Standard Isolation of Primary Adipose Cells from Mouse Epididymal Fat Pads Induces Inflammatory Mediators and Down-regulates Adipocyte Genes*

Received for publication, May 20, 2003, and in revised form, July 29, 2003
Published, JBC Papers in Press, September 15, 2003, DOI 10.1074/jbc.M305257200

Hong Ruan‡‡§, Mary Jane Zarnowski§, Samuel W. Cushman§, and Harvey F. Lodish‡‡**

From the ‡‡Whitehead Institute for Biomedical Research and §Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02142 and **Experimental Diabetes, Metabolism, and Nutrition Section, Diabetes Branch, NIDDK, National Institutes of Health, Bethesda, Maryland 20892

Isolation and subsequent in vitro culture of primary adipose cells are associated with down-regulation of GLUT4 mRNA and simultaneous induction of GLUT1 gene expression. Progressive loss of insulin-responsive GLUT4 contributes to the decrease in insulin-mediated glucose uptake in these cells when cultured in vitro. The mechanisms underlying these alterations are unknown. Here, we report that the standard procedure for isolating primary adipose cells from mouse adipose tissue triggers induction of many genes encoding inflammatory mediators including TNF-α, interleukin (IL)-1α, IL-6, multiple chemokines, cell adhesion molecules, acute-phase proteins, type I IL-1 receptor, and multiple transcription factors implicated in the cellular inflammatory response. Secretion of TNF-α protein was also significantly induced during the 2-h collagenase digestion of adipose tissue. Isolated primary adipose cells exhibit dramatic changes in expression of multiple mRNAs that are characteristic of TNF-α-treated 3T3-L1 adipocytes including down-regulation of many genes important for insulin action and triglyceride synthesis. Addition of TNF-α to primary adipose cells in culture did not change the kinetics or the extent of the repression of adipose-cell-abundant genes. Moreover, TNF-α-neutralizing antibody failed to block the changes in gene transcription in isolated primary adipose cells. Also, the standard isolation procedure induced the expression of NF-κB family members and their target genes in primary adipose cells prepared from TNF-α−/− mice to the same extent as in cells isolated from wild-type mice and resulted in almost identical changes in global gene expression when these cells were cultured in vitro. Thus, these data suggest that the standard isolation procedure-triggered reprogramming of gene expression in primary adipose cells that results in decreased insulin sensitivity does not require TNF-α, at least in this in vitro model system, but may be dependent on other inflammatory cytokines produced by these cells.

Insulin resistance is a fundamental defect that precedes the development of the cluster of abnormalities associated with type 2 diabetes (1–4). Initially, the reduced insulin sensitivity is compensated by an over-production of insulin from β-cells. When insulin resistance progresses and β-cells are no longer able to produce sufficient insulin, overt type 2 diabetes develops (5). Thus, improving the overall in vivo insulin sensitivity appears to be a key factor in the treatment of type 2 diabetes. Previous studies on the beneficial effects of the thiazolidinedione class of insulin-sensitizing compounds in the treatment of type 2 diabetes underscore this notion (6–8). Because obesity with or without overt hyperglycemia is associated with insulin resistance (9, 10), attention has focused on abnormalities in adipose tissue that could lead to decreased systemic insulin sensitivity.

Many adipose cell-secreted factors have been implicated in the pathogenesis of insulin resistance in vivo (11). Among them, tumor necrosis factor-α (TNF-α) is of particular interest, because it is highly induced in adipose tissues of obese animals and human subjects and induces insulin resistance both in cell culture and in vivo (12). However, it remains largely unknown which factors and pathways trigger the expression of TNF-α in disease settings such as obesity and diabetes. In addition, other potential adipose tissue-derived autocrine/paracrine and endocrine factors, and the signaling pathways that these factors might utilize to induce insulin resistance in vivo, still remain elusive.

One attractive cell model of insulin resistance is primary adipose cells. When these cells are cultured in vitro, they gradually lose the expression of insulin-responsive GLUT4 glucose transporters but increase the synthesis and cell-surface expression of GLUT1 (13). These changes in gene expression contribute to the development of insulin resistance in primary adipose cells in prolonged in vitro culture and mimic the insulin-resistant phenotype in adipose tissue in vivo. Yet the signals, as well as the molecular pathways these signals might use to initiate the changes in adipose cell gene expression, are unknown. Elucidating such mechanisms will provide new insight into our current understanding of the mechanisms of insulin resistance in vivo.

We have demonstrated previously (14) that NF-κB activation in 3T3-L1 adipocytes is obligatory for TNF-α-mediated repression of most adipocyte-abundant genes, as well as induction of many immune response, proinflammatory, and preadipocyte genes (14). However, whether NF-κB and its upstream activating signals play an essential role in the reprogramming of gene

* This work was supported in part by grants from the National Institutes of Health (to H. F. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by a postdoctoral fellowship from the American Diabetes Association. Currently holds a Postdoctoral Fellowship for Physician Scientists from the Howard Hughes Medical Institute.

‡‡ To whom correspondence should be addressed. Whitehead Inst. for Biomedical Research, 9 Cambridge Ctr, Rm. 601, Cambridge, MA 02142. Tel.: 617-258-5216; Fax: 617-258-6768; E-mail: lodish@wi.mit.edu.

