Differentiated 3T3L1 Adipocytes Are Composed of Heterogenous Cell Populations with Distinct Receptor Tyrosine Kinase Signaling Properties*

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Various studies have demonstrated that the platelet-derived growth factor (PDGF) receptor in adipocytes can activate PI 3-kinase activity without affecting insulin-responsive glucose transporter (GLUT4) translocation. To investigate this phenomenon of receptor signaling specificity, we utilized single cell analysis to determine the cellular distribution and signaling properties of PDGF and insulin in differentiated 3T3L1 adipocytes. The insulin receptor was highly expressed in a large percentage of the cell population (>95%) that also expressed caveolin 2 and GLUT4 with very low levels of the PDGF receptor. In contrast, the PDGF receptor was only expressed in ~10% of the differentiated 3T3L1 cell population with relatively low levels of the insulin receptor, caveolin 2, and GLUT4. Consistent with this observation, insulin stimulated the phosphorylation of Akt in the caveolin 2- and GLUT4-positive cells, whereas PDGF primarily stimulated Akt phosphorylation in the caveolin 2- and GLUT4-negative cell population. Furthermore, transfection of the PDGF receptor in the insulin receptor-, GLUT4-, and caveolin 2-positive cells resulted in the ability of PDGF to stimulate GLUT4 translocation. These data demonstrate that differentiated 3T3L1 adipocytes are not a homogeneous population of cells, and the lack of PDGF receptor expression in the GLUT4-positive cell population accounts for the inability of the endogenous PDGF receptor to activate GLUT4 translocation.

One of the most important biological actions of insulin is to increase glucose uptake into striated muscle and adipose tissue (1–4). In the basal state, the insulin-responsive glucose transporter (GLUT4) protein is primarily localized in various intracellular compartments with relatively low levels in the plasma membrane (5–8). Insulin triggers a large increase in the rate of GLUT4 vesicle exocytosis and a small decrease in the rate of GLUT4 internalization by endocytosis, resulting in a large increase in cell surface GLUT4 protein and subsequent increase in glucose uptake.

Currently, the molecular details of insulin signaling leading to GLUT4 translocation remain highly speculative and, in certain aspects, controversial. However, substantial data have established that the activation of the type IA PI 3-kinase and subsequent generation of PI 3,4,5-trisphosphate are essential for the insulin stimulation of GLUT4 translocation. For example, multiple studies using various pharmacological inhibitors, dominant-interfering mutants, expression of a phosphatidylinositol 5’-phosphatase, and expression of a constitutively active catalytic subunit are all consistent with a necessary PI 3-kinase activity for insulin-stimulated GLUT4 translocation (9–16).

However, despite the general agreement that PI 3-kinase activity is necessary for insulin-stimulated glucose uptake, a clear demonstration of a sufficient role for PI 3-kinase has not been forthcoming. Indeed, several lines of evidence suggest that one or more PI 3-kinase-independent signals may be required for insulin-stimulated GLUT4 translocation. For example, two naturally occurring insulin receptor mutations were unable to induce GLUT4 translocation and glucose uptake yet were fully capable of activating PI 3-kinase (17). Activation of the PI 3-kinase through integrin or interleukin 4 receptors had only a small stimulation of GLUT4 translocation and glucose uptake, which was markedly enhanced in the presence of insulin (18, 19). In this regard, the addition of a cell-permeable PI 3,4,5-trisphosphate analog had no effect on glucose uptake but fully stimulated glucose transport in the presence of insulin despite a complete inhibition of PI 3-kinase activity (20). More recently, a second insulin receptor-dependent signaling pathway has been identified that requires the recruitment of the CAP-Cbl complex to plasma membrane lipid raft microdomain (21).

Although it is likely that other signaling cascades, in addition to the activation of the PI 3-kinase pathway are required for this process, several studies have reported that activation of the PI 3-kinase by PDGF is sufficient to induce GLUT4 translocation and/or glucose uptake to a similar extent as insulin (22–24). In contrast, other studies have not detected a significant PDGF-stimulated GLUT4 translocation in 3T3L1 adipocytes (25–28).

To explore the potential basis for this difference in PDGF responsiveness, we have utilized single cell analysis to determine the distribution of PDGF and insulin receptors with respect to GLUT4 translocation-responsive versus nonresponsive 3T3L1 adipocyte cell populations.

