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Short Article

Respiratory Uncoupling in Skeletal Muscle Delays Death and Diminishes Age-Related Disease

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SUMMARY

Age-related disease, not aging per se, causes most morbidity in older humans. Here we report that skeletal muscle respiratory uncoupling due to UCP1 expression diminishes age-related disease in three mouse models. In a longevity study, median survival was increased in UCP mice (animals with skeletal muscle-specific UCP1 expression), and lymphoma was detected less frequently in UCP female mice. In *apoE* null mice, a vascular disease model, diet-induced atherosclerosis was decreased in UCP animals. In agouti yellow mice, a genetic obesity model, diabetes and hypertension were reversed by induction of UCP1 in skeletal muscle. Uncoupled mice had decreased adiposity, increased temperature and metabolic rate, elevated muscle SIRT and AMP kinase, and serum characterized by increased adiponectin and decreased IGF-1 and fibrinogen. Accelerating metabolism in skeletal muscle does not appear to impact aging but may delay age-related disease.

INTRODUCTION

Atherosclerosis, diabetes, hypertension, and cancer occur more frequently with increasing age. These age-related diseases are distinct from the process of aging, a postreproductive physiological decline that includes decreases in muscle strength, cardiopulmonary function, vision, and hearing as well as wrinkled skin and graying hair. Aging and age-related disease are associated but may not share the same mechanisms.

Disruption of insulin/IGF-1-like signaling prolongs life span in nematodes (Kenyon et al., 1993) and flies (Tatar et al., 2001). However, this issue is more complex in mice, where life span is increased in females with inactivation of one allele of the IGF-1 receptor, but males have glucose intolerance and survival is not significantly prolonged (Holzenberger et al., 2003). Inactivation of the insulin receptor in mouse adipose tissue increases life span (Bluher et al., 2003), but its inactivation in mouse liver causes diabetes (Michael et al., 2000), a disease that is probably contributing to decreases in human life span due to the current obesity epidemic (van Dam et al., 2006; Adams et al., 2006). Aging is impressively delayed in mutant mice with defects in anterior pituitary development (Flurkey et al., 2001; Brown-Borg et al., 1996). The opposite may occur in humans. Some investigators have reported premature mortality due mostly to atherosclerosis in people with defects in pituitary function (Tomlinson et al., 2001; Bates et al., 1996), although this may not apply to individuals with reduction of IGF-1 levels due to growth-hormone resistance or isolated growth-hormone deficiency (Laron, 2005; Oliveira et al., 2006).

In flies, JNK activation prolongs life span by antagonizing insulin signaling (Wang et al., 2005), but JNK activation in mice promotes diabetes (Hirosumi et al., 2002) and atherosclerosis (Ricci et al., 2004). Certain mutations that increase the activity of the tumor suppressor p53 in mice decrease cancer frequency but also accelerate aging (Tyner et al., 2002), raising the possibility that disease susceptibility and life span may be independently modulated. Given the difficulty of validating strategies to increase life span in humans and the possible dissociation between aging and age-related diseases, identifying a simple intervention affecting several age-related diseases is an attractive approach to decreasing the morbidity of growing old.

Altering the efficiency of mitochondrial respiration, the transfer of electrons from food to oxygen to synthesize ATP, may represent such an intervention. Uncoupling proteins (UCPs) are inner mitochondrial membrane anion transporters that disrupt ATP synthesis, releasing heat and causing a compensatory increase in oxygen consumption. UCP1, the first such protein to be identified, is responsible for heat production in brown fat. Transgenic mice with the murine *UCP1* cDNA driven by the rat myosin light chain 2 promoter, which ectopically express UCP1 in skeletal muscle, are protected from diet-induced (Li et al., 2000) and genetic (Bernal-Mizrachi et al., 2002) obesity. Overexpression of the related proteins UCP2 and UCP3 in mice also decreases obesity and improves insulin sensitivity (Clapham et al., 2000; Horvath et al., 2003; Choi et al., 2007). Less is known about the potential role of uncoupling in other disorders as well as in overall survival,

