Lipid Infusion Decreases the Expression of Nuclear Encoded Mitochondrial Genes and Increases the Expression of Extracellular Matrix Genes in Human Skeletal Muscle*[*]

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The association between elevated plasma free fatty acid (FFA) concentrations and insulin resistance is well known. Although the cause and effect relationship between FFAs and insulin resistance is complex, plasma FFA is negatively correlated with the expression of peroxisome proliferator activated receptor-γ cofactor-1 (PGC-1) and nuclear encoded mitochondrial genes. To test whether this association is causal, we infused a triglyceride emulsion (or saline as control) into healthy subjects to increase plasma FFA for 48 h followed by lipid infusion (p < 0.05). PGC-1 mRNA, along with mRNAs for a number of nuclear encoded mitochondrial genes, were reduced by lipid infusion (p < 0.05). Microarray analysis also revealed that lipid infusion caused a significant overexpression of extracellular matrix genes and connective tissue growth factor. Quantitative reverse transcription PCR showed that the mRNA expression of collagens and multiple extracellular matrix genes was higher after the lipid infusion (p < 0.05). Immunoblot analysis revealed that lipid infusion also increased the expression of collagen and the connective tissue growth factor protein. These data suggest that an experimental increase in FFAs decreases the expression of PGC-1 and nuclear encoded mitochondrial genes and also increases the expression of extracellular matrix genes in a manner reminiscent of inflammation.

The association between elevated plasma lipid concentrations and insulin resistance is well known. Although the cause and effect relationship between lipids and insulin resistance is complex, an experimental increase in plasma free fatty acid (FFA) concentrations induces insulin resistance in skeletal muscle in healthy humans (2, 3). Earlier studies explored the possibility that the Randle cycle could explain lipid-induced insulin resistance (2, 3). Although the concept that FFA and glucose compete with one another as oxidative fuels in skeletal muscle has withstood the test of time, more recent studies have shown that FFA and FFA metabolites inhibit insulin signaling (4), glucose transport (4), and the activities of various enzymes involved in glucose metabolism (2). Although insulin-resistant states such as obesity and type 2 diabetes are characterized by day-long elevation of plasma FFA levels due to excess lipolysis (5–7), paradoxically the rate of fat oxidation in skeletal muscle is reduced, not enhanced (8, 9). An increased supply of lipids to skeletal muscle in the face of decreased fat oxidation would be expected to lead to elevated intramyocellular lipid levels. Consistent with this thesis, triglyceride (10), fatty acyl-CoA (11, 12), diacylglycerol (11, 12), and ceramide concentrations (13) are increased in insulin-resistant skeletal muscle. Studies using computerized tomography and magnetic resonance spectrometry techniques show a positive correlation between increased intramyocellular triglyceride content and insulin resistance (14, 15). However, those studies provide no information regarding the biochemical or molecular basis of insulin resistance.

Recent studies have shown that there are pronounced patterns of change in skeletal muscle gene expression from insulin-resistant subjects (16–18). Because insulin-resistant subjects have chronic increases in plasma FFAs, it could be argued that chronic exposure to increased FFA might lead to changes in skeletal muscle gene expression that, in turn, could produce or contribute to insulin resistance. We (16) and others (17, 18) have found previously that insulin-resistant subjects had decreased expression of nuclear encoded mitochondrial genes accompanied by the decreased expression of peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1), the transcriptional coactivator that drives the expression of many genes coding for proteins in mitochondria. Moreover, PGC-1 expression is inversely correlated with plasma FFA concentrations (16). Therefore, we set out to test the hypothesis that an experimental increase in plasma FFA concentrations would

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§ The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1 (a flow diagram of steps used in microarray data analysis) and supplemental Tables I and II (presenting information on the genes and gene sets analyzed).

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1 The abbreviations used are: FFA, free fatty acid; CTGF-, connective tissue growth factor; HGF, hepatocyte growth factor; MAS, Microarray Analysis Suite (software); PGC-1, peroxisome proliferator-activated receptor-γ coactivator-1; Q-RT-PCR, quantitative real time PCR; SAM, Statistical Analysis of Microarrays (software); TGF-β1, transforming growth factor-β1.
reduce the expression of nuclear encoded mitochondrial genes along with their transcriptional coactivator PGC-1. Conducting this study using a global gene expression profiling allowed us to test this hypothesis and at the same time identify novel targets of increased FFA in skeletal muscle.

MATERIALS AND METHODS

Subjects—Seven normoglycemic, normal glucose-tolerant Mexican American subjects without a family history of diabetes took part in this study. All subjects had normal glucose tolerance, as assessed by a 75-g oral glucose tolerance test. Subjects received a history, physical examination, and screening blood tests to ensure that they were healthy. No subject was taking any medication known to affect glucose metabolism. All subjects gave informed written consent to participate in the study, which was approved by the Institutional Review Board of the University of Texas Health Science Center at San Antonio.

