Quantifying carbon sources for de novo lipogenesis in wild-type and IRS-1 knockout brown adipocytes

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Abstract **Studies were conducted to evaluate the flux of various carbon sources to lipogenesis during brown adipocyte differentiation. ^{13}C labeling and isotopomer spectral analysis quantified the contribution of metabolites to de novo lipogenesis in wild-type (WT) and insulin receptor substrate-1 knockout (KO) brown adipocytes. Both glucose (Glc) and glutamine (Gln) provided substantial fractions of the lipogenic acetyl CoA for both WT and KO cells in standard media, together contributing 60%. Adding acetoacetate (AcAc; 10 mM) to the medium resulted in a large flux of AcAc to lipid, representing 70% of the lipogenic acetyl CoA and decreasing the contribution of Glc plus Gln to 30%. For WT cells, the fractional synthesis of new fatty acids during 4 days of differentiation was 80% of the total. Similarly, 80% of the lipidic glycerol was derived from Glc in the medium; Gln was not a precursor for glycerol. When Gln was removed from the medium, the contribution of Glc to fatty acid synthesis doubled, replacing most of the contribution of Gln and maintaining total lipogenesis. Conversely, removal of Glc dramatically decreased lipogenesis. These results indicate that Glc's distinct role in lipid synthesis during differentiation cannot be replaced by other carbon sources, consistent with the role of Glc supplying NADPH and/or glycerol for triglyceride synthesis.**—Yoo, H., G. Stephanopoulos, and J. K. Kelleher. **Quantifying carbon sources for de novo lipogenesis in wild-type and IRS-1 knockout brown adipocytes. *J. Lipid Res.* 2004.** 45: **1324–1332.**

Supplementary key words insulin receptor substrate-1 • ^{13}C • isotopomer • gas chromatography-mass spectrometry • adipogenesis • glucose • glutamine • acetoacetate • acetate

The differentiation of brown adipocyte cells from a fibroblast-like precursor is evolutionarily related to prehibernation fat accumulation and thus linked to total body energy metabolism (1). Adipogenesis occurs under conditions of excess nutrients and accompanying hormones and involves changes in gene expression and cell signaling, leading to a substantial increase in de novo synthesis and storage of triglyceride. Cell surface receptors, especially for insulin and insulin-like growth factor-1, provide a mechanism for hormonal response to nutrient abundance. Recent investigations of adipogenesis have focused on gene expression and cell signaling events associated with this conversion (2–4). The importance of the insulin receptor substrate-1 (IRS-1) signaling pathway for differentiation has been demonstrated by the finding that an IRS-1 knockout (KO) preadipocyte cell line is unable to differentiate under the standard condition in which wild-type (WT) cells accumulate triglyceride and express adipocyte-specific genes, including UCP-1 and fatty acid synthase (5). A consequence of the IRS-1 KO is that these cells are deficient in insulin-stimulated glucose (Glc) uptake via the Glc transporter GLUT4. The studies of in vitro adipogenesis are normally conducted under constant nutrient conditions using cell culture media in which Glc (25 mM) and glutamine (Gln; 2–4 mM) provide the major carbon sources for cell metabolism. In the work presented here, we investigate the role of the nutrient and hormonal environment on the process of lipogenesis accompanying brown adipocyte differentiation.

Animal studies support the concept that Glc is a major carbon source for brown adipose lipogenesis. Lipid synthesis measured with $^{3}H_2O$ increases on Glc administration and is insulin sensitive (6). However, ketone bodies may also supply carbon for brown adipose lipogenesis. Investigations using rats and slices of rat brown adipose tissue demonstrated that acetoacetate (AcAc) was used by brown adipose tissue for both oxidation and de novo lipo-

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Abbreviations: AC, acetate; AcAc, acetoacetate; Dex, dexamethasone; Glc, glucose; Gln, glutamine; G3P, glycerol-3-phosphate; IBMX, isobutylmethylxanthine; IRS-1, insulin receptor substrate-1; ISA, isotopomer spectral analysis; KO, knockout; MTBSTFA, *N*-methyl-*N*-[*tert*-butyldimethylsilyl]trifluoroacetimide; PEPCK, phosphoenolpyruvate carboxykinase; SIM, selected ion monitoring; TBDMS, *tert*-butyldimethylsilyl; WT, wild-type.

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genesis. In fasted animals, the incorporation of ^{14}C-labeled β-hydroxybutyrate into lipids in brown adipose tissue was 10-fold greater on a weight basis than that into liver or white adipose tissue (7). In addition, incorporation of β-hydroxybutyrate into fatty acid was increased in brown adipose tissue of cold-adapted rats (8). In concert with these fluxes, increased levels of mitochondrial enzymes required for the conversions of AcAc to acetyl CoA, 3-oxoacid CoA transferase, and acetoacetyl-CoA thiolase have been found in brown adipose tissue from suckling and weanling rats (9, 10). In addition to fatty acid synthesis, nutrients are required for the glycerol backbone of the triglyceride. In most cells, Glc supplies the glycerol-3-phosphate (G3P) precursor for the triglyceride backbone. Brown adipocytes may also use the glyceroneogenesis pathway involving G3P synthesis from three carbon compounds via phosphoenolpyruvate carboxykinase (PEPCK) (11). A third possible route for G3P formation in brown adipocyte cells is from glycerol via glycerokinase. A futile cycle involving glycerokinase and triglyceride hydrolysis has been suggested as an additional heat-generating mechanism in brown adipocyte cells (12, 13).

A standard procedure for in vitro differentiation of brown preadipocytes has been adopted from the procedure used for white adipocyte differentiation (14). The procedure involves incubating confluent preadipocytes in dexamethasone (Dex), indomethacin, and isobutylmethylxanthine (IBMX). Dex, in brown preadipocytes, appears to repress the expression of the insulin-insensitive Glc transporter GLUT1 and to stimulate the level of the insulin-sensitive Glc transporter GLUT4 in a dose-dependent manner. In the absence of Dex, brown preadipocytes may differentiate by transporting Glc primarily through GLUT1 (15, 16). Thus, both the nutrient environment and the hormonal conditions inducing differentiation may affect the intracellular metabolic environment that allows the cells to rapidly accumulate triglyceride during differentiation.