¶ The abbreviations used are: TNF-α, tumor necrosis factor-α; IL, interleukin; RT, reverse transcriptase; NF-κB, nuclear factor κB; IRS, insulin receptor substrate; PPAR-γ, peroxisome proliferator activator receptor-γ; CEBP-β, CCAAT/enhancer binding protein-β; TRAF, TNF-α receptor-associated factor.
expression in primary adipose cells is not known. To begin to understand the mechanisms underlying the coordinate changes in gene expression in isolated primary adipose cells, we sought to identify the immediate early changes in gene expression and to determine whether TNF-α is involved in initiating these changes.

Here, we report that the standard procedure for isolating primary adipose cells from mouse adipose tissue induces genes encoding a variety of inflammatory mediators including TNF-α, IL-1α, IL-6, multiple chemokines, and several transcription factors implicated in the induction of acute-phase cytokines. Secretion of TNF-α protein is also significantly induced during the 2-h collagenase digestion. These inflammatory responses are accompanied by dramatic changes in primary adipose cell gene expression that are characteristic of TNF-α-treated 3T3-L1 adipocytes. Hence, we used adipose cells isolated from TNF-α−/− mice to provide a direct and definitive test for the role of TNF-α in the reprogramming of gene expression in isolated primary adipose cells. Herein we report these results.

**EXPERIMENTAL PROCEDURES**

**Isolation and Culture of Primary Adipose Cells—** 8- to 9-week-old male C57BL/6J and FVB mice were used to obtain primary adipose cells essentially as described previously (15). Briefly, the epididymal fat pads were removed, minced, and digested using collagenase at 37 °C for 2 h. The primary adipose cells were then washed extensively and incubated at 37 °C in a KRBH buffer (Krebs-Ringer-bicarbonate-HEPES buffer, pH = 7.4) or Dulbecco’s modified Eagle’s medium containing 5% bovine serum albumin. Primary adipose cells and conditioned medium were taken at various times as indicated in the figure legends and were flash-frozen in liquid nitrogen and stored in −80 °C until use.

**Antibody—** TNF-α neutralizing antibody was purchased from R&D Systems (catalog number AF-410-NA; Minneapolis, MN). The manufacturer selected this line of TNF-α neutralizing antibody by functional screening and assessed the specificity of the antibody by showing that it does not cross-react with other cytokines tested so far. When used at 0.02–0.05 μg/ml, this antibody caused 50% inhibition of the biological effects of TNF-α activity at a concentration of 0.25 ng/ml, as demonstrated by the mouse L-929 cytotoxicity assay (according to R&D product sheet for AF-410-NA).

**Enzyme-linked Immunosorbent Assay—** Conditioned media were collected from various primary adipose cell cultures. The protein levels of TNF-α, IL-6, and IL-8 were determined using Quantikine M kits from R&D Systems.

**Real-time Quantitative RT-PCR—** Total RNA was isolated from fresh epididymal fat pads, primary fat cells immediately after collagenase digestion, and collagen-digested primary adipose cells cultured in vitro for up to 24 h. mRNA was quantified by Taqman chemistry-based real-time quantitative RT-PCR. Following a standard protocol for preparing a primary adipose cell culture, we digested freshly harvested mouse epididymal fat pads with crude collagenase at 37 °C for 2 h, followed by extensive washes using KRBH buffer supplemented with 5% bovine serum albumin. Because the ectodomains of certain plasma membrane proteins including TNF-α can be readily released by proteolysis, and the resulting bioactive molecules may potentially have significant impact on adipose cell biology and function, we monitored the production of TNF-α during and after collagenase treatment by determining the concentration of TNF-α in the supernatant of the digestion mixture and subsequent cell culture. After collagenase addition to mouse adipose tissue equivalent to −106 adipose cells/ml, the concentration of TNF-α in the conditioned medium increased dramatically within 1 h, reaching 0.93 ng/ml (Fig. 1A, top panel). At the end of the 2-h collagenase digestion,
The steady state mRNA levels of multiple important inflammatory mediators were determined using oligonucleotide microarrays and are shown as raw data (average difference, according to Affymetrix) obtained from Affymetrix GENECHIP output files after normalization as described under “Experimental Procedures.” Before collagenase digestion, total RNA samples were prepared from freshly isolated mouse adipose tissue; after collagenase digestion, total RNA samples were prepared from primary adipose cells after a 2-h collagenase digestion at 37°C. N.D., hybridization signals were not detected on the microarray (A calls, according to Affymetrix). Fold induction was calculated by determining the ratio of the average difference from the two data sets (After collagenase/Before collagenase). No-fold change was calculated for genes whose expression levels were undetectable in freshly isolated adipose tissue and is indicated with an asterisk (*). No changes in mRNA levels were seen in the 12 control genes before and after the 2-h collagenase digestion.

