Oleate-Induced Formation of Fat Cells with Impaired Insulin Sensitivity

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ABSTRACT: Exogenous FA cause lipid accumulation in preadipocytes. We investigated whether the fat cells thus formed are metabolically distinct from adipocytes differentiated with standard methylisobutylxanthine, dexamethasone, and insulin (MDI) hormonal cocktail by comparing their expression of adipogenic genes, accumulation of TAG, lipogenesis, lipolysis, glucose uptake, and the effects of insulin on selected metabolic activities. Cells exposed to oleate began to accumulate TAG in parallel or prior to the induction of adipogenic genes, whereas cells treated with MDI expressed adipogenic genes before TAG accumulation. Oleate-treated fat cells also showed exaggerated basal lipolysis and weak response to insulin in both lipolysis regulation and glucose uptake. These findings were associated with increased basal phosphorylation of perilipin, increased Glut-1 but decreased Glut-4 expression, and reduced insulin-induced Akt phosphorylation. We suggest that this unique fat cell phenotype might be a mimetic of what can happen to fat cells formed *in vivo* under the influence of circulating FA and might be a useful model for *in vitro* studies of obesity-related insulin resistance in adipocytes.

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Exogenous FA have been shown to enhance preadipocyte differentiation, but the metabolic functions of fat cells thus formed have not been reported (1,2). We now show that fat cells formed by treating 3T3-L1 preadipocytes with oleic acid (oleate), while expressing adipocyte-specific genes to some extent, exhibit impaired insulin sensitivity in several important metabolic aspects as compared with MDI (methylisobutylxanthine, dexamethasone, and insulin)-induced adipocytes.

METHODS

Cells culture. 3T3-L1 Preadipocytes were treated with DMEM containing 10% FBS and 17 nM insulin +/– FA (oleate, palmitate, linoleate, 0.5 mM, all complexed to BSA at a 3:1 molar ratio). Control adipocytes were differentiated by treatment with an MDI hormone cocktail for 48 h and then switched to DMEM with 10% FBS and 17 nM insulin (3). All cells were incubated in low-glucose (5 mM) DMEM with 0.5% BSA for 24 h before metabolic experiments.

Cell cycle analysis. After treatments, cells were trypsinized and fixed with 70% ethanol. Samples were stained with propidium iodide (5 μg/mL in PBS with 20 μg/mL RNase) for 30 min at 37°C and then analyzed using FACScan flow cytometry (BD Biosciences, San Jose, CA).

Lipolysis, lipogenesis, glucose uptake, Western analysis, and statistical analysis. These steps were performed as previously described (4,5). RNA isolation and reverse transcription were done as described before (4). Real-time PCR was done by mixing 2 μL of first strand cDNA with an adequate amount of water and 10 μL TaqMan universal PCR master enzyme mix from Roche (Brunburg, NJ) using the Rotor-Gene 3000A instrument (Corbett Robotics, San Francisco, CA).

RESULTS AND DISCUSSION

Oleate-induced growth arrest and lipid accumulation. Figure 1A shows the changes in cell morphology before and after being treated with oleate, linoleate, and a mixture of palmitate, linoleate, and oleate (PLO, 1:1:1 molar ratio) for 4 d. All FA were added at 0.5 mM with 0.14 mM BSA. Palmitate alone added at the same concentration was found to induce apoptosis (data not shown). For comparison, a microphotograph of cells differentiated with standard MDI protocol is also shown (7 d post-differentiation). These results indicated that all the exogenous FA caused intracellular lipid accumulation, with oleate being the most efficient in forming large, coalescent lipid droplets. Since oleate is mitogenic in selected cell types (6–8), we performed fluorescence-activated cell sorting (FACS) analysis to assess whether this might occur in our cell systems. As shown in Figure 1B, a similar cell population distribution pattern was found for postconfluent preadipoctyes before and after being treated with oleate or MDI: ~75–80% at G1/G0, ~15% at G2/M and ~5% at S phase. Such a distribution pattern is typical for growth-arrested fibroblasts (9). To further test whether the cells maintained at a growth-arrested state after being treated with oleate, we performed real-time PCR analysis for the expression of G0S2 gene, a novel marker for growth arrest and differentiation in 3T3-L1 cells (10). As shown in Figure 1C, treatment with FA or MDI increased G0S2 mRNA substantially as compared with growing preadipocytes. Together, these data suggest that treating 3T3-L1 preadipocytes with oleate alone or in combination with other FA resulted in the formation of fat cells that mimic