The studies presented here focus on the role of nutrients in the synthesis of triglyceride during the brown adipocyte differentiation process using the WT and IRS-1 KO cell lines developed by Kahn and coworkers (5). This model provides the opportunity to compare the two cell types to assess their ability to accumulate lipid under a variety of nutrient conditions. In white adipose 3T3-L1 cells, the IRS-1 KO cells retain partial ability to differentiate (17). Thus, we specifically tested the hypothesis that alternations in nutrients could affect the lipogenic ability of the IRS-1 KO brown adipose cells. We used ^{13}C-labeled metabolites to investigate the carbon sources for lipogenesis and glycerol backbone. We analyzed labeling data with isotopomer spectral analysis (ISA) to estimate parameters associated with lipogenesis. This method has been used previously to assess the synthesis of triglyceride fatty acids in differentiating 3T3-L1 white adipocyte cells (18). Our studies provide insight into the role of nutrients in brown adipocyte differentiation to complement the extensive work on signaling and transcriptional changes under way in other laboratories (3, 5, 19).

MATERIALS AND METHODS

Materials

Biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). ^{13}C-labeled chemicals were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). Tissue culture media were obtained from Invitrogen, Co. (Carlsbad, CA).

Cell culture, adipocyte differentiation, and lipid isolation

Brown preadipocyte cells were cultured essentially as described by Fasshauer et al. (20) and as outlined in **Fig. 1A**. All studies were conducted on 10 cm^2 (surface area) six-well plates containing 4 ml of medium. WT and IRS-1 KO brown preadipocyte cells (kindly provided by Dr. C. R. Kahn, Joslin Diabetes Center, Boston, MA) were cultured until confluence (day 0) in "differentiation medium" (DMEM containing 25 mM Glc and 4 mM Gln, supplemented with 10% fetal bovine serum, 20 nM insu-

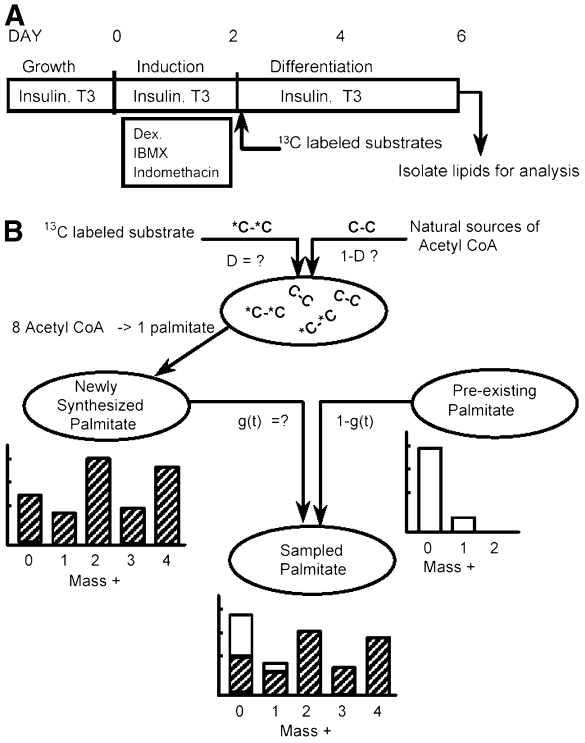

Fig. 1. Protocol for brown adipose cell differentiation and isotopomer spectral analysis (ISA). A: Standard differentiation protocol for wild-type (WT) or insulin receptor substrate-1 knockout (IRS-1 KO) brown adipocytes. Cells were seeded into a well of 10 cm^2 surface area with differentiation medium and grown to confluence. On day 0, the medium was changed to induction medium containing the following induction chemicals: dexamethasone (Dex), isobutylmethylxanthine (IBMX), and indomethacin. On day 2, cells were returned to differentiation medium containing ^{13}C-labeled substrates. On day 6, lipids were extracted from the cells for analysis. Insulin and thyroid hormone (T3) were present in the medium throughout the whole cell culture period. B: ISA model for the de novo biosynthesis of palmitate. Substrate labeled with ^{13}C is converted to ^{13}C-labeled acetyl CoA, which is mixed with acetyl CoA derived from natural sources in the intracellular pool of acetyl CoA at a constant ratio of D to 1 − D. One molecule of palmitate is synthesized from eight molecules of acetyl CoA in the homogeneous pool. When total cellular palmitate is sampled at the end of the incubation after the time period t, the fraction of palmitate resulting from de novo biosynthesis is g(t).

lin, and 1 nM thyroid hormone). On day 0, the medium was changed to "induction medium," which is differentiation medium supplemented with 0.125 mM indomethacin, 0.25 mM IBMX, and 5 μM Dex (in some studies, Dex was omitted from the induction medium, which is then designated "Dex−"). After 48 h, the medium was changed back to differentiation medium [in some studies, 10 mM sodium AcAc or 2 mM sodium acetate (AC) was added to differentiation medium, which is then designated "AcAc+" or "AC+"]. For all ^{13}C-labeling experiments, Glc, Gln, added AcAc, or added AC in the medium was individually replaced with the corresponding U-^{13}C-labeled carbon source, and DMEM was replaced with DMEM base medium (from Sigma Chemical Co.) with no Glc or Gln (buffered with 44 mM sodium bicarbonate at pH 7.2). The medium was replaced on day 4 with the same differentiation medium used on day 2.