TABLE I

| GenBank™ accession number | Gene name | Before collagenase digestion | After collagenase digestion | Fold induction |
|---------------------------|-----------|------------------------------|----------------------------|---------------|
| Chemokines                |           |                              |                            |               |
| J04596                    | Chemokine (C-C motif) ligand 1 | 78                           | 5187                       | 66.2          |
| X53798                    | Chemokine (C-C motif) ligand 2 | N.D.                         | 655                        | *             |
| U27267                    | Chemokine (C-C motif) ligand 5 | N.D.                         | 50                         | *             |
| M33266                    | Chemokine (C-C motif) ligand 10 | N.D.                        | 208                        | *             |
| L12030                    | Chemokine (C-C motif) ligand 12 | 143                         | 350                        | 2.4           |
| AV139913                  | Chemokine (C-C motif) ligand 12 | N.D.                         | 201                        | *             |
| M19681                    | Chemokine (C-C motif) ligand 2 | 239                          | 335                        | 14.6          |
| J04491                    | Chemokine (C-C motif) ligand 3 | N.D.                         | 133                        | *             |
| X2502                     | Chemokine (C-C motif) ligand 4 | N.D.                         | 124                        | *             |
| X70658                    | Chemokine (C-C motif) ligand 7 | 76                           | 628                        | 8.3           |
| U77462                    | Chemokine (C-C motif) ligand 11 | 76                           | 200                        | 2.6           |
| Acute phase proteins      |           |                              |                            |               |
| U60438                    | Serum amyloid A2               | N.D.                         | 344                        | *             |
| X03505                    | Serum amyloid A3               | N.D.                         | 1237                       | *             |
| U77630                    | Adrenomedullin                 | 46                           | 460                        | 10            |
| M28845                    | Early growth response 1        | 528                          | 2905                       | 5.5           |
| X87644                    | Immediate early response 3     | 236                          | 2155                       | 9.1           |
| X83601                    | Pentaxin-related gene          | N.D.                         | 120                        | 3.6           |
| Cell Adhesion Molecules   |           |                              |                            |               |
| M72332                    | Selectin, platelet             | N.D.                         | 519                        | *             |
| M80778                    | Selectin, endothelial cell     | N.D.                         | 127                        | *             |
| M90551                    | Intercellular adhesion molecule| N.D.                         | 576                        | *             |
| X53177                    | Integrin α-4                   | N.D.                         | 60                         | *             |
| U125884                   | Vascular cell adhesion molecule| 33                           | 120                        | 3.6           |
| Transcription factors     |           |                              |                            |               |
| AF017128                  | fos-like antigen 1             | N.D.                         | 218                        | *             |
| M21065                    | Interferon regulatory factor 1 | 204                          | 2435                       | 11.9          |
| X61800                    | CEBP-δ                         | 392                          | 1065                       | 2.7           |
| M61007                    | CEBP-β                         | 517                          | 1278                       | 2.5           |
| U20735                    | Jun-B oncogene                 | 366                          | 1409                       | 3.8           |
| X12761                    | Jun oncogene                   | 187                          | 688                        | 3.7           |
| A1837104                  | STAT 3                         | 222                          | 446                        | 2.0           |
| Control mRNAs             |           |                              |                            |               |
| X56123                    | Talin                          | 366                          | 362                        | 1.0           |
| M28729                    | Tubulin-α1                     | 4985                         | 5703                       | 1.1           |
| M28739                    | Tubulin, β2                    | 657                          | 635                        | 1.0           |
| M12481                    | β-Actin                        | 1981                         | 1298                       | 0.65          |
| Z45745                    | ATP-binding cassette 8         | 269                          | 233                        | 0.9           |
| M19381                    | Calmodulin                     | 2713                         | 2434                       | 0.9           |
| M73329                    | Phospholipase C-α              | 556                          | 502                        | 0.9           |
| M17516                    | Lactate dehydrogenase A-4      | 5317                         | 4827                       | 0.9           |
| X66405                    | Procollagen, type VI, α-1      | 1947                         | 1869                       | 1.0           |
| M18194                    | Fibronec                      | 472                          | 474                        | 1.0           |
| AB005823                  | 1-Acyl-sn-glycerol-3-phosphate acyltransferase | 313 | 319 | 1.0 |
| U35312                    | Nuclear receptor co-repressor  | 281                          | 288                        | 1.0           |

The steady state mRNA levels of multiple important inflammatory mediators were determined using oligonucleotide microarrays and are shown as raw data (average difference, according to Affymetrix) obtained from Affymetrix GENECHIP output files after normalization as described under “Experimental Procedures.” Before collagenase digestion, total RNA samples were prepared from freshly isolated mouse adipose tissue; after collagenase digestion, total RNA samples were prepared from primary adipose cells after a 2-h collagenase digestion at 37°C. N.D., hybridization signals were not detected on the microarray (A calls, according to Affymetrix). Fold induction was calculated by determining the ratio of the average difference from the two data sets (After collagenase/Before collagenase). No-fold change was calculated for genes whose expression levels were undetectable in freshly isolated adipose tissue and is indicated with an asterisk (*). No changes in mRNA levels were seen in the 12 control genes before and after the 2-h collagenase digestion.

The steady state mRNA levels of multiple important inflammatory mediators were determined using oligonucleotide microarrays and are shown as raw data (average difference, according to Affymetrix) obtained from Affymetrix GENECHIP output files after normalization as described under “Experimental Procedures.” Before collagenase digestion, total RNA samples were prepared from freshly isolated mouse adipose tissue; after collagenase digestion, total RNA samples were prepared from primary adipose cells after a 2-h collagenase digestion at 37°C. N.D., hybridization signals were not detected on the microarray (A calls, according to Affymetrix). Fold induction was calculated by determining the ratio of the average difference from the two data sets (After collagenase/Before collagenase). No-fold change was calculated for genes whose expression levels were undetectable in freshly isolated adipose tissue and is indicated with an asterisk (*). No changes in mRNA levels were seen in the 12 control genes before and after the 2-h collagenase digestion.

The isolated adipose cells were washed extensively after collagenase digestion and incubated in growth medium at a concentration of ~2 × 10^5 cells/ml, a steady but lower release of TNF-α continued as measured by the increasing amount of TNF-α in the conditioned medium (Fig. 1A, top panel). At the end of the incubation, the concentration of TNF-α in conditioned media reached 0.17 ng/ml.

