Exercise training and calorie restriction increase SREBP-1 expression and intramuscular triglyceride in skeletal muscle

Kristen J. Nadeau, Lindsay B. Ehlers, Lina E. Aguirre, Russell L. Moore, Korinne N. Jew, Heidi K. Ortmeier, Barbara C. Hansen, Jane E. B. Reusch, and Boris Draznin

Division of Pediatric Endocrinology, Department of Pediatrics, University of Colorado Health Sciences Center; Division of Endocrinology, Metabolism and Diabetes, Department of Medicine, University of Colorado Health Sciences Center, Denver; Department of Integrative Physiology, University of Colorado, Boulder, Colorado; Geriatric Research, Education and Clinical Center, Baltimore Veterans Affairs Health Care Center; Departments of Medicine and Physiology, University of Maryland School of Medicine, Baltimore, Maryland; Departments of Internal Medicine and Pediatrics, University of South Florida, College of Medicine, Tampa, Florida; and Veterans Affairs Research Service, Denver Veterans Affairs Medical Center, Denver, Colorado

Submitted 9 November 2005; accepted in final form 26 January 2006

Nadeau, Kristen J., Lindsay B. Ehlers, Lina E. Aguirre, Russell L. Moore, Korinne N. Jew, Heidi K. Ortmeier, Barbara C. Hansen, Jane E. B. Reusch, and Boris Draznin. Exercise training and calorie restriction increase SREBP-1 expression and intramuscular triglyceride in skeletal muscle. Am J Physiol Endocrinol Metab 291: E90–E98, 2006. First published January 31, 2006; doi:10.1152/ajpendo.00543.2005.—Intramuscular triglyceride (IMTG) deposition in skeletal muscle is associated with obesity and type 2 diabetes (T2DM) and is thought to be related to insulin resistance (IR). Curiously, despite enhanced skeletal muscle insulin sensitivity, highly trained athletes and calorie-restricted (CR) monkeys have increased IMTG. Sterol regulatory element-binding proteins (SREBPs) are transcription factors that regulate the biosynthesis of cholesterol and fatty acids. SREBP-1 is increased by insulin in skeletal muscle in vitro and in skeletal muscle of IR subjects, but SREBP-1 expression has not been examined in exercise training or calorie restriction. We examined the relationship between IMTG and SREBP-1 expression in animal models of exercise and calorie restriction. Gastrocnemius and soleus muscle biopsies were obtained from 38 Sprague-Dawley rats (18 control and 20 exercise trained). Triglyceride content was higher in the gastrocnemius and soleus muscles of the trained rats. SREBP-1c mRNA, SREBP-1 precursor and mature proteins, and fatty acid synthase (FAS) protein were increased with exercise training. Monkeys (Macaca mulatta) were CR for a mean of 10.4 years, preventing weight gain and IR. Vastus lateralis muscle was obtained from 12 CR monkeys. In addition, phosphorylation of ERK1/ERK2 was increased in skeletal muscle of CR animals. In summary, SREBP-1 protein and SREBP-1c mRNA are increased in interventions that increase IMTG despite enhanced insulin sensitivity. CR and exercise-induced augmentation of SREBP-1 expression may be responsible for the increased IMTG seen in skeletal muscle of highly conditioned athletes.

There is strong evidence that increased levels of intramuscular triglyceride (IMTG) are associated with obesity and type 2 diabetes (T2DM) and are thought to be related to insulin resistance (2, 18, 22, 38, 39, 48, 50, 52, 63, 67). Some evidence indicates that increased IMTG in itself contributes to insulin resistance via damaging effects of fatty acyl-CoA, ceramides, or diacylglycerols on the insulin-signaling cascade (13, 29, 40, 57, 72). Similarly, most interventions that improve insulin sensitivity decrease IMTG (23, 31, 54, 65–67, 71).

Curiously, it is also well supported that endurance-trained athletes have increased IMTG despite enhanced skeletal muscle insulin sensitivity (21, 27, 33, 43). The exercise training effect is not limited to elite athletes. Even moderate exercise training (1 h of cycle exercise at 45% of VO2max) in previously sedentary, elderly subjects increases IMTG (65). At the same time, exercise also improves generalized insulin sensitivity and normalizes insulin levels (6, 15, 16). Thus hyperinsulinemia is not the cause of increased IMTG with exercise. The paradox of increased IMTG with exercise training also suggests that the IMTGs themselves may not be directly causative of insulin resistance.