Study Design—Subjects were studied on two occasions separated by 3–4 weeks in random order, once with an infusion of Liposyn III (20% triglyceride emulsion largely composed of soyein oil) and once with saline as a control. Following an overnight fast, subjects reported to the General Clinical Research Center at 8 a.m., a forearm vein was catheterized, and either Liposyn III (80 ml/h) or saline was infused for 48 h. During this time, subjects were ambulatory and consumed a weight-maintaining (50% carbohydrate, 30% fat, and 20% protein) diet. After 48 h of lipid or saline infusion, an antecubital vein was catheterized, and a primed (25 μCi), continuous (0.25 μCi/min) infusion of [3H]glucose was begun to measure rates of glucose appearance and disappearance. A hand vein was catheterized and placed in a heated box to arterialized venous blood for the measurement of arterial glucose concentrations. One hour later, a percutaneous biopsy of the vastus lateralis muscle was performed as described previously (19). Biopsy specimens (75–150 mg) were frozen immediately in liquid nitrogen and stored in liquid nitrogen until they were processed. One hour after the muscle biopsy (2 h after the start of tritiated glucose), a primed continuous (80 milliunits/ml·min) insulin infusion was started and continued for 240 min to quantify the effects of insulin on glucose disposal (20). Throughout the insulin infusion, an infusion of 20% glucose was adjusted to maintain euglycemia (20).

Muscle Biopsy Processing—For mRNA analyses, muscle biopsy specimens were homogenized directly in RNaseout solution (Tel-Test Inc., Friendswood, TX), using a Polytron homogenizer (Brinkmann Instruments Westbury, NY). RNA pellets were stored in ethanol/sodium chloride, and prior to use, total RNA was purified with RNaseasy and Oligo (dT)1 treatment (Qiagen, Chatsworth, CA). For immunoblot analysis, detergent lysates of muscle were prepared as described previously (19).

Microarray Analysis, Including Target Preparation, Hybridization, Staining, Scanning, and Analysis of Image—RNA was prepared for hybridization to Affymetrix (Santa Clara, CA) HG-U133A arrays according to the manufacturer's instructions. Total RNA was used as a template for the synthesis of double-stranded cDNA (Superscript double-stranded cDNA synthesis kit; Invitrogen), which was used as a template for biotin-labeled cRNA synthesis (Enzo BioArray High Yield RNA transcription labeling kit; Affymetrix). Purified (RNasey kit; Qiagen), fragmented (35–200 nucleotides), biotinylated cRNA was hybridized to HG-U133A GeneChips overnight for 16 h at 45 °C in a rotating incubator. Following hybridization, the probe arrays were washed and stained using the GeneChip Fluidics station protocol EukGE-ES2. The protocol consisted of non-stringent and stringent washes followed by a staining procedure whereby the hybridized cRNA was fluorescently labeled using anti-biotin antibodies and a streptavidin-phycocerythrin (SAPE) solution. The intensity of bound dye was measured with an argon laser confocal scanner (GeneArray scanner; Agilent). The probe arrays were scanned twice, and the stored images were aligned and analyzed using the GeneChip software Microarray Analysis Suite (MAS 5.0) (Affymetrix). The present call by MAS 5.0 software was 26 ± 1.5% of total genes. The 3/5' glyceraldehyde-3-phosphate dehydrogenase and actin expression ratios were <3 (acceptable) for all but two chips; however, all chips yielded values for the spiked controls (BIOB, BIOEX) that were within the acceptable range, because all positive results were subsequently confirmed using quantitative real time PCR and/or immunoblot analysis, all chips were included in the analyses.

Microarray Data Expression and Analysis—A flow diagram of the steps used in analysis of the microarray data is given in supplemental Fig. 1, which is available in the on-line version of this article. The Affymetrix data acquisition programs in MAS 5.0 automatically generate a cell intensity (CEL) file from the stored images that contain a single intensity value for each probe cell on the array. The CEL files were imported into the R software package (www.r-project.org), and the probe level data were converted to expression measures using the Affy package (21) from Bioconductor. Expression values for each mRNA were obtained by the Robust Multi-array Analysis (RMA) method of Irizarry (1), which adjusts for the background on the raw intensity scale, carries out a non-linear quantile normalization of the perfect match values, log transforms the background-adjusted perfect match values, and carries out a robust multi-chip analysis of the quantile normalized log transformed values (1). CEL files were normalized together, and the expression values obtained were submitted to analysis with the Statistical Analysis of Microarrays (SAM) software (22) to identify those genes that were significantly increased or decreased.