In contrast, this standard isolation procedure did not affect the secretion of IL-6, as no detectable IL-6 protein was present in the supernatant of the digestion mixture throughout the 2-h collagenase treatment (Fig. 1A, bottom panel). To determine whether adipose tissue expresses the IL-6 gene, we first assessed IL-6 mRNA levels using oligonucleotide microarrays. IL-6 mRNA was barely detectable in fresh adipose tissue but was highly induced at the end of the collagenase digestion (Table I). The IL-6 transcript remained at high levels when the isolated primary adipose cells were cultured in vitro for up to 24 h (see below). Consistent with the expression kinetics of IL-6 mRNA, the protein levels of IL-6 increased significantly in the conditioned medium 2 h after in vitro culture, peaking at the end of the 24-h incubation with a concentration of 4 ng/ml (2 ×
10^5 cells/ml; Fig. 1A, bottom panel). IL-6 is normally induced by nuclear factor-xB (NF-xB), which is activated in response to a variety of extracellular stimuli such as tissue injury, oxidative stress, and inflammatory cytokines. Because TNF-α is secreted by adipose cells during collagenase digestion (Fig. 1A, top panel), TNF-α-mediated NF-xB activation is thus a potential inducer of IL-6 gene expression in the cultured primary adipose cells. The delayed kinetics of IL-6 induction in adipose cells during collagenase treatment and isolation, compared with that of TNF-α, is in accordance with this potential relationship. However, other adipose cell-secreted factors triggered by collagenase digestion and/or oxidative stress could also play a role in the induction of IL-6 mRNA and protein secretion (see below).

In many cell types such as immune cells, TNF-α is not expressed in resting cells but is rapidly induced in response to a number of extracellular stimuli such as pathogens. The induction of TNF-α in these cells is independent of de novo protein synthesis. To determine whether TNF-α gene transcription in adipose cells is induced by the standard isolation procedure including collagenase treatment, we used real-time quantitative RT-PCR to measure the steady state levels of TNF-α mRNA in fresh adipose tissue, as well as in isolated adipose cells after collagenase digestion. TNF-α mRNA is readily detectable in epididymal fat pads freshly harvested from lean mice and is induced over 420-fold following a 2-h collagenase treatment. The levels of TNF-α transcript remained at this high level even 4 h after completing the collagenase digestion. Notably, although the levels of TNF-α mRNA dropped 90% when cells were incubated in growth medium for 24 h, the TNF-α transcript still remained about 40-fold higher than fresh adipose tissue (Fig. 1B). Thus, our data indicate that the standard method for isolating primary adipose cells from adipose tissue triggers TNF-α gene transcription and protein secretion and that adipose cell-derived TNF-α may potentially affect the expression of at least a set of genes, including IL-6, in isolated primary adipose cells in culture.

**Standard Procedure for Isolating Primary Adipose Cells from Adipose Tissue Triggers Major and Rapid Changes in Gene Expression That Are Characteristic of TNF-α-treated 3T3-L1 Adipocytes**—To determine whether adipose cell gene transcription in general is significantly altered during standard preparation of isolated cells, we examined gene expression profiles in isolated adipose cells cultured *in vitro* for up to 24 h. We performed a self-organizing feature map (SOM) analysis of the data collected from oligonucleotide microarrays to identify clusters of genes with distinct patterns of expression kinetics during the 24-h period. The SOM analysis shows that this standard procedure for isolation of primary adipose cells evoked major and rapid changes in adipocyte gene expression, including down-regulation of essential adipose cell-abundant genes and up-regulation of immune response and pre-adipocyte genes. Strikingly, the isolated adipose cells exhibited distinct patterns of gene expression kinetics that are identical to those seen in 3T3-L1 adipocytes treated with TNF-α. Here, we focused on a subset of genes whose encoded proteins play a critical role in adipocyte function.

First, we assessed the expression pattern of key adipocyte proteins that are essential for insulin-stimulated glucose uptake and metabolism. As shown in Fig. 2, the steady state mRNA levels of insulin receptor substrate-2 (IRS-2), phosphatidylinositol 3-kinase, AKT-2, and c-Cbl-associated protein, whose encoded proteins are involved in transducing insulin signals in adipose cells, were down-regulated 80% or more by 24 h in culture relative to the beginning of the incubation (Fig. 2, A–D, empty squares). Similarly, the mRNA levels of many effector molecules of insulin signaling, such as GLUT4, glycogen synthase, fatty acid synthase, phosphoenolpyruvate carboxykinase, and diacylglycerol acyltransferase were repressed at least 80% compared with the beginning of the incubation (Fig. 2, E–J, empty squares). In addition, the mRNA levels of two adipocyte master transcription factors, PPAR-γ and CEBP-α, were repressed 95 and 77%, respectively, in primary adipose cells after a 24-h culture *in vitro* (Fig. 2, K–L, empty squares).

