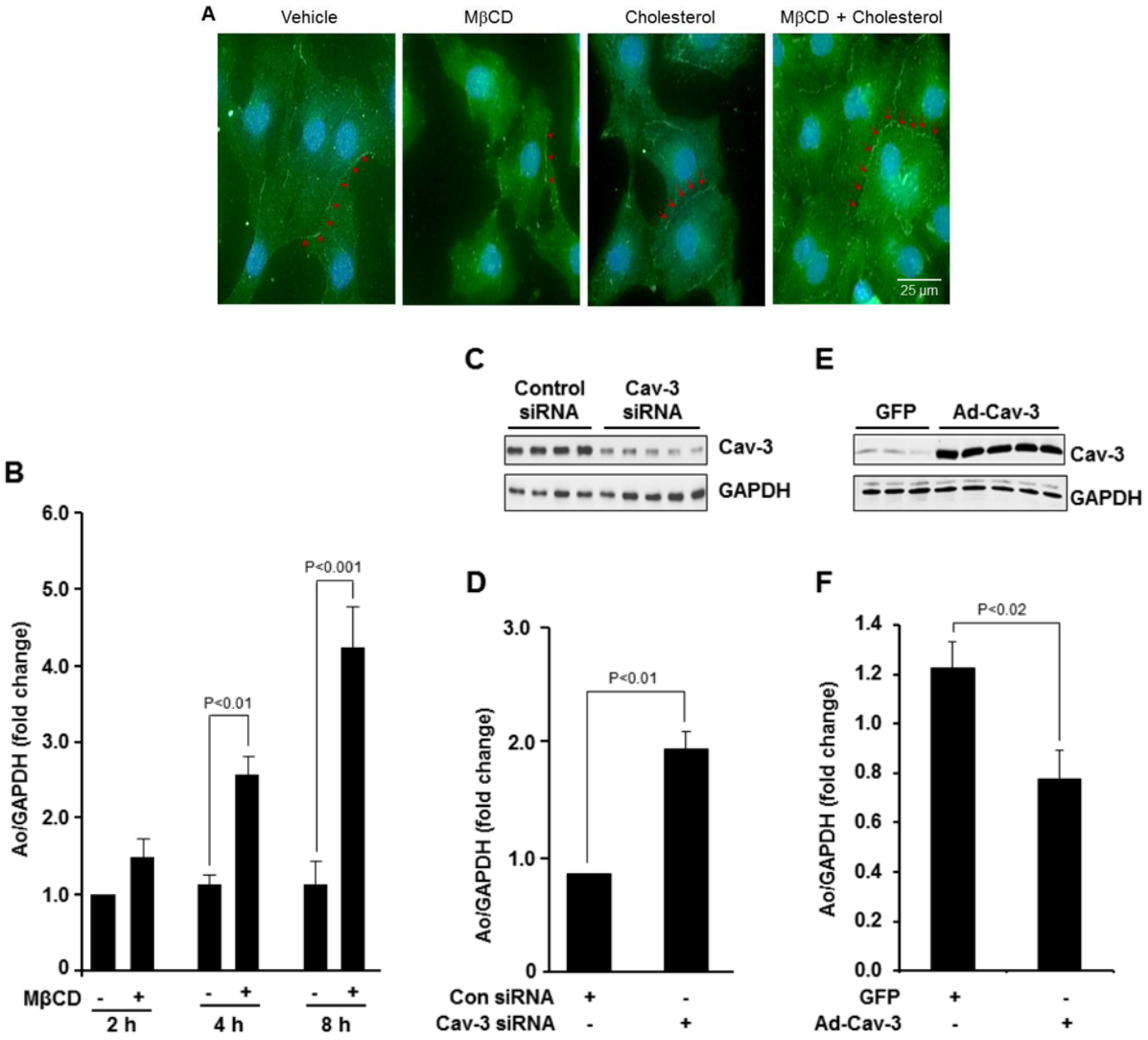

Figure 1. Caveolae disruption by MβCD or siRNA-mediated knockdown of Cav-3 upregulates Ao gene expression

