Original Article

Paradoxical Changes in Muscle Gene Expression in Insulin-Resistant Subjects After Sustained Reduction in Plasma Free Fatty Acid Concentration

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Lipid oversupply plays a role in developing insulin resistance in skeletal muscle, decreasing expression of nuclear-encoded mitochondrial genes, and increasing extracellular matrix remodeling. To determine if a decrease in plasma lipid content reverses these abnormalities, insulin-resistant subjects with a family history of type 2 diabetes had euglycemic clamps and muscle biopsies before and after acipimox treatment to suppress free fatty acids. Free fatty acids fell from 0.584 ± 0.041 to 0.252 ± 0.053 mmol/l ($P < 0.001$) and glucose disposal increased from 5.28 ± 0.46 to 6.31 ± 0.55 mg · kg^{-1} · min^{-1} ($P < 0.05$) after acipimox; intramuscular fatty acyl CoA decreased from 10.3 ± 1.9 to 4.54 ± 0.82 pmol/mg muscle ($P < 0.01$). Paradoxically, expression of PGC-1– and nuclear-encoded mitochondrial genes decreased after acipimox, and expression of collagens I and III α-subunits (82- and 21-fold increase, respectively, $P < 0.05$), connective tissue growth factor (2.5-fold increase, $P < 0.001$), and transforming growth factor-β1 increased (2.95-fold increase, $P < 0.05$). Therefore, a reduction in lipid supply does not completely reverse the molecular changes associated with lipid oversupply in muscle. Changes in expression of nuclear-encoded mitochondrial genes do not always correlate with changes in insulin sensitivity. *Diabetes* 56:743–752, 2007

Lipid oversupply leading to tissue lipid accumulation has received significant attention as a potential etiological factor in the development of insulin resistance in skeletal muscle. This excess of lipids either can derive from the diet (1), a decreased ability of muscle to use lipid (2,3), or both. Lipid oversupply leads to an increase in intramyocellular lipid in the form of triglycerides, diacylglycerol, fatty acyl CoA, and ceramides (4–12). The latter three can interfere directly or indirectly with various aspects of insulin signaling (9–19), thus producing insulin resistance. Diacylglycerol and fatty acyl CoAs can activate protein kinase C isoforms that produce insulin resistance by means of serine phosphorylation of insulin receptor substrate 1 (9,14). Ceramides activate phosphatases that inactivate Akt and also may activate protein kinase C (15–18).

Mitochondrial dysfunction, which is thought to be involved in the pathogenesis of insulin resistance (20,21), is characterized by a coordinated pattern of decreased nuclear-encoded mitochondrial gene expression, potentially driven by reduced expression of the transcriptional coactivator PGC-1 (22,23). We observed an inverse correlation between plasma free fatty acids (FFAs) and PGC-1 mRNA (L.J.M., Mary-Elizabeth Patti, unpublished data), suggesting that lipid oversupply itself might result in suppression of nuclear-encoded mitochondrial gene expression and mitochondrial dysfunction (23). Experimental lipid oversupply produced by an infusion of a triglyceride emulsion is well known to produce insulin resistance (24–26) accompanied by decreased insulin signaling and glycogen formation. Therefore, we further showed that lipid oversupply brought about by a lipid infusion also suppresses PGC-1 mRNA and produces a decrease in nuclear-encoded mitochondrial gene expression (27). In light of these data, the first purpose of the present study was to determine whether an experimental decrease in plasma FFAs and tissue lipid content in insulin-resistant subjects would improve PGC-1 and nuclear-encoded mitochondrial gene expression in skeletal muscle.

In our previous study, which was designed to determine whether experimental lipid oversupply alters nuclear-encoded mitochondrial gene expression, we showed that lipid infusion produced profound increases in the mRNA and protein expression of a variety of extracellular matrix proteins (27). Subsequently, we went on to show that collagen expression was increased in skeletal muscle from insulin-resistant obese nondiabetic and type 2 diabetic individuals (28). These changes in gene expression appeared to be driven by an increase in the expression of connective tissue growth factor (CTGF) (CTGF/CCN2), a member of the CCN gene family that mediates fibrotic responses (29). Therefore, the second purpose of this study was to determine whether a decrease in plasma FFAs also induces changes in expression of extracellular matrix genes.

Acipimox, a nicotinic acid analog that reduces plasma FFAs by suppressing lipolysis (30), improves insulin sensitivity (31,32). In the present study, we used acipimox

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CTGF, connective tissue growth factor; FFA, free fatty acid; OGTT, oral glucose tolerance test; TGF, transforming growth factor.

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treatment to produce a sustained decrease in plasma FFAs in insulin-resistant individuals who are genetically predisposed to develop type 2 diabetes. Such individuals, who have two or more primary relatives with type 2 diabetes, are characterized by insulin resistance, increased plasma FFAs, and decreased insulin signaling in skeletal muscle (33,34). We hypothesized that in these insulin-resistant subjects, acipimox would decrease plasma FFAs, concomitantly decrease myocellular fatty acyl CoAs while improving insulin sensitivity, and increase expression of PGC-1–and nuclear-encoded mitochondrial genes. We further hypothesized that reduced FFA levels would result in decreased expression of extracellular matrix genes.

RESEARCH DESIGN AND METHODS

Ten subjects with at least two first-degree relatives with type 2 diabetes took part in the study. Data for in vivo glucose metabolism and plasma FFA and adiponectin concentrations for 8 of the 10 subjects were included in a previous publication (35). None of the subjects had any significant medical problems, and their weight was stable for at least 3 months before the study. No subject was taking any medications known to affect glucose metabolism. None of the subjects participated in any heavy exercise, and they were instructed not to engage in vigorous exercise for at least 3 days before the study. The purpose, nature, and potential risks of the study were explained to all subjects, and written consent was obtained before their participation. The protocol was approved by the Institutional Review Board of the University of Texas Health Science Center at San Antonio.

Study design. Three weeks before study, all subjects met with a dietitian and were instructed to consume a weight-maintaining diet containing 50% carbohydrate, 30% fat (10% saturated fat, 10% polyunsaturated fat, and 10% monounsaturated fat), and 20% protein. During the week before the start of acipimox treatment, all subjects received *1*) a 75-g oral glucose tolerance test (OGTT) and *2*) a euglycemic insulin clamp study (36) in combination with 3-[^{3}H]glucose to examine hepatic and peripheral tissue sensitivity to insulin and with vastus lateralis muscle biopsy for assessment of gene expression changes and fatty acyl CoA concentrations (see below). The euglycemic insulin clamp study was started at 0600 h and the OGTT at 0800 h after a 10- to 12-h overnight fast.

After completion of these studies, subjects were started on acipimox, 250 mg orally every 6 h (0600, 1200, 1800, and 2400 h) for 7 days. The last dose of acipimox was administered at 0600 h on day 8. At 0600 h on day 5, all subjects were admitted to the General Clinical Research Center, where they remained until completion of the study on day 8. While in the General Clinical Research Center, subjects received a weight-maintaining diet of the same composition and were encouraged to ambulate freely. There was no change in body weight in any subject between the pre- and post-acipimox studies. The OGTT was repeated on day 7 and the euglycemic-hyperinsulinemic clamp/vastus lateralis muscle biopsy study was repeated on day 8 after a 10- to 12-h overnight fast. Acipimox (Olbetam, Pharmacia Upjohn, Milan, Italy) was used under investigational new drug no. 60,687 (to R.A.D.).