In addition to proteins involved in energy metabolism, the expression of many adipose cell-secreted factors was altered when primary adipose cells were cultured *in vitro* (Fig. 3, A–D, empty squares). Among them, the expression levels of leptin, resistin, and ACRP30 were all down-regulated whereas the mRNA level of IL-6 did not further increase from the already high level induced by the collagenase treatment (Table I). In contrast, other TNF-α family members such as Fas antigen and many proteins involved in TNF-α signaling including TNF-α receptor-associated proteins (TRAF-2, 3, and 4) and TNF-α...
Isolation of Primary Adipose Cells Induces Inflammation

Leptin

Resistin

ACRP30

TNFR-p75

TRAF-2

TRAF-3

IL-6

TRAF-4

AKT-1

Figure 3. Standard isolation of primary adipose cells triggers major and rapid changes in the expression of genes encoding adipocyte-secreted proteins and proteins involved in TNF-α signaling. At the end of the collagenase digestion, isolated primary adipose cells were washed extensively and then incubated in vitro for up to 24 h without (empty squares) or with (filled triangles) TNF-α (1 nmol/liter). The mRNA levels of the indicated genes were determined as described in the legend for Fig. 2. Time 0 indicates the beginning of the in vitro culture. ACRP30, adipocyte complement-related protein of 30 kDa.

receptor-2 were highly induced when adipose cells were cultured in vitro (Fig. 3, F–J, empty squares). Notably, whereas adipocyte-abundant AKT-2 (19, 20) mRNA was repressed in cultured primary adipose cells, the mRNA encoding AKT-1, a ubiquitously expressed isofrom of AKT, increased steadily (Fig. 3E, empty squares). This AKT isoform switch may contribute, in part, to the loss of insulin response in cultured primary adipose cells.

We have shown previously that in 3T3-L1 adipocytes NF-κB activation is obligatory for TNF-α-mediated repression of most of the adipocyte-abundant genes and induction of a subset of genes. Among the 22 genes shown in Figs. 2 and 3, the mRNA levels of phosphatidylinositol 3-kinase, AKT-2, c-Cbl-associated protein, GLUT4, glycogen synthase, phosphoenolpyruvate carboxykinase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase, PPAR-γ, and CEBP-α were all repressed by TNF-α in 3T3-L1 adipose cells in a NF-κB-dependent manner, because the expression levels of these genes were unaffected by TNF-α in 3T3-L1 adipose cells expressing a dominant inhibitor of NF-κB activation (IxBa-DN). Similarly, induction of Fas antigen by TNF-α requires NF-κB activation.2 TNF-α also repressed the expression of fatty acid synthase, resistin, and ACRP30 and induced TNF-R2 and TRAF-4 in 3T3-L1 adipocytes after multiple hours of incubation. However, TNF-α caused extensive cell death in adipose cells expressing IxBa-DN after 2 h of incubation, and so we could not determine whether the effects of TNF-α on the expression of these genes were dependent on NF-κB activation. The hybridization signals for the remaining six genes (IRS-2, leptin, IL-6, AKT-1, TRAF-2, and TRAF-3) were low in our previous experiments on TNF-α-treated 3T3-L1 adipocytes, and we did not further investigate the cause for this observation, although technical limitations are likely to be involved. Nevertheless, these data indicate that the standard procedure for isolating primary adipose cells from adipose tissue triggers rapid reprogramming of adipose cell gene expression that is characteristic of TNF-α-treated 3T3-L1 adipocytes.

TNF-α Is Not Necessary for the Reprogramming of Gene Expression in Isolated Primary Adipose Cells—Because the isolated primary adipose cells exhibited dramatic changes in gene expression that were similar to the TNF-α-induced changes in 3T3-L1 adipocytes, and because significant amounts of TNF-α were secreted from primary adipose cells during collagenase digestion, we hypothesized that primary adipose cell–derived TNF-α triggers the changes in global gene expression during isolation of primary adipose cells. To test this hypothesis, we first determined whether the dynamics of the primary adipose cell response to the standard isolation procedures, especially the collagenase exposure, would be further altered by the addition of TNF-α. We assessed the expression patterns of key adipocyte genes in collagenase-digested primary adipose cells incubated with TNF-α (1 nmol/liter) for up to 24 h. As shown in Figs. 2 and 3 (filled triangles), the expression kinetics of genes that are normally induced or repressed in primary adipose cells during in vitro incubation were not significantly affected by the presence of exogenous TNF-α, although the induction of some genes was further enhanced by TNF-α (data not shown). This suggests that the standard method for adipose cell isolation may activate the same pathways utilized by TNF-α to affect adipocyte gene expression. Alternatively, the standard isolation procedure could trigger cellular pathways in addition to those activated by TNF-α, and these different pathways could converge at the level of transcriptional machinery and result in transcriptional changes that are characteristic of TNF-α.

Because primary adipose cells continue to release a significant amount of TNF-α when they are cultured in vitro, we used a TNF-α-neutralizing antibody to test whether the TNF-α secreted by isolated adipose cells in culture is responsible for the changes in gene expression. As shown in Fig. 4, A and B, semi-quantitative RT-PCR analysis of mRNA levels indicates that glyceraldehyde 3-phosphate dehydrogenase was downregulated, and GLUT1 was induced in primary adipose cells following a 24-h incubation in vitro (compare lanes 1 with lanes 2 in Fig. 4, A and B), consistent with our microarray data. Addition of increasing amounts of TNF-α-neutralizing antibody (0.008–0.4 μg/ml) did not block the changes of the expression of these two representative genes (compare lanes 3–6 with lanes 2 in Fig. 4, A and B). According to the efficacy of the TNF-α-neutralizing antibody, and as described under “Experimental Procedures,” the highest amount of antibody we used in this study is sufficient to fully inhibit the biological activities of TNF-α at a concentration of 0.75 ng/ml, which is about 4-fold the concentration seen at the end of the 24-h incubation period (Fig. 1A, top panel). Thus, our data suggest that TNF-α produced during incubation of adipose cells did not significantly contribute to the induction of the changes in global gene transcription in adipose cells.