On day 6, the experiment was terminated by removing the medium and adding 1.0 ml of 2% perchloric acid for 10 min at room temperature to each well (21). After removing the acidic solution, cellular lipid was isolated twice by adding 1.3 ml of hexane-isopropanol (3:2) mixture for 30 min at room temperature, then 20 μg of triheptadecanoin in 10 μl of hexane-isopropanol (3:2) was added to the organic solvent for the quantification of total fatty acids. Combined hexane-isopropanol solution was evaporated and redissolved in 400 μl of methane-benzene (2:1) mixture, 100 μl of which was mixed with 100 μl of BF_3/methanol. The mixture was vortexed in a glass vial and incubated at 75°C for 2 h to derivatize the entire fatty acid moiety in lipids into the corresponding methyl esters. The reaction product was then purified by hexane extraction (3 × 0.3 ml) against 0.2 ml of saturated aqueous NaCl solution. Combined hexane solution was treated with 100 mg of sodium sulfate to remove water. The hexane solution was then diluted appropriately before injection into the GC-MS instrument.

For ISA analysis of the glycerol portion of the lipids, glycerol was derivatized with *tert*-butyldimethylsilyl (TBDMS) group and the mass isotopomer distribution was determined by GC-MS as described by Flakoll et al. (22). Upon separation of the organic phase after methylation of the fatty acid moiety of lipids, 0.2 ml of the aqueous solution was evaporated to dryness and dissolved in the mixture of 100 μl of pyridine and 100 μl of *N*-methyl-*N*-[*tert*-butyldimethylsilyl]trifluoroacetimide [MTBSTFA (Pierce)]. The mixture was incubated at room temperature for 30 min before GC-MS analysis.

GC-MS for fatty acid quantification and ISA analysis

Samples with fatty acid methyl esters in 1 μl of hexane were injected into a Hewlett-Packard model 6890A gas chromatograph connected to JMS-GCmate II (JEOL, Peabody, MA) and equipped with a DB-XLB [60 m × 0.25 mm (inner diameter) × 0.25 μm] capillary column (J & W Scientific, Folsom, CA). Helium flow was maintained at 1.0 ml/min via electronic pressure control. The injection port temperature was 230°C. The temperature of the column was started at 100°C for 1 min, increased to 250°C at 25°C/min, and held for 5 min. The temperature was then increased to 300°C at 25°C/min and held for 1 min. For general detection of fatty acid methyl ester, m/z = 100–330 was scanned and recorded with scan speed of 0.51 s and interscan delay of 0.2 s. Myristate (m/z = 242), palmitate (m/z = 270), palmitoleate (m/z = 268), oleate (m/z = 296), and stearate (m/z = 298) methyl esters were detected above the detection limit, together with the internal standard, heptadecanoate methyl ester (m/z = 284). For quantification of total fatty acids, the intensities of M0 peaks ("Mx" denotes the isotopomer with m/z = M + x, where M is the base mass) of fatty acid methyl esters were normalized by that of heptadecanoate methyl ester. Various known amounts of tripalmitin were derivatized and measured together with the samples to construct a standard curve for calculating the absolute amounts of the fatty acids. Protein amounts of the replicate cell culture samples were measured using a protein assay kit (Sigma Diagnostics, St. Louis, MO) after extraction of protein from the cell cultures with 1% Triton X-100 (0.7 ml per well) for 20 min at 37°C and centrifugation (18,000 *g* for 2 min).

Because palmitate was the major fatty acid under all experimental conditions, the analysis of methyl palmitate was used for representative ISA analysis on fatty acid synthesis in brown adipocytes. For detection of mass isotopomers of methyl palmitate in ^{13}C-labeling studies, relative intensities of the molecular ions and their isotopomers [M0, M1, M2, . . . M16 (m/z = 270–286)] were monitored by selected ion monitoring (SIM). For detection of mass isotopomers of the TBDMS derivative of lipidic glycerol in ^{13}C-labeling studies, the same instrumental setup used for fatty acid methyl esters was used except for the following GC temperature profile: temperature was held at 100°C for 0.5 min, increased to 300°C at 25°C/min, and held for 1.5 min. Relative intensities of M − 57 ions (M − *tert*-butyl) and their isotopomers (m/z = 377–384) were also monitored by SIM (22).

Assay of the concentrations of Glc, Gln, and lactate in the culture medium

The concentrations of Glc and lactate in media samples were measured with a YSI 2300 STAT Glc/L-lactate analyzer (Yellow Springs Instruments, Yellow Springs, OH). Gln concentration was measured by TBDMS derivatization and GC-MS and corrected for the spontaneous decomposition of Gln at 37°C. The mixture of 100 μl of each medium sample plus 40 μl of 10 mM [U-^{13}C]Gln was first acidified by adding 100 μl of 2% perchloric acid. Then, the mixture was loaded onto a column with 2 ml of Dowex 50Wx8-400 cation-exchange resin. After washing the column with 1 ml of distilled water, Gln was eluted with 3 ml of 6 M NH_4OH aqueous solution. The eluent was evaporated and dissolved into 50 μl of dimethylformamide by sonication before mixing with 70 μl of MTBSTFA. The mixture was then incubated at 70°C for 30 min before injection into the GC-MS instrument. Standard Gln solutions of known concentration were used for the construction of a standard curve. The GC-MS setup was the same as in the glycerol analysis described above except for the following GC temperature profile: temperature was held at 140°C for 2 min, increased to 200°C at 25°C/min, and held for 1 min; temperature was then increased to 250°C at 3°C/min and held for 7.9 min; temperature was increased again to 300°C at 5°C/min and held for 5 min. Relative intensities of M − 57 (M − *tert*-butyl) ions from natural Gln (m/z = 431) and [U-^{13}C]Gln (m/z = 436) were used for the calculation of Gln concentrations of the media samples.