The mechanism controlling increased IMTG in insulin resistance and in exercise training has not been determined. One possibility is through upregulation of the lipid synthesis pathway in muscle. Sterol regulatory element-binding proteins (SREBPs) are a group of membrane-bound transcription factors that regulate expression of the genes involved in the production and uptake of cholesterol, fatty acids, triglycerides, phospholipids and the low-density lipoprotein (LDL) receptor (28). There are three isoforms of SREBP designated SREBP-1a, SREBP-1c, and SREBP-2. Whereas SREBP-1a can stimulate all SREBP-responsive genes, SREBP-1c preferentially activates fatty acid synthetic genes, and SREBP-2 activates mainly cholesterol synthesis genes (11). SREBPs are made as precursor proteins, requiring cleavage of the NH2-terminal domain, before the mature form can reach the nucleus and operate as a transcription factor (28).

Expression of SREBP-1 is enhanced by insulin in three major insulin target tissues: liver, fat, and skeletal muscle (20, 24, 34, 45, 59), and the levels of SREBP-1 are increased in the presence of hyperinsulinemia (7, 30, 60). Furthermore, we and others have previously shown that SREBP-1 is increased by insulin in skeletal muscle in vitro (45) and in skeletal muscle of insulin-resistant animals (46) via the MAP kinase signaling pathway (36, 45). In skeletal muscle, the MAP kinase pathway...
remains intact in the setting of insulin resistance (8, 37), and hyperinsulinemia increases SREBP-1 despite defects in the muscle phosphatidylinositol 3-kinase pathway of insulin signaling (2, 8, 14, 19, 32, 35). Thus activation of SREBPs may be involved in the increased IMTG of insulin resistance, possibly through excess signaling by the hyperinsulinemia of this disease state. SREBP-1 expression has not been examined in the setting of exercise training.

Fasting and refeeding are also known to impact SREBP-1 expression. In muscle tissue, SREBP-1c mRNA is decreased in soleus, gastrocnemius, and white quadriceps muscle after short-term fasting (6–24 h) (5). In addition, after a 48-h fast, SREBP-1c mRNA is increased with refeeding in the same muscle groups (5), and after an 18-h fast, refeeding increases gastrocnemius SREBP-1c mRNA and SREBP-1 precursor and mature protein (12). However, long-term calorie restriction may have different effects on SREBP-1 expression than a short-term fast. Interestingly, Ortmeyer et al. (47) recently showed that monkeys subjected to long-term calorie restriction, like trained athletes, have increased IMTG. The relationship between SREBP-1 and IMTG with long-term calorie restriction, which, like exercise training, also increases insulin sensitivity, has not been examined.

We hypothesized that SREBP-1 expression would be enhanced in interventions that increase IMTG, even without hyperinsulinemia. To test this hypothesis, we examined models of exercise training and chronic calorie restriction. First, we measured SREBP-1 expression in skeletal muscle from Sprague-Dawley rats after a 20-wk exercise intervention and then in skeletal muscle from rhesus macaques after long-term (10.4 yr on average) calorie restriction.

MATERIALS AND METHODS

Materials

The spectrophotometer used was the DU 640 by Beckman (Fullerton, CA). The densitometer was the Flour-S MultiImager from Bio-Rad (Hercules, CA), with the Quantity One version 4.1.1 software being used. Also from Bio-Rad were the 12% Tris/HCl gel, the Kaleidoscope Prestained Standards, the polyvinylidene (PVDF) membranes, the iCycler system for quantitative real-time RT-PCR (qrt-RT-PCR), the iScript cDNA Synthesis kit, the 1X SYBR Green RT-PCR supermix, 96-well PCR plates, and optical sealing tape. The MagicMark Western Standard was from Invitrogen (Carlsbad, CA). The DNase/RNase free water and phospho-buffered saline were from GIBCO-BRL (Grand Island, NY). The tissue homogenizer (Tissuezumizer), Wheaton Tenbroeck tissue grinders, shell vials, and 96-well plates, the iCycler system for quantitative real-time RT-PCR (qRT-PCR), the iScript cDNA Synthesis kit, the 1X SYBR Green RT-PCR supermix, 96-well PCR plates, and optical sealing tape. The MagicMark Western Standard was from Invitrogen (Carlsbad, CA). The DNase/RNase free water and phospho-buffered saline were from GIBCO-BRL (Grand Island, NY). The tissue homogenizer (Tissuezumizer), Wheaton Tenbroeck tissue grinders, shell vials, and 96-well tissue culture plates were from Fisher (Pittsburgh, PA), as were the chloroform, ethanol, isopropanol, formamide, and formaldehyde. The N-Evap 111 was from Organon (Berlin, MA). The Kinetic Microplate Reader was from Molecular Devices (Sunnyvale, CA), and the SPF 3.1.2 SOFTmax Pro software was used.