Because TNF-α was highly induced during collagenase digestion (Fig. 1A, top panel), it is possible that this brief exposure to high levels of TNF-α during collagenase treatment is sufficient to trigger major changes in gene expression in pri-

H. Ruan and H. F. Lodish, unpublished data.
primary adipose cells. Thus, we used primary adipose cells isolated from TNF-α−/− mice to ascertain whether TNF-α is responsible for initiating the reprogramming of gene expression in isolated primary adipose cells. We first confirmed that TNF-α was indeed absent in primary adipose cells derived from TNF-α−/− mice. As shown in Fig. 5A, primary adipose cells isolated from wild-type mice released significant amounts of TNF-α (0.225 ng/ml) at the end of the standard collagenase digestion. When these primary adipose cells were washed extensively and cultured in vitro, the concentration of TNF-α in the conditioned medium reached 0.057 ng/ml at the end of the 24-h incubation; lane 2, no TNF-α neutralizing antibody; lanes 3-6, increasing amounts of TNF-α neutralizing antibody: 0.008, 0.04, 0.2, and 0.4 µg/ml, respectively.

Next, we measured the production of IL-6, which is normally induced by a variety of NF-κB-inducing signals including TNF-α, wild-type and TNF-α−/− adipose cells. Interestingly, the secretion of IL-6 was induced to the same extent in TNF-α−/− adipose cells as that seen in wild-type adipose cells (Fig. 5B). We then assessed the gene expression profiles of adipose cells derived from TNF-α−/− and wild-type mice after these cells had been cultured for 24 h in vitro. Strikingly, identical patterns of changes in gene expression were observed in both types of adipose cells (Table II). The absence of TNF-α did not prevent isolation process-induced reprogramming of adipocyte gene expression, including down-regulation of key adipocyte genes and induction of genes encoding inflammatory mediators. Additionally, NF-κB was activated in both TNF-α−/− and wild-type adipose cells, as determined by the induction of members of the NF-κB family including p65 (Rel A), NF-κB-1 (p105/p50), and NF-κB-2 (p100/p52) and NF-κB target genes such as IκB-α, vascular cell adhesion molecule-1, and IL-6 (Tables I and II).

Thus, our data suggest that although TNF-α may contribute to the induction of changes in global gene expression during isolation of primary adipose cells, other isolation procedure-triggered proteins and/or TNF-α-independent pathways also play a role, perhaps even the predominant role, in NF-κB activation and the induction of the coordinated changes in adipose cell gene expression that result in decreased insulin sensitivity.

**Standard Procedure for Isolating Primary Adipose Cells Leads to Induction of mRNAs Encoding Multiple Inflammatory Mediators, in Addition to TNF-α—As an initial step in identifying TNF-α-independent factors that may activate NF-κB and/or regulate global gene expression in isolated primary adipose cells, we measured the steady state mRNA levels of several important cytokines, chemokines, and members of the TNF-α superfamily that have been implicated in the inflammatory process and NF-κB activation, using oligonucleotide microarrays. The results in Table I show that the mRNAs encoding nine different chemokines, IL-1α, IL-6, TNF-α, and the type I IL-1 receptor are all up-regulated during the 2-h collagenase digestion, in contrast to the constant levels of dozens of control mRNAs (Table I). Notably, hybridization signals...**
corresponding to the mRNAs encoding TNF-α superfamily members were not detected in the total RNA samples prepared from primary adipose cells (data not shown).

Multiple genes encoding transcription factors that have been implicated in the induction of synthesis of acute phase cytokines were also rapidly induced in primary adipose cells during the 2-h collagenase digestion. Among them, CEBP-β and CEBP-δ, whose mRNA levels were induced 2.5- and 2.7-fold (Table I), respectively. These contribute to the induction of IL-1β, TNF-α, IL-6, IL-8, and other cytokines in macrophages and monocytes (21–24), and thus may participate in the induction of those cytokines in adipose cells, as well. Other transcription factors including NF-κB family members (p65, NF-κB-1, and NF-κB-2; see Table II), signal transducers and activators of transcription 3, fos-like antigen 1, Jun-B, and Jun also induced in primary adipose cells following the 2-h collagenase digestion are immediately involved in the induction of the pro-inflammatory cytokines (Table I).

Thus, our data suggest that the standard collagenase procedure for isolating primary adipose cells triggers induction of a variety of inflammatory mediators that could potentially have profound impact on NF-κB activation and on adipocyte gene expression. Interestingly, preliminary data (not shown) indicate that secretion of TNF-α and IL-6 occur at similar levels even if collagenase is not added to the disrupted adipose tissue prior to incubation. Therefore, the isolation procedure-triggered effects on adipose cell gene expression may not involve collagenase but only removal of adipose tissue from the animals.

**DISCUSSION**

We report three major findings, which, taken together, describe major transcriptional changes occurring in primary adipose cells following a standard isolation procedure involving collagenase digestion. First, we demonstrated, for the first time, that the standard procedure for isolating primary adipose cells is a potent inducer of TNF-α gene transcription and protein secretion, as well as multiple other important inflammatory mediators. Second, we found that the isolation procedure triggered changes in global gene expression in primary adipose cells that are characteristic of TNF-α-treated 3T3-L1 adipocytes. Third, we showed that adipose cell-derived TNF-α, which was highly induced during the standard isolation procedure, is dispensable for the reprogramming of gene expression in cultured primary adipose cells and that primary adipose cells exhibited extensive plasticity in gene transcription in response to a variety of inflammatory mediators.