ISA

The flux of carbon sources to fatty acids was evaluated by ISA, a stable isotope method for estimating the fractional contribution and the fractional new synthesis of carbon sources to lipogenesis (18, 23). This method uses the mass isotopomer distribution of a polymer produced in part by de novo synthesis, as illustrated in Fig. 1B. In this study, we analyzed palmitate synthesized from ^{13}C-enriched precursors. The ISA model produces estimated values for the two parameters controlling the flux of ^{13}C to the sampled palmitate. D is the fractional contribution of a ^{13}C-labeled precursor to the lipogenic acetyl CoA, and g(time) is the fractional amount of newly synthesized palmitate in the sample. The parameter g(time) is equivalent to the fractional synthesis of the product at the specified time. The model used here assumes that the flux of the precursors to the lipogenic acetyl CoA pool is constant during the time course of ^{13}C incubation. ISA

uses equations for the probability of the appearance of each isotopomer based on test values for D and g(time). These probabilities are compared with the fractional abundance determined for each palmitate isotopomer to obtain the best-fit solution. The fit is obtained by weighted nonlinear regression, with the weights proportional to the inverse of the standard deviation of the isotopomer measurement. In practice, the weighting ensures that the parameter estimates will not be strongly affected by the least reliable measurements.

RESULTS AND DISCUSSION

Quantifying total fatty acid content of WT and IRS-1 KO brown adipocytes

Confluent WT and IRS-1 KO brown adipocytes were cultured under the standard condition or one of two modified conditions: AcAc+ or Dex− (see Materials and Methods for details). Under the standard condition, WT cells produced approximately three times as much total fatty acids per protein mass as IRS-1 KO cells by day 6 (**Fig. 2**). Under AcAc+ or Dex− conditions, WT and IRS-1 KO cells produced similar amounts of fatty acids as in the standard condition. Removing any one of the induction chemicals (IBMX, Dex, or indomethacin) from the induction medium for WT cells did not alter the total fatty acid amounts noticeably, but the absence of two or more induction chemicals reduced fatty acid production of WT cells to that of IRS-1 KO cells under the standard condition (data not shown). Previous studies comparing the amount of total fatty acids synthesized in WT and IRS-1 KO cells used Oil Red O staining to demonstrate qualitatively the decreased triglyceride accumulation in the KO cells (20). The data in Fig. 2 quantify the total fatty acid amounts in both cell lines, allowing calculations of amounts of total fatty acids per milligram of protein. The finding that the Dex− condition did not alter the total fatty acid accumulation suggests that any shift in the population of GLUT1 and GLUT4 does not affect total fatty acid synthesis (15, 16).

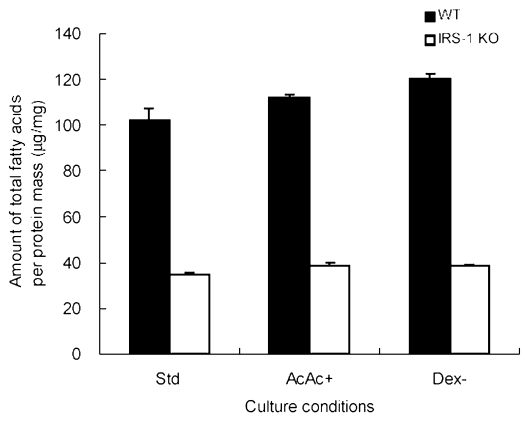

Fig. 2. Quantifying fatty acid synthesis in WT and IRS-1 KO cells. Amounts of total fatty acids under three culture conditions on day 6 measured by GC-MS using triheptadecanoin as the internal standard (conditions are as described in Materials and Methods and Fig. 1A). Conditions are as follows: Std, induction medium from day 0 to day 2, differentiation medium from day 2 to day 6; AcAc+, addition of 10 mM acetoacetate (AcAc) from day 2 to day 6; Dex−, induction medium without Dex from day 0 to day 2. Data shown are means ± SEM (n = 3) in WT and IRS-1 KO brown adipocytes. No significant difference was found among the three conditions in either WT or IRS-1 KO cells.

Lipogenesis under varied conditions for differentiation: adding AcAc and removing Dex

To determine the flux of various carbon sources to lipogenesis, cells were incubated in ^{13}C-labeled substrates for the 4 day differentiation period, days 2–6 (Fig. 1A). Among the three conditions studied, the experiment adding AcAc during differentiation (AcAc+) was performed to quantify the contribution of this carbon source to brown adipocyte lipogenesis under well-defined conditions and to test the hypothesis that alternative carbon sources could substitute for Glc in fatty acid synthesis of the IRS-1 KO cells. The Dex− experiment was conducted to test the hypothesis that the contribution of Glc to lipogenesis could be altered by the removal of Dex. For each of the three conditions, the fractional contribution and fractional new synthesis of Glc, Gln, and AcAc, when present, were estimated by the ISA parameters D and g(4 day) (Fig. 1B). A sample ISA experiment showing the mass isotopomer distribution and the fit of the model to the data demonstrates the key features of the analysis (**Fig. 3**).

The results of the ISA analysis indicate that brown adipocytes can use a variety of carbon sources for de novo lipogenesis (**Fig. 4A**). Glc (25 mM) and Gln (4 mM) are the major lipogenic carbon sources in the differentiation medium. ISA analysis indicated that both Glc and Gln make substantial contributions of carbon for lipogenesis under all conditions examined. AcAc (10 mM) was readily converted to lipid, indicating that these cell lines have a large capacity for AcAc utilization, consistent with the results in animal studies (9, 10). The activity of acetoacetyl-CoA syn-

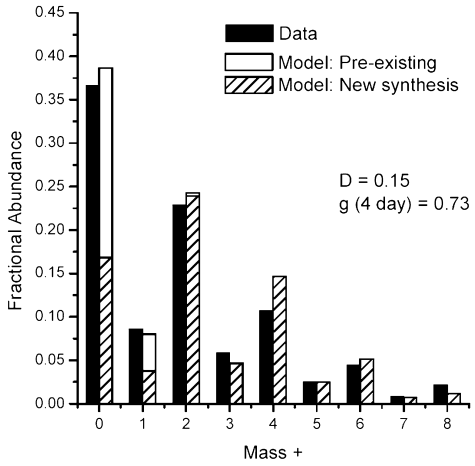

Fig. 3. ISA of palmitate synthesis in brown adipose cells. Representative isotopomer distribution of methyl palmitate [sampled on day 6 from WT brown adipocytes under the AcAc+ condition with [U-^{13}C]glutamine (Gln) from day 2 to day 6] compared with its fit by the ISA model with D = 0.15 and g(4 day) = 0.73.