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Abbreviations: Akt, protein kinase B; aP2, FA binding protein; C/EBPα, CCAAT/enhancer-binding protein-α; DGAT-2, DAG acyltransferase-2; FACS, fluorescence-activated cell sorting; GPDH, glycerol-3-phosphate dehydrogenase; MDI, methylisobutylxanthine, dexamethasone, and insulin; PCR, polymerase chain reaction; PLO, palmitate, linoleate, and oleate; PPARγ, peroxisome proliferator-activated receptor-γ.

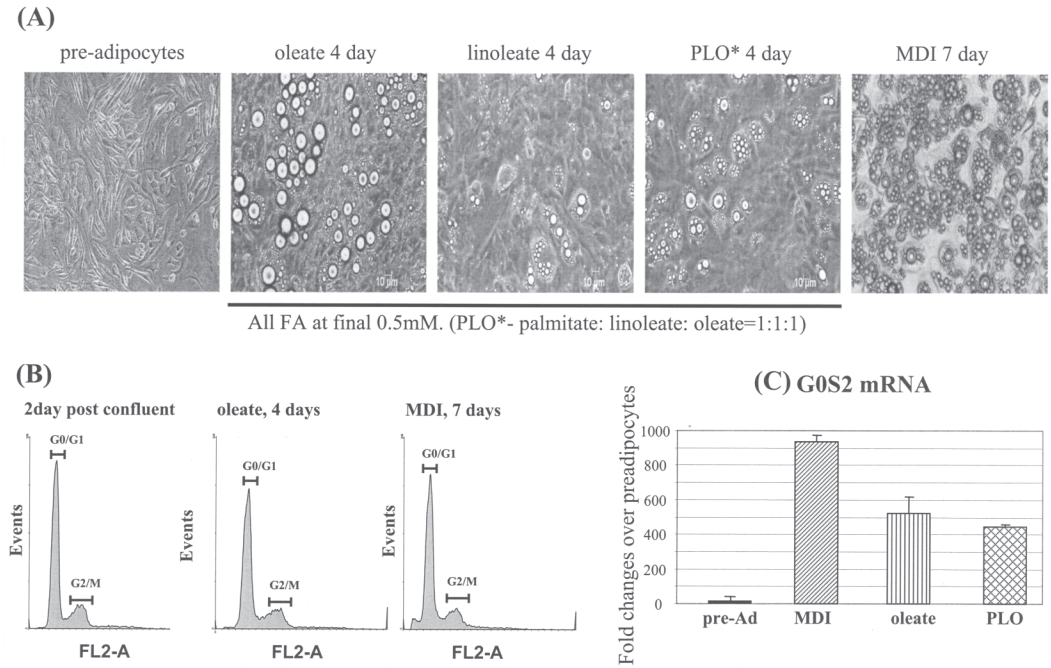

FIG. 1. Effects of FA on cell morphology and growth arrest. (A) Morphological lipid accumulation; (B) fluorescence-activated cell sorting (FACS) analysis of cell cycle population distribution; (C) steady-state expression of G0S2 mRNA in 3T3-L1 preadipocytes (pre-Ad) before and after being treated with FA [oleate or palmitate-linoleate-oleate (PLO)] or the methylisobutylxanthine, dexamethasone, and insulin (MDI) cocktail. FL2-A, propidium iodide.

the adipocytes differentiated with the standard MDI protocol. We then further characterized this new type of fat cells at the molecular and metabolic levels.

