Requirement of vimentin filament assembly for β3-adrenergic receptor activation of ERK MAP kinase and lipolysis

Naresh Kumar‡, Jacques Robidoux‡, Kiefer W. Daniel‡, Gabriel Guzman‡, Lisa M. Floering‡, Sheila Collins‡¶
‡Program in Endocrine Biology, Division of Biological Sciences
CIIT Centers for Health Research, Research Triangle Park, 27709, USA
and
¶Department of Psychiatry and Behavioral Sciences
Duke University Medical Center, Durham, NC 27710

Running title: Vimentin links βAR to ERK activation and lipolysis

Address correspondence to: Sheila Collins, CIIT Centers for Health Research, 6 Davis Drive, Box 12137, Research Triangle Park, NC 27709 USA, TEL: 919-558-1378, FAX: 919-558-1305, e-mail: scollins@ciit.org

Catecholamine stimulation of β-adrenergic receptors (βAR) in adipocytes activates the cAMP-dependent protein kinase to promote liberation of fatty acids as a fuel source. The adipocyte β3AR also activates Extracellular signal-Regulated Kinases (ERK)-1 and -2 through direct recruitment and activation of Src kinase. This pathway together with cAMP-dependent protein kinase contributes to maximal β3AR-stimulated lipolysis. In a search for other molecules that might associate with β3AR upon agonist stimulation, we identified vimentin using a proteomics approach. Immunoprecipitation of β3AR from adipocytes in the absence or presence of the β3AR agonist CL316243, followed by Western blotting for vimentin confirmed this specific interaction. Since vimentin has also been identified on lipid droplets, the functional consequences of blocking the expression or structural integrity of vimentin intermediate filaments on β3AR regulation of ERK activation and lipolysis was assessed. Following disruption of intermediate filaments with beta,beta'-iminodipropionitrile, as confirmed by confocal microscopy, β3AR-stimulated ERK activation was blocked and lipolysis was reduced by more than 40%. Independently, depletion of vimentin by shRNA completely inhibited β3AR-mediated ERK activation and significantly reduced lipolysis. By contrast, disruption of actin-containing microfilaments by cytochalasin D or microtubules by nocodazole had no effect on either lipolysis or ERK activation. These results indicate that vimentin plays an essential role in the signal transduction pathway from β3AR to the activation ERK, and its contribution to lipolysis.

β-adrenergic receptors (βARs) are members of the large family of G-protein-coupled receptors (GPCR) that mediate diverse physiological responses to adrenaline and noradrenaline. Of the three βAR subtypes, β1AR is found predominantly in heart and brain (1); β2AR is widely expressed (2); β3AR is mainly found in adipose tissue (3). All three βAR subtypes couple to the heterotrimeric G-protein Gs (4), leading in turn to elevations in intracellular cAMP and activation of PKA. However, there is evidence that these receptors can also couple to Gi (5-7) leading to ERK activation. In the case of β1AR and β2AR, coupling to Gi is dependent on the initial coupling to Gs, PKA activation and receptor phosphorylation (5,6). For β3AR, it is unique in that it can couple interchangeably to both Gs and...
Vimentin links βAR to ERK activation and lipolysis

Gi without a requirement for receptor phosphorylation (7,8). Several Gi-coupled receptors stimulate ERK activation by utilizing Src kinase recruitment to multi-protein complexes that include the receptor. β3AR activates ERK by a mechanism that depends on a series of proline-rich motifs in its third intracellular domain and carboxyl terminus that are conserved among the mammalian homologues (9). It is through these regions that the Src kinase is recruited to the β3AR. This interaction triggers Src catalytic activity, which together are necessary steps in ERK activation (9). One of the functional consequences of this β3AR to ERK cascade in adipocytes is lipolysis (10), wherein ERK functions together with PKA to produce maximal lipolytic capacity (11).