Reagents from Qiagen included the RNeasy minikit, DNase set (RNase free) and proteinase K. The amplification grade DNase I, and 0.24– to 9.5-kb RNA ladder were from Invitrogen (Carlsbad, CA). The SREBP-1 monoclonal IgG antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), and the phospho-p44/42 MAP kinase (Thr202/Tyr204) polyclonal antibody was from Cell Signaling (Beverly, MA). The anti-α-actin monoclonal antibody was from Sigma (St. Louis, MO), and the purified mouse anti-fatty acid synthase (FAS) monoclonal antibody was from BD Transduction Laboratories (San Jose, CA). The enhanced chemiluminescence (ECL) Western Blotting Analysis System was from Amersham Biosciences (Piscataway, NJ). The mammalian lysis buffer was from Pierce (Rockford, IL). The Triglyceride Reagent, Free Glycerol Reagent, and all other reagents and chemicals were from Sigma.

Rat SREBP-1c and 36β (acidic ribosomal phosphoprotein P0) primers were provided by Dr. Victor Sorribas (University of Zaragoza, Zaragoza, Spain), who selected, designed, and tested all primers using Beacon Designer IDT technology (Coralville, IA). Primer sequences were forward rat SREBP-1c forward primer (5’T-GACA-CCTGGGAAGATCTACGT-3’), rat SREBP-1c reverse primer (5’T-GCCGGGCACTTACGAA-3’), rat 36β forward primer (5’T-ACCTTCCCCACTGGCTGAA-3’), and rat 36β reverse primer (5’T-TCCTCCGACTCTCCTTTGC-3’). Primers were produced by Integrated DNA Technologies (Coralville, IA).

Methods

Exercise training. A 20-wk involuntary exercise protocol in Sprague-Dawley rats was used as a model of long-term vigorous exercise, as previously described (9). Briefly, female Sprague-Dawley rats aged 3–4 mo were randomly assigned to either a sedentary (n = 18) or a running group (n = 20). All rats were housed in the same facility with a 12:12-h light-dark cycle and fed standard rodent chow (Teklad 8640 rodent diet by Harlan) and water ad libitum. Rats in the trained group underwent 20 wk of forced treadmill running. During the first 8 wk, daily running speed was gradually increased from 20 to 35 m/min, and running time was gradually increased from 20 min at the end of week 1 to 1 h at the end of week 8. From 8 wk until time of death, the training protocol was 1 h/day, 5 days/wk of treadmill running up a 10% grade at 20 m/min for 15 min, 28 m/min for 30 min, and 35 m/min for 15 min. This training protocol is the standard used in our laboratory and results in a significant increase in skeletal muscle citrate synthase activity (25–40%) (61), a classic marker of exercise training (9, 10).

This study was conducted under guidelines accepted by the American Physiological Society and received prior approval from the Institutional Animal Care and Use Committee at the University of Colorado’s Boulder campus.

Rat characteristics after training are listed in Table 1. There were no significant differences between the sedentary or trained groups regarding weight or tibial length at the start or finish of the study. Average age at the time of death was similar in both groups (~8 mo). Rats were killed in the morning in a nonfasted state, 24 h after the last exercise bout. Gastrocnemius and soleus muscle samples were obtained and rapidly frozen in liquid nitrogen.