Prior to treatment for 10 min with 5 mM MβCD and/or 1 mM cholesterol, lipid rafts/caveolae were labeled by incubating cells for 10 min in culture medium containing 1μg/ml cholera toxin. The effects of MβCD and/or extracellular cholesterol on the integrity of lipid rafts in NRVM were visualized by immunofluorescent staining of cholera toxin (A). The location of intact lipid rafts/caveole is indicated by green arrows. Addition of cholesterol to the culture medium prevented the disruption of lipid rafts/caveolae by MβCD. For determinations of Ao gene expression, NRVM were treated with MβCD (5 mM) for various times (2 – 8 h), as indicated (B). After MβCD treatment, cells were harvested, RNA was isolated and expression of Ao mRNA was determined using real-time RT-PCR in a multiplex system with GAPDH (for normalization). The effects of siRNA-mediated down-regulation of Cav-3 expression in NRVM, in which myocytes were transfected with either duplex siRNA (150 nM) targeted against Cav-3 (Cav-3 siRNA) or a random sequence (control siRNA) (C). 48 h after transfection, cells were harvested and protein levels were analyzed in immunoblots probed with a Cav-3 or GAPDH antibody (D). NRVM were also infected with adenovirus expressing GFP (control) or Cav-3 for 48 h (E). Cav-3 expression was verified using Western blot analysis and effects on Ao gene expression are also given

(F). Results are expressed as means ± SEM. N = 4 experiments. MβCD, methyl-β-cyclodextrin; Anti-CT, anti-cholera toxin antibody staining.