In an effort to further understand this mechanism of β3AR signal transduction, we have extended our previous findings to search for other protein molecules in addition to Src that interact with β3AR. Using 2-dimensional gel electrophoresis followed by electrospray ionization mass spectrometry-mass spectrometry (ESI-MS/MS), we have identified vimentin as one such molecule that associates with β3AR upon agonist stimulation. Vimentin-type intermediate filaments (IFs), characteristic of many vertebrate cells of mesenchymal origin, have been shown to undergo reorganization during the differentiation of preadipocytes into adipocytes (12). During this reorganization, vimentin filaments surround the nascent lipid droplet, forming a cage-like structure around them. Similar structures of vimentin filaments also have been reported in cholesterol loaded macrophages (13) and the foam cells of atherosclerotic lesions (14). It also has been reported that disruption of vimentin IFs during adipose differentiation of 3T3-L1 cells inhibits lipid droplet accumulation (15). Here we show that acutely disrupting vimentin IFs filaments in differentiated adipocytes resulted in greater than 40% reduction in lipolysis stimulated by the selective β3AR agonist CL316,243 (CL). Activation of ERK MAP kinase by CL was also blocked by disruption of vimentin IFs. Thus, while the structural features of the receptor and vimentin that contribute to this interaction are as yet unknown, these results indicate that in mature differentiated adipocytes vimentin and its filament assembly plays a significant role in the signal transduction pathway from β3AR to ERK activation and lipolysis.

EXPERIMENTAL PROCEDURES

Chemicals and plasmids. The β3AR-selective agonist CL316,243 (CL) was a gift from American Cyanamid Co. (Pearl River, NY). BRL49653 (BRL) was gift from Glaxo Wellcome Inc. PP2, SB202190, and PD098059 were from Calbiochem (La Jolla, CA). Isobutylmethylxanthine (IBMX), insulin, dexamethasone, cytochalasin D, H89, nocodazole, urea, CHAPS, salbutamol and forskolin (Fsk) were from Sigma-Aldrich (St. Louis, MO). Beta,beta'-Iminodipropionitrile (IDPN) was from Acros Organics (New Jersey, USA). The HA-β3AR expression vectors for wild type (wt) and C-terminal tail mutant (tail mutant) were previously described (9). Anti-p42/44- and anti-phospho-p42/44- MAPK antibodies were from Cell Signaling Technology (Beverly, MA). Anti-HA antibodies were from Upstate Biotechnology. Fatty acid-free bovine serum albumin (FAF-BSA) was from ICN. 4-20% Tris-glycine gels were from NOVEX (Invitrogen, Carlsbad, CA). 4-12% Bis-Tris Zoom gels were from NuPage (Invitrogen, Carlsbad, CA). A protease inhibitor cocktail was purchased from Roche Applied Science.

Cell culture and transfection. The C3H10T1/2 (T1/2) cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. Cells were transfected with 10 µg of HA-β3AR plasmid and 3 µg of Src using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) in 100mm dish as per manufactures’ instructions. On day 2, cells were transfected and induced to differentiate (1 µM rosiglitazone, 200 nM insulin) for four days. The 3T3-L1 cells were differentiated 1 day after confluence in DMEM containing 10% FBS with the addition of 0.5 mM IBMX, 0.4 µg/ml dexamethasone, 5 µg/ml insulin, and 1 µM rosiglitazone to the medium. Three days later, medium was replaced but without additives with media replenished every 3 days. Cells were treated with 10 µM CL or...
Vimentin links βAR to ERK activation and lipolysis

20 µM FSK (10 min) without or with prior treatment for 1 hr with either 1% IDPN (v/v), 1 µg/ml cytochalasin D or 2 µM nocodazole.

Immunoprecipitation and immunoblotting: Cells were washed with PBS and lysed in 25 mM HEPES buffer, pH 7.4 (containing 150 mM NaCl, 5 mM β-glycerophosphate, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 5 mM EDTA, 5 mM EGTA, 0.9% Triton X-100 and 0.1% IGEPAL, and 1 complete mini tablet of protease inhibitors per 10 ml) for 15 min. The supernatant was recovered after centrifugation at 16,200 x g for 15 minutes at 4°C. For immunoprecipitation, 1 mg of protein above mentioned supernatant was incubated with 2-4 µg of antibody and 50 µl of protein G-agarose beads overnight at 4°C on a rotating platform. After centrifugation, beads were washed four times with lysis buffer. Bound proteins were eluted with SDS sample buffer, resolved by SDS-PAGE, and transferred onto nitrocellulose membranes. Nitrocellulose membranes were incubated at room temperature for 1 h in blocking buffer containing Tris-buffered saline with 0.1% Tween (TBS-T) and 4% bovine serum albumin, followed by incubation with the indicated antibodies in the blocking buffer. After being washed three times for 5 min each with TBS-T, the membrane was incubated with alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit IgG (Sigma, St. Louis, MO) antibodies followed by washing with TBS-T. Immuneactive bands were visualized with ECF substrate (GE Healthcare, Piscataway, NJ).