Calorie restriction. Male rhesus monkeys (Macaca mulatta) underwent caloric restriction as previously described (25). Briefly, the monkeys were individually housed and maintained in accordance with National Academy of Sciences guidelines for care and use of laboratory animals. After they reached mature adult stature, monkeys were placed on a protocol to stabilize body weight by weekly caloric adjustment. The calorie-restricted monkeys were given Purina monkey chow in restricted amounts to keep body weight at ~10–11 kg for a mean of 10.4 yr (8–13 yr). Thus weight gain was prevented by this caloric restriction, protecting against middle-age onset obesity, which otherwise occurs in this species (25). The daily caloric load required to maintain young adult weight was 40% less than that ingested by ad

Table 1. Characteristics of Sprague-Dawley rats

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Sedentary Value</th>
<th>Exercise Trained Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>283 ± 4</td>
<td>275 ± 8</td>
<td>0.381</td>
</tr>
<tr>
<td>Tibial length, cm</td>
<td>38.0 ± 0.2</td>
<td>37.7 ± 0.5</td>
<td>0.571</td>
</tr>
<tr>
<td>Age, days</td>
<td>243.9 ± 9.1</td>
<td>233.4 ± 5.1</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SE. NS, not significant. Rat characteristics are listed after 20 wk of exercise training, as described in MATERIALS AND METHODS, compared with sedentary control rats of comparable age.
libitum-fed monkeys. Calorie-restricted monkeys remained normoglycemic (fasting plasma glucose 69 ± 3 mg/dl) and normoinsulinemic (fasting plasma insulin 51 ± 12 μU/ml). This is in sharp contrast with the ad libitum-fed monkeys that gained weight, became hyperinsulinemic, and frequently became hyperglycemic. Complete characteristics of all groups of monkeys have been published previously (62).

Euglycemic hyperinsulinemic clamp studies were performed after an overnight fast, as previously described in 12 monkeys (6 calorie restricted and 6 controls) (62). Insulin-stimulated glucose disposal rates were not significantly different in calorie-restricted vs. control monkeys and were significantly higher than in obese or obese diabetic monkeys (62). Biopsies of the vastus lateralis muscle were taken both before and after insulin administration. Muscle samples were rapidly frozen, lyophilized, and stored in liquid nitrogen.

Preparation of total cellular extracts. One-hundred milligrams of muscle tissue were ground in a liquid nitrogen-cooled mortar and pestle with mammalian lysis buffer. The slurry was then homogenized on ice with a Tissuemiser at 30,000 rpm followed by centrifugation at 14,000 rpm for 10 min, and the supernatant was retained. Protein concentration was quantified using a bichinchoninic acid standard curve on a DU 640 spectrophotometer.

Western blotting. The protein lysates were boiled for 10 min, and 25 μg of protein per lane dissolved in Laemmli buffer were resolved by SDS-polyacrylamide gel electrophoresis (12% Tris/HCl gel). MagicMark and Kaleidoscope standards were used to identify the molecular weight. The protein was then electrotransferred to PVDF membranes using standard Western blotting technique (100 V for 1–1.5 h for SREBP-1, ERK1/2, and α-actin, and 100 V for 4 h for FAS). Membranes were prehybridized with nonfat powdered milk and hybridized with mouse monoclonal anti-SREBP-1 antibody in a 1:500 dilution, anti-FAS antibody in a 1:250 dilution, anti-phospho-p44/42 MAP kinase antibody in a 1:1,000 dilution, or anti-total-p44/42 MAP kinase antibody in a 1:1,000 dilution. The membranes were then incubated with anti-mouse or anti-rabbit antibody conjugated with horseradish peroxidase in a 1:1,000 dilution, and protein was detected by chemiluminescence with the ECL Western Blotting Analysis System. The hybridized membranes were exposed to X-ray film, and the band intensity was measured with the densitometer.

As a control, membranes were then hybridized with mouse monoclonal anti-α-sarcosomic actin antibody in a 1:1,000 dilution and anti-mouse antibody in a 1:100,000 dilution and developed as described for SREBP-1.