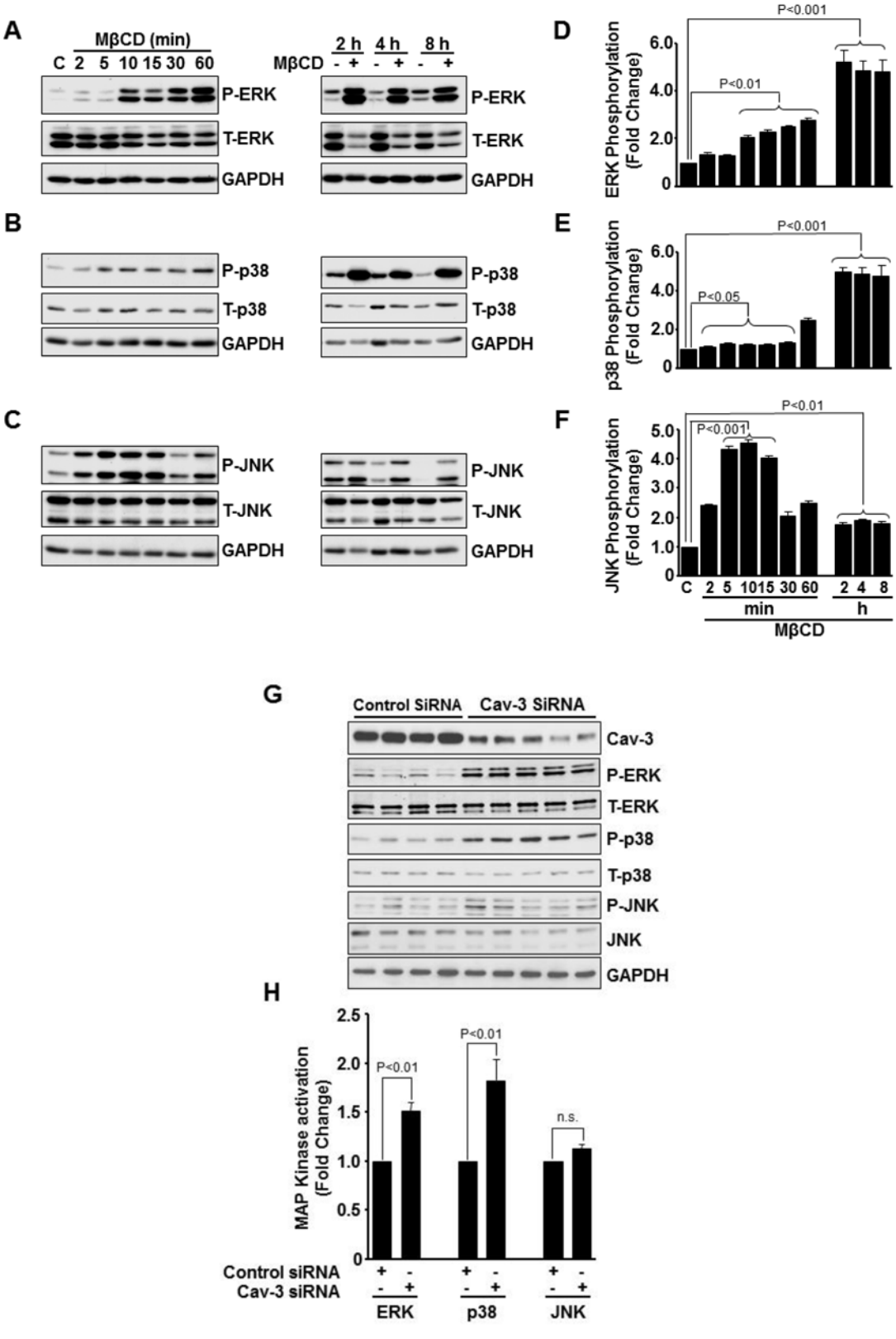

Figure 2. MAP kinases are activated by disruption of caveolae using MβCD or Cav-3 knockdown by siRNA

NRVM were treated with MβCD (5 mM) for different times (2 min - 8 h), as indicated. After MβCD treatment, cells were harvested and lysates were analyzed by immunoblotting using phospho-ERK1/2, phospho-p38 and phospho-JNK antibodies, as indicated (A, B and C, respectively). Bar graphs show fold changes in ERK (D), p38 (E) and JNK (F) phosphorylation after MβCD treatment (D, E and F, respectively). The effects of siRNA-mediated Cav-3 knockdown on MAP-kinase pathways via immunoblots prepared from cardiac myocytes transfected with siRNA targeted against Cav-3 or control siRNA are shown (G). Cell lysates were resolved by SDS-PAGE and analyzed in immunoblots probed with phospho and pan antibodies for ERK, p38 and JNK. Cav-3 and GAPDH expression were quantified in immunoblots probed with respective antibodies. Results of densitometric analyses from pooled data show levels of ERK, p38 and JNK phosphorylation in Cav-3 siRNA or control siRNA-transfected cells (H). Results are expressed as means ± SEM. N = 4 experiments. MβCD, methyl-β-cyclodextrin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

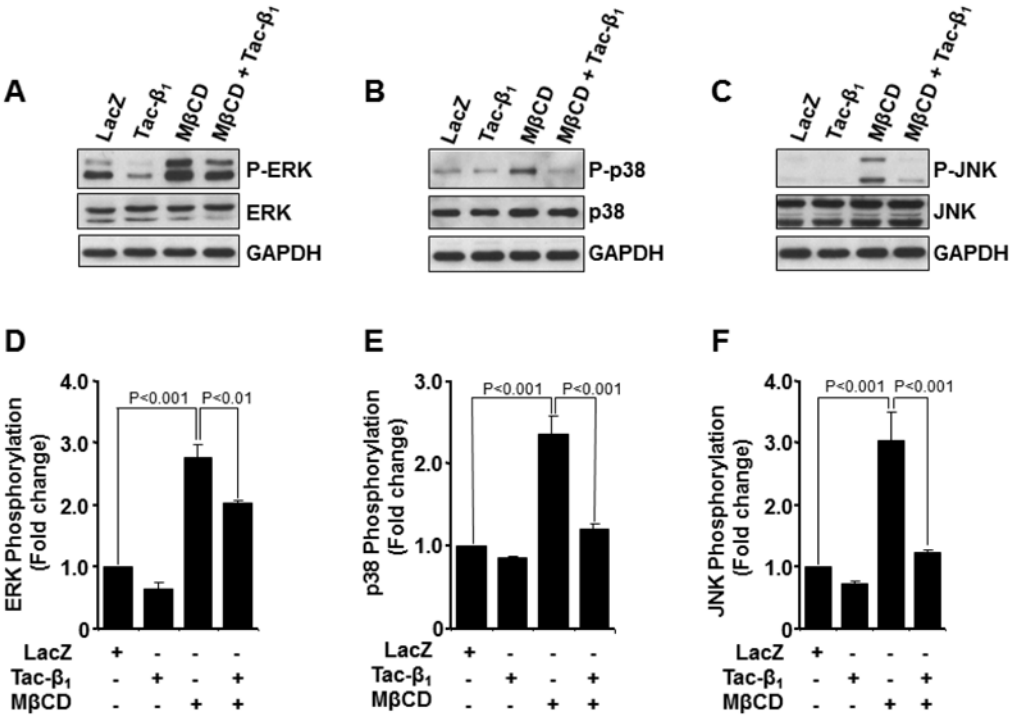

Figure 3. β_1-integrin is required for caveolae disruption-induced phosphorylation of ERK1/2, p38, and JNK

