Lipid Rafts/Caveolae Are Essential for Insulin-like Growth Factor-1 Receptor Signaling during 3T3-L1 Preadipocyte Differentiation Induction*

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Lipid rafts/caveolae are found to be essential for insulin-like growth factor (IGF)-1 receptor signaling during 3T3-L1 preadipocyte differentiation induction. In 3T3-L1 cells, IGF-1 receptor is located in lipid rafts/caveolae of the plasma membrane and can directly interact with caveolin-1, the major protein component in caveolae. Disruption of lipid rafts/caveolae by depleting cellular cholesterol with cholesterol-binding reagent, β-methylcyclodextrin or filipin, blocks the IGF-1 receptor signaling in 3T3-L1 preadipocyte. Both hormonal induced adipocyte differentiation and mitotic clonal expansion are inhibited by lipid rafts/caveolae disruption. However, a nonspecific lipid binding reagent, xylazine, does not affect adipocyte differentiation or mitotic clonal expansion. Further studies indicate that lipid rafts/caveolae are required only for IGF-1 receptor downstream signaling and not the activation of receptor itself by ligand. Thus, our results suggest that localization in lipid rafts/caveolae and association with caveolin enable IGF-1 receptor to have a close contact with downstream signal molecules recruited into lipid rafts/caveolae and transmit the signal through these signal molecule complexes.

Lipid rafts are plasma membrane microdomains, principally composed of cholesterol and sphingolipids, which form liquid-ordered domains of decreased membrane fluidity (1–6). With integration of caveolins into lipid rafts, these microdomains will form caveolae, which are flask-shaped vesicular invaginations in the plasma membrane (2, 4, 7). Caveolae are a specific form of lipid rafts and are now considered to be broader than just vesicular membrane invaginations (1, 7). The long saturated tails of sphingolipids impart the lipid rafts a high degree of order further stabilized by interacting cholesterol. This property leads a light buoyant density on sucrose density gradient centrifugation.

Cholesterol is an essential component in lipid rafts/caveolae. In caveolae, cholesterol binds directly to caveolins and facilitates the integration of caveolins into membrane (8, 9). Depletion of cellular cholesterol with cholesterol-binding reagents, such as methylcyclodextrin or filipin, will remove cholesterol from lipid rafts/caveolae, disassemble the striated caveolin coats, and eventually lead to the disruption of both lipid rafts and caveolae (1, 7, 10–15).

During recent years, more and more reports confirmed that many signaling molecules are found to be enriched in lipid rafts/caveolae, which serve as platforms and play an important role in regulating signal cascade (1–4, 7, 16, 17). Signal molecules, such as heterotrimeric G-proteins (18), protein kinase C (19, 20), Shc (21), SOS (22), Raf1 (22, 23), and Src family tyrosine kinases (19, 24–26), are recruited into caveolae by caveolins, which, through the scaffolding domain, interact with the caveolin-binding motifs in these signal molecules (17). These clusters of signal molecules can form “preassembled signaling complexes” on the plasma membrane. In addition, many growth factor receptors (epidermal growth factor receptor, platelet-derived growth factor receptor, insulin receptor, etc.) (12, 20, 21, 27, 29) are found to be located in lipid rafts/caveolae. Thus, the enrichment of receptors and signal molecules in lipid rafts/caveolae enables them to be in close contact with each other and makes lipid rafts/caveolae the gateways for signals entering into the cells.

Lipid rafts/caveolae are indicated to be important for insulin receptor signaling (30, 31). Insulin receptors are found to be located in caveolae of adipocyte plasma membrane (27), and many signal molecules involved in insulin receptor signal cascade are also found to be enriched in caveolin-enriched plasma membrane domain (32). In 3T3-L1 adipocytes, caveolin-1 is phosphorylated by insulin receptor (33, 34) and is an activator of insulin receptor signaling (35). In addition, caveolin-enriched lipid raft microdomains and lipid rafts are required for insulin signaling and Glut4 translocation (36–39). These results provide compelling evidence that lipid rafts/caveolae are essential for insulin receptor signaling.