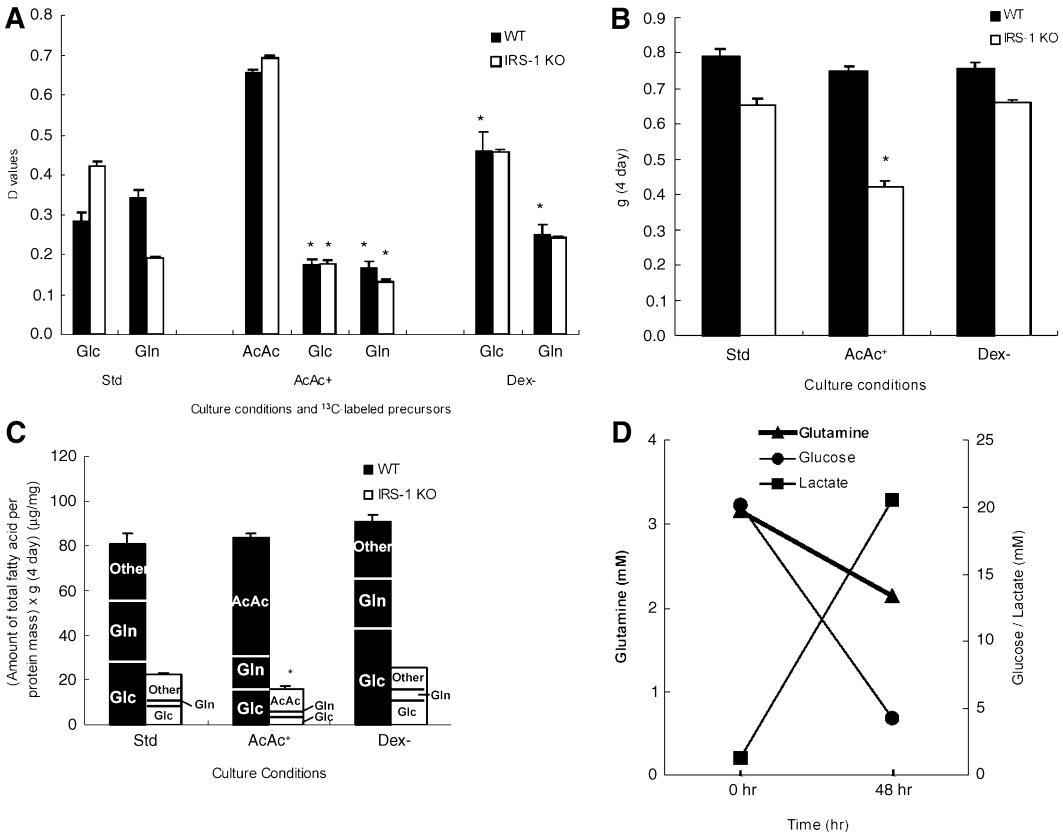

Fig. 4. Flux of carbon sources to lipids in brown adipose cells. A: Fractional tracer contribution (D values) for palmitate synthesis from glucose (Glc), Gln, or AcAc in WT and IRS-1 KO brown adipocytes on day 6 (^{13}C labeling from day 2 to day 6) under standard (Std), AcAc+, or Dex− conditions. B: Fractional synthesis [g(4 day) values] for the same conditions as in A. C: Partitioning of fatty acid synthesis among substrates − the products of g(4 day) × the amount of total fatty acids per protein mass (μg/mg) partitioned for the corresponding D values. Data shown are means ± SEM (n = 6). Asterisks indicate significant differences between the standard condition and each of the modified conditions with the same ^{13}C-labeled precursors at $P \leq 0.01$. D: Consumption of Gln and Glc from the medium and production of lactate into the medium for WT brown adipocytes under the standard condition within 48 h (days 4–6).

thetase has been correlated with the incorporation of AcAc carbon into lipid, which was increased in the presence of Glc and insulin (9). AcAc displaced Glc and Gln as lipogenic carbon sources, as indicated by the high $D_{(AcAc)}$ values and the decreases in the D values for these substrates in the presence of AcAc. When Dex was removed from the induction medium (Dex− condition), the fractional contribution of Glc to lipogenesis increased in the WT cells, consistent with the finding that this condition increases the expression of GLUT1 (15, 16) (Fig. 4C).

D values estimate the fractional contribution of a substrate to the lipogenic acetyl CoA pool. To examine the total flux of a substrate to fatty acids, it is necessary to consider the fraction of total fatty acids that was newly synthesized during the isotope incubation period. The ISA term for this variable, g(4 day) (Fig. 1B), was estimated as shown in Fig. 4B. Except for IRS-1 KO cells in the presence of AcAc, 60–80% of the total fatty acids in WT and IRS-1 KO cells was synthesized during the differentiation period (days 2–6). The total flux to fatty acids per milligram of protein was determined by multiplying g(4 day) with the total content of fatty acids (Fig. 4C). This calculation assumes that there is little turnover of the newly synthesized fatty acids during the 4 day experiment. However, it should be noted that the well-differentiated WT cells undergo clonal expansion as part of the differentiation process [clonal expansion of adipocytes during differentiation is reviewed by Darlington, Ross, and MacDougald (24)]. Because the protein mass of WT cells per well is approximately twice that of IRS-1 KO cells by day 6, the differences in total lipogenic flux between WT and IRS-1 KO cells shown in Fig. 4C would be further increased. The isotopic flux of each substrate to fatty acids may be calculated as follows: (flux of substrate per milligram of protein) = $D_{(substrate)} \times g(4\ day) \times$ (total amount of fatty acids per milligram of protein). In Fig. 4C, the total flux is partitioned among the ^{13}C-labeled substrates and other carbon sources. For each of the three conditions, the D values for all carbon sources are expected to sum to 1 according to the ISA model (Fig. 1B). Sources of carbon for lipogenic acetyl CoA other than the compounds investigated as ^{13}C substrates are grouped together and labeled "Other." This term includes metabolites in the medium as well as intracellular metabolites. Although the fractional