EXPERIMENTAL PROCEDURES

Materials—The monoclonal phosphotyrosine (PY20), polyclonal caveolin 1, and monoclonal caveolin 2 antibodies were purchased from Transduction Laboratories (Lexington, KY), and the phospho-Akt was purchased from New England Biolabs (Beverly, MA). The monoclonal PDGF receptor β subunit antibody was obtained from Biogenesis (San-
down, NH), and fluorescent secondary antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). All other antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) except for the polyclonal antibody against rat GLUT4 (IAO2), which was generated as previously described (29). The human PDGF β receptor was kindly provided by Dr. Andrius Kazlauskas (Harvard Medical School, Boston, MA).

Cell Culture and Transient Transfection by Electroporation—3T3L1 adipocytes (American Type Culture Collection, Manassas, VA) were grown and differentiated as described previously (30). Briefly, 9–10-day postdifferentiation adipocytes were electroporated and 18 h later serum-starved for 2 h in Dulbecco's modified Eagle's medium containing 25 mM glucose and 0.1% bovine serum albumin. The cells were left untreated or stimulated with either insulin (100 nM) or PDGF (20 or 50 ng/ml) for the time indicated at 37 °C.

Immunoblotting—3T3L1 cells were washed twice with ice-cold PBS, and cell extracts were prepared by solubilization in lysis buffer (25 mM HEPES, pH 7.4, 1% Nonidet P-40, 10% glycerol, 50 mM NaF, 10 mM NaP2O4, 137 mM NaCl, 1 mM Na3VO4, 10 μg/ml aprotinin, 1 μg/ml pepstatin, 5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Clarified whole cell lysates were separated on 8% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA), and subjected to Western blotting.

Immunofluorescence Microscopy—Intact cell immunofluorescence was performed by washing the cells once with ice-cold PBS, followed by fixation with 4% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA) and 0.2% Triton X-100 in PBS at room temperature for 10 min. The cells were then blocked with 5% donkey serum. Plasma membrane sheets were prepared by the method of Robinson et al. (31). The membranes were fixed in 2% paraformaldehyde at room temperature for 20 min and blocked with 5% donkey serum. The cells or plasma membrane sheets were then incubated with primary mouse monoclonal

Fig. 1. Differentiated 3T3L1 adipocytes are responsive to insulin and PDGF-BB but not to PDGF-AA. Differentiated 3T3L1 adipocytes were either left untreated (C, lane 1) or incubated with 100 nM insulin (I, lane 2), 20 ng/ml PDGF-AA (AA, lane 3), or 20 ng/ml PDGF-BB (BB, lane 4) for 10 min at 37 °C. Whole cell extracts were prepared, and 20 μg were subjected to phosphotyrosine (PY20) immunoblotting as described under “Experimental Procedures.”

Fig. 2. The PDGFβ receptor, but not the insulin receptor or GLUT4, is in a subpopulation of 3T3L1 adipocytes that have low levels of caveolin 2 expression. A, differentiated 3T3L1 cells were fixed, permeabilized, and subjected to confocal immunofluorescence microscopy for the insulin receptor (α), the PDGFβ receptor (d), and GLUT4 (g). Each of these proteins was co-stained with caveolin 2 (b, e, and h), and merged images are presented in c, f, and i as described under “Experimental Procedures.” B, plasma membrane sheets were prepared from the differentiated 3T3L1 adipocytes and subjected to confocal immunofluorescence microscopy for the insulin receptor (α), the PDGFβ receptor (d), and caveolin 2 (b and e). The merged images are presented in c and f.
and activate all three PDGF receptor isoforms, whereas the PDGF-AB, and PDGF-CC). The PDGF-BB ligand can interact with at least four functional ligands (PDGF-AA, PDGF-BB, PDGF ligand chains (PDGF-A, PDGF-B, and PDGF-C) that can form at least four functional ligands (PDGF-AA, PDGF-BB, PDGF-AB, and PDGF-CC). The PDGF-BB ligand can interact with and activate all three PDGF receptor isoforms, whereas the PDGF-αα homodimer is the universal receptor capable of interacting with all the known ligands. To determine which functional PDGF receptor(s) are present in differentiated 3T3L1 adipocytes, we performed phosphotyrosine immunoblots from control and insulin-, PDGF-AA-, and PDGF-BB-stimulated cells (Fig. 1). As expected, insulin stimulation resulted in a marked increase in tyrosine phosphorylation of IRS1 and the insulin receptor β subunit (Fig. 1, lanes 1 and 2). In contrast, PDGF-AA had no effect on tyrosine phosphorylation, whereas PDGF-BB induced the tyrosine phosphorylation of the PDGF receptor (Fig. 1, lanes 3 and 4). Based upon the ligand specificity of the PDGF receptors, these data indicate that differentiated adipocytes express the PDGFαα and/or PDGFββ receptors.