IGF-1** receptor tyrosine kinase signaling (along with glucocorticoid and cAMP signaling) is required for 3T3-L1 preadipocyte differentiation induction (40–45). High level insulin or IGF-1 at physiological concentration activates the IGF-1 receptor on the plasma membrane, leading to the initiation of the differentiation program (40, 41). Two events occur after the activation of IGF-1 receptor in 3T3-L1 preadipocytes: mitotic clonal expansion and adipocyte differentiation. Previously, we have identified that these two events are both activated by the IGF-1 receptor signaling and can be separately blocked without affecting the other (41, 46, 47). The mitotic clonal expansion is activated by IGF-1 receptor through a signal pathway involv-

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1 The abbreviations used are: IGF-1, insulin-like growth factor-1; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; Mes, 4-morpholineethanesulfonic acid.
ing the activation of ERK1 and -2, whereas adipocyte differentiation is initiated through the reversible tyrosine phosphorylation of the adapter protein c-Crk by IGF-1 receptor tyrosine kinase and tyrosine phosphatase. These results suggest that IGF-1 receptor activates two separate signal pathways in 3T3-L1 preadipocytes simultaneously.

Although some of the cellular functions for IGF-1 receptor and insulin receptor are different, structural and signaling similarities between these two receptors have long been recognized. Recently, it has been reported that IGF-1 may also induce caveolae-tyrosine phosphorylation and its translocation in the lipid rafts (48). Since in 3T3-L1 cells IGF-1 receptor activates more than one signal pathway at the same time, it is likely that IGF-1 receptor on plasma membrane interacts or cross-talks with several signaling pathways. With their preassembled signaling complexes on the intracellular side, lipid rafts/caveolae provide the structural foundation for simultaneous activation or cross-talk of multiple signal pathways by IGF-1 receptor.

In the present study, we reported that IGF-1 receptor was located in lipid rafts/caveolae in 3T3-L1 preadipocyte and adipocyte. The integrity of lipid rafts/caveolae was essential for IGF-1 receptor signal transduction during 3T3-L1 preadipocyte differentiation induction. Disruption of lipid rafts/caveolae by cholesterol depletion effectively blocked the downstream signaling of IGF-1 receptor but not IGF-1 receptor activation itself.

EXPERIMENTAL PROCEDURES

Materials—Anti-IGF-1 receptor β-subunit antibody was purchased from Oncogene Research Products. Anti-phosphotyrosine antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-caveolin-1 α isofrom, anti-clathrin light chain, anti-insulin receptor β-subunit, anti-ERK, and anti-p-ERK (against the critical Tyr residue-phosphorylated peptide) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-c-Crk antibody was from Transduction Laboratories. Horseradish peroxidase conjugate, FITC conjugate, or rhodamine conjugate conjugate secondary antibodies, β-methylcycloexodrin, filipin, xylazine, dexamethasone, 1-methyl-3-isobutylxanthine, and insulin were from Sigma. Dulbecco’s modified Eagle’s medium and protein A-agarose were from Invitrogen.

Cell Culture, Differentiation Induction of 3T3-L1 Preadipocytes, and Oil Red-O Staining—3T3-L1 preadipocytes were cultured and induced to differentiate following the protocol described previously (41–44). The differentiated 3T3-L1 adipocytes were stained with Oil Red-O to show triglyceride droplets (41).

Double Immunochemistry Staining—For caveolin-1 and IGF-1 receptor double immunofluorescence staining, 3T3-L1 preadipocytes were cultured on glass coverslips. The coverslips were rinsed with phosphate-buffered saline and fixed for 10 min in 3.7% formaldehyde and 0.18% Triton X-100 in phosphate-buffered saline solution. The fixed cells were incubated in blocking buffer (1% bovine serum albumin in Tween/Tris-buffered saline) for 30 min at room temperature. The cells were then incubated with anti-Cav-1 antibody (rabbit) and anti-IGF-1Rβ antibody (mouse) in blocking buffer for 1 h at room temperature. After washing, the coverslips were incubated with rhodamine conjugate anti-mouse and FITC conjugate anti-rabbit secondary antibodies. The cells were visualized by confocal microscope (Bio-Rad).