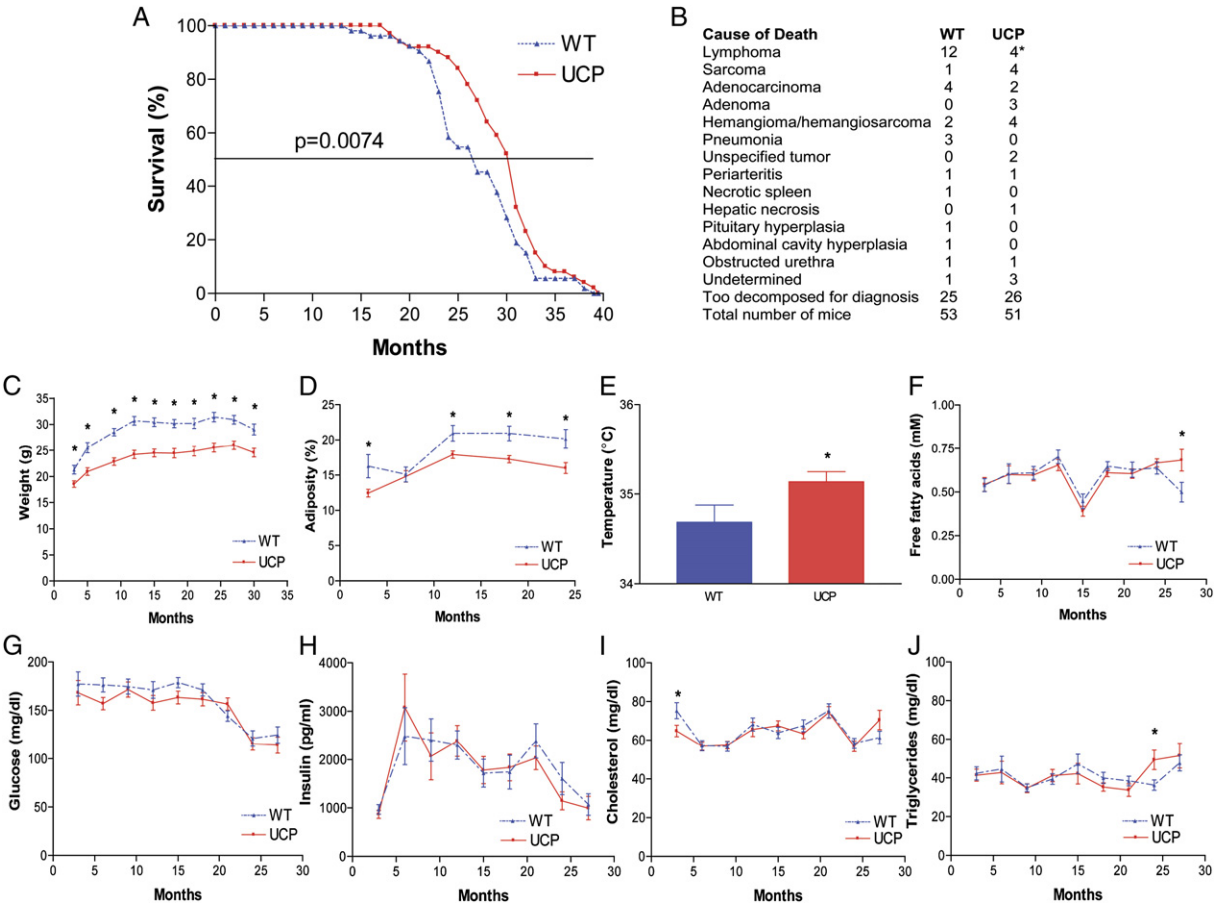

Figure 1. Increased Survival and Fewer Cancer Deaths in Mice with Respiratory Uncoupling in Skeletal Muscle

(A) Survival curves for *UCP1* transgenic (UCP, solid red line) and wild-type (WT, dashed blue line) mice. The p value represents a comparison of median survival. For comparison of curves by log-rank testing, p = 0.0262.