Caveolin is a major protein component of caveolae structures and plays an important role in the assembly of a signaling cascade leading to GLUT4 translocation (21, 35–38). Caveolae are also highly induced during adipocyte differentiation and can occupy up to 20% of the cell surface (39, 40). Thus, the expression of caveolin is not only a marker for adipocyte differentiation but only the caveolin-positive cell population is expected to display insulin-responsive GLUT4 translocation. As expected, the majority of the insulin receptor-positive adipocytes were found to co-express caveolin 2 (Fig. 2A, a–c). However, there was a small percentage of cells that had relatively low levels of caveolin 2. These cells also had relatively low levels of insulin receptor. Some of these cells had a fibroblast-like morphology, but others were more rounded, typical of adipocytes. In any case, only a small percentage of the cells were positive for the PDGF receptor (Fig. 2A, d). Importantly, these PDGF receptor-positive cells had very low levels of caveolin 2 expression (Fig. 2A, d–f). GLUT4 expression was also relatively high in the same cell population that co-expressed caveolin 2 (Fig. 2A, g–i).

We and others have recently observed that caveolin can be detected as torus or donut-shaped structures in isolated plasma membranes from 3T3L1 adipocytes (21, 36). As previously observed, the insulin receptor was found to partially co-localize in these donut-shaped caveolin 2-positive structures in isolated plasma membrane sheets (Fig. 2B, a–c). In contrast, the plasma membrane sheets that we detected containing the PDGF receptor were essentially devoid of these caveolin 2

![Fig. 3. Insulin receptor and PDGFβ receptor are expressed in distinct cell populations.](Image)

A. Differentiated 3T3L1 cells were fixed, permeabilized, and subjected to confocal immunofluorescence microscopy for the insulin receptor (a) and the PDGFβ receptor (b) of the merged image is presented in c. B. Differentiated 3T3L1 cells were either untreated (a, d, and g) or examined for the expression of the insulin receptor (b, e, and h) or the PDGF receptor (c, f, and i). In parallel, the cells were also treated with Oil Red O to identify the lipid-loaded adipocyte cell population (d–f). The merged images are presented in g–i.

**RESULTS AND DISCUSSION**

There are two isoforms of the PDGF receptor (PDGFRα and PDGFRβ) that can homo- and heterodimerize to generate three functional PDGF receptors (32–34). In addition, there are three PDGF ligand chains (PDGF-A, PDGF-B, and PDGF-C) that can form at least four functional ligands (PDGF-AA, PDGF-BB, PDGF-AB, and PDGF-CC). The PDGF-BB ligand can interact and activate all three PDGF receptor isoforms, whereas the PDGF-αα homodimer is the universal receptor capable of interacting with all the known ligands. To determine which functional PDGF receptor(s) are present in differentiated 3T3L1 adipocytes, we performed phosphotyrosine immunoblots.

**Fig. 4. Insulin and PDGF activate Akt in different cell populations.** Differentiated 3T3L1 adipocytes were either left untreated (a–c) or treated with either of 100 nM insulin (d–f) or 50 ng/ml PDGF-BB (g–i) for 5 min at 37 °C. The cells were then fixed, permeabilized, and subjected to confocal immunofluorescence microscopy with a phospho-Akt antibody (a, d, and g) or an insulin receptor antibody (b, e, and h). The merged images are presented in c, f, and i.
Differentiated 3T3L1 adipocytes were electroporated with the human PDGF receptor-, caveolin-, and GLUT4-positive 3T3L1 adipocytes. With 100 nM insulin (transfected 3T3L1 adipocytes were either left untreated or incubated with 100 nM insulin (d–f) or 50 ng/ml PDGF-BB (g–i) for 5 min at 37 °C. The cells were then fixed, permeabilized, and subjected to confocal immunofluorescence microscopy. The merged images are presented in a–c). In the basal state, the phosphoserine Akt antibody displayed a low level of immunoreactivity in the entire cell population visualized (Fig. 4, a–c). Insulin stimulation resulted in the vast majority of insulin receptor-positive cells displaying an increase in phospho-Akt immunofluorescence (Fig. 4, d–f). Although there was an increase in phospho-Akt immunofluorescence throughout the cell, it was primarily localized at the cell surface membrane, indicative of Akt translocation to the plasma membrane (41–44). In contrast, PDGF stimulation resulted in only a fraction of the cell population undergoing Akt phosphorylation (Fig. 4, g–i). These cells had a more central localization of phospho-Akt and had relatively lower levels of insulin receptor immunoreactivity. Thus, taken together, these data demonstrate that the insulin and PDGF receptor are distributed in distinct cell populations of differentiated 3T3L1 adipocytes. Furthermore, insulin and PDGF responsiveness in these cell populations is specifically defined by the presence of these receptor subtypes.