Protein kinase A activity assay. Cells were treated with various cytoskeletal disrupting agents followed by stimulation with either CL or Fsk (as a positive control) for 10 min. Cells were washed twice with phosphate-buffered saline (PBS) and incubated in KRB containing 2% FAF-BSA at 37°C for 3 hr before being replaced with fresh buffer containing 1% IDPN, nocodazole or cytochalasin D for 1 hr. Following this pre-incubation, cells were provided with either CL (10 µM) or Fsk (20 µM) and collected 2 hr later. Glycerol released into the buffer was measured by the GPO-Trinder method (Sigma, St. Louis, MO).

Lipolysis. Differentiated T1/2 and 3T3-L1 adipocytes were gently washed with PBS and incubated in KRB containing 2% FAF-BSA at 37°C for 3 hr before being replaced with fresh buffer containing 1% IDPN, nocodazole or cytochalasin D for 1 hr. Following this pre-incubation, cells were provided with either CL (10 µM) or Fsk (20 µM) and collected 2 hr later. Glycerol released into the buffer was measured by the GPO-Trinder method (Sigma, St. Louis, MO).

2-Dimensional gel electrophoresis and mass spectrometry. The samples were immunoprecipitated as described above with anti-HA antibody and the proteins were eluted with 0.05 %SDS and 1X NuPAGE sample reducing agent. Protein samples were cleaned up using ReadyPrep 2-D cleanup kit (Bio-Rad Laboratories). The final pellet was suspended in 155 µl sample rehydration buffer (Rehydration Buffer: 9 M urea, 2% CHAPS, 0.5% Ampholytes, and 0.001% Bromophenol Blue) and absorbed overnight onto 7-cm pH 3–10
nonlinear immobilized pH gradient (IPG) ZOOM strips (Invitrogen, Carlsbad, CA). Isoelectric focusing was carried out using the ZOOM IPGRunner system (Invitrogen, Carlsbad, CA) and the Bio-Rad 3,000-V power supply (Bio-Rad Laboratories, Hercules, CA) using the following voltage step protocol: 200 V for 25 minutes, 450 V for 20 minutes, 750 V for 20 minutes, and 2,000 V for 90 minutes. For the second dimension, focused IPG strips were equilibrated in NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA) in the presence of NuPAGE Sample Reducing Agent (Invitrogen, Carlsbad, CA) for 15 minutes, and further incubated in LDS sample buffer in the presence of 125 mM iodoacetamide for 15 minutes. The strips were placed on 4–12% Bis-Tris gels. The gels were stained for protein using Sypro Ruby staining. Proteomic analysis was performed at the Michael Hooker UNC/Duke Proteomics Center by nanoelectro-spray ionisation tandem mass spectrometry (nanoESI-MS/MS) method according to their standard methods (16).

Inhibition of vimentin expression by small hairpin RNA (shRNA). The shRNA-containing virus was generated by the BLOCK-iT Lentiviral RNAi Expression System (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Briefly, the short hairpin DNA oligos (Vimentin oligo 1: CACCGCCAAGATCTGCTCAATGTTCGAAAACATTGAGCATCTTGG, Vimentin oligo 2: AAAACCAGATCTGCTCAATGTTCGAAAACATTGAGCATCTTGG, Scramble oligo1: CACCGTCCACATTTGATCTACGAAATCACGAATATGAATTTGCAGATCTTGG, Scramble oligo 2: AAAAGTCCCACATTGATACTGTATTCGATACGAATACGATATCAATGTGGGCACG) were synthesized and sub-cloned into a pENTR/U6 vector to yield pENTR/U6-vimentin. The lentiviral vector, pLenti6-si-vim, was generated by left-right recombination between the pENTR/U6 entry construct and pLenti6/BLOCK-iT-DEST. These cloned constructs were cotransfected with virus packaging vectors into HEK 293FT cells to produce virus. Two days later, supernatants of cultures were harvested and centrifuged at 26,000xg for 5 hr at 4°C. The pellet was suspended in PBS. The differentiated T1/2 cells were infected at multiplicity of infection (moi) of 280. Cells were used after 48 hr to measure vimentin expression, ERK activation and lipolysis as described above.