For caveolin-1 and clathrin double immunofluorescence staining, 3T3-L1 preadipocytes cultured on glass coverslips were induced to differentiate into adipocytes following the standard differentiation protocol as previously described (41–44). After adipocytes on coverslips were rinsed, fixed, and blocked as mentioned before. The cells were then incubated with anti-Cav-1 antibody (rabbit) and anti-clathrin antibody (mouse) in blocking buffer for 1 h at room temperature. After washing, the coverslips were incubated with rhodamine conjugate anti-rabbit and FITC conjugate anti-mouse secondary antibodies. The cells were visualized by confocal microscope (Bio-Rad).

Sodium Carbonate Extraction and Sucrose Density Gradient Fractionation—Cell extracts/Caveolae—The precipitated materials were centrifuged at 39,000 rpm in a SW41 rotor (Beckman). Fractions were collected from the top of the gradient, and proteins were precipitated with 12.5% trichloroacetic acid, dissolved in 1× Laemmli SDS sample buffer containing 20 mM dithiothreitol, and subjected to Western immunoblot.

Immunoprecipitation and Western Immunoblot—10-cm 3T3-L1 cell monolayers were treated as described in Fig. 4. Cell extracts were prepared and immunoprecipitated as described before (41). 500 μg of cell extract was immunoprecipitated with 1 μg of anti-IGF-1R antibody. The immunoprecipitated samples were subjected to SDS-PAGE and Western blot analysis, which was conducted as described previously (46).

Analysis of IGF-1 Receptor Signaling—Two-day postconfluent 3T3-L1 preadipocytes were fed with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum for 48 h to minimize the serum effect. 10 mM β-methylcycloexodrin was added to the cells for 45 min to deplete the cellular cholesterol and disrupt lipid rafts/caveolae (12–14). The cells were then treated with 10 μg/ml insulin (to activate the IGF-1 receptor) or 0.5 mM 3-isobutyl-1-methylxanthine (to inhibit cAMP phosphodiesterase and increase cellular cAMP concentration). At the indicated time point, the cells were harvested for immunoprecipitation analysis of IGF-1 receptor and its autophosphorylation or harvested directly in 1× boiling SDS sample buffer with 20 mM dithiothreitol for analysis of ERK and its activation. In control cells, the experiments were conducted in the same way without the β-methylcycloexodrin treatment.

Effect of β-Methylcycloexodrin, Filipin, and Xylazine on the 3T3-L1 Preadipocyte Differentiation and Mitotic Clonal Expansion—Postconfluent 3T3-L1 preadipocytes were pretreated with cholesterol binding reagents (β-methylcycloexodrin or filipin) or nonspecific lipid binding reagent (xylazine) for 24 h and then were induced to differentiate following the standard differentiation induction protocol. Lipid binding reagents were removed after differentiation induction medium was changed during the first 4 days (i.e. 2 days of induction with 1-methyl-3-isobutylxanthine, dexamethasone, and insulin and an additional 2 days with insulin alone) and removed after day 4. During differentiation induction, cell numbers were counted to analyze the mitotic clonal expansion. By day 8, the cells were stained with Oil Red-O.

RESULTS

Co-localization and Association of IGF-1 Receptor with Caveolin—Since they are predominantly found in caveolae on plasma membrane, caveolins could be used to indicate caveolae on cell surface. Caveolin-1, the most abundant form of caveolins in caveolae, has two isoforms, α and β, which are produced by RNA alternative splicing (50). Using double immunofluorescence staining with rabbit anti-caveolin-1 (reacting only with α isoform) and mouse anti-IGF-1 receptor β-subunit antibodies, we could simultaneously reveal the location of caveolin-1 and IGF-1 receptor on the cell surface. As shown in Fig. 1, A and B, IGF-1 receptor was co-localized with caveolin-1α protein in 3T3-L1 preadipocytes. To validate this double immunofluorescence staining result, localization of caveolin-1 and clathrin (the component protein in coated pits, which are different plasma membrane structures from caveolae) was analyzed. Since clathrin was highly expressed in 3T3-L1 adipocyte, but not preadipocyte (Fig. 3A), 3T3-L1 adipocytes were used in double immunofluorescence staining for caveolin-1 and clathrin. Fig. 1, C and D, showed the confocal images of caveolin-1 and clathrin double immunofluorescence in 3T3-L1 adipocytes. Under the confocal microscope, ball-shaped 3T3-L1 adipocytes were revealed as a ring. Caveolin-1 staining and clathrin staining were quite different, and their two images were not well overlapped.