Prior to MβCD treatment, NRVM were infected with adenovirus (24 h) which resulted in expression of Tac-β_{1D} or control protein (*lacZ*). After 24 h of transfection, viral medium was replaced with serum free medium and allowed for overnight starvation before starting the MβCD treatment. After MβCD treatment (1 h), phosphorylation levels of ERK (A), p38 (B) and JNK (C) were determined. Bar graphs show fold changes in ERK (D), p38 (E) and JNK (F) phosphorylation after Tac-β_{1D} expression compared to control virus. Values are means ± SEM. N = 4 experiments. MβCD, methyl-β-cyclodextrin.

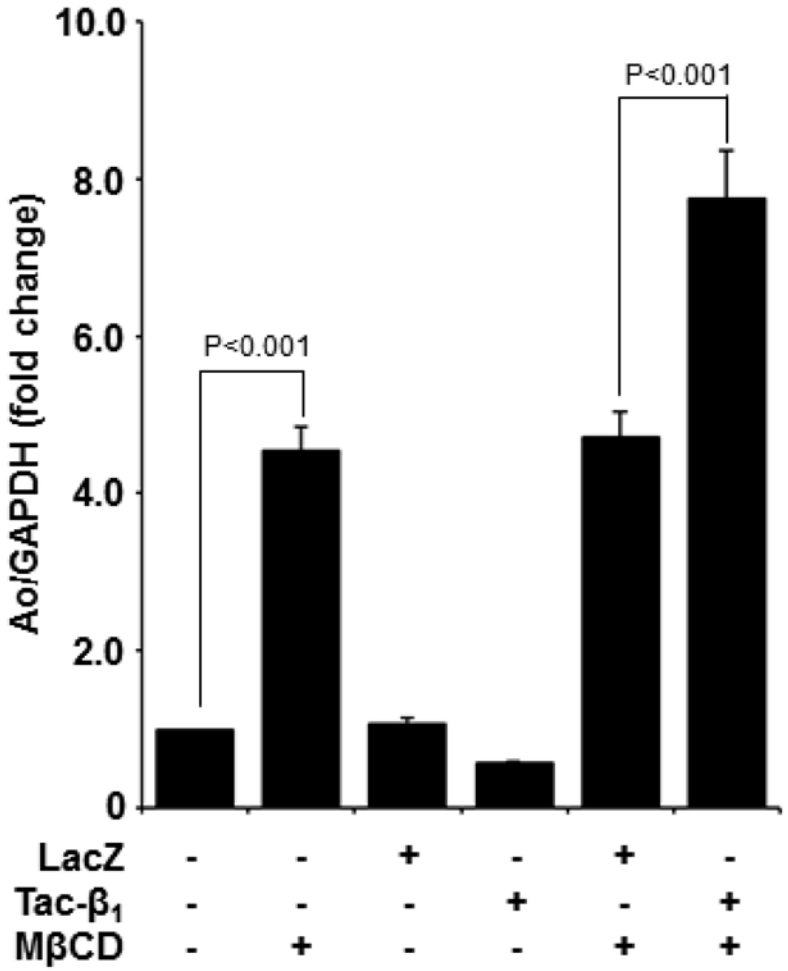

Figure 4. β_1-integrin dependent regulation of MβCD-induced Ao gene expression

Prior to MβCD treatment, NRVM were infected with adenovirus (24 h) which resulted in expression of Tac-β_{1D} or control protein (*lacZ*). After 24 h of transfection, viral medium was replaced with serum-free medium and allowed for overnight starvation before starting cyclodextrin treatment. After MβCD treatment, cells were harvested and RNA was isolated, and expression of Ao mRNA was determined using real-time RT-PCR. Tac-β_{1D} expression decreased basal Ao gene expression in control cells, but significantly increased Ao gene expression in the cells treated with MβCD. Results are expressed as means ± SEM. N = 4 experiments. MβCD, methyl-β-cyclodextrin.