Expression of adipogenic genes. As shown in Figure 2A, oleate induced a gradual increase in expression of the key adipogenic transcription factors, peroxisome proliferator-activated receptor-γ (PPARγ) and C/EBPα (CCAAT/enhancer-binding protein-α), in preadipocytes. After being normalized to the maximal expression level at maturation (day 7), the time courses of PPARγ and C/EBPα induction in cells treated with oleate were similar to that in cells treated with MDI, except that PPARγ expression was induced to a greater extent in the former during the early phase (day 0–2, Fig. 2A). Figure 2B shows that oleate also induced expression of selected differentiation marker genes, including FA binding protein (aP2), CD36, DAG acyltransferase-2 (DGAT-2), and glycerol-3-phosphate dehydrogenase (GPDH). Compared with MDI, oleate had a similar induction of GPDH, two- to threefold less CD36 and DGAT2 but two- to threefold more *aP2*. This suggests that fat cells formed after the two different treatments were similar but not identical and that MDI might be a more potent inducer for adipogenesis. Oleate, on the other hand, might specifically increase steady-state aP2 mRNA *via* alternative mechanisms as previously reported (11). Treatment with a combination of PLO gave a similar induction as that induced by oleate alone.

TAG accumulation. In the absence of oleate or MDI, little TAG was found in preadipocytes (Fig. 2C), consistent with a previous report that insulin alone does not induce adipogenesis (12). Cells treated with oleate began to accumulate TAG within 24 h, which reached a plateau at day 3–4 (Fig. 2C). On the other hand, TAG was undetectable in MDI-treated cells in the first 3 d but began to accumulate rapidly thereafter. These results indicate that TAG accumulation was tightly associated with adipogenesis in MDI-treated cells but not in oleate-treated cells.

Metabolic characterization. As shown in Figure 3A, cells treated with oleate or MDI both showed a higher basal lipogenesis rate as compared with the untreated preadipocytes. Insulin further stimulated lipogenesis two- to threefold (Fig. 3A), indicating that the insulin-sensitive lipogenic pathway was developed to a similar extent in both cell types. On the other hand, Figure 3B shows that oleate-treated cells had a greater basal glucose uptake than the MDI-treated cells. However, glucose uptake in the oleate-treated cells was not responsive to insulin stimulation at or near physiological concentration (170 nM) and only moderately responsive at supraphysiological concentration (1.7 μM), as compared with cells treated with MDI that showed a 3.5-fold increase in response to 170 nM insulin but with no further increase when insulin was increased to 1.7 μM. This difference suggests that the insulin signaling pathways were not activated properly in oleate-treated cells. Figure 3C shows that oleate-treated cells also had a greater basal lipolysis as compared with cells treated with MDI. However, isoproterenol-stimulated lipolysis was blunted, indicating dampened sensitivity to β-agonist as compared with that in the MDI-treated cells. Insulin also had no effect on isoproterenol-stimulated lipolysis in oleate-treated cells although it greatly suppressed that in the MDI-treated cells (Fig. 3C).