Due to the large difference in the protein amount of IGF-1 receptor and caveolin-1 (on plasma membrane, caveolin-1 is much more abundant than IGF-1 receptor), the immunofluorescence signal of IGF-1 receptor had to be highly amplified by...
Lipid Rafts/Caveolae Essential for IGF-1 Receptor Signaling

Blockade of 3T3-L1 Preadipocyte Differentiation Induction by Lipid Rafts/Caveolae Disruption—To analyze the function of lipid rafts/caveolae in IGF-1 receptor signaling, cholesterol binding reagent, β-methylcyclodextrin or filipin, was used to deplete the cellular cholesterol, which is essential for the structural integrity of lipid rafts/caveolae. As a control to cholesterol-binding reagents, a general lipid binding reagent, xylazine, was also used in the experiments. Following the reports by other researchers (12–14), 10 mM β-methylcyclodextrin was used to treat 3T3-L1 preadipocytes. After the treatment, lipid rafts/caveolae were significantly disrupted, as indicated by dislocation of caveolin in density gradient separation (Fig. 2). As a control, xylazine had no effect on the integrity of lipid rafts/caveolae (result not shown). Thus, only cholesterol-binding reagent (β-methylcyclodextrin), but not general lipid-binding reagent (xylazine), disrupted lipid rafts/caveolae.

IGF-1 receptor signaling plays a key role in inducing 3T3-L1 preadipocytes to differentiate into adipocytes (40, 41, 46). To study the function of lipid rafts/caveolae in IGF-1 receptor signaling during 3T3-L1 preadipocyte differentiation induction, cells were induced to differentiate in the presence of cholesterol-binding reagent (β-methylcyclodextrin or filipin) or control reagent (xylazine). Since these reagents were used at different concentrations, the concentrations used in experiments were determined by their effect on cell proliferation of dividing 3T3-L1 preadipocytes. In order to compare the results of these reagents, we used these reagents at the concentrations that had similar effect on cell proliferation (Fig. 6, A, C, and E). At high concentrations, they all severely inhibited cell proliferation of exponentially growing cells and did not allow cells to survive the differentiation induction process (results not shown). Therefore, in our experiments, these reagents were used at concentrations at which cells could proliferate and survive the differentiation induction process (Fig. 6).

A

B

C

D

Fig. 1. Co-localization of IGF-1 receptor with caveolin-1. Double immunofluorescence staining was conducted on 3T3-L1 cells as described under “Experimental Procedures.” A and B, IGF-1 receptor was detected by rhodamine red fluorescence (IGF-1R) and caveolin-1 by FITC green fluorescence (Caveolin-1). These two images were merged into one overlapped picture (merged) to show the relative location of IGF-1 receptor and caveolin-1. A and B, high and low magnification view, respectively. C and D, caveolin-1 was detected by rhodamine red fluorescence (Caveolin-1), and clathrin was detected by FITC green fluorescence (Clathrin). The overlapped picture of these two images is shown (merged). C, high magnification view; D, low magnification view.
Before the differentiation induction, cells were pretreated with \( \beta \)-methylcyclodextrin or filipin at the indicated concentrations for 24 h to deplete cellular cholesterol. With this treatment \((e.g.~4~\text{mM} \beta \text{-methylcyclodextrin for 24~h})\), significant amounts of lipid rafts/caveolae were disrupted (results not shown). \( \beta \)-Methylcyclodextrin or filipin was then kept in the differentiation induction medium to maintain a low cellular cholesterol level. Since the major triglyceride accumulation in the 3T3-L1 adipocyte differentiation process occurred after day 4, the reagent was removed from the culture medium after the initial 4-day induction period to prevent any side effect on the lipid accumulation. The effect on 3T3-L1 adipocyte differentiation by these lipid-binding reagents was evaluated by the triglyceride accumulation in the cells after differentiation induction (Fig. 5A). At 2.5 mM, \( \beta \)-methylcyclodextrin significantly reduced the triglyceride accumulation in the cells. At 4 mM \( \beta \)-methylcyclodextrin almost completely inhibited the adipocyte differentiation. For filipin, a similar effect on adipocyte differentiation was observed (Fig. 5B). At 1.5 \( \mu \text{g/ml} \) (~2.3 \( \mu \text{M} \)), filipin completely blocked the induced adipocyte differential-
tion. At a concentration of 1 μg/ml (~1.5 μM), filipin still exhibited some inhibitory effect on adipocyte differentiation (results not shown). In contrast, xylazine used in the same condition as a control had no detectable effect on adipocyte differentiation (results not shown). In contrast, xylazine used in the same conditions as described under “Experimental Procedures” and homogenized with 3× ultrasonic pulse, F, insoluble material; S, soluble extract. C, caveolin-1 co-immunoprecipitation with IGF-1 receptor. B, immunoprecipitation with lysate buffer as a blank control; C, cell lysate without immunoprecipitation as control for caveolin-1. +Insulin, 0, 30′ 2h and 4h, same as in A. IgG, the antibody used for immunoprecipitation recognized by the secondary antibody in Western blot.