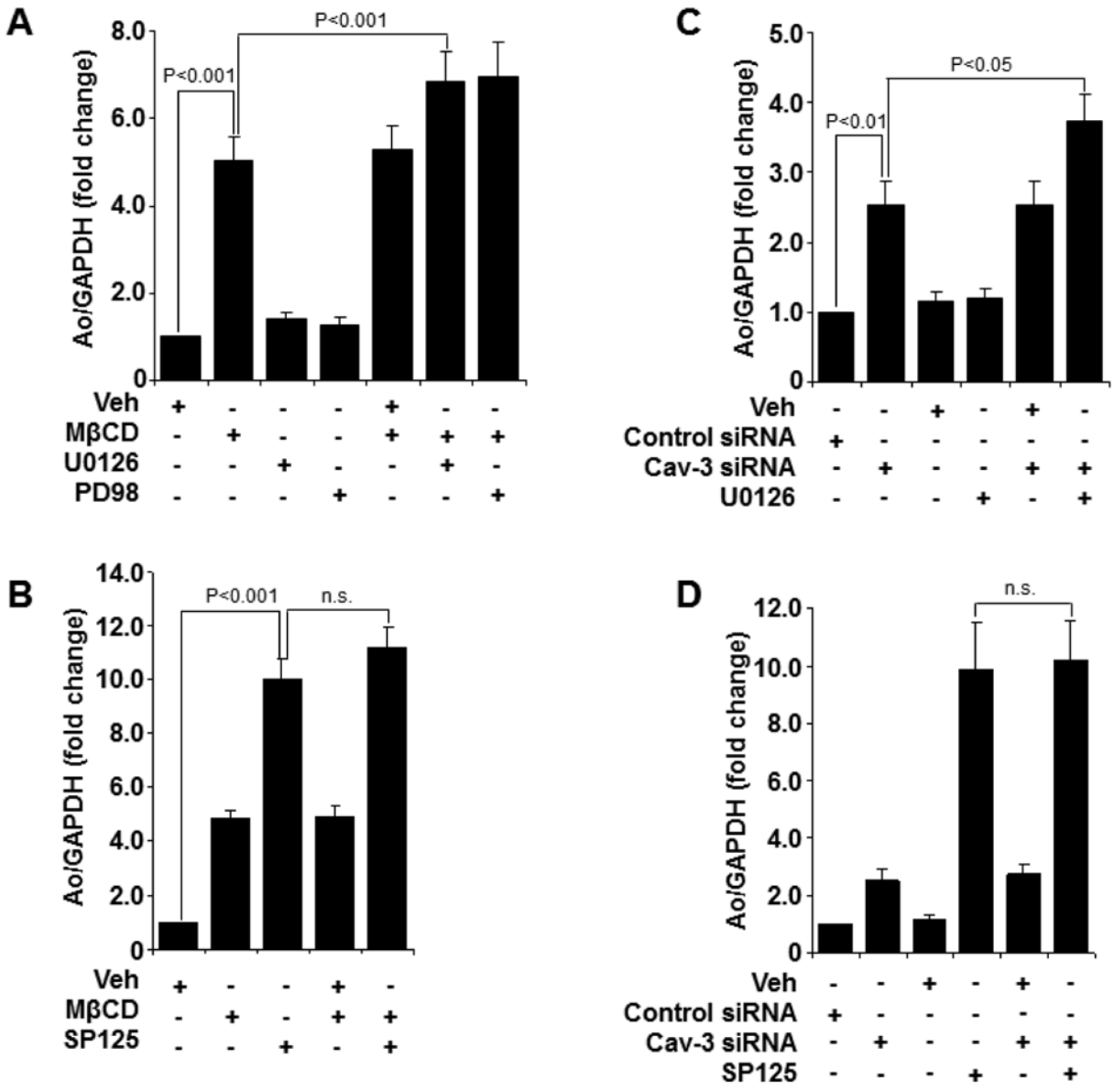

Figure 5. ERK negatively regulates MβCD or Cav-3 siRNA-induced Ao gene expression, whereas JNK negatively regulates basal Ao gene expression independent of caveolae

NRVM were treated with MβCD in the presence and absence of specific MAP kinase cascade inhibitors (1 h pretreatment) for ERK (10 μM U0126) and JNK1/2 (20 μM SP600125) (A and B, respectively). After 8 h of MβCD treatment, cells were harvested, RNA was isolated and expression of Ao mRNA was determined using real-time RT-PCR. Control and Cav-3 siRNA transfected NRVM were incubated with inhibitors for ERK (10 μM U0126); JNK1/2 (20 μM SP600125) (C and D, respectively). After 8 h of incubation, cells were harvested, total RNA was isolated and Ao mRNA expression was determined using real-time RT-PCR. Results are expressed as means ± SEM. N = 4 experiments. MβCD, methyl-β-cyclodextrin; PD98, PD98059; SP125, SP600125; Veh, vehicle.

