UCP-mediated energy depletion in skeletal muscle increases glucose transport despite lipid accumulation and mitochondrial dysfunction

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Han, Dong-Ho, Lorraine A. Nolte, Jeong-Sun Ju, Trey Coleman, John O. Holloszy, and Clay F. Semenkovich. UCP-mediated energy depletion in skeletal muscle increases glucose transport despite lipid accumulation and mitochondrial dysfunction. Am J Physiol Endocrinol Metab 286: E347–E353, 2004. First published November 12, 2003; 10.1152/ajpendo.00434.2003.—To address the potential role of lipotoxicity and mitochondrial function in insulin resistance, we studied mice with high-level expression of uncoupling protein-1 in skeletal muscle (UCP-H mice). Body weight, body length, and bone mineral density were decreased in UCP-H mice compared with wild-type littermates. Forelimb grip strength and muscle mass were strikingly decreased, whereas muscle triglyceride content was increased fivefold in UCP-H mice. Electron microscopy demonstrated lipid accumulation and large mitochondria with abnormal architecture in UCP-H skeletal muscle. ATP content and key mitochondrial proteins were decreased in UCP-H muscle. Despite mitochondrial dysfunction and increased intramyocellular fat, fasting serum glucose was 22% lower and insulin-stimulated glucose transport 80% higher in UCP-H animals. These beneficial effects on glucose metabolism were associated with increased AMP kinase and hexokinase activities, as well as elevated levels of GLUT4 and myocyte enhancer factor-2 proteins A and D in skeletal muscle. These results suggest that UCP-H mice have a mitochondrial myopathy due to depleted energy stores sufficient to compromise growth and impair muscle function. Enhanced skeletal muscle glucose transport in this setting suggests that excess intramyocellular lipid and mitochondrial dysfunction are not sufficient to cause insulin resistance in mice. Uncoupling protein; insulin resistance; lipotoxicity; mitochondria

HYPERGLYCEMIA, DYSLIPIDEMIA, HYPERTENSION, and abdominal obesity comprise the metabolic syndrome, which affects about a quarter of adults in the United States (11). The metabolic syndrome is both common and lethal, because it is a powerful predictor of cardiovascular events. However, it remains a syndrome, a collection of clinical disorders lacking a clear unifying cause. Insulin resistance is involved in the pathophysiology of the metabolic syndrome, but the discrete mediators of impaired insulin signaling are unknown.

Insulin resistance is usually defined by the inability of insulin to appropriately stimulate glucose transport in peripheral tissues such as skeletal muscle. Progressive loss of the ability of insulin to stimulate glucose transport in muscle is associated with development of the metabolic syndrome and, in genetically susceptible individuals, type 2 diabetes (13). The fact that exercise is an effective therapy for both the prevention and treatment of diabetes (24) suggests that muscle plays a role in the metabolic decompensation that characterizes insulin-resistant states. Intramyocellular fat has been implicated in skeletal muscle insulin resistance (18).

Since the 1960s, fatty acids have been known to inhibit skeletal muscle glucose metabolism (36). Associational studies have demonstrated an inverse relationship between insulin sensitivity and skeletal muscle neutral lipid content (15, 31, 32, 34). These observations prompted the notion that lipotoxicity contributes to defective glucose transport in skeletal muscle. Lipids have been proposed to disrupt glucose metabolism in several ways, including activation of protein kinase C isoforms (20), direct effects on enzymes such as hexokinase (18), and disruption of trafficking of the insulin-responsive glucose transporter GLUT4 (21). Insulin resistance is known to be associated with defective fat oxidation in skeletal muscle (22), providing a potential mechanism for the accumulation of intramyocellular lipid. Correlations between mitochondrial function and insulin sensitivity have led to the suggestion that defective mitochondria (one site of fatty acid oxidation) could be responsible for lipid accumulation and defective glucose transport in muscle (33).