Confocal fluorescence microscopy. Differentiated T1/2 cells were replated on Lab-Tek chamber slides coated with poly-L-Lysine and allowed to attach and recover for overnight. Cells were incubated for 1 h with the indicated cytotoxic disrupting agent. The coverslips were then rinsed with Cyto- PG buffer (CB: 10 mM MES pH 6.1, 150 mM NaCl, 5 mM EGTA, 5 mM MgCl2, 5 mM glucose) at room temperature and simultaneously fixed and permeabilized by immersion in cold methanol (-20°C) for 10 min. Cells were rinsed again with CB, then placed in blocking solution (1% BSA and 2% FBS in Tris-buffered saline, TBS), followed by incubation for 1 hr at room temperature with antibodies as indicated. The cells were washed with TBS and incubated with Alexa Fluor 594-conjugated anti-mouse or anti-rabbit IgG or Alexa Fluor 488-phalloidin and incubated for 1 h at room temperature. Finally, the cells were washed three times in PBS and mounted in an anti-fade reagent (Molecular Probes, Carlsbad, CA). The cells were observed, and images were acquired with an LSM510 confocal laser-scanning microscope using a Zeiss 63X oil immersion lens. Two independent experiments were performed, and 10 or more fields per sample were analyzed in each experiment.

RESULTS

Association of vimentin with β3-adrenergic receptor

We previously reported that in response to agonist, β3AR in adipocytes recruits Src to a series of intracellular proline-rich motifs in the receptor, which leads to activation of ERK1/2 (9). One of the functional consequences of this β3AR-dependent Src activation of ERK1/2 is augmentation of lipolysis (11). Since there are multiple proline motifs in the intracellular domains which appear to cooperate with one another to activate ERK1/2, we set out to determine whether other molecules are brought to this complex. Differentiated T1/2 adipocytes
were transfected with HA-tagged β3AR and treated with the selective β3AR agonist CL. Following immunoprecipitation with anti-HA antibody, samples were separated on 2-D gel and were silver-stained. There were two categories of spots that were of interest. In the first, four spots (one major and three minor) significantly increased in the intensity in response to CL. In the second group, two minor spots appeared only upon CL stimulation. The major spot, which had increased by 2.5-fold with CL stimulation, was identified as vimentin by ESI/MS/MS. The minor spots had too little mass to be identified at this time; however, vimentin was pursued further as it also has been shown to be present on lipid droplets (17).

Since the ability of β3AR to recruit Src and activate ERK is dependent on its proline-rich motifs, we wanted to test whether vimentin is also part of this structural complex. Cells were transfected or not with HA-tagged wild type β3AR or a mutant β3AR in which the C-terminal PXXP sequences had been mutated (9), and immunoprecipitations with anti-HA antibody were performed using whole cell lysates. The samples were separated on 1-D SDS-PAGE followed by Western blotting with anti-vimentin antibody. As can be seen in Figure 1, by virtue of its abundance some vimentin was recovered even from mock-transfected ‘control’ cells. Therefore, all results were corrected for this background level. Even though we routinely observed 35-40% transfection efficiency (determined by GFP-expressing vector) there was a 23.7 ± 6.1 (n = 4) – fold increase in the association of vimentin with the HA-tagged wild type β3AR in response to CL treatment (Figure 1A). However, there was no such association of vimentin with the C-terminal PXXP mutated β3AR. Nor was there any increase in cells expressing HA-tagged β3AR in response to the selective β2AR agonist salbutamol. As shown in Figure 1B, pretreatment of the cells with a selective inhibitor of Src kinase (PP2) before addition of CL blocked the recruitment of vimentin to the wild type β3AR. These results are consistent with our earlier observation that Src is recruited to the β3AR in its catalytically active form (9), and supports a role for Src activation in the association of vimentin with β3AR.