RNA isolation from rat skeletal tissue. Total RNA was isolated from skeletal muscle tissue using the Qiagen RNeasy kit, following the manufacturer’s recommendations. Briefly, 10–30 mg of frozen rat gastrocnemius or soleus muscle tissue were homogenized until completely uniform using a rotor/stator tissue homogenizer in a guanidine isothiocyanate buffer at a speed of 30,000 rpm for 2 min. Samples were then treated with proteinase K to inactivate nucleases. Lysate was then mixed with ethanol and applied to an RNeasy minicolumn. The column was subsequently incubated with 5 units of DNase I for 15 min at room temperature. This step was followed by two washes with RW1 and RPE (Qiagen Kit) buffers. RNA was eluted in 30 μl of RNase-free water (Qiagen kit).

After RNA isolation experiments, total RNA purity and quality were assessed by measuring the optical densitometry, using a spectrophotometer, and by resolving RNA on 6% formaldehyde-1.2% agarose gels. RNA was visualized using ethidium bromide and a 0.24–to-9.5-kb RNA ladder to verify lack of degradation.

qRT-PCR. For all qRT-PCR, assays first-strand cDNA was synthesized from 1 μg of total RNA using the iScript cDNA Synthesis kit, following the manufacturer’s recommendations. Briefly, the reactions were incubated as follows: 5 min at 25°C, 30 min at 44°C, 5 min at 95°C, and then stored at −20 to −80°C until use. Rat 36B4 forward and reverse primers were used to normalize all assays at a concentration of 240 nM total primers. Each qRT-PCR reaction for SREBP-1c was carried out in a 20-μl volume in 1× SYBR Green supermix PCR reagents, 50 ng of reverse-transcribed total RNA, 120 nM of the forward rat SREBP-1c primer and 120 nM of rat SREBP-1c reverse primers (240 nM total primers), and 10 μl of 2× SYBR Green PCR Master Mix. Each qRT-PCR reaction for the housekeeping gene was carried out in a separate 20-μl volume in 1× SYBR supermix PCR reagents, 50 ng of reverse-transcribed total RNA, 120 nM of the forward rat 36B4 primer and 120 nM of rat 36B4 reverse primers (240 nM total primers), and 10 μl of 2× SYBR Green PCR supermix.

qRT-PCR was performed on a Bio-Rad iCycler iQ Real-Time PCR detection system in 96-well plates. Triplicate amplifications were performed for each control and sample. All 96-well PCR plates were sealed with optical tape to prevent evaporation. The iCycler protocol was as follows: 1 cycle of 95°C for 90 s, 40 cycles of 95°C for 30 s followed by 60°C for 60 s, and 1 cycle of 95°C for 60 s. A melt curve of 80 cycles, starting at 55°C and increasing by 0.5°C every 10 s, was done to determine primer specificity. Expression levels were calculated by the relative method by comparing the target amount of rat 36B4 mRNA, according to the manufacturer’s recommendations.

Muscle triglyceride analysis. Fifty milligrams of tissue per animal were weighed and homogenized in a hand-held tissue homogenizer, using a volume of distilled water equal to eight times the tissue weight in milligrams. The protocol for triglyceride extraction was as follows: 5 M NaCl, methanol, and chloroform were added to the homogenate and mixed, and the ternary phase was broken with water and chloroform after 5 min of incubation. The three-phase system was then separated by centrifugation at top speed. The aqueous and protein phases were reextracted with 9:1 chloroform-methanol wash solvent and separated by centrifugation. The organic solvent was dried using a stream of nitrogen gas with an N-Evap, and the triglyceride was resuspended in isopropanol containing 2% Triton X-100.

Glycerol concentration was measured relative to a standard curve made with glycerol and SDS. Standards and samples were loaded on a 96-well plate, and Free Glycerol Reagent was added to each well. The plate was incubated at 37°C and read on a Kinetic Microplate Reader at 540 nm. Triglyceride Reagent was added to each well and incubated at 37°C. The plate was read again on the Microplate Reader at 540 nm, and the background glycerol was subtracted.

Data analysis. The results are expressed as means ± SE. An unpaired t-test with a P value of <0.05 was used to determine statistical significance.

RESULTS

Exercise Training

Exercise training increases skeletal muscle triglyceride content. Initially, we compared the triglyceride content of control (sedentary) and exercise-trained rats. Triglyceride content was significantly higher in the gastrocnemius muscle of trained rats (sedentary) and exercise-trained rats. Triglyceride content of control rats (22 ± 2% of 37 nmol/mg, P < 0.001; Fig. 1). Similarly, there was a significant increase in triglyceride in the soleus muscle of trained rats vs. sedentary controls (372 ± 74 vs. 216 ± 37 nmol/mg, P < 0.001; Fig. 1).