We recently showed that skeletal muscle expression of uncoupling protein-1 (UCP1) enhances insulin sensitivity in transgenic mice (26). Two transgenic lines were established that showed a dose-dependent relationship between UCP1 expression and insulin sensitivity. UCP-L mice, with low-level expression of UCP-1 in muscle, are protected from diet-induced (26) and genetic (7) obesity as well as several features of the metabolic syndrome (7, 26). UCP-H mice, with high-level expression in skeletal muscle, were actually shown to be more insulin sensitive than UCP-L mice (26). Here we report that UCP-H animals have impaired growth and muscle dysfunction due to energy depletion. Unexpectedly, insulin-stimulated glucose transport is increased in the muscles of these animals despite the presence of defective mitochondria and increased stores of intramyocellular lipid, suggesting that enhanced insulin sensitivity can occur in the presence of conditions thought to contribute to insulin resistance.

METHODS

Animals. The generation of skeletal muscle UCP1 transgenic mice was described previously (26). Two independent founder lines (UCP-L, for low expression, and UCP-H, for high expression), each

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with the mouse UCP-1 cDNA driven by the rat myosin light-chain 2 promoter, were established. In UCP-H mice, the subject of this report, ectopic expression of UCP-1 mRNA and protein was shown to be limited to skeletal muscle. UCP-1 protein is 3-fold higher and oxygen consumption 2.5-fold higher in UCP-H compared with UCP-L muscle (26). These animals of mixed genetic background (C57BL/6 and CBA) were maintained in the hemizygous state by mating UCP-H males with wild-type females, since UCP-H females rarely delivered pups. Tail DNA was used for genotyping by PCR with the following primers: 5′/H11032 ATAAGGTGTCCTAGGGACCATCA-3′ (upstream), 5′/H11032 ACAGCTTGGTACGCTTGGATACTG-3′ (downstream). All studies were performed using littermates maintained on a chow diet with a fat content of 4.5%. The Washington University Animal Studies Committee approved these experiments.

Fig. 1. Body measurements for mice with high-level expression of uncoupling protein-1 (UCP-H, solid bars) and wild-type mice (open bars). Body weight (A), body length (B), bone mineral density (C), and adiposity (D) were determined in 6–8 males and 6–8 females of each genotype at the age of 8 mo. Mineral density and adiposity were determined in live mice by dual-energy X-ray absorptiometry. Data are presented as means ± SE. For each panel, comparisons were made by ANOVA. *Significant P values by Tukey’s multiple comparison test: A, males P < 0.001, females P < 0.01; B, males P < 0.001, females P < 0.001; C, males P < 0.001, females P < 0.01.

Fig. 2. Grip strength, muscle mass, and muscle triglyceride content in UCP-H (solid bars) and wild-type (open bars) mice. Determinations were performed in 14–20 UCP-H and 10–18 wild-type mice with equal numbers of each sex. EDL, extensor digitorum longus. Data were pooled by genotype, since there were no sex-specific differences for these parameters. Grip strength was assayed by use of a forelimb grip strength transducer. Triglyceride content was determined chemically. Data are presented as means ± SE. *P < 0.0001 for A–D; *P = 0.0118 for E, by unpaired, two-tailed t-test.
Analytical procedures. Blood was obtained from the lateral saph- neous vein of mice after a 4-h fast. Serum was isolated and assayed for glucose, insulin, cholesterol, triglycerides, and nonesterified fatty acids as previously described (27). Bone mineral density and adiposity were determined on anesthetized, living mice by dual-energy X-ray absorptiometry by use of a small animal densitometer, as described (7).

Muscle characterization. Forelimb strength was determined using a grip strength meter for mice (Ugo Basile, Comerio, Italy catalog no. 47106). Mice were accustomed to handling and placement of forelimbs on the apparatus trapeze for several days before actual measurements were performed. For data acquisition, the tail was gently retracted while each mouse grasped the force transducer (trapeze). The peak force in grams achieved at the time the animal lost its grip was stored and displayed by a peak preamplifier. Multiple determinations, each separated by 20–30 min, were recorded during several daily sessions to generate the mean strength value for each mouse.

For tissue triglyceride content, muscles were homogenized and extracted with chloroform-methanol. The lipid was taken to dryness by nitrogen stream and quantified by scanning densitometry performed within the linear response range of the film.