During 3T3-L1 preadipocyte differentiation induction, growth-arrested confluent cells were induced to reenter into a cell division process called mitotic clonal expansion. This mitotic clonal expansion is induced only by IGF-1 receptor signal (46). Since IGF-1 receptor signal-induced adipocyte differentiation was blocked by lipid rafts/caveolae disruption (Fig. 5), mitotic clonal expansion was also inhibited in the similar condition. Our results indicated that the presence of cholesterol binding reagents (β-methylcyclodextrin or filipin) in the differentiation induction medium markedly decreased the IGF-1 receptor signaling-induced mitotic clonal expansion (Fig. 6, B and D). At 4 mM β-methylcyclodextrin, mitotic clonal expansion was completely blocked. However, exponentially growing 3T3-L1 preadipocytes could still divide in the presence of β-methylcyclodextrin or filipin, although the rate of cell division was slowed down, especially at the high concentration (Fig. 6, A and C). This result indicated that the inhibition of mitotic clonal expansion during the 3T3-L1 preadipocyte differentiation process by β-methylcyclodextrin or filipin was due to the blockade of IGF-1 receptor signaling rather than the cell mitotic machinery. In comparison, xylazine had a very similar effect on slowing the proliferation rate of exponentially growing 3T3-L1 preadipocytes (Fig. 6E) but had no effect on IGF-1 receptor signal-induced mitotic clonal expansion during the differentiation induction process (Fig. 6F). With these results, it was clear that cholesterol binding reagents, β-methylcyclodextrin and filipin, could block both IGF-1 receptor signal-induced adipocyte differentiation and mitotic clonal expansion, whereas the nonspecific lipid binding reagent, xylazine, had no effect on either adipocyte differentiation or mitotic clonal expansion.

Lipid Rafts/Caveolae Disruption Blocks Downstream Signaling of IGF-1 Receptor but Not the Activation of Receptor Itself—Cellular responses (adipocyte differentiation and mitotic clonal expansion) induced by IGF-1 receptor signal are long term effects: mitotic clonal expansion is induced in the first 48 h after hormonal stimulation, and adipocyte phenotype appears around 96 h after induction. Thus, it is difficult to analyze the mechanism of lipid rafts/caveolae in IGF-1 receptor signaling by using these cellular responses. Previously, we have reported that ERK1 and -2 were activated by IGF-1 receptor signaling within minutes after stimulation, and their activation was essential for mitotic clonal expansion (46). This activation of

![Fig. 4: Association of IGF-1 receptor with caveolin-1 protein.](http://www.jbc.org/)

**A.** Western blot analysis of IGF-1 receptor autophosphorylation after stimulation with high level insulin. +Insulin, 2-day postconfluent 3T3-L1 preadipocytes stimulated with insulin; 0, control prior to insulin stimulation; 30′, 4h, 8h, and 24h, 30-min, 4-h, 8-h, and 24-h insulin stimulation time, respectively. IP, immunoprecipitation; IB, Western blot. α-IGF-1Rβ, antibody against IGF-1 receptor β subunit; α-P-Tyr, antibody against phosphotyrosine. B, extraction of IGF-1 receptor and caveolin-1 by Triton X-100 and ultrasonic homogenization. Cells were lysed with Triton buffer as described under “Experimental Procedures” and homogenized with 3× ultrasonic pulse. F, insoluble material; S, soluble extract. C, caveolin-1 co-immunoprecipitation with IGF-1 receptor. B, immunoprecipitation with lysate buffer as a blank control; C, cell lysate without immunoprecipitation as control for caveolin-1. +Insulin, 0, 30′ 2h and 4h, same as in A. IgG, the antibody used for immunoprecipitation recognized by the secondary antibody in Western blot.