The expression values also were assembled into “gene sets” for analysis (supplemental Table II, available in the on-line version of this article), similar to that described by Mootha et al. (17). In particular, our gene set analysis approach was based on the comparison of statistics comprised of the sum of the average differences (lipsoid minus saline) for each gene in a particular set divided by the variance of the average differences. The method is briefly described here. Assume a set consisting of N genes, with n subjects studied under each of two conditions. For gene j, the mean difference in expression (dij) for that gene between conditions 1 and 2 is given by Equation 1,

\[
d_{ij} = \frac{\sum_{i=1}^{n} (X_{ij} - X_{ni})}{n}
\]

where Xij is the expression value for subject 1, condition 1, for instance. Then, as shown in Equation 2,

\[
M = \frac{\sum_{i=1}^{n} d_{ij}}{s^2}
\]

the sum of the average differences in gene expression across all of the genes in the set (normalized for the variance, s2, of the average differences) is the statistic M. In the case where Xij = Xni (that is, there is no difference in gene expression between conditions 1 and 2 for all i and j) the value of M will be 0. In practice, M is calculated for each gene set based on the observed expression values for the genes in that set. An empirical distribution of expected M values for a given gene set is then derived by selecting a subset of N genes randomly 10,000 times from the entire number of genes called “permute” by MAS 5.0 software. The observed M for the gene set is then compared with the distribution of expected values to determine statistical significance. The gene sets used in the analysis either were annotated previously (17) or independently in our laboratory.

A separate analysis, including gene normalization to specific samples, was conducted with GeneSpring 5.1 software (Silicon Genetics, CA) using the CHP file generated in the MAS 5.0 software. The CHP file is an output file generated from the analysis of each probe array. Filtering tools in the GeneSpring software were used to identify significantly up-regulated and down-regulated genes affected by the lipid infusion.

Quantitative TaqMan Real Time PCR (Q-RT-PCR)—Muscle expression of various genes was determined using the one-step Q-RT-PCR from the total RNA used for the microarray analysis. Q-RT-PCR was performed on the ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA) using TaqMan One Step RT-PCR Master Mix reagents and the Assay On Demand gene expression primer pair and probes (Applied Biosystems). To determine the efficiencies of each primer pair and probe set, a standard curve was generated by serial dilution of an RNA sample taken from a healthy subject. Each sample was run in duplicate, and the mean value of the duplicate was used to calculate the mRNA expression of the gene of interest and an endogenous control. The quantity of the gene of interest in each sample was normalized to that of 18 S ribosomal RNA using the comparative (2−ΔΔCT) method (23). Statistical comparisons were done using paired t tests.

Immunoblot Analysis and Immunofluorescence Staining—Detergent lysates of muscle biopsies were resolved by SDS-polyacrylamide gel electrophoresis as described (19). Proteins were transferred to nitrocellulose membranes, and the membranes were probed with various anti-
Bodies. Membranes were developed using Western Lightning reagents (PerkinElmer Life Sciences) and digitized and quantified using a VersaDoc 5000 imaging system (Bio-Rad). Monoclonal antibodies directed against collagens and procollagens were a generous gift of Dr. Nirmala Sundarraj at the University of Pittsburgh. Rabbit anti-con-nective tissue growth factor (CTGF) antibody was obtained from Torrey Pines Biologs (Houston, TX). Five-micrometer frozen sections of muscle biopsy specimens were probed using anti-collagen I and collagen III monoclonal antibodies (gift of Dr. Sundarraj), each at a dilution of 1:500. After exposure to fluorescein isothiocyanate-conjugated goat anti-mouse IgG, images were digitized using Spot v.3.5 software (Diag-nostic Instruments, Inc., Sterling Heights, MI). Statistical comparisons were done using paired t tests.

Other Analyses—Plasma insulin and FFA concentrations were determined by radioimmunoassay (Diagnostic Products, Los Angeles, CA) and enzymatic kit (NEFA-C, Wako Pure Chemicals, Osaka, Japan), respectively. Plasma samples were deproteinized by the Somogyi method for the calculation of glucose-specific activity, which was used to calculate the rates of glucose metabolism (20). The statistical signifi-cance of difference between means for in vivo data was determined using paired or non-paired Student’s t tests where appropriate (see above for statistical analysis of microarray data).

RESULTS

Subject Characteristics and in Vivo Data—Seven healthy subjects (three men and four women) with a mean (±S.E.) age of 43 ± 6 years and a body mass index of 24.5 ± 1.3 kg/m² participated in the study. The fasting plasma glucose concentration was within the normal range at 92 ± 2 mg/dl. Each subject had a normal oral glucose tolerance and was studied on two occasions with 48 h of Liposyn III (60 ml/hr) or saline control infusion.