GLUT4 and δ-aminolevulinate synthase were quantified as described (2, 25). For citrate synthase, the primary antibody (Alpha Diagnostics International) was used at a dilution of 1:5,000 and the secondary antibody (donkey anti-rabbit, Jackson ImmunoResearch) was used at 1:2,000 and the secondary antibody (donkey anti-mouse, Jackson ImmunoResearch) at 1:5,000. Monoclonal antibodies to cytochrome oxidase subunit I (used at a dilution of 1:10,000), cytochrome oxidase subunit IV (1:2,500), NADH ubiquinol oxidoreductase (1:5,000), ATP synthase (α-subunit) (1:5,000), and succinate ubiquinol oxidoreductase (1:5,000) were purchased from Molecular Probes (Eugene, OR). For each, the secondary antibody (donkey anti-mouse, Jackson ImmunoResearch) was used at a dilution of 1:10,000.

Western blotting. Extracts were subjected to SDS-PAGE under reducing conditions, blotted, and detected by chemiluminescence. Signals were quantified by scanning densitometry performed within the linear response range of the film.

Table 1. Mitochondrial enzyme levels in skeletal muscle of wild-type and UCP-H mice

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Wild Type</th>
<th>UCP-H</th>
<th>P Value</th>
</tr>
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<tbody>
<tr>
<td>Cytochrome oxidase</td>
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| Subunit I                      | 0.66±1.10 | 0.27±0.08 | 0.01*
| Subunit IV                     | 0.92±0.12 | 0.50±0.11 | 0.02*
| NADH ubiquinol oxidoreductase  | 0.88±0.05 | 0.57±0.03 | <0.001*
| δ-Aminolevulinate synthase     | 0.94±0.09 | 0.64±0.08 | 0.013*
| ATP synthase (α-subunit)       | 0.78±0.67 | 0.67±0.22 | 0.665
| Citrate synthase               | 0.81±0.20 | 0.55±0.18 | 0.362
| Succinate ubiquinol oxidoreductase | 0.98±0.05 | 1.04±0.06 | 0.419

Skeletal muscle protein content of mitochondrial enzymes was determined by Western blotting followed by quantitative densitometry with muscles from 5–6 different mice of each genotype. UCP-H mice with high-level expression of uncoupling protein-1. Data are expressed as means ± SE in arbitrary densitometric units. P values were calculated by unpaired, two-tailed t-test.
RESULTS

We previously showed that mice with low-level skeletal muscle expression of UCP1, UCP-L mice, have normal body length, normal exercise tolerance, and less adiposity than littermates. UCP-H mice had a different phenotype. At 8 mo of age, UCP-H animals of both sexes weighed ~30% less than their wild-type littersmates (Fig. 1A). Nasal-anal length was 14% less (Fig. 1B), consistent with decreased linear growth, and bone mineral density was 21% lower (Fig. 1C) in UCP-H mice. Adiposity was unaffected by genotype in these chow-fed mice (Fig. 1D), perhaps reflecting a striking increase in intramuscular fat in the UCP-H mice (see below).

UCP-H mice appeared weak and would not run on a treadmill. Their muscle weakness was quantified with a grip strength meter. Forelimb grip strength was decreased by 36% in UCP-H mice compared with littersmates (Fig. 2A). Data for males and females are presented together for both grip strength and muscle mass because we did not detect sex-dependent differences for these measurements. Muscle weights for extensor digitorum longus, epitrochlearis, and gastrocnemius muscles were ~75% lower in UCP-H mice (Fig. 2B, C, and D). Chemically determined triglyceride content of gastrocnemius muscle was increased fivefold in UCP-H mice (Fig. 2E).

The presence of intramyocellular fat in UCP-H mice was confirmed with use of electron microscopy (Fig. 3). In wild-type muscle, lipid droplets were seen in some sections (Fig. 3A) and normal-appearing mitochondria were present (Fig. 3C). In UCP-H mice, muscle architecture was disrupted, and numerous large lipid droplets were present in every low-power field (Fig. 3B). Figure 3D shows a higher-power view of lipid droplets in apposition to large, abnormal-appearing mitochondria.

To confirm that mitochondrial function was impaired in UCP-H mice, we performed quantitative Western blotting for key mitochondrial markers (Table 1). The contents of cytochrome oxidase subunits I and cytochrome oxidase subunit IV (components of complex IV of the respiratory chain), NADH ubiquinol oxidoreductase (complex I of the respiratory chain), and δ-aminolevulinate synthase (rate limiting for the synthesis of heme, required for electron transfer) were significantly decreased by 32–60% in UCP-H muscle. ATP synthase and citrate synthase levels tended to be lower as well, but these differences were not significant. The level of succinate ubiquinol oxidoreductase (complex II of the respiratory chain) was identical in wild-type and UCP-H muscle.