Altered metabolic characteristics are associated with

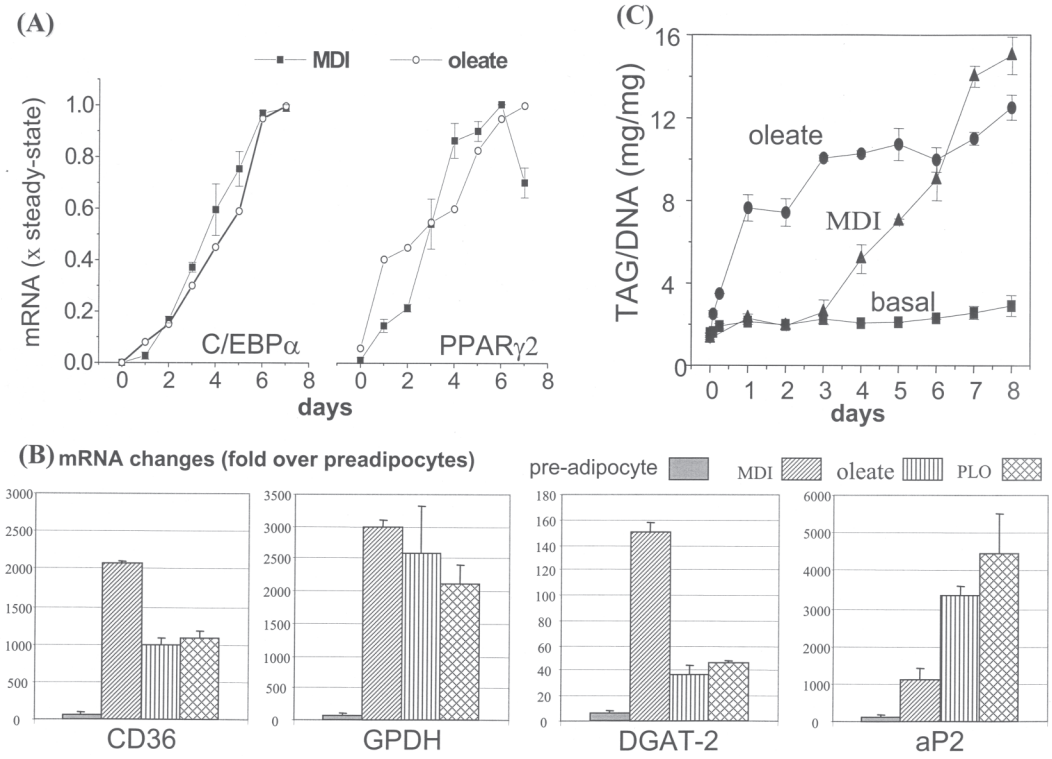

FIG. 2. Effects of FA on adipogenic gene expression and TG storage. (A) Time-dependent expression of CCAAT/enhancer-binding protein-α (C/EBPα) and peroxisome proliferator-activated receptor-γ (PPARγ) in preadipocytes treated with oleate or MDI, normalized to the steady-state expression levels on day 7; (B) time-dependent TAG accumulation (mean ± SE, $n = 3$); (C) mRNA expression of selected adipogenesis-related genes on day 7. GPDH, glycerol-3-phosphate dehydrogenase; DGAT-2, DAG acyltransferase-2; aP2, FA binding protein; for other abbreviations see Figure 1.

changes in the expression or phosphorylation of key functional proteins. First, we measured the expression/phosphorylation of perilipin, a protein that coats the lipid droplets in fat cells and facilitates lipolysis when phosphorylated (13). Consistent with the exaggerated basal lipolysis (Fig. 3C), we found that oleate-treated cells had a higher level of basal perilipin phosphorylation than MDI-treated cells (Fig. 4A). Second, we measured the expression of Glut-1 and Glut-4, the transporter proteins

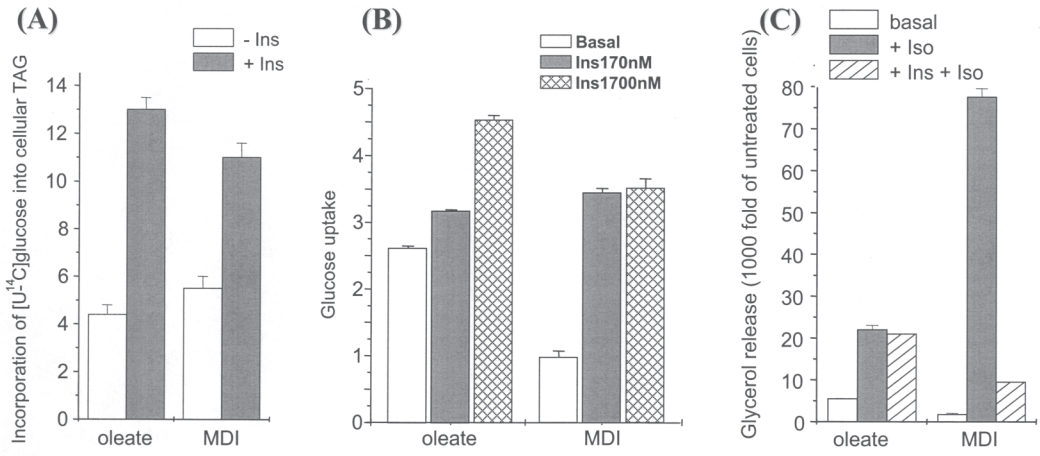

FIG. 3. Effects of oleate on metabolic functions and cellular response to insulin. (A) Incorporation of [U-^{14}C] glucose into cellular lipids, (B) uptake of 2-deoxy [2,6-^{3}H] glucose, (C) glycerol release, with or without insulin (170 nM, Ins) or isoproterenol (0.5 μM, Iso). Results are presented as fold changes over the untreated cells, for which the basal rates of lipogenesis, glucose uptake, and glycerol release were 0.82 pmol/μg DNA/min, 0.06 nmol/μg DNA/min, and 0.8 pmol/μg TAG/min, respectively (mean ± SE, $n = 3$, Duncan's test). For abbreviation see Figure 1.