![Fig. 5: Effect of cholesterol-binding reagents or general lipid-binding reagent on hormonal induced 3T3-L1 adipocyte differentiation.](http://www.jbc.org/)

**A.** Effect of β-methylcyclodextrin on 3T3-L1 adipocyte differentiation. Postconfluent 3T3-L1 preadipocytes were induced to differentiation in the presence of β-methylcyclodextrin as described under “Experimental Procedures.” The β-methylcyclodextrin was used at concentrations indicated. Day 8 cells were stained with Oil Red O to reveal the triglyceride droplets. Control, standard differentiation induction. +β-MCD, cells induced in the presence of β-methylcyclodextrin, 2.5 mM, 3.0 mM, and 4.0 mM, β-methylcyclodextrin concentrations. B, effect of filipin on 3T3-L1 adipocyte differentiation. All of the experiments were carried out in the same conditions as described for A. +Filipin, cells induced in the presence of filipin, 1.2 μg/ml, 1.35 μg/ml, and 1.5 μg/ml, filipin concentrations. Control, standard differentiation induction. C, effect of xylazine on 3T3-L1 adipocyte differentiation. All of the experiments were carried out in the same conditions as described for A. +Xylazine, cells induced in the presence of xylazine, 5 μg/ml, 20 μg/ml, and 50 μg/ml, xylazine concentrations. Control, standard differentiation induction.
ERK1 and -2 by IGF-1 receptor signal provides a feasible cellular response to analyze the function of lipid rafts/caveolae in IGF-1 receptor signal transduction.

Since disruption of lipid rafts/caveolae blocked IGF-1 receptor signal-induced adipocyte differentiation and mitotic clonal expansion, IGF-1 receptor signaling might be blocked at the activation of IGF-1 receptor by ligand and/or the downstream signal transduction of the activated IGF-1 receptor. If lipid rafts/caveolae are required for the activation of IGF-1 receptor by its ligand, disruption of lipid rafts/caveolae with cholesterol binding reagent will interfere with the receptor activation by ligand and reduce the receptor autophosphorylation. However, as shown in Fig. 7B, IGF-1 receptor autophosphorylation stimulated by ligand (high level of insulin) was not affected by the treatment of β-methylcyclodextrin. On the other hand, disruption of lipid rafts/caveolae greatly affected the downstream signal transduction of IGF-1 receptor. The activation of ERK1 and -2 by IGF-1 receptor signal was significantly reduced by β-methylcyclodextrin treatment (Fig. 7C). These results suggested that in the IGF-1 receptor signaling process lipid rafts/caveolae are required for IGF-1 receptor’s downstream signal transduction but not the activation of the receptor per se.

To rule out the possibility that β-methylcyclodextrin treatment directly inhibits the kinases activating ERK1 and -2, not
**Fig. 7. Effect of cholesterol-binding reagent, β-methylcyclodextrin, on IGF-1 receptor signaling.** The experiments were carried out as described under “Experimental Procedures.” A, samples before immunoprecipitation were subjected to SDS-PAGE and Western blotted by anti-IGF-1 receptor β subunit antibody to show total IGF-1 receptor. Control, cells without β-methylcyclodextrin treatment and; +β-MCD, cells with β-methylcyclodextrin treatment. +Insulin, insulin stimulation; 0, 5, 10, and 30 min, stimulation time. B, ligand-induced IGF-1 receptor autophosphorylation in the presence or absence of β-methylcyclodextrin. IGF-1 receptor was immunoprecipitated from cell extracts as described under “Experimental Procedures.” Control, + Insulin, and the time points (0, 1, 5, 10, and 30 min), same as in A. IP, immunoprecipitation; IB, Western blot; α-IGF-1Rβ, antibody against IGF-1 receptor β subunit; α-P-Tyr, antibody against phosphotyrosine. B, immunoprecipitation of lysis buffer as a blank control; C, effect of β-methylcyclodextrin treatment on ERK activation by IGF-1 receptor or 1-methyl-3-isobutylxanthine. Experiments were carried out as described under “Experimental Procedures.” The cells were treated in the same conditions as in B. The ERK activation was analyzed by phosphorylation on the activation site. Control, +β-MCD, + Insulin, and the time points (0, 1, 5, 10, and 30 min) are the same as in B. +MIX, stimulation with 1-methyl-3-isobutylxanthine. α-pERK, antibody against tyrosine-phosphorylated ERK1 and -2; α-ERK, antibody against ERK1 and -2.