OGTT. After obtaining baseline blood samples for determination of plasma glucose, adiponectin, and FFAs, subjects underwent a 75-g OGTT.

Hyperinsulinemic-euglycemic clamp. Insulin sensitivity was assessed with a euglycemic insulin clamp, as previously described (36), using an insulin infusion rate of 80 mU $\cdot$ min^{-1} $\cdot$ m^{-2} body surface area, with infusion of 3-[^{3}H]glucose for measuring glucose disposal and endogenous glucose production. A needle biopsy of the vastus lateralis was obtained under local anesthesia in the fasted condition, immediately before the hyperinsulinemic-euglycemic clamp. Thirty minutes after the start of insulin infusion, a second percutaneous muscle biopsy was obtained from the contralateral vastus lateralis muscle. After acipimox treatment, biopsies were performed in contralateral legs at sites several centimeters distant to the pretreatment biopsy sites to avoid any effects of the biopsy procedure itself on the tissue. Continuous indirect calorimetry, using a ventilated hood system (Deltatrac II; Sensor Medics, Yorba Linda, CA), was performed during the last 40 min of the basal period and during the last 30 min of the insulin clamp.

Analytical determinations. Plasma glucose was measured by the glucose oxidase method (Beckman Instruments, Fullerton, CA). Plasma insulin concentration was measured by radioimmunoassay (Diagnostic Products, Los Angeles, CA). Tritiated glucose specific activity was determined on deproteinized plasma samples as previously described (22). Plasma FFA concentration was determined by an enzymatic calorimetric quantification method (Wako Chemicals, Nuess, Germany). Plasma adiponectin concentration was measured by radioimmunoassay (Linco Research, St. Charles, MO).

Fatty acyl CoA analysis. Eight of the 10 volunteers had sufficient biopsy material for assay of long-chain fatty acyl CoA concentrations in addition to mRNA assay. Long-chain acyl CoA standards were purchased from Avanti (Polar lipids; Alabaster, AL). Acetonitrile was from Burdick and Jackson (Muskegon, MI). Other chemicals and solvents were from Sigma (St. Louis, MO).

Sample extraction. Frozen muscle specimens (30–100 mg wet weight) were successively homogenized together with a C12 internal standard in a glass-glass homogenizer using 2 ml of 100 mmol/l KH_2PO_4, pH 4.6, 2 ml of 2-propanol, and 4 ml acetonitrile. Saturated aqueous ammonium sulfate (200 μl) was added and the sample was re-homogenized. The resulting suspension was vortexed for 5 min and then centrifuged for 5 min at 1,900*g*. The supernatant was kept on ice, while the precipitate was re-homogenized using one-half volumes of the above solvents. The two resulting supernatants were combined, and 5% trichloroacetic acid was added to 40 ml. After periodic re-homogenization of the cold extracts for 5 min, the samples were centrifuged for 1 h at 3,000*g* at 4°C. Elution solvent (300 μl; 70% buffer A:30% acetonitrile, see below) was added, and the supernatant was transferred to small tubes. The pH of the resulting extract was between 5 and 6. Samples were injected on the high-performance liquid chromatography (see below) directly. In the present work, aliquots of 100 μl were injected immediately after addition of 2 μl of 1.5 mmol/l EDTA, which reduced and stabilized the baseline.

High-performance liquid chromatography. All procedures were carried out on a Thermo Finnigan Surveyor high-performance liquid chromatography System coupled with a Photodiode Array Detector and controlled by Xcalibur Software 1.3 (Bellefonte, PA). The separation was carried out using a Waters SunFire C18 3.5 μm, 2.1 × 100 mm column (Waters, Milford, MA) and a C18 10-mm precolumn (Waters). Chromatography was performed using a gradient system including two mobile phases: *1*) 10 mmol/l ammonium acetate/10 mmol/l potassium phosphate, pH 5.8, and *2*) 100% acetonitrile. The starting conditions were 70% A:30% B, with a gradient to 60% A:40% B over 5 min; 54% A:46% B at 14 min; 38% A:62% B at 19 min; and finally back down to 70% A:30% B after 30 min. The flow rate was variable, starting at 0.3 ml/min and reaching a flow of 0.5 ml/min at 14 min and 0.6 ml/min at 19 min, where it was sustained until 30 min. Thereafter, it was reduced to 0.3 ml/min. Separation occurred with a column temperature of 40°C. The program was a modification of the procedure original of Corkey et al. (37).

Gene expression analysis. RNA was isolated from muscle biopsies using a commercial kit (RNA STAT-60 Total RNA/mRNA Isolation Reagent; Tel Test, Friendswood, TX) and purified before use (RNeasy Mini kit; Qiagen, Valencia, CA). Quantitative real-time PCR was used to quantify mRNA. The specific genes analyzed and the primers used for this analysis are given in Table 1. A Bio-Rad ICycler (Bio-Rad Laboratories, Hercules, CA) was used to quantify mRNA levels relative to the average value for β-actin, GAPDH, and cyclophilin as controls. Three genes were chosen as controls to minimize variability. Relative changes in mRNA levels were analyzed by the ΔΔCt method (38).

Calculations. Under steady-state postabsorptive conditions, the rate of endogenous glucose appearance is calculated as the [3-^{3}H]glucose infusion rate (dpm/min) divided by the steady-state plasma [3-^{3}H]glucose specific activity (dpm/mg). During the euglycemic insulin clamp, the rate of total-body glucose appearance was calculated using Steele's equation (39), using a distribution volume of 250 ml/kg. Endogenous glucose production was calculated by subtracting the exogenous glucose infusion rate from glucose appearance. The rate of insulin-mediated total-body glucose disposal was determined by adding the rate of residual endogenous glucose production to the exogenous glucose infusion rate.

Statistical analysis. The effect of acipimox on muscle fatty acyl CoA concentrations was assessed by paired *t* test. The effect of acipimox on mRNA expression was expressed as fold change compared with pretreatment values, and statistical significance compared with no change (value of 1.0) was assessed by the *z* test. Correlation analysis was performed by the Pearson product-moment method.

RESULTS

Subject characteristics and OGTT. Subject characteristics are given in Table 2. All subjects had normal glucose tolerance and had at least two primary relatives with type 2 diabetes. Subjects were only mildly overweight, with an average BMI of 26.5 ± 0.8 kg/m^2. After acipimox treatment, the baseline fasting plasma FFA concentration was reduced from 0.584 ± 0.041 to 0.252 ± 0.053 mmol/l ($P <$ 0.01). Fasting plasma insulin, glucose, and adiponectin concentrations did not significantly change after acipimox