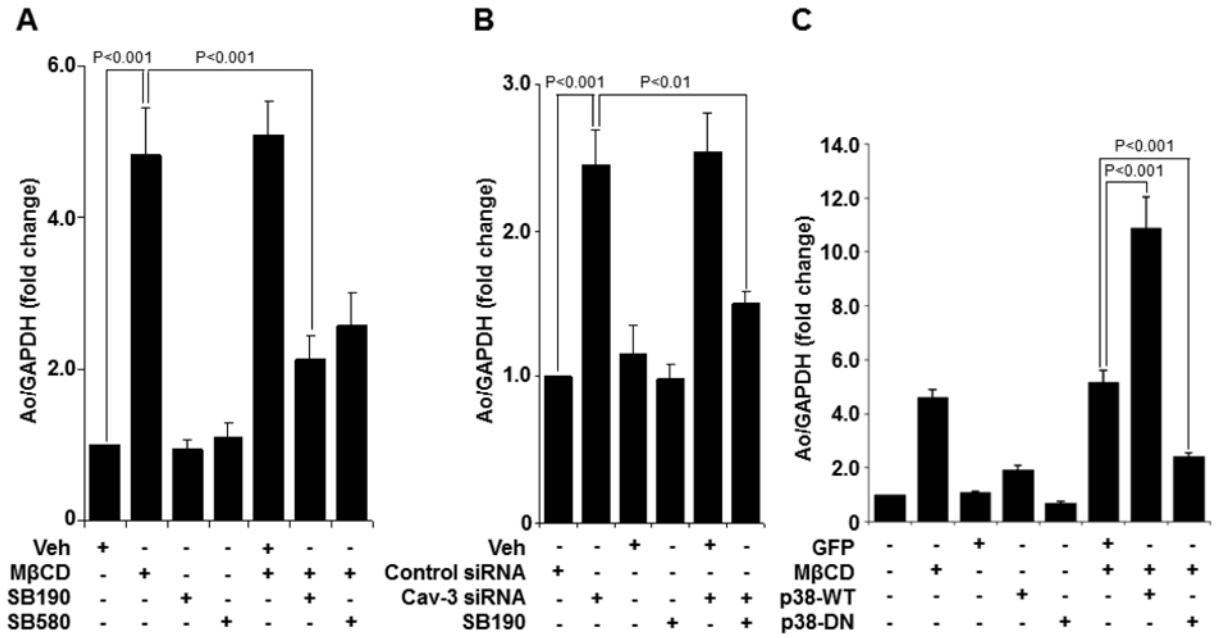

Figure 6. p38 mediates both MβCD and Cav-3 siRNA-induced Ao gene expression

NRVM were treated with MβCD in the presence and absence of specific p38 kinase inhibitors SB202190 (10 μM) or SB203580 (10 μM) (1 h pretreatment). After 8 h of MβCD treatment, cells were harvested, RNA was isolated and Ao mRNA expression was determined using real-time RT-PCR (A). Control and Cav-3 siRNA transfected NRVM were incubated with p38 inhibitor SB202190 (10 μM). After 8 h of treatment, cells were harvested, RNA was isolated and Ao mRNA expression was determined. Cells were transfected with adenoviruses expressing wild-type (p38-WT) and dominant-negative (p38-DN) forms of p38α (B). After 24 h, the viral medium was replaced with serum-free medium and cells were starved overnight prior to MβCD treatment. After MβCD treatment, cells were harvested and RNA was isolated, and expression of Ao was determined (C). Results are expressed as means ± SEM. N = 4 experiments. MβCD, methyl-β-cyclodextrin; SB190, SB20190; SB580, SB203580; Veh, vehicle.