contributions from Glc and Gln for IRS-1 KO cells are comparable to those in WT cells (Fig. 4A), the absolute fluxes of the carbon sources in IRS-1 KO cells are much lower than those in WT cells under all three conditions, as shown in Fig. 4C. Thus, despite the changes in substrate use, the IRS-1 KO cells were not able to overcome the defect in total lipogenesis (20).

To further explore the quantitative use of Glc and Gln for lipid synthesis, the changes in concentration in the medium of WT cells under the standard condition were measured during the period of the most active lipogenesis, from day 4 to day 6 (Fig. 4D). Gln consumption of WT cells from the medium amounted to 4.1 μmol per well over 48 h. Also, it was estimated that 2.0 μmol of Gln was used for fatty acid synthesis during the last 2 days of differentiation.[2] These results indicate that an isotopic flux of 49% of the Gln consumed from medium was used for the synthesis of fatty acids during the 48 h period. In parallel with the result from Gln analysis, 63 μmol of Glc was consumed and 77 μmol of lactate was produced per well over the 48 h period (Fig. 4D), consistent with Glc's contribution to lipogenesis (Fig. 4C; see Fig. 6 below).

Under standard and Dex− conditions, in which Glc and Gln were the major carbon sources in the medium, the sum of $D_{(Glc)}$ and $D_{(Gln)}$ was considerably less than 1, indicating that other carbon sources supplied nearly 40% of the acetyl units used for de novo fatty acid synthesis. On the other hand, addition of 10 mM AcAc made the sum of $D_{(AcAc)}$, $D_{(Glc)}$, and $D_{(Gln)}$ equal to 1.00 in both WT and IRS-1 KO cells. AcAc was able to entirely displace the contribution of the other sources and reduced the contribution of both Glc and Gln. This finding suggests that both WT and IRS-1 KO cells have a high capacity to metabolize AcAc to acetyl CoA without affecting total fatty acid synthesis (Fig. 2).

Lipogenesis in the absence of Glc or Gln

The studies presented in Fig. 4 demonstrate substantial isotopic flux of both Glc and Gln to the lipogenic acetyl CoA during the differentiation of WT brown adipocytes. To further explore this issue, we removed either Gln (Gln−) or Glc (Glc−) from the standard medium from day 2 to day 6 and examined the flux of ^{13}C-labeled Glc and Gln to lipogenic acetyl CoA. The results of this ISA analysis are summarized in **Fig. 5**. When Gln was removed, the fractional contribution of Glc was almost doubled [$D_{(Glc)} = 0.51 \pm 0.03$] from that in WT cells with 25 mM Glc and 4 mM Gln [$D_{(Glc)} = 0.28 \pm 0.02$], and amounted to 81% of the sum of the two D values [$D_{(Glc)} + D_{(Gln)} = 0.63 \pm 0.03$]. In parallel with these findings, total fatty acid production in WT cells under the Gln− condition was similar to that in WT cells under the standard condition (Fig. 5B). Thus, Glc's flux to lipogenic acetyl CoA can largely compensate for the absence of Gln in WT cells. In contrast, when Glc was removed from the medium (Glc−), Gln provided only 24 ± 1% of the carbon sources for fatty acid synthesis. This D value was lower than $D_{(Gln)}$ (0.34 ± 0.02) in WT cells under the standard condition. With Glc removed from the medium, total fatty acid production in WT cells under the Glc− condition was reduced to a level as low as that in IRS-1 KO cells under the standard condition, which was not restored even after the addition of AcAc (Fig. 5B). These results indicate that Glc is required to support triglyceride synthesis from Gln during the differentiation of brown adipocytes.

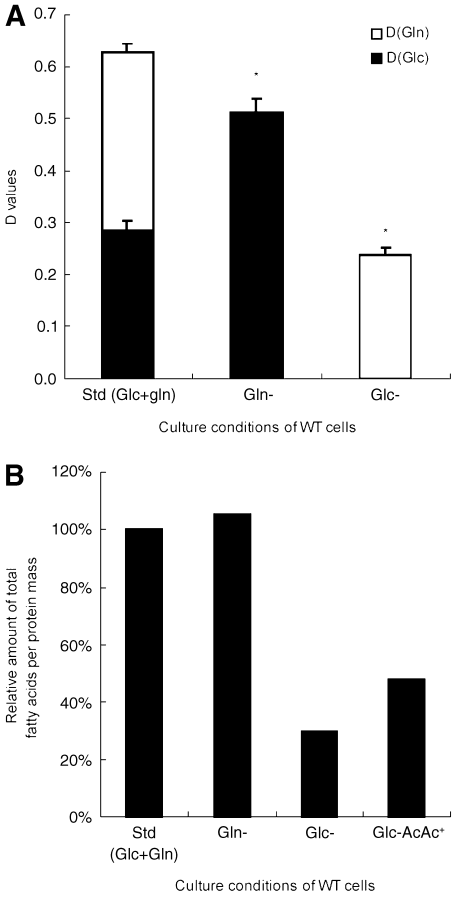

Fig. 5. Effect of the absence of Glc or Gln on the flux of carbon sources to fatty acids. A: D values for palmitate synthesis from either Glc or Gln in WT brown adipocytes under standard (Std), Gln−, and Glc− conditions on day 6 (^{13}C labeling of Glc or Gln from day 2 to day 6). Data shown are means ± SEM ($n \geq 4$). Asterisks indicate significant differences between the conditions with the same ^{13}C-labeled precursors at $P \leq 0.01$. B: Relative amounts of total fatty acids per protein mass in WT cells − the effect of the absence of Glc. Conditions were the same as in A except for Glc−AcAc+, which indicates the addition of 10 mM AcAc and the absence of Glc from day 2 to day 6.