Role of vimentin filament assembly in lipolysis
Vimentin has been identified as one of many molecules associated with lipid droplets in adipocytes (17). We have also shown recently that the β3AR-Src-ERK1/2 activation process contributes to a significant fraction of lipolysis (11). Therefore, we measured lipolysis following stimulation with CL in cells treated or not with IDPN, which selectively disrupts vimentin intermediate filaments (18). CL-stimulated lipolysis was reduced by more than 40% in T1/2 cell treated with IDPN as compared to untreated cells (Figure 2A), while there were no significant differences in basal levels of lipolysis. Moreover, disruption of vimentin filament assembly did not significantly affect FSK-stimulated lipolysis, as it is primarily mediated by PKA activation. Disruption of other cytoskeletal components, actin and tubulin, by cytochalasin D and nocodazole, respectively, had no effect on CL-stimulated lipolysis. Similar results were seen in another model of adipocytes, 3T3-L1 (Figure 2B). The specificity of IDPN in our hands to collapse vimentin filament assembly was confirmed by confocal microscopy (Figure 3). There was no effect of IDPN on actin and tubulin assembly (data not shown). Also shown in Figure 3 is that neither cytochalasin D nor nocodazole disrupted vimentin assembly (nocodazole did lead to some reorganization of vimentin filament assembly as reported by others (19)). Together these results strongly suggest that vimentin filament assembly is a necessary component contributing to lipolysis mediated by β3AR.

Regulation of ERK activity and lipolysis by vimentin filaments
Recently, we reported that β3AR-stimulated lipolysis requires activation of PKA as well as ERK (11). Since we observed that disruption of vimentin intermediate filaments resulted in substantially decreased CL-stimulated lipolysis, we measured the effect of vimentin disruption on PKA activation. There was no change in CL-mediated PKA activation in cells treated with IDPN as compared to
untreated cells (Figure 4A). Another kinase pathway in adipocytes, downstream of PKA, is p38 MAPK, and is also activated by βARs (20). This pathway was also unaffected by vimentin disruption (Figure 4B) (although basal activity was increased by nocodazole and cytochalasin D, as these compounds are known activators of this stress kinase (21)). It was possible that a decrease in lipolysis observed by disruption of vimentin filaments was due to the interference in association of vimentin with other proteins at the lipid droplet. However, disruption of vimentin filaments with IDPN essentially blocked CL-stimulated ERK activation (Figure 4C and 4D). Moreover, removal of IDPN and provision of fresh medium for 3 hr allowed restoration of ERK activation by CL (Figure 4C and 4D). This effect of IDPN on ERK activation by CL was deemed specific based on the following observation. In cells in which actin or tubulin assembly had been disrupted there was no effect on ERK activation by CL (Figure 4C and 4D), although there seemed to be a small but insignificant increase in basal ERK phosphorylation.

These results were also strongly supported by lipolysis studies in which specific inhibitors of either the PKA (H89) or ERK (PD098059) pathway were used in T1/2 and 3T3-L1 adipocytes (Figure 5A and 5B). In IDPN-treated cells, H89 completely blocked glycerol release in response to CL, whereas with PD098059 there was no further effect. However, pretreatment of control cells (i.e. not treated with IDPN) with H89 showed a residual response to the lipolytic action of CL that is due to the ERK pathway as previously reported (11).

A second approach was employed to test the requirement for vimentin in the β3AR-dependent activation of ERK and lipolysis. Using a previously published sequence for knockdown of vimentin by siRNA duplex (22), we confirmed its efficacy in proliferating T1/2 cells (Figure 6A); however, the efficiency was less than 10% once the cells were fully differentiated. Therefore this sequence was used to produce a lentiviral vector for delivery of shRNA to differentiated cells. Infection of differentiated T1/2 cells with pLenti6-si-vim suppressed vimentin expression by more than 60% (61±7.4%, n=3, p<0.01), whereas pLenti6-scramble had no effect (Figure 6B). Using this protocol, pLenti6-si-vim in differentiated T1/2 cells resulted in 21% decrease in CL-stimulated lipolysis (Figure 6C) and total inhibition of ERK activation (Figure 6D). Since the small molecule IDPN, which can act on 100% of the cells to disrupt vimentin filament integrity, decreased CL-stimulated lipolysis by 40%, this 21% decrease by the shRNA approach is consistent with the fact that vimentin expression was reduced by approximately half (60%). These results confirm that vimentin is required for CL-stimulated both ERK activation and lipolysis.