TABLE 1
Primers used in quantitative real-time PCR

CTGF	Forward: TAGCCTCAATTTCTGAACAC
	Reverse: TTGACGGACTGTCATTCTAT
CPT1β (64)	Forward: GAGGCCTCAATGACCAGAATG
	Reverse: GTGGACTCGCTGGTACAGGAA
Citrate synthase (64)	Forward: TTGGCTGACCTGATACCTAAGG
	Reverse: CACCATACATCATGTCCACAGTG
FN (65)	Forward: CCTCAATTGTTGTTCGCTGGA
	Reverse: GCTACCTTCTACTGATGGCGAATAG
COLIα1 (65)	Forward: CCTCAAGGGCTCCAACGAG
	Reverse: TCAATCACTGTCTTGCCCCA
COL3α1	Forward: TCTCCTGGAAGATTGGTTTA
	Reverse: CACCATAACATGCGTCTTTA
TGFβ1 (65)	Forward: GCGTGCTAATGGTGGAAACC
	Reverse: CGGAGCTCTGATGTGTTGAAGA
PORIN	Forward: CAGTGCCAAATCAAAGCTGA
	Reverse: CCTGATGTCCAAGCAAGGTT
COX6	Forward: AAGGACGTTGGTGTTGAGGT
	Reverse: TTTCTTTGATCAGCCACACG
COX7	Forward: GTTTGTACTTTGGATCTGCATT
	Reverse: TGGCATATGAGTTCTAGTTTGA
NRF1	Forward: TGACATTGGAACAGTGACAT
	Reverse: AATGCAGTTTCTTCACCAAT
NRF2	Forward: AAATTGAGATTGATGGAACAGAGAA
	Reverse: TATGGCCTGGCTTACACATTCA
MtTFA	Forward: AGATTCCAAGAAGCTAAGGGTGATT
	Reverse: TTTCAGAGTCAGACAGATTTTTCCA
PGC1α	Forward: TTTCCTTTTGCCATGGAATC
	Reverse: GAAAGAACCGCTGAACAAGC
PGC1β	Forward: CTGCATTGATAACAGCCTTA
	Reverse: AAAGGACAGCAGTTTCAAGT
GAPDH	Forward: AACCTGCCAAATATGATGAC
	Reverse: TCATACCAGGAAATGAGCTT
β-ACTIN	Forward: AAACTGGAACGGTGAAGGTG
	Reverse: AGAGAAGTGGGGTGGCTTTT
Cyclophilin	Forward: GCCATGGAGCGCTTTGG
	Reverse: CCACAGTCAGCAATGGTGATC
NADH Fe-S	Forward: TTACTCCAAAGCCTGTTGTT
	Reverse: CCACATAAATCACCATGACA
NADH α1	Forward: AATTGGCGACTTCGATATTA
	Reverse: CAGCACTGAAGTTGTTCAAA
NADH flavor	Forward: TCATGAATAAGCCCTCAGAT
	Reverse: CACAAACACGTCAAAATCAT
NQO1 (64)	Forward: GGAGTAAGAAGGCAGTGCTTTC
	Reverse: GGCCAGAGAATGACATTCATGT
SOD2 (66)	Forward: GCTTGTCCAAATCAGGATCCA
	Reverse: GCGTGCTCCCACACATCA
OGG1 (66)	Forward: ATTCCAAGGTGTGCGACTGCT
	Reverse: GCGGGCGATGTTGTTGTT
GPX1	Forward: AGTTTGGGCATCAGGAGAACG
	Reverse: CAGGAAGGCGAAGAGAGGGT

CTGF, connective tissue growth factor; CPT1B, carnitine palmitoyltransferase 1B; CS, citrate synthase; FN, fibronectin; COLIα1, collagen I α1-subunit; COLIIIα1, collagen III α1; PORIN, porin; COX6, cytochrome C oxidase subunit 6; COX7, cytochrome C oxidase subunit 7; NRF1, nuclear respiratory factor 1; NRF2, nuclear respiratory factor 2; MtTFA, mitochondrial transcription factor A; PGC1α, peroxisome proliferative–activated receptor-γ coactivator 1α; PGC1β, peroxisome proliferative–activated receptor-γ coactivator 1β; GAPDH, glyceraldehyde phosphate dehydrogenase; β-ACTIN, β-actin; NADH Fe-S, NADH dehydrogenase iron-sulfur protein; NADHα1, NADH dehydrogenase α1 subunit; NADH flavo, NADH dehydrogenase flavoprotein subunit 1; NQO1, NAD(P)H quinone oxidoreductase; SOD2, superoxide dismutase 2; OGG1, 8-oxoguanine DNA glycosylase; GPX1, glutathione peroxidase 1. Where noted, primers were used as reported in the literature. Otherwise, primers were custom designed or, in the case of GPX1, courtesy of Dr. A. Civitarese.

TABLE 2
Subject characteristics

	Acipimox treatment	
	Before	After
Age (years)	41 ± 4	—
Sex (F/M)	4/6	—
BMI (kg/m^2)	26.5 ± 0.8	26.4 ± 0.8
Fasting insulin (pmol/l)	38 ± 4	40 ± 6
Fasting glucose (mmol/l)	5.2 ± 0.1	5.2 ± 0.1
Fasting FFAs (mmol/l)	0.584 ± 0.041	0.252 ± 0.053§
Clamp FFAs (mmol/l)	0.156 ± 0.016†	0.125 ± 0.014†§
Fasting adiponectin (μg/ml)	7.4 ± 0.8	7.0 ± 1.0

Data are means ± SE. †$P < 0.01$ basal vs. clamp; §$P < 0.01$ after vs. before acipimox.

treatment (Table 2). During the OGTT, the mean plasma glucose (7.6 ± 0.3 vs. 7.0 ± 0.5, $P < 0.01$) and FFA (0.288 ± 0.031 vs. 0.154 ± 0.02, $P < 0.01$) concentrations were significantly reduced after acipimox treatment. The mean plasma insulin concentration during the OGTT did not change significantly after acipimox treatment (Table 2).

Effects of acipimox on FFA and insulin action in vivo. To determine how acipimox altered insulin action in vivo, euglycemic clamps (80 mU/m^2 per min) with indirect calorimetry were performed before and after 7 days of treatment. These data are given in Table 3. The steady-state plasma glucose concentrations during the 120-min euglycemic insulin clamp were similar before and after acipimox treatment (5.1 ± 0.1 vs. 5.1 ± 0.1 mmol/l). The steady-state plasma insulin concentrations during the insulin clamp studies also were similar before and after acipimox (870 ± 53 vs. 838 ± 65 pmol/l). After acipimox treatment, fasting plasma FFA concentrations (determined before the start of the euglycemic clamp) decreased from 0.584 ± 0.041 to 0.252 ± 0.053 mmol/l ($P < 0.001$ vs. before acipimox). Before acipimox, the plasma FFAs during the euglycemic clamp declined from 0.584 ± 0.041 to 0.156 ± 0.016 mmol/l ($P < 0.001$). After acipimox, FFAs decreased from 0.252 ± 0.053 to 0.125 ± 0.014 mmol/l ($P < 0.01$) during the clamp.

In keeping with the lack of effect of acipimox on fasting plasma glucose, fasting endogenous glucose production was unaffected by acipimox (Table 3). However, whereas before acipimox treatment insulin infusion during the clamp decreased endogenous glucose production to 0.190 ± 0.073 mg · kg^{-1} · min^{-1}, after acipimox insulin suppressed endogenous glucose production to 0.013 ± 0.010 mg · kg^{-1} · min^{-1} ($P < 0.05$ vs. before acipimox). In a similar manner, acipimox increased insulin stimulation of glucose disposal, from 5.28 ± 0.46 mg · kg^{-1} · min^{-1} before to 6.31 ± 0.55 mg · kg^{-1} · min^{-1} ($P < 0.01$) after acipimox. Acipimox increased the rate of systemic glucose oxidation under basal conditions from 1.33 ± 0.08 to 1.52 ± 0.09 mg · kg^{-1} · min^{-1} ($P < 0.05$) and decreased lipid oxidation from 0.77 ± 0.07 to 0.66 ± 0.04 mg · kg^{-1} · min^{-1} ($P < 0.05$). Finally, acipimox treatment had no effect on insulin-stimulated glucose oxidation (2.90 ± 0.18 mg · kg^{-1} · min^{-1} before or 2.87 ± 0.22 mg · kg^{-1} · min^{-1} after acipimox) but increased insulin-stimulated nonoxidative glucose metabolism from 2.51 ± 0.35 to 3.12 ± 0.43 mg · kg^{-1} · min^{-1} ($P < 0.01$).