IGF-1 receptor signaling, the effect of β-methylcyclodextrin on ERK1 and -2 activation stimulated by 1-methyl-3-isobutylxanthine was analyzed. 1-Methyl-3-isobutylxanthine increases the intracellular cAMP concentration by inhibiting cAMP phosphodiesterase, which is a plasma membrane-independent process. Fig. 7C showed that β-methylcyclodextrin treatment had no effect on 1-methyl-3-isobutylxanthine-stimulated ERK activation. The ERK enzyme system was not adversely affected by β-methylcyclodextrin treatment. Thus, it is clear that disruption of lipid rafts/caveolae by cholesterol depletion only inhibited the membrane-dependent IGF-1 receptor’s downstream cAMP signaling.

The phosphotyrosine protein band of smaller molecular weight was insulin receptor β-subunit (Fig. 7B). It could be detected by insulin receptor antibody (results not shown). This is probably due to the insulin and IGF-1 hybrid receptor (52), which could be immunoprecipitated by IGF-1 receptor antibody.

**DISCUSSION**

In many ways, caveolae are lipid rafts enriched with structural protein caveolins, which are the defining protein components in caveolae. Although caveolins have three types, general types caveolin-1 and -2 and muscle-specific caveolin-3 (7), cells completely lack caveolae in caveolin-1 knockout mice (53). This result from caveolin-1-deficient mice has demonstrated the importance of caveolin-1 in the formation of caveolae and provided support for using caveolin-1 as an indicator for caveolae. By using immunofluorescence staining (Fig. 1), sucrose density gradient centrifugation (Figs. 2 and 3), and co-immunoprecipitation (Fig. 4), we have identified that IGF-1 receptor was located in lipid rafts/caveolae in 3T3-L1 cells. To provide further support, we have also shown that IGF-1 receptor and insulin receptor, which has been indicated to be located in caveolae of adipocyte plasma membrane (26, 51), were associated with the same membrane structures in 3T3-L1 adipocytes (Fig. 3B). These results provided strong evidence that IGF-1 receptor was located in lipid rafts/caveolae in 3T3-L1 preadipocytes and adipocytes.

It was consistently observed that, besides in caveolae, IGF-1 receptor also appeared to be in the membrane structure slightly lighter than caveolae. The peak of IGF-1 receptor and insulin receptor was in fractions 6 and 7, whereas the peak of caveolin was in fractions 7 and 8 (Figs. 2 and 3B). However, in
high level insulin-stimulated adipocytes, IGF-1 receptor, insulin receptor, and caveolin were better correlated in density gradient separation (Fig. 3B). These results were observed in several independently repeated experiments (results not shown). Thus, it is likely that IGF-1 receptor was also located in the caveolin-free lipid rafts around the caveolae and might further converge into caveolae upon ligand stimulation. Currently, we are investigating this translocation induced by ligand.

Although lipid rafts and caveolae are important in signal transduction (1–7), their roles in IGF-1 receptor cellular signaling are not fully understood. The identification of IGF-1 receptor in lipid rafts/caveolae provided us an opportunity to study the role of lipid rafts/caveolae in IGF-1 signaling. In 3T3-L1 preadipocytes, IGF-1 receptor signal is essential for inducing two cellular responses: adipocyte differentiation and mitotic clonal expansion. However, these two cellular responses can be separately blocked by inhibitors without affecting the other (41, 46). PD98059, an inhibitor of MEK-1, blocked mitotic clonal expansion but not adipocyte differentiation, whereas vanadate, a protein-tyrosine phosphatase inhibitor, only blocked adipocyte differentiation. Since lipid rafts/caveolae are on the plasma membrane, which is on the top of the IGF-1 receptor signal cascade, disruption at the level of lipid rafts/caveolae will more likely block all of the cellular responses induced by IGF-1 receptor signaling. Our results clearly support this hypothesis. Disruption of lipid rafts/caveolae by cholesterol-binding reagents led to the blockade of both cellular responses simultaneously (Figs. 5 and 6). Taken together, these results composed a hierarchy of IGF-1 receptor signaling system in 3T3-L1 cells. The signal generated by IGF-1 receptor requires the assistance of lipid rafts/caveolae on plasma membrane to transmit into the cell and activates different signal pathways, which lead to adipocyte differentiation and mitotic clonal expansion, respectively.