Exercise training increases SREBP-1c mRNA. We then examined muscle SREBP-1 expression in exercise-trained and sedentary rats. As SREBP-1c is the isoform most related to triglyceride production in vivo, we assessed the effect of exercise on SREBP-1c mRNA levels using qRT-PCR. SREBP-1c mRNA was significantly higher in gastrocnemius tissue from trained rats than sedentary controls (8.92 ± 0.56, P < 0.002; Fig. 2). Similarly, soleus tissue from trained rats expressed significantly more SREBP-1c than sedentary controls (1.63 ± 0.23 vs. 1.04 ± 0.04, P < 0.03; Fig.
2). There were no significant differences in 36B4 mRNA from gastrocnemius of soleus tissue of trained or control rats.

**Exercise training increases SREBP-1 precursor and mature protein.** To determine whether the increased SREBP-1c mRNA expression translated to increased SREBP-1 protein, we used immunoblotting. Western blotting studies from gastrocnemius of trained rats revealed significantly higher SREBP-1 precursor protein levels than sedentary controls (0.38 ± 0.02 vs. 0.26 ± 0.02, P = 0.001; Fig. 3). Similarly, SREBP-1 precursor expression was significantly higher in soleus muscle of trained rats than in sedentary controls (0.39 ± 0.03 vs. 0.29 ± 0.02, P = 0.03; Fig. 3).

Activation of SREBP-1 requires its cleavage and the entry of the mature protein into the nucleus. Therefore, we also assessed the amounts of mature (or active) SREBP-1. Expression of the mature SREBP-1 protein was significantly higher in the gastrocnemius and soleus muscles of trained rats than in sedentary controls (0.56 ± 0.07 vs. 0.37 ± 0.07, P = 0.03 and 0.39 ± 0.03 vs. 0.29 ± 0.02, P = 0.01, respectively; Fig. 4).

**Exercise training increases FAS protein.** To evaluate the physiological relevance of increased SREBP-1, we assessed expression of FAS protein, an enzyme stimulated by SREBP-1 (24). Expression of FAS protein was significantly higher in the gastrocnemius muscle of trained rats than in sedentary controls (0.32 ± 0.03 vs. 0.56, P = 0.001; Fig. 5). Similarly, there was a trend toward an increase in FAS in the soleus muscle of exercise-trained rats compared with sedentary controls [0.45 ± 0.07 vs. 0.36 ± 0.07, P = not significant (NS); Fig. 5].
Chronic calorie restriction increases skeletal muscle triglyceride content. To assess the effect of calorie restriction on muscle triglyceride, vastus lateralis muscle biopsies were obtained from four calorie-restricted yet normally insulin-sensitive male rhesus monkeys (10 ± 0.1 kg) and three normal control monkeys (8 ± 0.8 kg) (47). Skeletal muscle triglyceride was significantly lower in the control (4 ± 2 nmol/mg dry wt) than in the calorie-restricted (43 ± 11 nmol/mg dry wt) monkeys (P < 0.05).

Chronic calorie restriction increases SREBP-1 precursor and mature protein. To determine whether SREBP-1 is increased in calorie-restricted monkeys with high muscle triglyceride, we examined muscle SREBP-1 expression in both groups. Western blotting studies revealed that SREBP-1 precursor protein was significantly higher in vastus lateralis muscle of calorie-restricted monkeys than controls (0.66 ± 0.10 vs. 0.34 ± 0.07, P = 0.019; Fig. 6). Similarly, vastus lateralis SREBP-1 mature protein was significantly higher in calorie-restricted than in control monkeys (0.70 ± 0.09 vs. 0.43 ± 0.08, P = 0.038; Fig. 7).