Although in our differentiated 3T3L1 adipocytes the PDGF receptor is found in a relatively small subset of cells that also has low levels of the insulin receptor, caveolin 2, and GLUT4, other studies have reported that PDGF receptor activation can result in GLUT4 translocation (22–24). Therefore, to determine if the PDGF receptor has the capacity to induce GLUT4 translocation when present in the appropriate cell context, we next transfected the human PDGFRβ into differentiated 3T3L1 adipocytes (Fig. 5). Using a low voltage electroporation technique that primarily results in the transfection/expression of adipocytes and excludes fibroblasts (45), the human PDGF receptor was found to be expressed in insulin receptor-, caveolin-, and GLUT4-positive adipocytes (Fig. 5A, a–i). In the basal state, neither the untransfected nor human PDGF receptor-transfected adipocytes displayed significant immunoreactivity with the phospho-Akt antibody (Fig. 5B, d–f). Insulin stimulation

![Image of Figure 5](http://www.jbc.org/)

**FIG. 5. Transfection of the human PDGFRβ receptor in insulin receptor-, caveolin-, and GLUT4-positive 3T3L1 adipocytes.** A, differentiated 3T3L1 adipocytes were electroporated with the human PDGFRβ receptor cDNA as described under “Experimental Procedures.” The cells were then subjected to confocal immunofluorescence microscopy with a human-specific PDGFRβ receptor antibody (a, d, and g), an insulin receptor antibody (b), a caveolin 1 antibody (e), or the GLUT4 antibody (h). The merged images are presented in c, f, and i. B, the PDGFRβ receptor-transfected 3T3L1 adipocytes were either left untreated (a–c) or incubated with 100 nM insulin (d–f) or 50 ng/ml PDGF-BB (g–i) for 5 min at 37 °C. The cells were then fixed, permeabilized, and subjected to confocal immunofluorescence microscopy with a human-specific PDGFRβ receptor antibody (a, d, and g) or the phospho-Akt antibody (b, e, and h). The merged images are presented in c, f, and i. C, quantitation of the number of 3T3L1 adipocytes displaying cell surface phospho-Akt immunofluorescence.
resulted in the phosphorylation of Akt in both the untransfected and human PDGF receptor-transfected cells (Fig. 5 B, d–f). However, the insulin stimulation of Akt phosphorylation and translocation to the plasma membrane was somewhat reduced in the PDGF receptor-expressing cells. Quantitation of the number of cells displaying phospho-Akt rim fluorescence indicated that 79% of the non-PDGF receptor-expressing adipocytes were insulin-responsive, which was reduced to 45% in the human PDGF receptor-expressing cell population (Fig. 5 C). In any case, expression of the human PDGF receptor resulted in a PDGF stimulation of phospho-Akt immunoreactivity similar to that of insulin (Fig. 5C). In these conditions, 78% of the human PDGF receptor-expressing adipocytes displayed phospho-Akt at the plasma membrane (Fig. 5C).

The ability of the expressed PDGF receptor to activate Akt in the insulin-responsive cell population provided an assay to determine if the PDGF receptor could couple to GLUT4 translocation. As typically observed (46, 47), expression of a GLUT4-EGFP fusion protein resulted in a perinuclear and small cytoplasmic vesicle distribution (Fig. 6A, b). Insulin stimulation resulted in a strong continuous cell surface GLUT4-EGFP fluorescence characteristic of GLUT4 translocation (Fig. 6A, d).