ATP levels were significantly decreased in UCP-H muscle, consistent with the presence of defective mitochondria (Fig. 4A). ADP levels were lower (Fig. 4C) and AMP levels higher (Fig. 4B) in UCP-H muscle. The enzyme activity of AMP-activated protein kinase (AMP kinase), a key mediator of muscle metabolic flux in response to increased energy demand, was 114% higher in extracts of UCP-H muscle (Fig. 4D). This value probably underestimates the authentic induction in intact muscle, because high levels of AMP (missing from in vitro assays) allosterically activate AMPK (46).

Compared with wild-type animals, UCP-H mice at 2–3 mo of age have lower fasting blood glucose and lower glucose levels during glucose tolerance testing and are more sensitive to insulin as determined by insulin tolerance tests (26). As expected, fasting glucose levels for the 8-mo-old mice in the current study were 22% lower in UCP-H compared with wild-type animals (Table 2). Insulin, cholesterol, and triglyceride levels were not significantly different, but serum free fatty acids were increased by ~50% in UCP-H mice.

UCP-H animals had enhanced insulin sensitivity, as reflected by an 80% increase in insulin-stimulated glucose trans-
port in isolated muscle (Fig. 5A). Commensurate with the increase in glucose transport, GLUT4 protein was 84% higher (Fig. 5B) and hexokinase enzyme activity (Fig. 5C) was 91% higher in UCP-H muscle. Members of the MEF2 transcription factor family, MEF2A and MEF2D, are known to participate in hormonally regulated expression of GLUT4. Protein levels for MEF2A and MEF2D mirrored changes in GLUT4, with 85% higher levels of MEF2A (Fig. 5D) and 79% higher levels of MEF2D (Fig. 5E) in UCP-H compared with wild-type muscle.

**DISCUSSION**

Lipotoxicity is an attractive potential link between obesity and insulin resistance. However, a direct cause and effect relationship between muscle lipid and impaired insulin action has not been established. In the current work, we demonstrate increased insulin sensitivity in mice with a mitochondrial myopathy and increased intramyocellular triglyceride content. These results provide evidence that the molecular machinery required to move glucose into muscle in response to insulin can be induced even in the setting of defective ATP production and intracellular fat.

Our previous studies of UCP-L mice, with relatively low expression of UCP-1 in skeletal muscle, showed increased glucose transport in the setting of modestly increased muscle lipid (7, 26). However, it is unlikely that the phenotype in UCP-H mice (the subject of the current work) and that in UCP-L animals represent a continuum. UCP-L animals had normal levels of high-energy phosphates in muscle (26), normal exercise tolerance (26), normal levels of GLUT4 protein in skeletal muscle (26), and normal mitochondria by electron microscopy (data not shown). All of these features were different in UCP-H mice, indicating that the level of uncoupling in these animals surpassed a threshold beyond which mitochondrial compensation is insufficient to maintain energy stores.

Depleted energy stores caused muscle weakness in UCP-H mice (Fig. 2A). Because exercise can decrease muscle triglyceride content, it is possible that relative inactivity in UCP-H mice could have contributed to the accumulation of intramyocellular lipid (Fig. 2E). However, the striking difference in triglyceride content between control and UCP-H mice is beyond the range expected with exercise, suggesting that the major contributor to this finding is the inability of defective mitochondria to oxidize fat.

Independent groups have described an inverse relationship between intramyocellular fat content and insulin sensitivity (15, 31, 32, 34). Enzymatic assays, neutral lipid staining in biopsy samples, CT imaging with determination of muscle attenuation, and proton magnetic resonance spectroscopy yield similar results (23), providing little doubt that obese type 2 diabetics with the highest degree of insulin resistance tend to have the highest amounts of intramuscular fat.

However, several lines of evidence suggest that the presence of lipid alone is not sufficient to disrupt insulin-mediated glucose transport. One group has generated muscle-specific lipoprotein lipase transgenic mice with increased muscle tri-
glycerides and normal insulin-stimulated glucose uptake (45). Insulin-sensitive endurance athletes have increased amounts of intramyocellular fat (14). Levels of GLUT4, the insulin-responsive glucose transporter, are not associated with cellular lipid content (12). Chronic training improves insulin sensitivity without affecting muscle triglyceride content (19). Niacin induces insulin resistance without affecting muscle lipid (35). In type 2 diabetes, intramuscular triglycerides are inversely correlated with insulin sensitivity at baseline but increase with insulin infusion without affecting insulin sensitivity (1).