Insulin infusion increases SREBP-1 protein in controls, but not with calorie restriction. To determine the effect of insulin administration on the skeletal muscle SREBP-1 expression, SREBP-1 protein was examined in a subset of both groups before and after a hyperinsulinemic euglycemic clamp (46). Interestingly, although insulin infusion significantly increased SREBP-1 protein in control monkeys (0.17 ± 0.02 vs. 0.26 ± 0.04, P = 0.04; Fig. 8), calorie-restricted monkeys showed no increase in SREBP-1 after exposure to insulin (0.54 ± 0.06 vs. 0.49 ± 0.05, P = NS; Fig. 8). Of note, this lack of SREBP-1 response to insulin does not indicate generalized insulin resistance, because the calorie-restricted animals were otherwise very sensitive to insulin (62). Instead, it likely indicates that maximal SREBP-1 expression has already occurred at baseline.

Chronic calorie restriction increases FAS protein. To assess signaling downstream of SREBP-1, expression of FAS protein was measured by Western blotting. FAS protein levels were significantly higher in vastus lateralis muscle of calorie-restricted monkeys than controls (2.83 ± 0.39 vs. 1.23 ± 0.33, P = 0.026; Fig. 9).

Chronic calorie restriction activates the ERK1/ERK2 pathway. Finally, we looked for a mechanism to increase SREBP-1 in calorie restriction. We have previously shown that SREBP-1 is increased via ERK1/ERK2 in hyperinsulinemia (45). On the basis of these data, we examined the activity of the ERK1/ERK2 pathway in calorie restriction. Despite equal amounts of total ERK1/ERK2 (Fig. 10A), ERK1 phosphorylation increases with calorie restriction compared with controls (0.24 ± 0.05 vs. 0.08 ± 0.03, P = 0.02; Fig. 10B), as does ERK2 phosphorylation (0.65 ± 0.11 vs. 0.15 ± 0.05, P = 0.003; Fig. 10C).
DISCUSSION

Even though increased levels of IMTG are commonly found in obesity and T2DM, which are two well-defined insulin-resistant states (1, 2, 5–9, 11), unexpectedly, increased IMTG is also observed in two models of enhanced insulin sensitivity: calorie restriction and exercise. Both the physiological significance and the biochemical mechanism underlying these paradoxical observations remain largely enigmatic.

Increased IMTG with exercise has been reported previously by several groups (21, 27, 32, 43, 65). Exercise training increases both intramuscular lipid and oxidative capacity of vastus lateralis muscle (53). Increased IMTG with calorie restriction, however, was only recently reported (47). The present study is the first to begin to explain the recent observation that increased IMTG is associated with long-term calorie restriction and to suggest a mechanism that might explain the increased IMTG in exercise training as well. We found that, as is the case with T2DM and the metabolic syndrome (46), this increased IMTG in skeletal muscle of trained and calorie-restricted monkeys is associated with increased expression of SREBP-1c and FAS. The two yet-unanswered questions are whether the mechanism explaining increased IMTG in the setting of exercise or calorie restriction is different from the cause of increased IMTG seen with insulin resistance and whether the consequences of increased IMTG in these settings are the same.

It is not yet clear what is responsible for regulating SREBP-1. Because the compensatory hyperinsulinemia of insulin resistance increases SREBP-1 in multiple tissues (20, 24, 34, 45, 59), insulin is a likely culprit. In contrast, as the present study demonstrates, insulin levels are not elevated with calorie restriction or exercise, and yet the levels of skeletal muscle SREBP-1 are also increased. In addition, insulin sensitivity was normal in the calorie-restricted monkeys by insulin clamp (62). One potential mechanism is that exercise and chronic caloric restriction increase SREBP-1 expression in skeletal muscle through the MAP kinase signaling pathway. Our previous observations and the results from the literature indicate that in skeletal muscle, insulin increases SREBP-1 via the MAP kinase pathway (24, 45). Therefore, it is conceivable that MAP kinase activation also increases SREBP-1 in exercise and caloric restriction.

MAP kinase activation is a feasible mechanism to link both exercise and caloric restriction with SREBP-1 expression in...
skeletal muscle, as the MAP kinase pathway has been shown to be activated in both settings. For example, stimulation of the ERK1/2 kinase pathway is increased in human skeletal muscle after acute cycling exercise (68, 74) and marathon running (73). This appears to be a local, not systemic, effect of muscle contraction, because it occurs only in the exercising limb (68). This observation has also been confirmed in vitro with isolated contracting rat skeletal muscle (56, 69, 70). Thus skeletal muscle contraction might increase SREBP-1 directly via the MAP kinase pathway. Similarly, calorie restriction can activate the MAP kinase pathway. Mice subjected to a 30% reduction in calorie intake compared with control littersmates showed increased MAP kinase pathway activation (1). Our data confirm that MAP kinase activation may be the trigger increasing SREBP-1 in calorie restriction.