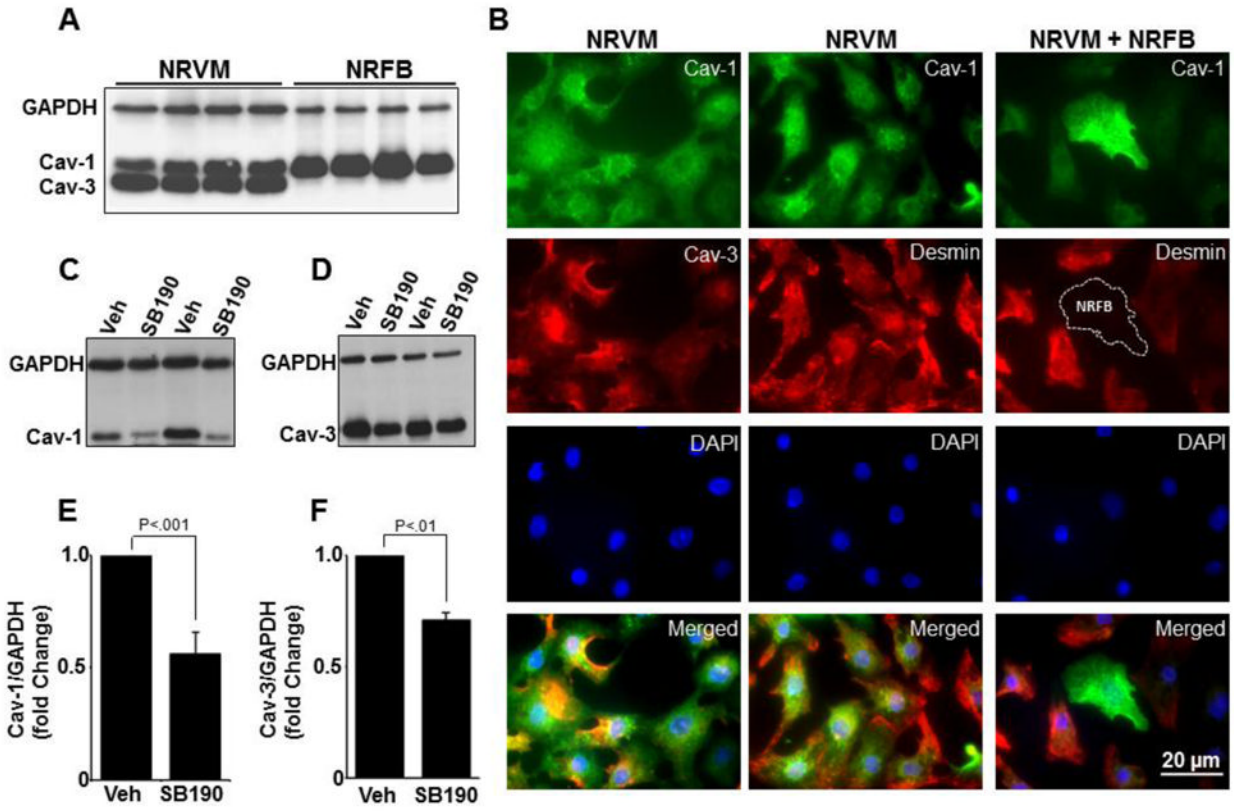

Figure 7. Cardiac myocytes express both Cav-1 and Cav-3 in a p38 dependent manner

Western blot analysis of cell lysates and immunofluorescent staining demonstrates that NRVM express both Cav-1 and Cav-3 (A), whereas, neonatal rat cardiac fibroblasts (NRFB) express only Cav-1 (A and B). Immunofluorescent staining, using primary antibodies, demonstrate perinuclear staining for Cav-1, whereas Cav-3 was also localized along the cell borders (B). Western blots and immunofluorescent staining also indicate Cav-1 protein expression was higher in NRFB, compared to NRVM. When NRVM were treated for 24 h with a specific p38 inhibitor (10 μM SB202190), analysis of cell lysates by Western blot showed that inhibition of p38 cascade significantly decreased Cav-1 (C and E), as well as Cav-3 expression (D and F). However, treatment with inhibitors for ERK (10 μM U0126), JNK1/2 (20 μM SP600125) had no effect on Cav-1 and Cav-3 protein expression (data not shown). N = 4 experiments. Veh, vehicle; SB190, SB20190; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NRVM, neonatal rat ventricular myocytes; NRFB, neonatal rat ventricular fibroblasts.