**DISCUSSION**

In this study we have shown that vimentin associates with β3AR in adipocytes upon agonist activation of the receptor, and intact vimentin filament assembly plays a critical role in the β3AR-stimulated increase in ERK activity and lipolysis. This work arose from our earlier observation that the β3AR directly recruits Src to its intracellular domains through conserved proline-rich motifs in the receptor, and this interaction is necessary for the activation of ERK (9). We have also shown that one of the roles of ERK in the adipocyte following β3AR stimulation is to contribute to the maximal lipolytic response (11). However, neither the target(s) of ERK that is/are phosphorylated to promote lipolysis, nor the detailed mechanism of ERK activation by β3AR is understood. A related unresolved issue is the apparent requirement for multiple proline-rich PXXP binding motifs in the receptor: even though there are three to four PXXP motifs in all the species homologues of the β3AR, disrupting just one or two is sufficient to abrogate signaling to ERK (9). This observation suggests that additional molecules are required in this complex and/or the stoichiometry and binding of Src at multiple motifs is necessary for proper signal transduction. We have identified one of these molecules recruited to the β3AR-Src complex as vimentin.
Vimentin links βAR to ERK activation and lipolysis

Vimentin is a vital component of intermediate filaments (IFs): a structure shown to be involved in many cellular signaling pathways (23). In adipocytes vimentin IFs have been shown to be required for their differentiation from preadipocytes into mature lipid-laden cells (12). During adipocyte differentiation vimentin IFs undergo reorganization to form a cage-like structure around the lipid droplets (12). However, compensatory mechanisms must also exist because, in mice lacking vimentin, adipocyte differentiation evidently proceeds normally (24).

In our studies the interaction of β3AR with vimentin was dependent on Src kinase activity, although it is not clear how the β3AR-vimentin interaction is modulated by Src. Nevertheless, there is precedent for interaction between vimentin and Src family kinases. For example, it was previously demonstrated by Valgeirsdottir et al that tyrosine phosphorylation of vimentin in response to PDGF requires activated Src (25), while in microglial cells the Src family member Yes was shown to colocalize with vimentin (26).

In a recent study (11), we have shown that lipolysis stimulated by β3AR is largely dependent on PKA activation (required for HSL phosphorylation and translocation) with a lesser contribution from the ERK pathway (target(s) still unidentified). This role of ERK in the regulation of lipolysis is supported by our current results with the identification of vimentin as a necessary interacting partner of β3AR; thus providing one component linking β3AR to ERK and lipolysis. We show that by interfering either qualitatively (by IDPN) or quantitatively (by shRNA) with vimentin IF integrity, these maneuvers led to inhibition of both β3AR agonist-stimulated ERK activation and lipolysis. β3AR-stimulated PKA activity and HSL translocation were unaffected (data not shown). A role for vimentin IFs in lipolysis may not be totally unexpected since a previous proteomic analysis of lipid droplet associated proteins in 3T3-L1 adipocytes showed that vimentin is present on lipid droplets (17). In addition, disruption of vimentin filament assembly prevented accumulation of lipid droplets in differentiating 3T3-L1 adipocytes (15). In this latter case there was also increased turnover of lipids, indicating that vimentin filaments are involved not only in lipid storage, but are also required for the accessibility of these lipids to be acted upon by lipases. Our results here support this idea.

Although it is presently unclear how vimentin IFs are recruited to β3AR, or how they lead to ERK activation and lipolysis in adipocytes, at least one other connection between vimentin and ERK has been observed. Recently it was shown that newly synthesized vimentin interacts with ERK MAP kinase at sites of axon injury in dorsal root ganglion neurons, and in this situation vimentin is instrumental in signal propagation by serving as a scaffold to connect activated impotin β and dynein (27). For our part, we also do not rule out other effects of vimentin on lipolysis since, being present on the lipid droplet, it may be associated with other proteins that are themselves the target of ERK or of other signaling pathways.