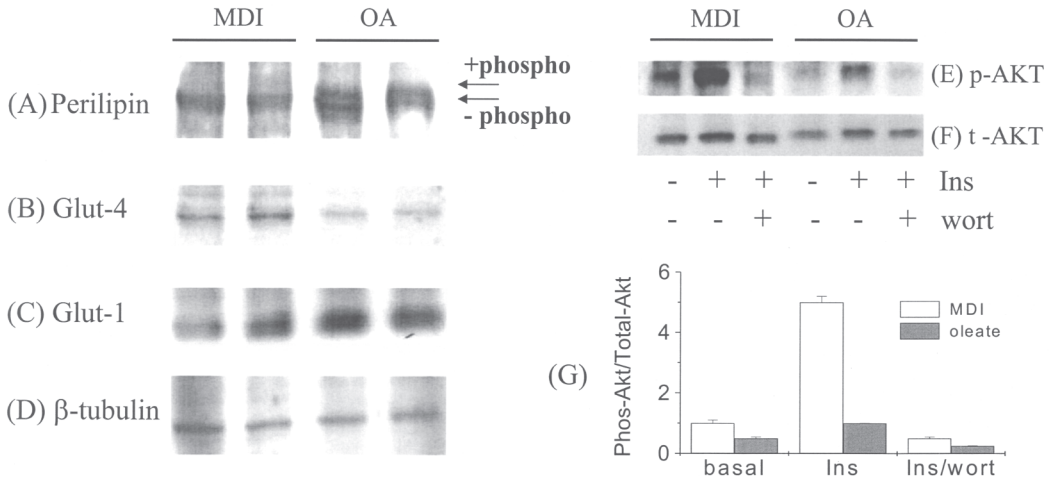

FIG. 4. Western analysis of selected proteins as labeled. Cells were prepared as in Figure 2 and used directly (A–D) or after incubation with insulin (170 nM, 15 min) with or without wortmanin (500 nM, wort) (E,F). Cells were lysed using RIPA buffer containing inhibitors for proteases and phosphatases, and 20 μg crude protein lysate of each sample was loaded for electrophoresis. Results are representative of three independent experiments. OA, oleate; p-AKT, phosphorylated protein kinase B; t-AKT, total protein kinase B protein; for other abbreviations see Figures 1 and 3.

that control the basal and insulin-stimulated glucose uptake, respectively (12). As shown in Figures 4B and 4C, oleate-treated cells had a greater expression of Glut-1 but less of Glut-4 compared with MDI-treated cells. This is consistent with the elevated basal, but dampened insulin-stimulated, glucose uptake in the former (Fig. 3B). Finally, we showed that insulin-stimulated protein kinase B (Akt) phosphorylation was also dampened in oleate-treated cells (~twofold), compared with a more sensitive response in MDI-treated cells (~fivefold) (Fig. 4E–G). This might also contribute to the poor insulin sensitivity with regard to lipolysis and glucose uptake in oleate-treated cells, based on the pivotal role of Akt in control of insulin signaling (14). The insulin-stimulated lipogenesis, on the other hand, appeared to be normally developed in these cells (Fig. 3A).

In summary, we showed that oleate induced the formation of a unique fat cell phenotype that exhibited some of the features of adipocytes, as shown by the expression of adipogenic genes, active lipogenesis, and lipolysis. However, these cells were also relatively insensitive to insulin and β-agonist. It is almost certain that they also differ from normal adipocytes in other functional aspects that still remain to be determined. Because elevation of blood FA concentration is common after a high-fat diet, and high-fat diets are known to be associated with increased fat cell formation *in vivo* (15–18), it is possible that fat cells thus formed may bear similar features to those of oleate-treated preadipocytes *in vitro*, with augmented fat storage but impaired hormonal sensitivity. In this regard, this work might provide a useful *in vitro* model for studying the effects of FA on adipose tissue development.

ACKNOWLEDGMENT

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