Impaired mitochondrial function, which can lead to lipid accumulation because of reduced fatty acid oxidation, has also been implicated in insulin resistance. Mitochondrial enzymes tend to be decreased in insulin-resistant states (4, 43, 44), and skeletal muscle oxidative capacity is directly correlated with insulin sensitivity (23, 38, 39). Reduced mitochondrial oxidation and phosphorylation activity determined using NMR are associated with skeletal muscle insulin resistance in elderly humans (33). However, the energy-producing capacity of muscle mitochondria isolated from elderly humans does not appear to be reduced (37). In addition, defective insulin secretion and regional adiposity may be more important than skeletal muscle mitochondrial function in the development of glucose intolerance with aging (5), raising the possibility that the link between mitochondrial dysfunction and insulin resistance is tenuous. Consistent with this idea, human mitochondrial mutations cause myopathy and diabetes that is due to insulin secretory failure, usually in the absence of insulin resistance (3, 6, 41).

UCP-H mice had physiological, morphological, and biochemical evidence of a mitochondrial myopathy. In addition to decreased weight, these animals had decreased bone mineral density and short stature (Fig. 1), also seen in humans with myopathies (17). They were weak with decreased muscle mass (Fig. 2) and had structurally abnormal mitochondria (Fig. 3), low concentrations of the proteins required for oxidative function in mitochondria (Table 1), and low levels of ATP (Fig. 4). Despite these defects, serum glucose levels were decreased (Table 2), skeletal muscle had increased sensitivity to insulin (Fig. 5), and the key components of glucose transport and metabolism were increased in skeletal muscle (Fig. 5). Using a different promoter, Couplan et al. (8) also recently generated transgenic mice expressing UCP1 in skeletal muscle. Like UCP-H mice in the current study, these animals had decreased body weight and muscle mass. Unlike UCP-H mice, there was no effect on serum glucose, probably because UCP-H mice have a more pronounced phenotype. Adult body weight was ~25 g for male and ~18 g for female mice generated by Couplan et al. (8) and ~20 g for male and ~14 g for female UCP-H mice in the present study (Fig. 1A).

The proximate cause of enhanced glucose transport in UCP-H animals was an increase in GLUT4 (Fig. 5). Although many of the acute effects of insulin on glucose transport are mediated by the recruitment of transporters to the cell surface, decreased expression of GLUT4 can cause insulin resistance (40), and increased expression of GLUT4 promotes insulin-stimulated glucose transport (16). MEF2A and MEF2D, probably functioning as a heterodimer, are responsible for hormonal regulation of GLUT4 gene expression (28). Both were increased in UCP-H mice (Fig. 5). Although MEF2B has been implicated as a mediator of energy flux in muscle (9), chemical activation of AMP kinase causes a coordinate induction of MEF2A, MEF2D, and GLUT4 in myocytes (30). AMP kinase (increased in UCP-H mice), another key mediator of skeletal muscle energy flux, is activated by several different mechanisms (46) involving 5’-AMP (also increased in UCP-H mice). Metformin, an effective treatment for insulin resistance, appears to activate AMP kinase in association with the stimulation of skeletal muscle glucose transport and decreased skeletal muscle ATP content (29, 47). Collectively, these data suggest that energy depletion in UCP-H mice activated AMP kinase, leading to induction of MEF2A and MEF2D, resulting in increased GLUT4 expression and enhanced insulin-stimulated glucose transport, all in the setting of mitochondrial dysfunction and intramyocellular lipid accumulation.

Our results must be viewed within the context of our model, mice with a severe myopathy. Similar mechanisms may not be operative in humans with normal muscle function. Regardless, the results suggest that mitochondrial dysfunction and lipid accumulation in muscle may be markers rather than mediators of insulin resistance. They also show that glucose transport can be activated despite the presence of conditions implicated in insulin resistance. Modulating AMP kinase or the MEF2 family of transcription factors could represent a viable strategy for improving glucose tolerance in insulin-resistant states such as type 2 diabetes and the metabolic syndrome.

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