Fatty acyl CoAs. Under basal conditions, before acipimox treatment, the total fatty acyl CoA concentration in muscle was 10.3 ± 1.9 pmol/mg muscle (wet weight). The

TABLE 3
Effect of acipimox and insulin on glucose metabolism

	Endogenous glucose production	Glucose disposal	Glucose oxidation	Nonoxidative glucose metabolism	Fat oxidation
Pre-acipimox					
Basal	1.94 ± 0.09		1.33 ± 0.08	0.59 ± 0.10	0.77 ± 0.07
Insulin	0.1 ± 0.07	5.28 ± 0.46	2.90 ± 0.18†	2.51 ± 0.35†	0.18 ± 0.05†
Post-acipimox					
Basal	1.97 ± 0.10		1.52 ± 0.09§	0.46 ± 0.09	0.66 ± 0.04§
Insulin	0.01 ± 0.01§	6.31 ± 0.55†‡	2.87 ± 0.22†	3.12‡ ± 0.43†‡	0.18 ± 0.04†

Data are means ± SE. †$P < 0.01$ vs. basal, ‡$P < 0.05$ vs. pre-acipimox, §$P < 0.01$ vs. pre-acipimox.

species of fatty acyl CoAs that could be identified based on coelution with standards included palmitoleoyl, palmitoyl, oleoyl, and stearoyl. Linoleoyl and arachidonoyl CoA were coeluted with these high-performance liquid chromatography conditions and were quantified together. Before acipimox treatment, insulin infusion decreased muscle total fatty acyl CoA concentrations from 10.3 ± 1.9 to 4.06 ± 0.78 pmol/mg ($P < 0.01$). Acipimox treatment itself decreased basal total fatty acyl CoA levels to 4.54 ± 0.82 pmol/mg ($P < 0.01$ vs. pre-acipimox levels). After acipimox, insulin infusion decreased total fatty acyl CoA concentration to 2.76 ± 0.57 pmol/mg ($P < 0.05$ vs. basal post-acipimox). The concentration of the individual fatty acyl CoA species is given in Table 4. Before acipimox, insulin decreased the concentration of all fatty acyl CoA species that were identified; the decreases in palmitoyl, oleoyl, and linoleoyl plus arachidonoyl CoA were significant. Under basal conditions, acipimox significantly decreased palmitoleoyl, palmitoyl, oleoyl, and stearoyl CoA. Under basal conditions, either before or after acipimox treatment, there was no correlation between the plasma FFA concentration and muscle total fatty acyl CoA concentration. However, at the end of 2 h of insulin infusion, plasma FFA and muscle fatty acyl CoA concentrations were positively correlated, with this relationship reaching statistical significance after acipimox treatment ($R = 0.70$, $P < 0.05$, Fig. 1).

Effect of acipimox and insulin on mRNA expression of PGC-1 and nuclear-encoded mitochondrial genes. The mRNA levels for PGC-1α and -β isoforms as well as an array of nuclear-encoded mitochondrial genes were determined using quantitative real-time PCR. The effects of acipimox on basal (no insulin) mRNA levels are shown in Fig. 2. Surprisingly, PGC-1α and NRF-1 mRNA were significantly decreased after acipimox ($P < 0.05$). PGC-1β and mRNA decreased, but this decrease was not statistically significant. Other mRNAs that decreased under basal conditions after acipimox treatment included NRF-2, porin, cytochrome c oxidase subunit 6, mitochondrial transcription factor A (mtTFA), carnitine palmitoyl transferase 1B, NADH dehydrogenase flavoprotein 1, and citrate synthase.

The effects of insulin on expression of nuclear-encoded mitochondrial genes are shown in Fig. 3. Before acipimox treatment, insulin had little effect on mRNA expression for nuclear-encoded mitochondrial genes, except for PGC-1α mRNA, which increased 1.64 ± 0.35 ($P < 0.05$ vs. basal). After acipimox, however, in keeping with improved systemic insulin sensitivity, insulin increased the expression of several genes, including PGC-1α, NRF-1, NRF-2, and porin (all $P < 0.05$, Fig. 3).

Effect of acipimox and insulin on mRNA expression of extracellular matrix genes and genes involved in free radical scavenging. Acipimox treatment had profound effects on the suppression of extracellular matrix genes in skeletal muscle under basal conditions (Fig. 4). Under basal conditions after acipimox, both collagens I and III α-subunits, as well as fibronectin mRNA, increased many fold, with some individuals having increases of over 100-fold in the mRNA for the collagen genes. For example, the range in increase for the collagen 3α1 subunit was 2.21- to 104-fold, and the range for collagen 1α1 was 2.42- to 405-fold. Interestingly, after acipimox treatment, insulin suppressed collagen subunit mRNA (Fig. 4).

Because an increase in mRNA for matrix proteins often reflects the action of transforming growth factor (TGF)-β and CTGF, we also quantified the mRNA for these two genes. CTGF mRNA was increased 2.53 ± 0.47 by acipimox ($P < 0.001$, Fig. 5). Such an extracellular matrix gene, CTGF response often is in response to increase TGF-β signaling. To gain evidence regarding this potential mechanism, TGF-β1 mRNA was quantified. Under basal conditions, TGF-β1 mRNA was also increased 2.95 ± 1.11-fold ($P < 0.05$, Fig. 5) after acipimox treatment. After acipimox,

TABLE 4
Myocellular fatty acyl CoA concentrations

	Palmitoleoyl	Linoleoyl and arachidonyl	Palmitoyl	Oleoyl	Stearoyl	Total
Pre-acipimox						
Basal	0.19 ± 0.10	3.50 ± 1.33	1.89 ± 0.57	3.28 ± 1.18	1.49 ± 0.34	10.3 ± 1.9
Insulin	0.007 ± 0.005*	2.16 ± 0.65	0.57 ± 0.11*	0.69 ± 0.26*	0.64 ± 0.15†	4.06 ± 0.78†
Post-acipimox						
Basal	0.05 ± 0.05	1.50 ± 0.59‡	0.61 ± 0.19‡†	1.33 ± 0.31‡	1.05 ± 0.31	4.54 ± 0.82§
Insulin	0.07 ± 0.07	1.08 ± 0.45	0.44 ± 0.17	0.53 ± 0.17	0.63 ± 0.15	2.76 ± 0.57*

Data are means ± SE in units of picomoles per milligram of muscle wet weight. *$P < 0.05$, †$P < 0.01$ vs. basal, ‡$P < 0.05$ vs. pre-acipimox, §$P < 0.01$ vs. pre-acipimox.