It has been reported that β-methylcyclodextrin treatment does not inhibit insulin receptor-induced ERK1 and -2 activation in primary rat adipocytes (12). We observed similar results in 3T3-L1 adipocytes (results not shown). However, in 3T3-L1 preadipocytes, β-methylcyclodextrin treatment dramatically inhibited IGF-1 receptor-induced ERK1 and -2 activation (Fig. 7). It should be noted that 3T3-L1 adipocytes contain more cholesterol than preadipocytes, and under the same β-methylcyclodextrin treatment, less caveolin-1 was displaced from the low density centrifugation fractions in 3T3-L1 in adipocytes (results not shown). Thus, it is likely that lipid rafts/caveolae in adipocytes were not disrupted by β-methylcyclodextrin treatment as completely as in preadipocytes. Although IGF-1 receptor may employ a different signal pathway from insulin receptor to activate ERK1 and -2, we believe that the discrepancy between 3T3-L1 adipocyte and preadipocyte in the activation of ERK is more likely due to the incomplete disruption of lipid rafts/caveolae in adipocyte.

Based on our present studies, only the downstream signal transduction of the receptor required lipid rafts/caveolae (Fig. 7). A possible function of lipid rafts/caveolae in IGF-1 receptor signaling is to recruit intracellular signal molecules for the receptor. Interestingly, studies of the phosphorylation of c-Crk, an endogenous IGF-1 receptor tyrosine kinase substrate, suggest that physical contact between IGF-1 receptor and c-Crk is essential for the phosphorylation of the substrate by the receptor kinase (54, 55). IGF-1 receptor tyrosine kinase can only phosphorylate c-Crk that has bound to the receptor through its Src homology 2 domain. c-Crk protein with the Src homology 2 domain deleted is not phosphorylated by IGF-1 receptor tyrosine kinase. Recruitment of signal molecules by lipid rafts/caveolae not only brings the downstream signal molecules into the receptor but also allows the receptor to be in close contact with signal molecules of many signal pathways for which lipid rafts/caveolae act like a signaling hub.

Recent studies from caveolin-1 knockout mice indicate that the caveolin-1-deficient mice show some abnormalities in adiposity (50–56). The younger caveolin-1-deficient mice have a relatively intact adipocyte tissue except in a few places like in mammary gland and hypodermal fat layers and show similar body weight to their wild type littermates. However, the older caveolin-1-deficient mice have much smaller body sizes than their normal cohorts. This reduced body weight in older caveolin-1-deficient mice is due to reduced adiposity. The Cav-1 knockout mice also appear to be resistant to diet-induced obesity. These results indicated a relatively normal fetal development of adipose tissue in caveolin-1-deficient mice but a problem in adulthood adipose tissue metabolism. In our present studies, lipid rafts/caveolae appeared to be required for adipocyte differentiation induction. Therefore, the caveolaeless knockout mice are probably having problem in adulthood adipocyte differentiation.

Adulthood adipocyte differentiation has more and more been considered as one of the leading causes in obesity, especially in hyperplastic obesity (28, 45). New adipocytes can differenitized from the residual preadipocytes in adipose tissue throughout the life span. The phenotype of caveolin-1-deficient mice suggests that the lipid rafts/caveolae-dependent adipocyte differentiation mechanism in 3T3-L1 cells might more closely resemble adipocyte differentiation in adult animal rather than in embryonic development. Further studies are needed to verify this hypothesis. Taken together with the strong evidence from Cav-1 knockout mice, our present study has established the role of lipid rafts/caveolae in the adipocyte differentiation process.

REFERENCES

Lipid Rafts/Caveolae Are Essential for Insulin-like Growth Factor-1 Receptor Signaling during 3T3-L1 Preadipocyte Differentiation Induction

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