Another potential mechanism controlling SREBP-1 expression is dietary cholesterol intake. The Schoenheimer effect describes the process whereby a low dietary cholesterol intake increases cholesterol synthesis and high dietary cholesterol intake decreases cholesterol synthesis (58). Recent evidence shows that SREBP-1 regulation also may control this phenomenon. Insig proteins are required for the sterol-mediated processing of SREBP to its nuclear form (11). Cholesterol feeding reduces nuclear SREBP-1 and downstream lipogenic enzymes; this effect is blocked in Insig 1 and Insig 2 double knockouts (17). Similarly, lower cholesterol intake leads to increased nuclear SREBP-1. Therefore, a calorie-restricted diet, leading to absolute decreases in cholesterol intake, could lead to increased nuclear SREBP-1 expression.

If the pathophysiological consequences of increased skeletal muscle IMTG in insulin-resistant states include the pathogenesis of insulin resistance itself, their role in exercise and chronic caloric restriction is much less apparent. Although this issue is beyond the scope of the present investigation, it is likely that increased IMTG in exercised muscle may represent an important fuel depot that is ready for mitochondrial oxidation.

Exercise training increases the oxidative capacity of skeletal muscle in rats (3, 26, 42), guinea pigs (4), and humans (46, 53). Specifically, in rats undergoing treadmill running, the ability to oxidize fat and carbohydrate is doubled (3, 26, 42). When a muscle cell is geared for oxidation, as with exercise (21), the fat will be oxidized by mitochondria to supply the energy needs of exercise. Increasing fatty acid oxidation in L6 muscle cells can also increase their insulin sensitivity without decreasing their lipid content (49). In contrast, cells in subjects with T2DM are not poised to use lipids as fuel, and turnover of lipids is slow because their mitochondria have defects limiting oxidation (41, 55). Similarly, offspring of patients with T2DM who do not yet have diabetes also have impaired mitochondrial activity, implying that the mitochondrial defect occurs early on rather than as a consequence of the diabetes (51).

Increased IMTG may also be an important defense mechanism in the face of limited glycogen stores during chronic caloric restriction. Thus the metabolic milieu may be quite different in cells from insulin-sensitive vs. insulin-resistant subjects once fat is deposited. Because our study was not designed to examine lipid oxidation in exercise or caloric restriction, future studies are necessary to address this issue. Even though our study is the first to demonstrate that calorie restriction increases skeletal muscle SREBP-1, increased pre-cursor and mature SREBP-1 proteins have been found in adipose tissue from male Wistar rats upon refeeding after both short- and long-term calorie restriction (64). These investigators concluded that calorie restriction increases the capacity for fatty acid biosynthesis. Unfortunately, neither muscle triglyceride, SREBP-1, nor FAS were assessed in the aforementioned study. However, taken together with our present observation, these results support the notion that chronic calorie restriction increases SREBP-1, which could lead to increased IMTG deposition.

In summary, we found increased expression of SREBP-1 mRNA and protein, FAS protein, and increased IMTG in skeletal muscle of exercise-trained rats and chronically calorie-deprived monkeys. With better understanding of the mechanism leading to increased IMTG, the next logical step would be to evaluate why and how accumulation of IMTG in obesity and T2DM is associated with insulin resistance, whereas in exercise and chronic caloric restriction it is associated with normal insulin sensitivity.

ACKNOWLEDGMENTS
We express gratitude to Dr. Marc Goalstone for his expert advice and Dr. Victor Sorribas for providing primers for q-RT-PCR.

GRANTS
This work was supported in part by the Veterans Affairs Research Service (B. Draznin), National Institutes of Health/Division of Research Resources Grant no. 1K23 RR-020038-01 and National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases Grant no. 5-P30-DK-48520-10 (R. L. Moore), National Institutes of Health Grants R01-DIC-06741 and P01-56481 (J. E. B. Reusch), and National Heart, Lung, and Blood Institute Grant no. PHS-ROI-HL-72790 (R. L. Moore).

REFERENCES