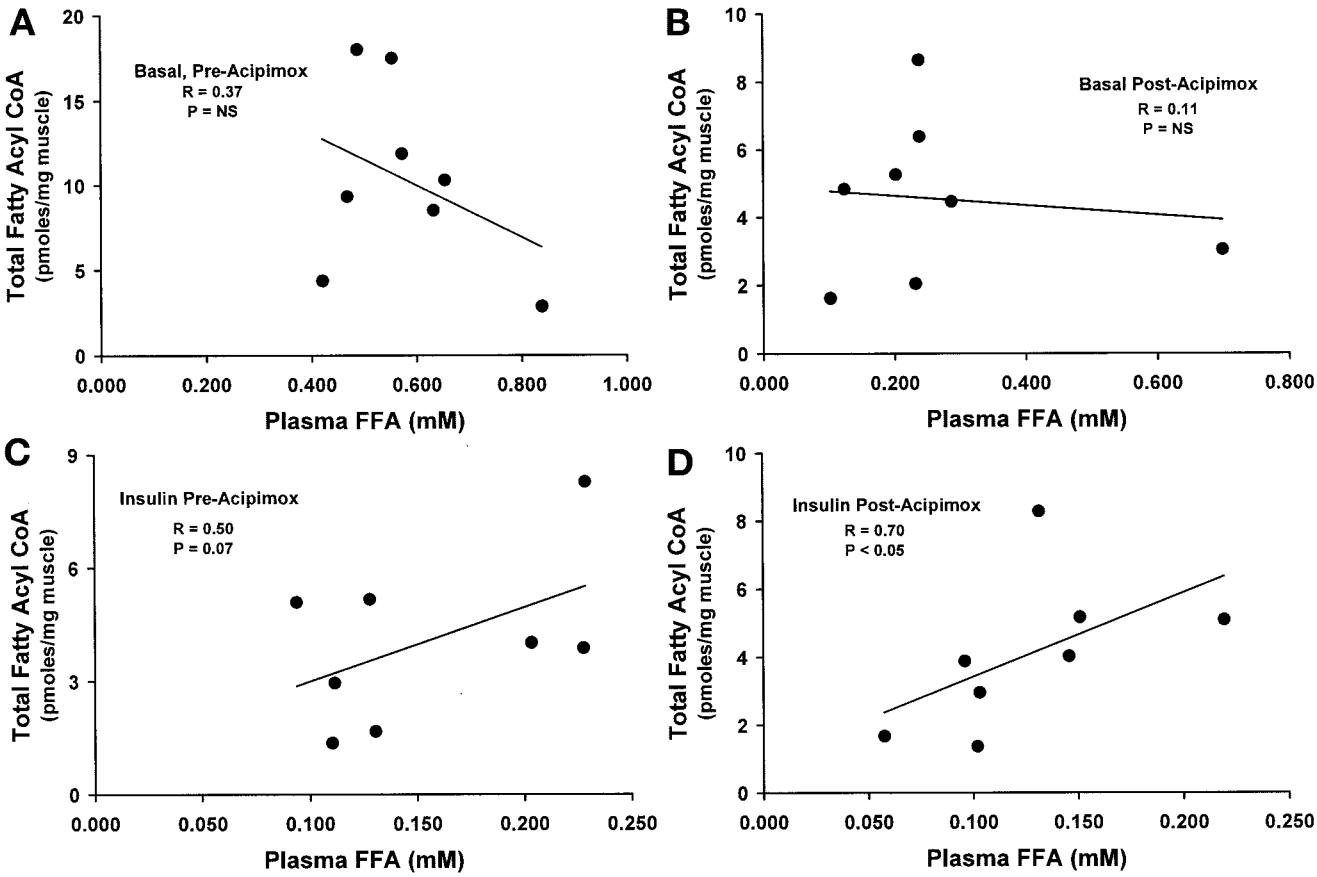

FIG. 1. Relationships between plasma FFA concentrations and muscle total fatty acyl CoA concentrations. Data for both basal and clamp (insulin) data are shown before (*A* and *C*) and after (*B* and *D*) acipimox treatment. Of the 10 subjects studied, 8 had sufficient biopsy material to complete fatty acyl CoA determinations.

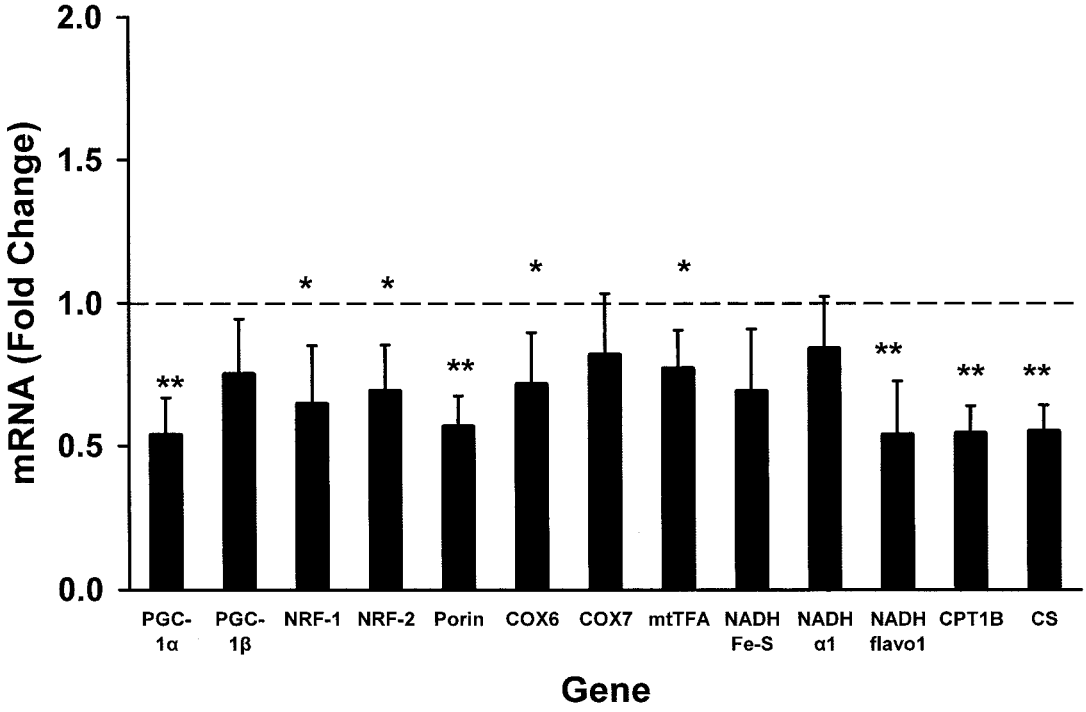

FIG. 2. Effect of acipimox treatment on basal mRNA expression of nuclear-encoded mitochondrial genes. The dashed line indicates no change in gene expression. *$P < 0.05$, **$P < 0.01$ vs. pre-acipimox. For abbreviations, see the Table 1 footnote.