In conclusion, we have demonstrated the association of vimentin with the agonist activated β3AR. We also show that vimentin filament assembly is necessary for β3AR-mediated ERK activation and lipolysis. This interaction of vimentin seems to be specific to β3AR, as we could not observe any significant interaction with another βAR that is capable of activating ERK, the β2AR. These results place vimentin in a position as a structural link between the β3AR-Src complex and ERK activation. As such, future mechanistic studies of the interactions between the β3AR and vimentin filaments in adipocytes may provide new insight into the formation of signaling complexes for the spatiotemporal integration of external stimuli.
Vimentin links βAR to ERK activation and lipolysis

References:


Vimentin links βAR to ERK activation and lipolysis


Acknowledgements

We thank Dr Wenhong Cao for helpful discussions and comments on the manuscript. This work was supported by National Institutes of Health awards R01-DK53092 and R01-DK57698 to SC.

Abbreviations: β-adrenergic receptor, βAR; cAMP-dependent protein kinase, PKA; disodium (R,R)-5-[2-[(3-chlorophenyl)-2-hydroxyethyl]-amino]-propyl]-1,3-benzodi-oxazole-2,2-dicarboxylate (CL316,243), CL; extracellular signal-regulated kinases 1 & 2, ERK; hormone-sensitive lipase, HSL; mitogen-activated protein kinase, MAPK; forskolin, Fsk; intermediate filaments, IFs; salbutamol, Sal; non-specific bands, NSB.

Figure Legends:

Figure 1. Agonist-stimulated association of vimentin with β3AR in T1/2 adipocytes. (A) T1/2 cells transfected with HA-tagged wild type or C-terminal tail mutant (Mut) β3AR or HA-tagged β2AR and stimulated with either CL (10 µM) or salbutamol (1 µM) for 10 minutes. (B) T1/2 cells transfected with HA-tagged wt β3AR pretreated with 1 µM PP2 for 30 minutes and stimulated with CL (10 µM) for 10 minutes. Lysates were used for immunoprecipitation with anti-HA antibody. The samples were run on SDS-PAGE and immunoblotted with anti-vimentin antibody. C represents untransfected control cells; CL: cells treated with CL316,243.

Figure 2. Disruption of vimentin filaments blunts β3AR-agonist stimulated lipolysis. Differentiated T1/2 (A) and 3T3-L1 (B) cells were serum-starved in Krebs buffer containing 2% fatty acid free BSA for 3hr. The cells were pretreated with indicated cytoskeletal disrupting agents for 1 hr. Lipolysis in intact adipocytes was assessed by measuring glycerol released into the media 2 hr after addition of agonists as detailed in the Experimental Procedures. Results are from 3 independent experiments, each experimental sample was done in duplicates. IDPN-cells pretreated with imniodipropionitrile, cyto-cells pretreated with cytochalasin D, noco-cells pretreated with nocodazole, B-Basal, CL-CL316,243 treated, Fsk-forskolin treated. (* for p < 0.001, # for p < 0.01 compared to untreated CL.).

Figure 3. Confocal microscopy of vimentin filaments treated with various cytoskeletal disrupting agents. Differentiated T1/2 cells were treated with indicated cytoskeletal disrupting agents for 1 hr before fixation in methanol (-20°C). The cells were stained with monoclonal antibody against vimentin and were visualized by indirect immunofluorescence as described in Experimental Procedures. C-untreated, IDPN-cells pretreated with immiodipropionitrile, cyto-cells pretreated with cytochalasin D, noco-cells pretreated with nocodazole. White stars show examples of lipid droplets; arrow indicates example of reorganized intermediate filaments.
Vimentin links βAR to ERK activation and lipolysis