Plasma FFA concentration (0.48 ± 0.02 mm) after saline increased to 1.73 ± 0.43 mm after lipid infusion (p < 0.01). Fasting plasma insulin concentrations were 4 ± 1 micro-units/ml after saline and 5 ± 1 micro-units/ml after lipid infusion. Basal rates of glucose appearance did not differ between the saline and lipid studies (1.92 ± 0.12 versus 2.07 ± 0.09 mg/(kg·min); p = 0.09). After 48 h of lipid or saline infusion, subjects received a 4-h euglycemic hyperinsulinemic clamp (80 microunits/m²·min) with triptized glucose. Steady state plasma insulin concentrations during insulin infusion were similar in the saline and lipid infusion studies (107 ± 4 versus 108 ± 5 micro-units/ml). After saline, insulin increased the rate of glucose disposal to 8.82 ± 0.69 mg/(kg·min). Lipid infusion decreased the rate of insulin-stimulated glucose disposal to 6.67 ± 0.66 mg/(kg·min); p = 0.005. During the saline study, insulin completely suppressed endogenous glucose production to −0.46 ± 0.17 mg/(kg·min). After the lipid infusion, there was a tendency for reduced suppression of endogenous glucose production (0.19 ± 0.36 mg/(kg·min); p = 0.06 versus saline).

Gene Set Expression Analysis—The present study was un-dertaken in part to test the hypothesis that an experimental increase in plasma lipids decreased the expression of nuclear encoded mitochondrial genes. Accordingly, using gene set analysis, we tested whether sets of such genes were decreased in muscle after the lipid infusion. The gene expression values obtained using the Robust Multi-array Analysis method were analyzed in the gene set analysis as described under “Materials and Methods.” A number of gene sets were significantly (p < 0.05) decreased in expression after the lipid infusion (Table 1). Gene set analysis revealed a significant decrease in the mitochondrial set, which included a set of co-regulated genes involved in oxidative phosphorylation, also yielded significance. In addition, the uncoupling protein gene set was significantly decreased in response to the experimental increase in lipids.

Global gene expression profiling also allowed us to test the hypothesis that the expression of other, perhaps unpredicted genes would be changed by a lipid infusion and the consequence increase in plasma FFA. A number of gene sets significantly increased in expression following the lipid infusion including, among others, the collagen, fibronection, and extracellular matrix gene set (Table II). Also significantly increased were the GLUCCO_HG-U133A set of gluconeogene-sis genes and the F6_BIO_HG-U133A set, which include the fatty acid biosynthesis genes.

Single Gene Expression Analysis—Expression values obtained using the Robust Multi-array Analysis method and analyzed using SAM revealed a number of genes that were significantly increased or decreased. Likewise, the Gene-Spring software also identified a number of differentially expressed genes. Lipid infusion significantly increased the expression of 198 individual genes using SAM and 90 genes with GeneSpring analysis (specific genes differentially expressed are given in supplemental Table I, a and b). Of the 198 genes increased in expression using SAM, 34 were concordant with results from the GeneSpring analysis (Table III). Many of these increasers were genes coding for extracel-lular matrix proteins including collagens, fibronection, lumicain, thrombospondin, and proteoglycans.

Of the genes decreased by lipid infusion, 137 were significantly decreased in expression using GeneSpring, and 97 were significantly decreased in expression using SAM analysis. Only one gene was concordantly decreased according to GeneSpring and SAM analysis (secretory pathway component 2015889). This gene encodes a protein of unknown function and is moderately similar to the VAPI protein, which is an endosomal membrane-associated protein containing a putative Ca²⁺/calmodulin-dependent kinase II phosphorylation site.

Because the gene set and single gene expression analyses identified significant increasers in extracellular matrix proteins, we looked at the microarray expression values of other genes that are related to extracellular matrix turnover and biosynthesis. Lipid increased the expression of lamin β1, proteoglycan 2, annexin A2, pannexin 1, tenascin XB, tissue inhibitor of metalloproteinases 1 (TIMP1), F-spondin 1, thrombospondin 4 (TSP4), and a number of matrix metalloproteinases (MMPs), all with p < 0.05 (Table IV) (without correcting for multiple testing).