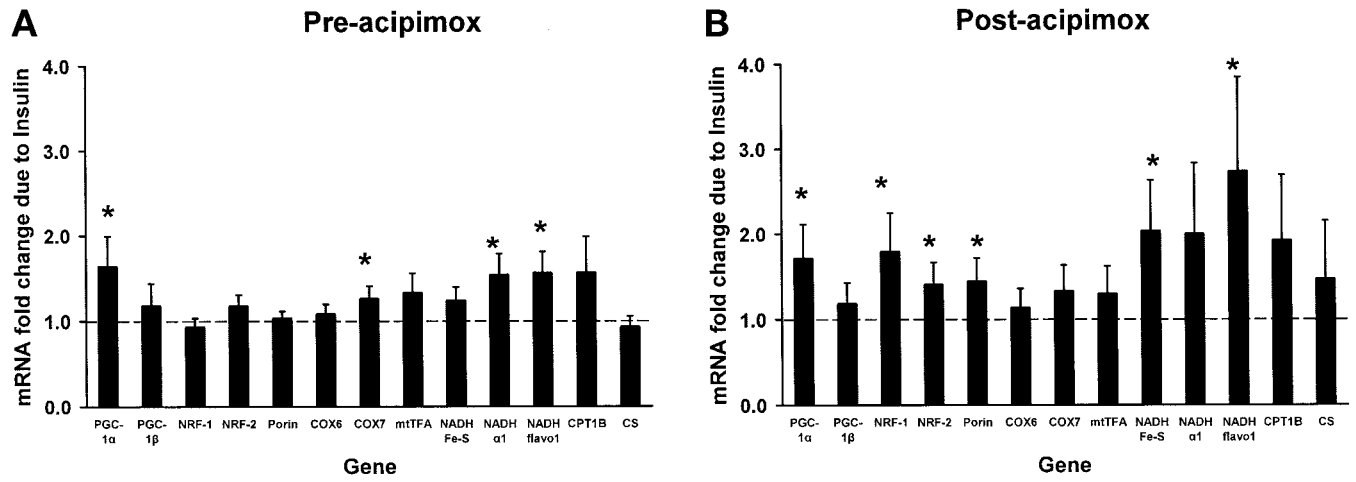

FIG. 3. Effect of insulin on mRNA expression of nuclear-encoded mitochondrial genes before (*A*) and after (*B*) acipimox treatment. The dashed line indicates no change in gene expression. **P* < 0.05, ***P* < 0.01 insulin vs. basal. For abbreviations, see the Table 1 footnote.

insulin significantly decreased the mRNA for CTGF (0.43 ± 0.15-fold insulin vs. basal, $P < 0.001$). Because of the increases in these indicators of an inflammatory response, we also assayed the mRNA expression for superoxide dismutase 2 (SOD2), 8-oxoguanine DNA glycosylase (OGG1), glutathione peroxidase 1 (GPX1), and NAD(P)H ubiquinone oxidoreductase (NQO1, cytoplasmic free radical scavenger). These results are shown in Fig. 6. Of these genes, only NQO1 increased significantly after acipimox. Interestingly, before acipimox, of these genes, insulin increased only NQO1 mRNA ($P < 0.05$), whereas after acipimox, insulin significantly increased not only NQO1, but also the mRNA for SOD2 and GPX1 (both $P < 0.05$).

DISCUSSION

Insulin resistance in skeletal muscle is associated with increased muscle lipid content and can be reproduced by experimental increases in plasma lipids. On a cellular level, insulin resistance is accompanied by increased myocellular fatty acyl CoA levels (31,40) and mitochondrial abnormalities (20,21), while on a molecular level it is characterized by decreased expression of nuclear-encoded mitochondrial genes (22,23) and increased expression of extracellular matrix genes and CTGF (27). The present study was undertaken to determine whether a decrease in plasma FFA concentration and improvement in insulin sensitivity brought about by treating genetically predisposed normal glucose tolerant insulin-resistant subjects with acipimox could reverse the biochemical and molecular abnormalities associated with lipid-induced insulin resistance.

In the present study, acipimox treatment for 7 days improved insulin-stimulated glucose disposal by ~20% in normal glucose tolerant insulin-resistant subjects with a strong family history of type 2 diabetes. This result is similar to that observed in previous studies using acipimox in insulin-resistant subjects (32,35). This improvement in insulin sensitivity was accompanied by a 60% decrease in plasma FFAs under postabsorptive conditions, without changing the fasting plasma insulin concentration. Thus, this pharmacological maneuver successfully brought

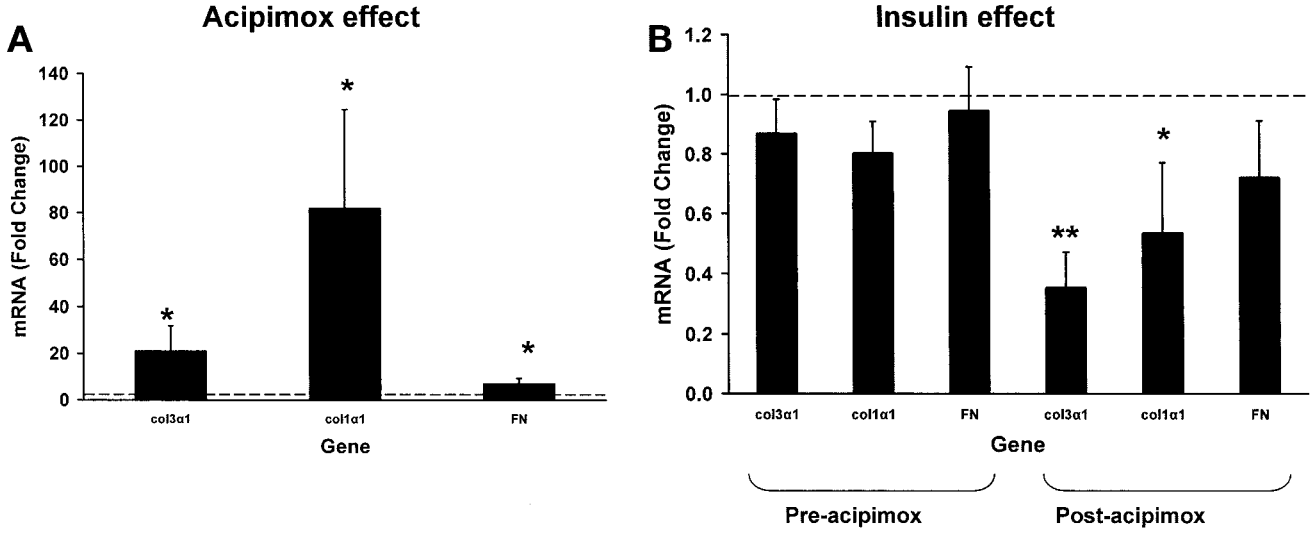

FIG. 4. Effect of acipimox treatment under basal conditions (*A*) or insulin before and after acipimox (*B*) on mRNA expression of extracellular matrix genes. The dashed line indicates no change in gene expression. **P* < 0.05, ***P* < 0.01 vs. pre-acipimox or basal vs. insulin. For abbreviations, see the Table 1 footnote.