Carbon sources for the glycerol backbone of cellular lipid

The studies removing Glc or Gln from the medium during differentiation raise the issue of the carbon sources for the glycerol backbone of the newly synthesized lipids

[2] It was assumed that one molecule of Gln provides a two-carbon unit of acetyl CoA for fatty acid synthesis and that palmitate is the representative fatty acid. Also, from Fig. 4C, it was calculated that 0.3 μmol of fatty acid was synthesized from Gln over a 4 day period in a well (2.8 mg of protein per well). Together with the measurement that 83% of the newly synthesized fatty acids during the 4 day differentiation period is produced from day 4 to day 6, Gln usage for fatty acid synthesis was estimated to be 2.0 μmol.

as an explanation for the limited lipogenic ability of WT cells under the Glc− condition. We considered two hypotheses for the failure of the WT cells under the Glc− condition to produce normal amounts of fatty acids. First, Glc may be required for adequate production of acetyl CoA such that lipogenesis will not proceed unless the acetyl units provided by Glc are available to supplement those from Gln and other sources. Second, Glc may be required to provide the glycerol backbone to the synthesis of lipids. To evaluate these hypotheses, we examined the labeling of the glycerol moiety of the lipids under the standard condition in the presence of 25 mM [U-^{13}C]Glc for WT cells (carbon from neither Gln or AcAc contributed to lipidic glycerol under any condition). The isotopomer distribution of TBDMS-glycerol indicated that ^{13}C-labeled Glc provided 79% of the glycerol backbone for WT cells, calculated as M3/(M0 + M3) after correction for natural abundance (**Fig. 6**). This percentage agrees well with the g(4 day) value of 0.79 for WT cells under the standard condition, indicating that 79% of the lipids was newly synthesized. This result also agrees with the finding that the amount of total fatty acids increased approximately 4-fold over the 4 day differentiation period (data not shown). Thus, Glc from the medium appears to be the sole carbon source for glycerol used for the de novo lipogenesis of WT cells under the standard condition.

Several routes for the synthesis of G3P, the immediate precursor for the glycerol backbone of lipids, have been proposed in brown adipose tissue. As the previous data indicate, carbon of G3P can be derived from Glc. A second route, glyceroneogenesis (glycerol synthesis from non-Glc sources), has been reported to be active in brown adipose tissue (11), but its quantitative significance has not been investigated. A third possibility is the recycling of glycerol via glycerokinase (25). We sought evidence that glyceroneogenesis and/or glycerol cycling could replace the role of Glc in supplying G3P for lipidic glycerol. WT cells were incubated during days 2–6 of differentiation in the absence of Glc but in the presence of Gln (4 mM) supplemented with one of the following: pyruvate (5 mM), lactate (5 mM) plus pyruvate (0.5 mM), or glycerol (10 mM). None of these conditions increased the synthesis of total fatty acids over those found for the Glc− condition. In addition, the possibility that Gln could form glycerol via glyceroneogenesis was investigated in the Glc− condition using [U-^{13}C]Gln. GC-MS analysis of lipidic glycerol was unable to detect the flux of Gln to glycerol in the Glc− condition. Thus, we found no evidence that alternative carbon sources could compensate for the role of Glc to stimulate de novo lipogenesis in the presence of Gln.

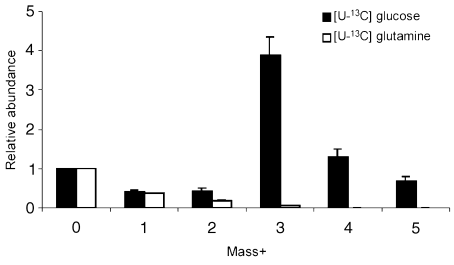

Fig. 6. Carbon sources for the glycerol backbone of lipids. Isotopomer distribution of the *tert*-butyldimethylsilyl derivative of glycerol from lipids of WT cells after 4 days of labeling with either [U-^{13}C]Glc or [U-^{13}C]Gln on day 6 under the standard condition. The large M3 value indicates that Glc, and not Gln, supplies carbon for glycerol. Data shown are averages of two independent determinations, with error bars indicating the ranges.

Lipogenesis in the presence of added AC

AC is a commonly used substrate for lipogenesis and has been used previously with ISA to quantify the contribution in lipogenesis of 3T3-L1 cells (18). To evaluate the contribution of AC to the lipogenic acetyl CoA pool, 2 mM AC was added to the differentiation medium from day 2 to day 6 for WT and IRS-1 KO brown adipocytes. For ISA analysis, AC, Glc, or Gln in the medium was replaced in individual wells with [U-^{13}C]AC, [U-^{13}C]Glc, or [U-^{13}C]Gln, respectively. The isotopomer distribution of methyl palmitate was evaluated by ISA to determine the D and g(4 day) values (**Fig. 7**). The data demonstrate that