Q-RT-PCR—To confirm mRNA expression changes revealed by either gene set or single gene microarray analysis, Q-RT-PCR was performed for a number of selected genes. Because co-regulated sets of nuclear encoded mitochondrial genes were decreased in expression after lipid infusion, we quantified mRNA expression by Q-RT-PCR for PGC-1 and nuclear respiration factor-1 (NRF-1), which are involved in transcriptional regulation of these sets of genes. After lipid infusion, PGC-1 expression was decreased to 0.7 ± 0.1-fold compared with that of saline control (p < 0.05); NRF-1 mRNA was unchanged. In addition, a number of mitochondrial genes were quantified,

Table I

| Gene Sets                                      | p < 0.05 |
|-----------------------------------------------|----------|
| Mitochondr. HG-U133A probes                  |          |
| Uncoupling proteins                          |          |
| Hypothetical proteins                        |          |
| Nuclear receptor                             |          |
| Progesterone                                 |          |
| MAP00550_peptidoglycan_biosynthesis          |          |
| MAP00630_glyoxylate_and_dicarboxylate_metabolism |         |
| MAP00910_nitrogen_metabolism                 |          |
| Extracellular matrix gene set                 |          |
| Cell motility and DNA binding gene set        |          |
including isocitrate dehydrogenase 3 β-subunit (IDH3B), NADH-ubiquinone oxidoreductase 1 a subcomplex 5 (NDUFA5), L-arginine:glycine amidinotransferase (GATM), cytochrome B5 (CYB5), and acyl-CoA dehydrogenase medium chain (ACADM).

The expression of all of the nuclear encoded mitochondrial genes was decreased (Fig. 1a), with significant decreases in mRNA expression for IDH3B (0.6 ± 0.1-fold) and GATM (0.6 ± 0.1-fold), both having a p < 0.05.

Collagen genes, fibronectin 1, and lumican were tested for increased expression by Q-RT-PCR. The mRNA expression levels for these genes (relative to 18S mRNA) were higher after the lipid infusion (Fig. 1b). The greatest increases in mRNA expression were found for Col1a1 and Col3a1, with 23.5 ± 7.3 and 11.4 ± 5.3-fold, respectively. Because the expression of extracellular matrix genes is regulated under many circumstances by CTGF (also called CCN2) (24), we examined the mRNA expression of this gene. Quantification of CTGF mRNA by Q-RT-PCR confirmed that CTGF expression was increased by 3.6 ± 1.2-fold after lipid infusion. CTGF expression itself is regulated by angiotensin II, TGF-β1 (24), and hepatocyte growth factor (HGF) (25). However, the mRNA expression levels of TGF-β1, angiotensinogen, angiotensin receptor 1, renin, angiotensin-converting enzyme, and HGF were unchanged using the microarray data. Because of the importance of TGF-β1 in regulating CTGF expression, we reexamined the expression of TGF-β1 mRNA using Q-RT-PCR and found it to be unchanged. Lipid infusion did not change the expression of 18S (17.56 ± 0.25 versus 17.62 ± 0.23; saline versus lipid).

Immunoblot Analysis and Immunofluorescence Staining—To determine whether changes in mRNA expression were translated into increased protein expression, immunoblot analysis was performed for collagen I α1 and collagen III α1 (Fig. 2, a and b). Lipid infusion increased expression of both collagen and their respective procollagens. Immunoblot analysis revealed that CTGF protein expression increased significantly (Fig. 2c) after lipid infusion. To further confirm the increases in collagen I and III protein expression and to visualize the location of the increased protein, thin sections of muscle biopsies were visualized by immunofluorescence microscopy (Fig. 3). Collagen I and especially collagen III protein was increased by lipid infusion.

**DISCUSSION**

The present study was undertaken in part to test the hypothesis that an experimental increase in plasma lipids decreases the expression of PGC-1 and nuclear encoded mitochondrial genes. The increase in FFA levels was brought about by a triglyceride infusion. As expected, lipid infusion resulted in a 30–40% reduction in insulin-stimulated glucose disposal as compared with a saline control infusion. We found that this experimental increase in plasma FFA also decreased the mRNA expression of a number of nuclear encoded mitochondrial genes. In addition, we found using Q-RT-PCR that mRNA expression of PGC-1, a transcriptional coactivator of nuclear encoded mitochondrial genes, was decreased by the lipid infusion.