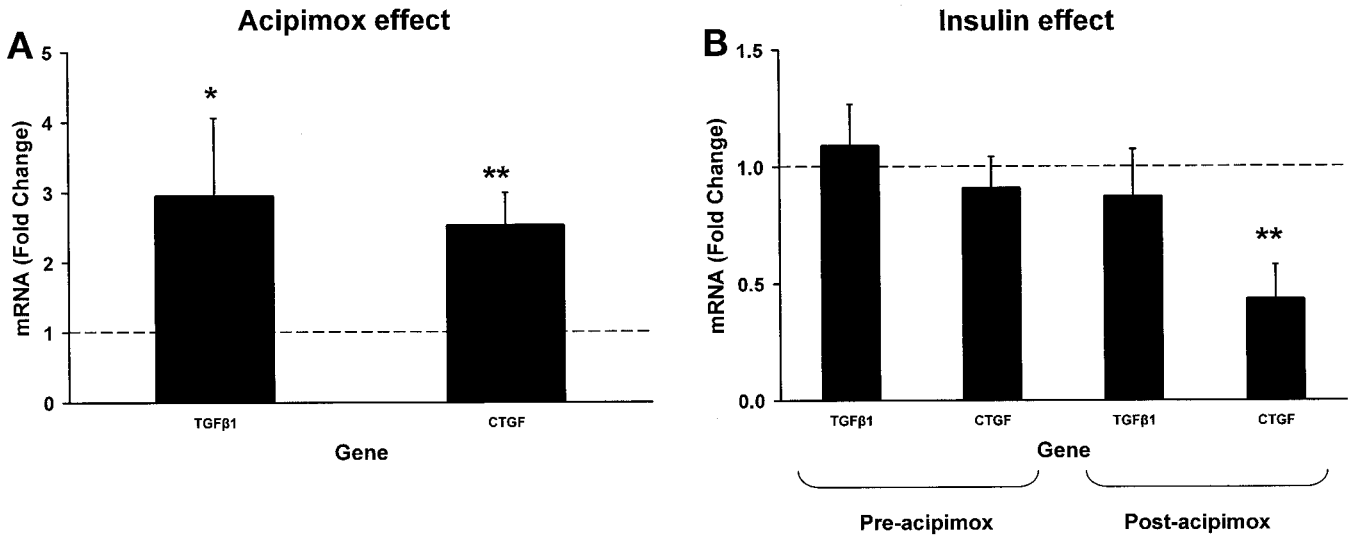

FIG. 5. Effect of acipimox treatment under basal conditions (*A*) or insulin before and after acipimox (*B*) on mRNA expression of CTGF and TGF-β1. The dashed line indicates no change in gene expression. *$P < 0.05$, **$P < 0.01$ vs. pre-acipimox or basal versus insulin. For abbreviations, see the Table 1 footnote.

about the changes required to test the hypotheses proposed in the present study.

One purpose of this study was to determine whether a decrease in plasma FFAs resulted in a decrease in myocellular fatty acyl CoAs. The results of the present study answer this question in the affirmative; after acipimox, total fatty acyl CoA concentrations in skeletal muscle decreased by ~50–60%. The decline in muscle fatty acyl CoAs is similar to what was observed after acipimox treatment of insulin-resistant patients with type 2 diabetes (31), who also had improved insulin sensitivity. This decline also is consistent with the hypothesis that increased levels of fatty acyl CoAs are linked with or causally related to insulin resistance. The decrease in fatty acyl CoA was not limited to any particular fatty acid chain length, since similar decreases were seen with all the species that could be detected using this method.

The results of this study also provide insight into the potential mechanism regulating myocellular fatty acyl CoA levels. Insulin infusion, before acipimox treatment, suppressed plasma FFA concentrations by ~75% and muscle fatty acyl CoA levels by 60%. Acipimox treatment by itself also decreased the postabsorptive levels of both plasma FFAs and fatty acyl CoA to a similar extent. These data suggest that fatty acyl CoA levels in muscle may closely track plasma FFA availability. Supporting this was the decreased effect of insulin, after acipimox, on already suppressed plasma FFA and muscle fatty acyl CoA concentrations. Despite this, there was no significant correlation between plasma FFA and muscle fatty acyl CoA content under basal conditions before or after acipimox treatment. However, under conditions where insulin further suppressed lipolysis after treatment with acipimox (in other words, during conditions of minimum plasma FFA availability), fatty acyl CoA concentrations in muscle were significantly correlated with plasma FFAs. Therefore, although the present data do not address abnormalities in fatty acid oxidation in insulin resistance, they emphasize

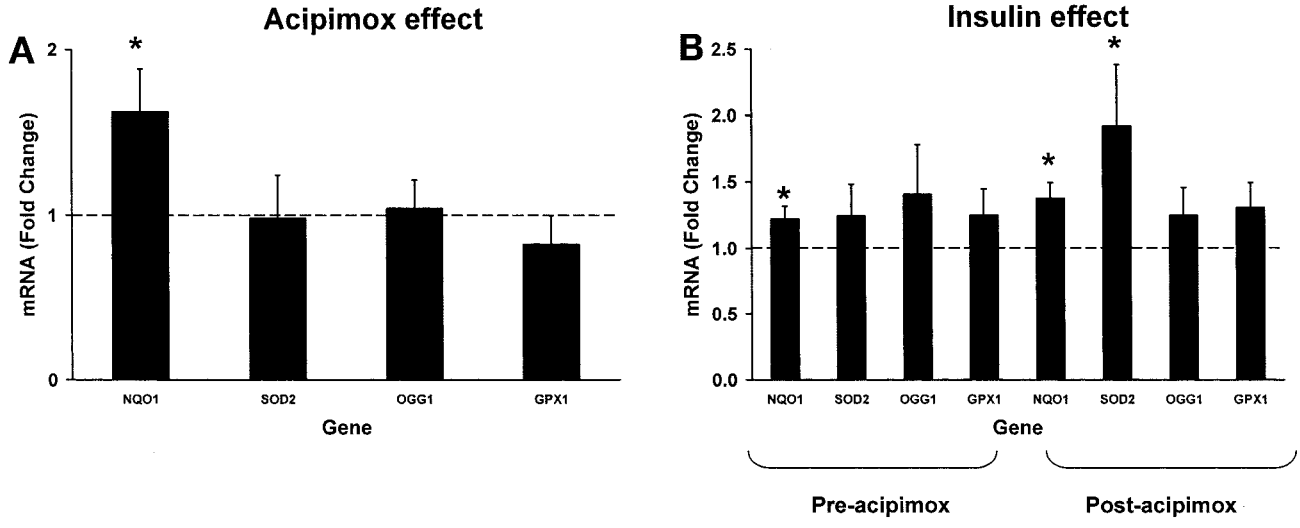

FIG. 6. Effect of acipimox treatment under basal conditions (*A*) or insulin before and after acipimox (*B*) on mRNA expression of genes involved in free radical scavenging or DNA repair. The dashed line indicates no change in gene expression. *$P < 0.05$ vs. pre-acipimox or basal vs. insulin. For abbreviations, see the Table 1 footnote.

that myocellular fatty acyl CoA concentrations track plasma FFA availability when plasma FFAs are maximally suppressed. Therefore, if increased myocellular fatty acyl CoA plays a causal role in the pathogenesis of insulin resistance, it follows that simply decreasing the plasma FFA concentration could be a viable means of improving insulin sensitivity.