Figure 4. Effect of various cytoskeletal disrupting agents on β3AR stimulated kinase activation. Differentiated T1/2 adipocytes were treated with indicated cytoskeletal disrupting agents, or allowed to recover for 3 h in fresh medium for IDPN treated cells (*), followed by stimulation with either 10µM CL (10 minutes) or 20 µM forskolin (10 minutes). The cells were lysed as described in Experimental Procedures. (A) PKA activity was measured in the whole-cell lysate using fluorescent Kemptide as substrate. (B) p38 MAPK activity was measured in whole cell lysates using GST-ATF2 as substrate and measuring the phosphorylation of ATF-2 with anti-phospho-ATF-2 antibody and input for assay is shown as total p38. (C) ERK activity measured by Western blotting of whole cell extracts by using anti-phospho-ERK or anti-ERK antibodies. (D) Quantification of phosphorylated ERK-1/2 is from three independent experiments. A representative experiment is shown above from 3 independent experiments. IDPN-cells pretreated with immiodipropionitrile, cytoD-cells pretreated with cytochalasin D, noco-cells pretreated with nocodazole, C-untreated, CL-CL316,243 treated, Fsk-forskolin treated.

Figure 5. Contribution of PKA and ERK to β3AR-agonist stimulated lipolysis. Differentiated T1/2 (A) and 3T3-L1 (B) cells were serum-starved in Krebs buffer containing 2% fatty acid free BSA for 3hr. The cells were pretreated with IDPN for 1 hr. H89 (40µM) or PD098059 (40µM) were added 30 minutes before addition of indicated agonists. Lipolysis in intact adipocytes was assessed by measuring glycerol released into the media 2 hr after addition of agonists as detailed in the Experimental Procedures. Results are from 3 experiments. IDPN-cells pretreated with immiodipropionitrile, H89-cells pretreated with H89, PD-cells pretreated with PD098059, B-Basal, CL-CL316,243 treated, Fsk-forskolin treated. (* for p < 0.001, # for p < 0.01 and ** for p < 0.001 compared to untreated CL).

Figure 6. Effect of depletion of vimentin by shRNA on lipolysis and ERK activation. (A) Proliferating T1/2 cells were reverse transfected with vimentin siRNA duplex; (B-D) differentiated T1/2 were infected with lentiviral vector carrying vimentin shRNA (L-vim) or scramble shRNA as control (L-scm). (B) The cells were lysed, samples were run on SDS-PAGE and immunoblotted with anti-vimentin antibody. (C) Lipolysis and (D) ERK activation and total ERK were measured as described above. A representative experiment is shown above from 1-3 independent experiments. (* for p < 0.001, and # for p < 0.05 compared to untreated CL).
Figure 1

A

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>β3AR</th>
<th>Mut-β3AR</th>
<th>β2AR</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP: HA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sal</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>β3AR</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP: HA</td>
<td></td>
</tr>
<tr>
<td>Lysate</td>
<td></td>
</tr>
<tr>
<td>CL</td>
<td>-</td>
</tr>
<tr>
<td>PP2</td>
<td>-</td>
</tr>
</tbody>
</table>

Vimentin
Figure 2

A

Glycerol release (fold change)

B

Glycerol release (fold change)
Figure 3
Figure 4

A

CL
IDPN
Cyto
Noco
Fsk

B

CL
IDPN
Cyto
Noco

(-)C
Kemptide
pKemptide
pATF-2
Total p38
Figure 4

C

<table>
<thead>
<tr>
<th></th>
<th>IDPN</th>
<th>Cyto</th>
<th>Noco</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

D

ERK phosphorylation (fold change)

<table>
<thead>
<tr>
<th></th>
<th>IDPN</th>
<th>Cyto</th>
<th>Noco</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* indicates significant difference.
Figure 5

A

Glycerol release (fold change)

B

Glycerol release (fold change)
Figure 6

A

0 2 5 10 20 50 pMol/well

B

Control L-vim L-scr

C

Glycerol release (fold change)

B CL B CL B CL

L-vim L-scr

D

CL - + - + - +

L-vim L-scr

pERK

ERK
Requirement of vimentin filament assembly for β3-adrenergic receptor activation of ERK MAP kinase and lipolysis
Naresh Kumar, Jacques Robidoux, Kiefer W. Daniel, Gabriel Guzman, Lisa M. Floering and Sheila Collins

J. Biol. Chem. published online January 24, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M605571200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2007/01/24/jbc.M605571200.citation.full.html#ref-list-1