In a recent study examining skeletal muscle gene expression differences among insulin-sensitive subjects without a family history of diabetes, insulin-resistant normal glucose tolerant subjects with a family history of type 2 diabetes, and patients with type 2 diabetes, we found decreased expression of a variety of metabolic and nuclear encoded mitochondrial genes involved in electron transport and oxidative phosphorylation (16). Mootha et al. have provided similar data using a microarray approach (17), and Højlund and colleagues found, using proteomics techniques, that insulin resistance is associated with decreased protein expression of the ATP synthase β-subunit (26). From these studies it was hypothesized that decreased PGC-1 expression might be responsible for the decreases in expression of nuclear encoded mitochondrial genes, because PGC-1 serves as a transcriptional co-activator for many of these genes (27). Moreover, in that study we found that PGC-1 expression was inversely correlated with plasma FFA levels (16). This suggested that the decrease in PGC-1 expression might be a consequence of increased plasma FFA concentrations that result from resistance to the antilipolytic effects of insulin in adipocytes (5–7). Results from the present study indicate that the inverse correlation between plasma FFA and PGC-1 may have a causal basis. It can be theorized that the decrease in PGC-1 expression and mitochondrial function observed in skeletal muscle from insulin-resistant subjects may be secondary to increased lipid supply to the muscle. Moreover, because the ability of skeletal muscle from insulin-resistant individuals to oxidize FFA is reduced, these two factors could combine to increase intramyocellular lipids and induce insulin-signaling defects. A similar decrease in the expression of nuclear encoded mitochondrial genes has been described in aging muscle (28). Because we also found significant reductions in a number of individual nuclear encoded mitochondrial genes as well as a coordinate reduction in mitochondrial genes as indicated by the gene set analysis, it can be hypothesized that increased lipid supply to muscle may have adverse effects on mitochondrial function. Importantly, the changes observed in the expression of nuclear encoded mitochondrial genes correlate with functional and morphological changes in the mitochondria in various states of insulin resistance, including aging, type 2 diabetes, obesity, and a family history of type 2 diabetes (29–31).

Global gene expression profiling also allowed us to identify novel targets of increased FFA in skeletal muscle. In this study, the most pronounced and consistent changes in gene expression produced by lipid infusion, regardless of the method used to express the data or statistical analysis, was a coordinated and marked increase in the expression of extracellular matrix-related genes, including collagenas, fibronectin, proteoglycans, laminin, matrix metalloproteinases, tissue inhibitor of metalloproteinases, and members of the thrombospondin family. Such a pattern is characteristic of an inflammatory response. There is an increasing body of evidence to suggest an inflammatory basis for insulin resistance (32, 33). The present results show for the first time that increased plasma FFA results in...
so we can now include increased plasma FFA concentrations as plasma lipids increases CTGF mRNA and protein expression, present study we show that an experimental increase in angiotensin II (acting through the angiotensin receptor 1), CCN family (24). CTGF expression is increased by TGF-

changes in gene expression in skeletal muscle that are consistent with an inflammatory response.

A number of avenues of investigation have led to the notion that such inflammatory responses can be mediated by the protein CTGF (also termed CCN2), a 38-kDa member of the CCN family (24). CTGF expression is increased by TGF-β1, angiotensin II (acting through the angiotensin receptor 1), HGF (25), and high glucose concentrations (24, 34–36). In the present study we show that an experimental increase in plasma lipids increases CTGF mRNA and protein expression, so we can now include increased plasma FFA concentrations as one of the potential regulators of CTGF expression. There is evidence that CTGF mediates fibrotic changes in atherosclerotic plaques (37), mesangial expansion in models of diabetic nephropathy (38, 39), fibrosis induced by cardiac myofibroblasts following myocardial infarction (40), and scleroderma and keloids (41). Of note, CTGF expression is increased in liver from Zucker obese rats in association with lipid abnormalities and fatty liver in this animal model of insulin resistance (36). Moreover, liver biopsies taken from nondiabetic and type 2 diabetic patients with non-alcoholic steatohepatitis have increased CTGF expression that correlates with the degree of