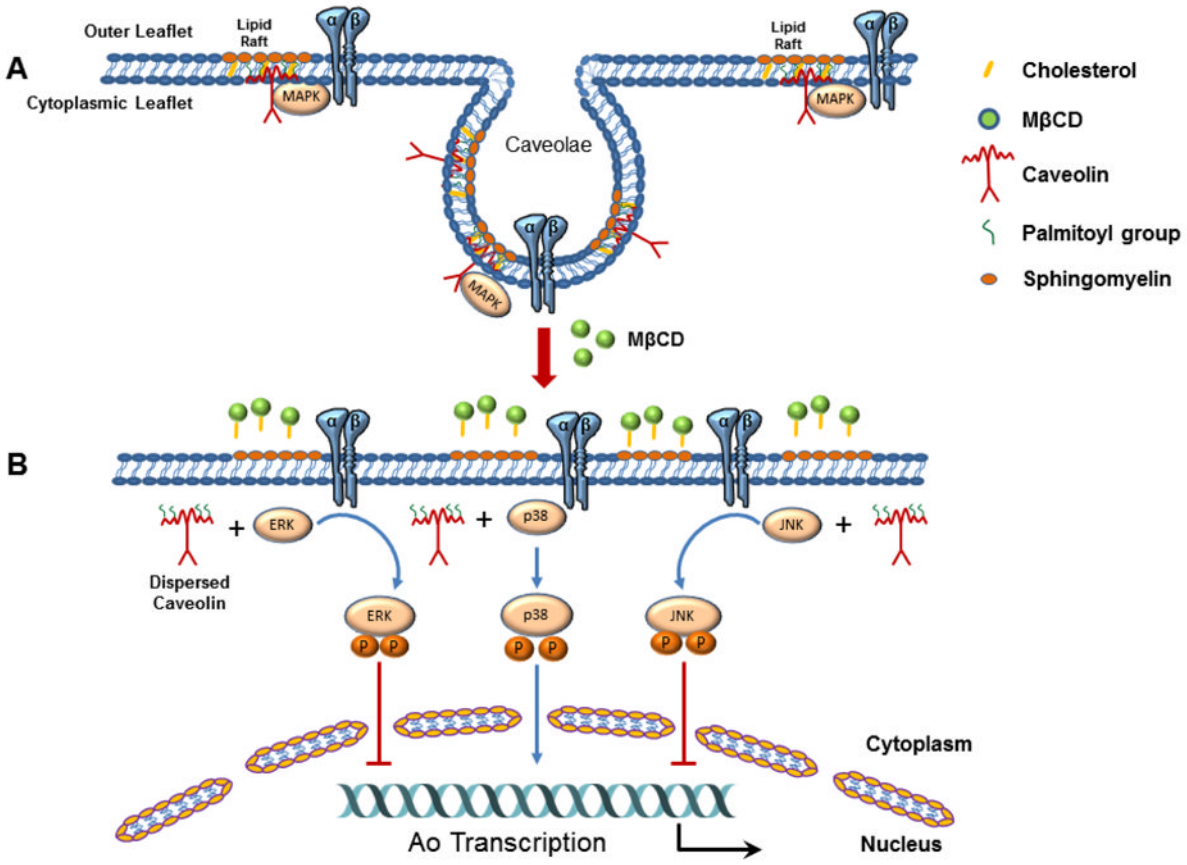

Figure 8. Model showing lipid raft/caveolae signaling and coupling to Ao gene expression in cardiac myocytes

Lipid rafts and caveolae are microdomains localized on the plasma membrane which known to integrate signaling pathways (A). Caveolae, or “little caves” are cholesterol and sphingolipid enriched invaginations of the plasma membrane. Results from this study suggest that caveolin-3 that is associated with caveolae stuctures, regulate MAPKs (ERK1/2, p38, JNK). Removal of cholesterol from the plasma membrane using MβCD, causes disruption of caveolae, as well as activation and release of MAPKs (B). Results from the present study indicate that activated forms of ERK1/2 and JNK serve as negative regulators of Ao gene expression, whereas p38 is a positive regulator. MβCD, methyl-β-cyclodextrin; MAPK, mitogen activated protein kinases.