Primary adipose cells have been used extensively as a model system to study the biology of adipose cells for over 40 years (25). It is widely recognized, however, that isolated primary adipose cells gradually lose their response to insulin stimulation when cultured in vitro, as determined by decreased insulin-stimulated GLUT4 translocation and glucose uptake and increased free fatty acid release. These alterations in cultured primary adipose cells essentially mimic the development of insulin resistance in isolated primary adipose cells.

Impaired tyrosine kinase activity of the insulin receptor and/or increased serine phosphorylation of IRS-1 have been implicated as a potential cause for the decreased insulin response in insulin-target cells. Here, we demonstrated that the mRNA levels of genes encoding these and other essential components of the insulin-signaling pathway, as well as the end effector molecules including metabolic enzymes, were significantly down-regulated in primary adipose cells following this
standard isolation procedure. Furthermore, the standard isolation procedure resulted in major changes in global gene expression, including down-regulation of adipocyte-abundant genes and up-regulation of immune response, growth-phase, and proadipocyte genes, characteristics that closely resemble those seen in cell models and animal models of insulin resistance such as TNF-α-treated 3T3-L1 adipocytes and db/db mice.

Because TNF-α induces major changes in adipocyte gene expression both in cell culture and in vitro, and because a significant amount of TNF-α is released from primary adipose cells during the standard isolation procedure, we hypothesized that primary adipose cell-secreted TNF-α is responsible for initiating the transcriptional changes in global gene expression in adipose cells. However, addition of TNF-α neutralizing antibody to the primary adipose cell culture after collagenase digestion had no effect on the changes in adipose cell gene expression; the same changes were seen in TNF-α−/− mice. Thus, our data provide direct and definitive evidence indicating that TNF-α is not the sole cause, or perhaps even the primary cause, of the observed transcriptional changes in primary adipose cells.

We then attempted to identify potential TNF-α-independent mediators that might initiate the transcriptional changes in primary adipose cells observed following the standard isolation procedure. We found that a group of signature inflammatory mediators were highly induced in primary adipose cells during the 2-h collagenase digestion, which strongly mimic the inflammation processes that result from tissue injury such as surgical trauma and ischemic necrosis. Among the inflammatory mediators that are immediately induced in the primary adipose cells at the end of the 2-h collagenase digestion, IL-1α is known to activate multiple cellular signal transduction pathways including IKK/NF-κB, p38, extracellular signal-regulated kinase 1/extracellular signal-regulated kinase 2 (p42/p44), and c-Jun NH₂-terminal kinase (26). The activation of NF-κB leads to induction of many proinflammatory cytokines and cell surface receptor proteins, as well as repression of adipocyte-abundant genes (14), and is sufficient to repress PPAR-γ-mediated gene transcription in reporter gene assays (27). The p38, extracellular signal-regulated kinase, and c-Jun NH₂-terminal kinase mitogen-activated protein kinases have also been implicated in the regulation of gene transcription and the steady state mRNA levels of multiple genes involved in inflammation (28–30). Furthermore, these cellular mitogen-activated protein kinases can independently activate the IKK complex and result in NF-κB activation (31). Notably, we have shown previously (14) that NF-κB activation is required for TNF-α-mediated repression of key adipocyte genes in 3T3-L1 adipocytes but that induction of many transcription factors and proinflammatory genes is not dependent on NF-κB. Thus other potential NF-κB-independent factors, especially those activated by the several inflammatory cytokines that are induced during collagenase treatment of primary adipocytes, may play an important role in reprogramming gene expression in primary adipose cells, as well.

On the other hand, the production of many cytokines and chemokines including IL-6, IL-1, and TNF-α can be further enhanced by IL-1 and/or TNF-α in an autocrine or paracrine fashion. The initial stimuli could thus be amplified and sustained from within the adipose tissue. In parallel, transcription factors implicated in the induction of the inflammatory cytokines such as CEBP-β, CEBP-δ, NF-κB, signal transducers and activators of transcription 3, Jun, and fos-like antigen-1 are also immediately up-regulated in primary adipose cells following the standard isolation procedure, indicating a potential cross-talk and positive feed-back loop between multiple signaling pathways.

The concurrent induction of multiple cytokines and inflammatory mediators with overlapping functions in primary adipose cells suggests one potential explanation for the observation that TNF-α is not essential for induction of the coordinate changes in gene expression during in vitro culture of primary adipose cells following the standard isolation procedure. The identification of which inflammatory mediator(s) is (are) responsible for the reprogramming of gene expression in primary adipose cells requires definitive tests in knockout animals lacking each protein or a combination of inflammatory mediators. Such genetic analyses of the role of inflammatory mediators in the regulation of gene expression in primary adipose cells, even confined to their potential involvement in the repression of adipocyte-abundant genes, are far from straightforward, given the extraordinary complexity and overlapping functions of the cytokines and their down-stream signaling pathways. Thus, in the current study we could not determine which inflammatory mediator(s) is (are) critical in initiating the changes in adipose cell gene expression.