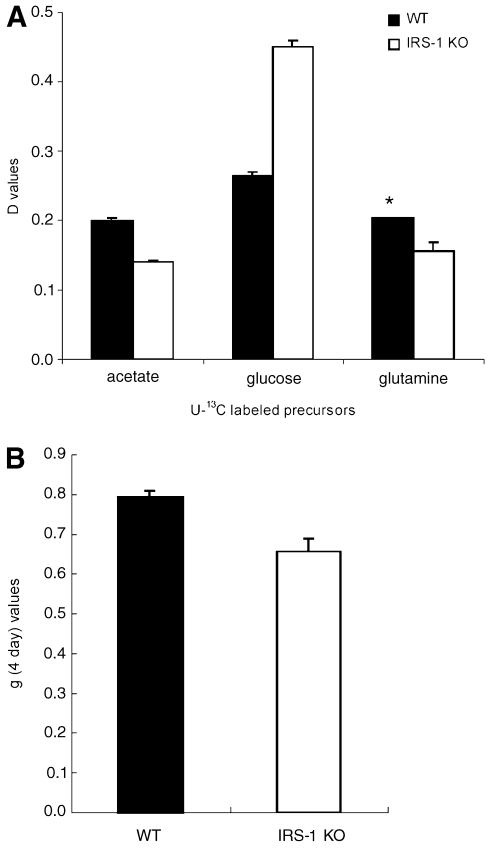

Fig. 7. Effect of acetate (AC) on the flux of carbon sources to fatty acids. A: Fractional tracer contribution (D values) for palmitate synthesis from Glc, Gln, or AC in WT and IRS-1 KO cells under standard + AC condition (AC+) on day 6 (^{13}C labeling from day 2 to day 6). Data shown are means ± SEM (n ≥ 3). Asterisks indicate significant differences between the standard condition (see Fig. 4A) and the AC+ condition with the same ^{13}C-labeled precursors at $P \leq 0.01$. B: Fractional synthesis [g (4 day) values] for the same conditions as in A.

AC is an effective carbon source for lipogenic acetyl CoA in both WT and IRS-1 KO brown adipocytes. Comparing the D values from Figs. 6A and 4A indicates that AC added to the medium displaced some of the contribution of Gln to lipogenic acetyl CoA in WT cells. This result was obtained by noting that the sum of D values of added carbon sources for WT cells in the presence of AC [$D_{(AC)} + D_{(Glc)} + D_{(Gln)} = 0.67 \pm 0.01$] was not different from that of WT cells under the standard condition [$D_{(Glc)} + D_{(Gln)} = 0.63 \pm 0.03$]. $D_{(Glc)}$ remained similar between the two conditions [standard, $D_{(Glc)} = 0.28 \pm 0.02$; AC+, $D_{(Glc)} = 0.26 \pm 0.01$], whereas $D_{(Gln)}$ was reduced (from 0.34 ± 0.02 to 0.20 ± 0.00), mainly as a result of the contribution from AC [$D_{(AC)} = 0.20 \pm 0.00$].

A different result was found for IRS-1 KO cells (Figs. 4A and 7A). The sum of D values of added carbon sources in IRS-1 KO cells under the AC+ condition (0.75 ± 0.02) was increased from those in IRS-1 KO cells under the standard condition (0.61 ± 0.01) by the same amount as $D_{(AC)}$ (0.14 ± 0.00). $D_{(Glc)}$ [standard, $D_{(Glc)} = 0.42 \pm 0.01$; AC+, $D_{(Glc)} = 0.45 \pm 0.01$] and $D_{(Gln)}$ [standard, $D_{(Gln)} = 0.19 \pm 0.00$; AC+, $D_{(Gln)} = 0.16 \pm 0.01$] remained similar. These results indicate that AC replaced the other carbon sources in fatty acid synthesis of the IRS-1 KO cells. When AC was added to the medium, the g(4 day) value for WT cells was 0.79, identical to that found for the standard condition (Figs. 4B and 7B). Likewise, for IRS-1 KO cells, the g(4 day) value was not affected by adding AC. These results indicate that the addition of 2 mM AC altered the fluxes of carbon to lipogenic acetyl CoA but did not affect the fractional synthesis of total fatty acids.

Physiology of lipogenesis in brown adipose cells

Taken together, the results of this study demonstrate the utilization of a number of carbon sources for de novo lipogenesis in brown IRS-1 KO preadipocytes and differentiating WT adipocytes. The finding that the contribution of Gln to lipogenic acetyl CoA was nearly equal to that of Glc was novel, as Gln has not been noted as a lipogenic carbon source in adipocytes. Low rates of Gln flux to triglyceride have previously been reported for white adipose tissue (26). Two routes are known for the flux of Gln to acetyl CoA. Glutaminolysis flux described by Newsholme and Carrie (27) involves TCA cycle metabolism to α-ketoglutarate followed by the flux of malate or oxaloacetate to pyruvate via malic enzyme or PEPCK and pyruvate kinase. Alternatively, the reductive carboxylation pathway metabolizes α-ketoglutarate to citrate by reversal of the NADH- or NADPH-coupled isocitrate dehydrogenase (28). The studies performed here do not distinguish between these pathways. However, isotopic methods can be used to determine the relative flux by each pathway (29), and this technique may be useful to clarify the role of Gln as a carbon source for lipogenesis. Although Gln was an important carbon source for lipogenesis, we found that neither Gln nor Gln plus AcAc could compensate for the removal of Glc from the medium (Fig. 5B).

In the absence of Glc, the amount of triglyceride fatty acids in WT cells was low, similar to that of the IRS-1 KO cells. These findings indicate a distinct role for Glc in lipid synthesis during differentiation. A distinct metabolic action of Glc is the source for G3P for the glycerol backbone of triglyceride and for NADPH generation in the pentose phosphate pathway. In this study, we found no evidence for alternatives to Glc for G3P synthesis, despite ample evidence for glyceroneogenesis and glycerokinase flux in brown adipose tissue (30). Thus, G3P synthesis remains a candidate for a pathway not duplicated by the other substrates investigated here. A second candidate for a distinct metabolic role of Glc is the production of lipogenic NADPH through the pentose phosphate pathway. However, NADPH may be produced both via Glc-6-phosphate dehydrogenase in the pentose phosphate pathway and via malic enzyme. Brown adipocyte differentiation induces increases in both of these enzymes, and thus both may play a role in generating NADPH for lipogenesis (31, 32). As shown here, isotopic tools, including ISA, may be applied to further explore and quantify lipogenic fluxes in brown adipose cells.

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