| Gene name | Gene symbol | Chromosome | Fold change |
|-----------|-------------|------------|-------------|
| Matrix metalloproteinase 2 | MMP2 | 16q13-q21 | 1.4 | >1.5 |
| Legumain | LGMN | 14q32.1 | 1.2 | >1.5 |
| Disabled homolog 2, mitogen-responsive phosphoprotein | DAB2 | 5p13 | 1.2 | >1.5 |
| Collagen, type VI, α3 | COL6A3 | 2q37 | 2.7 | >1.5 |
| Lumican | LUM | 12q13.3-q22 | 2.5 | >1.5 |
| Collagen, type III, α1 | COL3A1 | 2q31 | 12.3 | >1.5 |
| OGT-interacting protein, 106 kDa | OIP106 | 3p25.3-p24.1 | 1.1 | |
| Collagen, type I, α1 | COL1A1 | 17q21.3-q22.1 | 9.3 | >1.5 |
| Collagen, type I, α2 | COL1A2 | 7q22.1 | 3.7 | >1.5 |
| Collagen, type I, α2 | COL1A2 | 7q22.1 | 7.1 | >1.5 |
| Fibrillin 1 | FBN1 | 15q21.1 | 1.6 | >1.5 |
| Natural killer cell transcript 4 | NK4 | 16p13.3 | 2.9 | >1.5 |
| Thrombospondin 4 | THBS4 | 5q13 | 2.9 | >1.5 |
| Myosin-binding protein H | MYBPH | 1q32.1 | 1.4 | >1.5 |
| Myosin, heavy polypeptide 8, skeletal muscle | MYH8 | 17p13.1 | 1.7 | >1.5 |
| Complement component 1, s subcomponent | C1S | 12p13 | 1.5 | >1.5 |
| Follistatin-like 1 | FSTL1 | 3q13.33 | 1.7 | >1.5 |
| Fibrinectin 1 | FN1 | 2q34 | 2.0 | >1.5 |
| Collagen, type III, α1 | COL3A1 | 2q31 | 5.6 | >1.5 |
| Collagen, type IV, α1 | COL4A1 | 13q34 | 1.4 | >1.5 |
| Ribosomal protein L27a | RPL27A | 11p15 | 2.1 | >1.5 |
| Fibrinectin 1 | FN1 | 2q34 | 1.6 | >1.5 |
| Collagen, type V, α1 | COL5A1 | 9q34.2-q34.3 | 1.4 | >1.5 |
| Collagen, type V, α1 | COL5A1 | 9q34.2-q34.3 | 1.1 | >1.5 |
| Ribosomal protein S11 | RPS11 | 19q13.3 | 3.5 | >1.5 |
| Collagen, type VI, α1 | COL6A1 | 21q22.3 | 1.9 | >1.5 |
| Islet cell autoantigen 1, 69 kDa | ICA1 | 7p22 | 1.1 | >1.5 |
| Branched chain aminotransferase 1, cytosolic | BCAT1 | 12pter-q12 | 1.1 | >1.5 |
| Collagen, type III, α1 | COL3A1 | 2q31 | 12.6 | >1.5 |
| LOC158288 | NA | 9q34.3 | 1.1 | >1.5 |
| Similar to hypothetical protein FLJ20958 | NA | 9q34.3 | 2.0 | >1.5 |
| Hypothetical protein LOC147343 | LOC147343 | 18q21.1 | 1.1 | >1.5 |
| Chondroitin sulfate proteoglycan 2 (versican) | CSPG2 | 5q14.3 | 1.2 | >1.5 |
| Hypothetical protein FLJ20489 | NA | 3p13 | 1.1 | >1.5 |

*The word “concordant” in the title is defined as having expression levels increased using both SAM and GeneSpring analysis.

**Not available.

| Probe set identification | Gene name | Saline (mean ± S.E.) | Lipid (mean ± S.E.) | p Value |
|--------------------------|-----------|----------------------|---------------------|---------|
| 210427_x_at | Annexin 2A | 9.33 ± 0.17 | 10.15 ± 0.20 | 0.024 |
| 201590_x_at | Annexin 2A | 9.48 ± 0.14 | 10.25 ± 0.23 | 0.029 |
| 213994_x_at | P-spondin 1 | 5.13 ± 0.03 | 5.17 ± 0.03 | 0.016 |
| 211651_s_at | Laminin B1 | 5.53 ± 0.03 | 5.65 ± 0.03 | 0.010 |
| 201505_s_at | Laminin B1 | 5.17 ± 0.02 | 5.24 ± 0.03 | 0.030 |
| 203876_s_at | Matrix metalloproteinase 11 | 5.60 ± 0.04 | 5.69 ± 0.04 | 0.038 |
| 201069_at | Matrix metalloproteinase 2 | 7.21 ± 0.06 | 7.66 ± 0.08 | 0.006 |
| 219909_at | Matrix metalloproteinase 26 | 5.49 ± 0.02 | 5.53 ± 0.01 | 0.029 |
| 204715_at | Pancrexin 1 | 5.61 ± 0.05 | 5.78 ± 0.07 | 0.012 |
| 201654_s_at | Proteoglycan 2 | 5.74 ± 0.04 | 5.80 ± 0.04 | 0.007 |
| 221731_x_at | Proteoglycan 2 | 5.53 ± 0.04 | 5.74 ± 0.08 | 0.022 |
| 204620_x_at | Proteoglycan 2 | 5.58 ± 0.04 | 5.74 ± 0.06 | 0.024 |
| 211571_s_at | Proteoglycan 2 | 5.84 ± 0.03 | 5.90 ± 0.02 | 0.031 |
| 216333_x_at | Tenascin XC | 8.09 ± 0.11 | 8.35 ± 0.13 | 0.023 |
| 213451_x_at | Tenascin XB | 8.17 ± 0.15 | 8.46 ± 0.11 | 0.039 |
| 216654_at | Tenascin XB | 5.88 ± 0.06 | 5.98 ± 0.08 | 0.048 |
| 201666_at | Tissue inhibitor of metalloproteinases 1 | 6.34 ± 0.07 | 6.82 ± 0.21 | 0.019 |
| 204776_at | Thrombospondin 4 | 7.26 ± 0.09 | 8.69 ± 0.28 | 0.005 |