Despite this, our data suggest, at least indirectly, that the inflammatory mediators produced by various cell components within the adipose tissue following the standard isolation procedure contribute to the activation of NF-κB and perhaps other transcription regulatory proteins that result in repression of the adipocyte-abundant genes in primary adipose cells cultured in vitro. The primary cause of the cascade of events leading to the changes in global gene expression in primary adipose cells is likely related to the surgical/mechanical trauma in adipose tissue during the standard isolation procedure, because our preliminary data indicate that such coordinated changes in adipose cell gene transcription does not require collagenase treatment but may only involve surgical removal of adipose tissue from the animals. In response to a variety of triggering stimuli such as infection, surgical injury, and ischemic necrosis, resident tissue macrophages, T-lymphocytes, leukocytes, and endothelial cells initiate acute-phase inflammatory responses. Similarly, in adipose tissue, activated immune cells release a variety of inflammatory mediators including many cytokines, free radicals, and reactive oxygen species that trigger a cascade of transcriptional changes in response to the initial stimuli, through the activation of multiple common cellular mediators including NF-κB and other transcription regulators.

In summary, our data provide proof that adipose tissue is a major source of inflammatory mediators and that adipose cells themselves are highly responsive to a variety of inflammatory factors. These observations are intriguing, because they strongly suggest that different initiating factors, including TNF-α, may act in parallel to drive the development of insulin resistance in a number of clinical settings through diverse cellular pathways. Although we found that multiple TNF-α-independent inflammatory factors may potentially trigger the activation of NF-κB and other transcription regulatory proteins and induce the changes in global gene expression in isolated primary adipose cells, our study does not exclude TNF-α as an important mediator in vivo; rather, our data provide a basis for further investigation of the role of several adipocyte-derived factors in the induction of insulin resistance in vivo and for testing the therapeutic potential of inhibiting the activity of NF-κB in the treatment of insulin resistance in the context of obesity and type 2 diabetes.
Isolation of Primary Adipose Cells Induces Inflammation

47593

4. Olefsky, J. M., Ciaraldi, T. P., and Kolterman, O. G. (1985) *Am. J. Med.* 79, 12–22
5. LeRoith, D. (2002) *Am. J. Med.* 113, Suppl. 6A, 38–118
6. Olefsky, J. M., and Saltiel, A. R. (2000) *Trends Endocrinol. Metab.* 11, 362–368
7. Saltiel, A. R., and Olefsky, J. M. (1996) *Diabetes* 45, 1661–1669
8. Maggs, D. G., Buchanan, T. A., Burant, C. F., Cline, G., Gumbiner, B., Hsueh, W. A., Inzucchi, S., Kelley, D., Nolan, J., Olefsky, J. M., Polonsky, K. S., Silver, D., Valiquett, T. R., and Shulman, G. I. (1998) *Ann. Intern. Med.* 128, 176–185
9. Ferrannini, E. (1995) *Metabolism* 44, 15–17
10. Walker, M. (1995) *Metabolism* 44, 18–20
11. Trayhurn, P., and Beattie, J. H. (2001) *Proc. Nutr. Soc.* 60, 329–339
12. Moller, D. E. (2000) *Trends Endocrinol. Metab.* 11, 212–217
13. Gerrits, P. M., Olson, A. L., and Pessin, J. E. (1995) *J. Biol. Chem.* 268, 640–644
14. Ruan, H., Hacohen, N., Golub, T. R., Van Parijs, L., and Lodish, H. F. (2002) *Diabetes* 51, 1319–1336
15. Malide, D., Ramm, G., Cushman, S. W., and Slot, J. W. (2000) *J. Cell Sci.* 113 Pt 23, 4203–4210
16. Ruan, H., Miles, P. D. G., Ladd, C. M., Ross, K., Golub, T. R., Olefsky, J. M., and Lodish, H. F. (2002) *Diabetes* 51, 3176–3188
17. Kohonen, T. (1991) *Proc. IEEE.* 78, 1464–1480
18. Tamayo, P., Slonim, D., Mesirov, J., Zhu, Q., Kitareewan, S., Dmitrovsky, E., Lander, E. S., and Golub, T. R. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 2907–2912
19. Hill, M. M., Clark, S. F., Tucker, D. F., Birnbaum, M. J., James, D. E., and Macaulay, S. L. (1999) *Med. Cell Biol.* 19, 7771–7781
20. Cho, H., Mu, J., Kim, J. K., Thorvaldsson, J., Chu, Q., Crenshaw, E. B., III, Kae stner, K. H., Bartolomei, M. S., Shulman, G. I., and Birnbaum, M. J. (2001) *Science* 292, 1728–1731
21. Matsuzaka, T., Fujikawa, K., Nishio, Y., Mukaida, N., Matsushima, K., Kishimoto, T., and Akira, S. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 10193–10197
22. Pope, R. M., Leutz, A., and Ness, S. A. (1994) *J. Clin. Invest.* 94, 1449–1455
23. Godambe, S. A., Chaplin, D. D., Takova, T., and Bellone, C. J. (1994) *J. Immunol.* 153, 143–152
24. Wedel, A., and Ziegler-Heitbrock, H. W. (1995) *Immunobiology* 193, 171–185
25. Simpson, I. A., Hedo, J. A., and Cushman, S. W. (1984) *Diabetes* 33, 13–18
26. Dunne, A., and O'Neill, L. A. (2003) *Science's STKE* http://stke.sciencemag.org/cgi/content/full/sigtrans;2003/171/re3
27. Ruan, H., Pownall, H. J., and Lodish, H. F. (2003) *J. Biol. Chem.* 278, 28181–28192
28. Chang, L., and Karin, M. (2001) *Nature* 416, 37–40
29. Davis, R. J. (1995) *Mol. Reprod. Dev.* 42, 459–467
30. Lee, J. C., and Young, P. R. (1996) *J. Leukocyte Biol.* 59, 152–157
31. Lee, F. S., Hagner, J., Chen, Z. J., and Maniatis, T. (1997) *Cell* 88, 213–222