Since few cells expressed the endogenous PDGF receptor (Fig. 6A, a, c, and e), PDGF stimulation had a very minor effect on GLUT4-EGFP translocation (Fig. 6A, f). Quantitation of these data demonstrated that in the basal state ~15% of the cells displayed a plasma membrane GLUT4-EGFP rim fluorescence (Fig. 6B). Insulin stimulation resulted in ~73% of the adipocytes displaying GLUT4-EGFP translocation, whereas PDGF stimulation resulted in only 24% of the cells translocating GLUT4-EGFP (Fig. 6B). In cell overexpressing the human PDGF receptor, the number of cells displaying GLUT4 cell surface labeling in the basal state decreased to 7% (Fig. 6A, g and h, and B). Although insulin was still capable of inducing GLUT4 translocation, the number of cells was reduced to 40% (Fig. 6A, i and j) and (Fig. 6B). These data are consistent with the reduction in insulin-stimulated phospho-Akt activation in the cell population transfected with the human PDGF receptor (Fig. 5). Nevertheless, PDGF was now capable of inducing GLUT4-EGFP translocation to a similar extent as insulin (Fig. 6A, k and h, and B). Thus, these data demonstrate that expression of the PDGF receptor in the appropriate cell context will result in the coupling of the PDGF receptor to GLUT4 translocation. Importantly, however, the expression of the

FIG. 6. Activation of the PDGF receptor in transfected but not in wild type 3T3L1 adipocytes results in GLUT4 translocation. A, differentiated 3T3L1 adipocytes were electroporated with 50 μg of the GLUT4-EGFP expression plasmid with either 200 μg of the pcDNA3 empty vector (a–f) or the human PDGFr receptor cDNA (g–l). Eighteen hours later, the cells were serum-starved for 2 h and left untreated (a, b, g, and h) or stimulated with either 100 nm insulin (c, d, i, and j) or 50 ng/ml PDGF-BB (c, f, h, and l) for 30 min at 37 °C. The cells were then fixed, permeabilized, and subjected to confocal immunofluorescence microscopy with a PDGF receptor antibody (a, c, e, g, i, and k) or for the GLUT4-EGFP (b, d, f, h, j, and l). B, quantitation of the number of 3T3L1 adipocytes displaying cell surface GLUT4-EGFP fluorescence.
clearly demonstrate the need to take this diversity into account in the differentiated 3T3L1 adipocyte cell population and overexpression of the PDGF receptor can couple to GLUT4 translocation. The observation that activation of the endogenous PDGF receptor can induce GLUT4 translocation by some investigators probably reflects cells that are in an earlier stage of adipocyte differentiation. Similarly, it has been reported that overexpression of the PDGF and epidermal growth factor receptors can result in GLUT4 translocation (22, 24, 50). Consistent with these data, we have also observed that overexpression of the PDGF receptor itself has only a partial stimulatory effect and markedly decreased during adipocyte differentiation (48, 49). Thus, in our cell context, PDGF receptors are unable to couple to GLUT4. This findings are consistent with earlier studies demonstrating that the PDGF receptor mRNA and protein are expressed low levels of the insulin receptor, caveolin, and GLUT4. This findings are consistent with earlier studies demonstrating that the PDGF receptor mRNA and protein are markedly decreased during adipocyte differentiation (48, 49).

In summary, there is a substantial controversy about whether the PDGF receptor can couple to the translocation of GLUT4 in adipocytes (22–28). Our data demonstrate that in well differentiated 3T3L1 adipocytes, the PDGF receptor is primarily expressed in a distinct minor cell population that expresses low levels of the insulin receptor, caveolin, and GLUT4. This findings are consistent with earlier studies demonstrating that the PDGF receptor mRNA and protein are markedly decreased during adipocyte differentiation (48, 49). Thus, in our cell context, PDGF receptors are unable to couple to GLUT4 translocation. The observation that activation of the endogenous PDGF receptor can induce GLUT4 translocation by some investigators probably reflects cells that are in an earlier stage of adipocyte differentiation. Similarly, it has been reported that overexpression of the PDGF and epidermal growth factor receptors can result in GLUT4 translocation (22, 24, 50). Consistent with these data, we have also observed that overexpression of the PDGF receptor can couple to GLUT4 translocation. However, it is important to note that the PDGF receptor is substantially less effective than insulin under these conditions. Together, these data underscore the heterogeneity in the differentiated 3T3L1 adipocyte cell population and clearly demonstrate the need to take this diversity into account when analyzing the signal transduction and regulatory pathways controlling GLUT4 translocation.

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