*Significance tested by a Student’s paired two-tailed t test (not corrected for multiple testing).
fibrosis (36). We examined the mRNA expression of TGF-β1, components of the renin-angiotensin system, and HGF in the muscle biopsies to determine whether the increase in muscle CTGF expression was an autocrine response to an increase in expression of any of these factors. Lipid infusion did not alter the expression of any of these genes. Therefore, it is likely that muscle was responding to exogenous factors in response to the experimental increase in plasma lipids. A possible candidate is adipose tissue, which is now known to secrete a wide array of cytokines, including TGF-β and angiotensin (42, 43). Another possibility is that macrophage infiltration of muscle was the source of inflammatory cytokines. It is also possible that FFA itself or some intracellular metabolite such as fatty acyl-CoA, ceramides, or diacylglycerol can increase CTGF expression.

Because of the growing evidence of a relationship between inflammation and insulin resistance, it is tempting to speculate that the increase in extracellular matrix gene expression induced by lipid might be related to the insulin resistance. The relationship between mitochondrial dysfunction and insulin resistance has gained acceptance. The results of a recent study suggest that there is a connection between the extracellular matrix and mitochondrial function (44). A Col6α1−/− mouse is characterized by myopathy with latent mitochondrial dysfunction (44). In vitro, the defects in mitochondria were normalized by plating Col6α1−/− myofibers on culture dishes that had been coated with collagen VI. Although the defects in those animals are more profound than the mitochondrial dysfunction observed in type 2 diabetes (29), our results suggest there may be a previously unappreciated potential connection among lipids, inflammation, the extracellular matrix, mitochondrial function, and insulin resistance.

Several caveats exist for the interpretation of this data. First, the infusion of a triglyceride emulsion increases not only FFA but also glycerol, so the use of saline as a control would not allow an effect of glycerol to be ruled out. However, even when glycerol is used as a control, it is clear that triglyceride infusion produces insulin resistance (4), suggesting that the FFAs are the active components in this process. Second, triglyceride in-

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**FIG. 1.** mRNA expression for selected genes. A and B, Q-RT-PCR analysis of RNA from seven subjects for PGC-1 and a number of mitochondrial genes (A) and for collagens (COL), lumican (LUM), fibronectin (FN1), and CTGF (B). *, p < 0.05 versus control (---).
**FIG. 2.** Protein expression of collagens and CTGF. A–C, immunoblot analysis of skeletal muscle lysates for collagen (Col I at the top, COL1a1 at the bottom) and the procollagen I α1 subunit (ProCol I at the top, ProCOL1a1 at the bottom) (A), collagen (Col III at the top, COL3a1 at the bottom) and the procollagen III α1 subunit (ProCol III at the top, ProCOL3a1 at the bottom) (B), and CTGF (C). Muscle lysates from saline (S) and lipid (L) studies were resolved by SDS-PAGE, and proteins were transferred to nitrocellulose and probed with specific antibodies. Bands were quantified as described under “Materials and Methods.” Lysates are shown for six of seven subjects because of insufficient biopsy material on the seventh subject. *, *p < 0.05 versus saline control.

**FIG. 3.** Immunofluorescence staining of thin sections from vastus lateralis muscle biopsies taken after saline (left) or lipid (right) infusion. Collagen I staining is shown in the top row, and collagen III staining is shown in the bottom row. Details of immunostaining procedure can be found under “Materials and Methods.” Magnification is 40×.
fus results in a modest increase in insulin secretion (45), so it cannot be ruled out totally that some of the effects we observed may have been due to an increase in insulin. Finally, the subjects received more calories during the lipid infusion than during the saline control, and it is conceivable that increased caloric intake may have had effects on gene expression. Additional studies will be required to address these questions.

In summary, the results of the present study suggest that the decrease in expression of PGC-1 and nuclear encoded mitochondrial genes that characterize insulin-resistant skeletal muscle may be secondary, in part, to increased plasma FFA. In addition, increasing plasma lipids produces a robust increase to mitochondrial genes that characterize insulin-resistant skeletal muscle may be secondary, in part, to increased plasma FFA. In addition, increasing plasma lipids produces a robust increase.

Acknowledgments—We thank John Adams, Andrea Barrentine, Kathy Camp, and Sheila Taylor for excellent technical assistance and additional, increasing plasma lipids produces a robust increase to mitochondrial genes that characterize insulin-resistant skeletal muscle may be secondary, in part, to increased plasma FFA. In addition, increasing plasma lipids produces a robust increase.

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