In undertaking a previous experiment (23), we found that plasma FFA concentrations were inversely correlated with the mRNA expression of PGC-1α (L.J.M., Mary-Elizabeth Patti, unpublished data), and the decrease in PGC-1α mRNA was accompanied by decreased mRNA expression of a number of nuclear-encoded mitochondrial genes involved in electron transport and oxidative phosphorylation (22,23). Subsequently, we showed that increased plasma FFAs and induced experimental insulin resistance drove down the expression of PGC-1α and nuclear-encoded mitochondrial genes (27). Thus, we expected that in the current experiment, in which we improved insulin sensitivity by decreasing plasma FFAs, the mRNA expression of PGC-1 and nuclear-encoded mitochondrial genes would increase. In general, we found the opposite. The effect of acipimox treatment on PGC-1 and nuclear-encoded mitochondrial gene expression was clear; 9 of 13 mRNAs that were quantified decreased significantly in muscle obtained under postabsorptive conditions after acipimox. The decrease in citrate synthase provides at least indirect evidence against the notion that an increase in mitochondrial number was associated with an improvement in insulin sensitivity. It is possible that this pattern of decrease in mRNA expression is not reflected in protein abundance or activity, but unfortunately, there was not sufficient muscle biopsy material remaining after quantification of fatty acyl CoA concentration and the mRNA expression of multiple genes to perform determinations of protein concentration. Further studies integrating measurements of protein abundance and respiratory chain complex activities will be required to completely understand these events.

The present results with respect to the decrease in PGC-1 expression also differ from those in an earlier study by another group (41). In that study, lipolysis was decreased acutely in healthy subjects using nicotinic acid, and muscle PGC-1α mRNA increased acutely (within hours). Differences in study design make it difficult to compare those results with findings from the current study. The present study was undertaken to improve insulin sensitivity in insulin-resistant subjects by suppressing plasma FFA concentrations to test whether such a change was accompanied by increased PGC-1α expression. To do this required a longer-term suppression of FFAs than was used in the acute study of Watt et al. (41). Also, healthy subjects may respond differently than insulin-resistant subjects with a family history of type 2 diabetes. Regardless, it is conceivable that suppression of plasma FFAs in the present study may have led to an acute increase in PGC-1 expression that in fact led to increased PGC-1 protein and perhaps improved mitochondrial function, but a subsequent decrease in expression of PGC-1 and nuclear-encoded mitochondrial genes may have occurred as a compensatory homeostatic effort. Only detailed time course experiments can dissect these possibilities.

In an earlier experiment in which lipid was infused to create insulin resistance, we observed marked increases in mRNA and protein expression of extracellular matrix genes (27). Based on those results, we predicted that decreasing plasma FFAs would decrease the expression of extracellular matrix genes. Again, this prediction was not upheld by experimental evidence. Rather than decreasing the expression of these genes, acipimox treatment, which reduced plasma FFA and myocellular fatty acyl CoA concentrations, dramatically increased extracellular matrix gene expression. Also increased were TGF-β1 and CTGF mRNA, and these two factors often initiate a remodeling of the extracellular matrix. Taken together with our earlier results, the present data suggest that changes in insulin sensitivity in either direction induced by changes in plasma FFAs are accompanied by a remodeling of the extracellular matrix. Matrix turnover, with release or sequestering of growth factors such as TGF-β (42,43), as well as changes in cell-matrix interactions and reorganization of the cytoskeleton (44–46), can have profound effects on the behavior of cells. These findings suggest that insulin action at the cellular or molecular level may be connected to the composition and turnover of the extracellular matrix. It must be noted that many factors, in addition to TGF-β, can influence CTGF expression and its subsequent effects on extracellular matrix gene expression. CTGF expression is increased by high glucose (47,48), as well as being elevated in fatty liver disease (49,50), and atherosclerotic plaques (51). A possible link between fatty acid metabolism and CTGF expression is indicated by the observation that sphingosine 1-phosphate, signaling through sphingosine 1-phosphate receptors, directly increases CTGF mRNA in mesangial cells (52), smooth muscle (53), fibroblasts (54), and endothelial cells (55). Another possibility is that extracellular fatty acids are signaling directly through cell surface receptors, such as the toll-like receptors or others, which are present in skeletal muscle (56,57). Toll-like receptors, part of the innate immune system, engage saturated and polyunsaturated fatty acid ligands (58,59), and there is evidence that saturated and polyunsaturated fatty acids signal through toll-like receptor 4 (TLR4) to produce opposing effects (60). It should be recognized that, in the present study and the previous study infusing lipid (27), the composition of individual FFA species in the plasma have changed as dramatically as the total concentration. It is possible that engagement of different combinations of toll-like receptors by a different array of fatty acid ligands might produce similar effects on the extracellular matrix even though total FFA concentrations diverge. Intriguingly, in one study (61), lipopolysaccharide and polyunsaturated fatty acids, signaling through TLR4, activated nuclear factor κB and increased collagen production.

Despite the similarities between the results of our two studies, there were a number of differences in experimental design that lead to the opposite changes in insulin sensitivity. In the study by Richardson et al. (27), lipid was infused into healthy subjects for 48 h, increasing not only plasma FFAs, but also plasma triglyceride levels. In the present study, 7 days of acipimox treatment only altered plasma FFA concentrations, and the study was performed in insulin-resistant subjects with a strong family history of type 2 diabetes. In addition, with the design of the current study, a direct effect of acipimox cannot be ruled out. Regardless of these differences, the present study shows that improved insulin sensitivity can be dissociated, at least temporally, from an increase in mRNA expression of PGC-1 and other nuclear-encoded mitochondrial genes. Moreover, both increased and

decreased insulin sensitivity were associated with profound increases in expression of extracellular matrix genes and CTGF, indicating changes in the immediate environment of the cell, that is, the extracellular matrix, with which the cell constantly interacts.

Irrespective of their origin, since these changes in gene expression may have represented an inflammatory response, we also determined the mRNA expression for four genes encoding proteins involved in free radical scavenging or DNA repair (SOD2, OGG1, GPX1, and NQO1). Of these, mRNA expression for the cytoplasmic free radical scavenger NQO1 [NAD(P)H ubiquinone oxidoreductase 1] was increased by 50% after acipimox. If the protein and activity for this gene are also increased, this would be consistent with the inflammatory-like response in the muscle observed after 7 days of acipimox treatment.

Although our observations link changes in expression of genes encoding mitochondrial and extracellular matrix proteins, the present data cannot directly address the mechanisms connecting these phenomena. However, the results of some other recent studies may provide insight into this. First, a connection between the composition of the extracellular matrix and mitochondrial function was provided by data from a collagen 6–null mouse that is a model of Bethlem muscular dystrophy (62). The muscular dystrophy in the mouse is due to a severe disruption of mitochondrial function that can be restored in Col 6–null myoblasts by culturing the cells on a matrix containing collagen 6 (62). Second, a more recent study from van Waveren et al. (63) provided evidence, using cultured cells in which oxidative phosphorylation was inhibited chemically or by genetic manipulation, that dysfunction in oxidative phosphorylation increases CTGF expression and extracellular matrix turnover on a transcriptional level. This opens the possibility that changes in oxidative phosphorylation can affect extracellular matrix composition directly.

In summary, the present results confirm that a reduction in myocellular fatty acyl CoA concentrations is elicited by a decrease in lipolysis and plasma FFAs and that this is accompanied by improved insulin sensitivity. However, the changes in expression of nuclear-encoded mitochondrial and extracellular matrix genes are paradoxical when viewed in the context that of earlier findings. These seemingly paradoxical results will require further study to define the role of changes in nuclear mitochondrial gene expression in the setting of changing insulin sensitivity. The profound changes in expression of extracellular matrix gene expression associated with changes, either up or down, in insulin sensitivity provide new evidence in favor of a role for remodeling of the extracellular matrix in insulin sensitivity in skeletal muscle, although the mechanism responsible for such a role is not known.

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