(B) Probable cause of death as determined by necropsy for all 104 animals in (A). The difference in lymphoma detection was gender based, with no cases detected in UCP females and 8 cases detected in WT females (p = 0.0045 by Fisher's exact test).

(C and D) Body weight (C) and adiposity (D) were determined over time for the animals in (A).

(E) Body temperature as determined by telemetry using implanted sensors in 3-month-old littermate mice not included in the longevity study (WT, n = 6; UCP, n = 9; p = 0.0478 by two-tailed t test).

(F–J) Fasting free fatty acids (F), glucose (G), insulin (H), cholesterol (I), and triglycerides (J) were determined over time for the animals in (A).

Data are presented as mean ± SEM. *p < 0.05. Data are presented for males and females combined in this and subsequent figures; the same patterns were seen when sexes were analyzed separated.

although *UCP2* null mice have increased susceptibility to chemically induced colon tumors (Derdak et al., 2006).

Young animals with low-level expression of UCP1 in skeletal muscle have a mildly increased metabolic rate, normal food intake, normal growth, and normal levels of ATP and phosphocreatine in muscle (Li et al., 2000). We used these mice to determine whether respiratory uncoupling in skeletal muscle, a tissue that adapts to altered heat production and oxygen consumption during exercise, can affect age-related disease.

RESULTS AND DISCUSSION

Increased Survival in Uncoupled Mice

UCP1 transgenic mice were backcrossed five times with C57BL/6 mice, and the resulting wild-type (WT) and littermate *UCP1* transgenic (UCP) mice were provided free access to standard chow diet until their natural death (Figure 1A). Median survival was prolonged by 3 months in the UCP mice (30 months for UCP versus 27 months for WT; p = 0.0074 by Mann-Whitney test). Survival curves were different by log-rank testing (p = 0.0262). Data represent both sexes; the same trends were seen in males and females (see Figure S1 in the Supplemental Data available with this article online). Maximal life span was not affected; death occurred at 39 and 39.5 months for the longest lived WT and UCP mouse, respectively. Median survival for the longest lived 20% of each group was 33 months for WT mice and 35 months for UCP mice (p = 0.0753). Lymphoma, the most common probable cause of death detected at necropsy in WT mice (Figure 1B), was less frequent in UCP mice due to a gender-specific difference.

In females, lymphoma was detected in 8 of 25 WT and 0 of 22 UCP mice ($p = 0.0045$ by Fisher's exact test). In males, lymphoma was detected in 4 animals of each genotype. UCP mice weighed less (Figure 1C) and had lower adiposity (Figure 1D). Lower body weight in humans is associated with lower risk for lymphoma (Pan et al., 2005), analogous to our less frequent detection of lymphoma in UCP mice (Figure 1B).

Young UCP animals had core body temperatures ~0.5°C higher than WT mice (Figure 1E), reflecting the biochemical effect of uncoupling. Body temperature is reduced in some long-lived mutant mice (Hunter et al., 1999). Caloric restriction can decrease body temperature, but mice engineered to maintain a lower body temperature (Conti et al., 2006) have a survival phenotype similar to UCP mice with higher body temperature, suggesting that lower temperature is not required to increase median life span in the absence of caloric restriction. There were minimal genotype differences in levels of fatty acids, glucose, insulin, cholesterol, and triglycerides over time between WT and UCP mice (Figures 1F–1J). UCP mice had significantly increased metabolic rates compared to WT mice, but there were no differences in food intake, fecal fat, or caloric output in feces (Figure S2).

Separate cohorts of young (~3 months) and old (>12 months) WT and UCP mice were further characterized (Figure 2). Transgenic expression of UCP1 was sustained with age. Western blots showed similar expression of UCP1 in the skeletal muscle of young and old transgenic mice (Figure 2A, right). Sir2 is an NAD-dependent deacetylase that mediates increased survival with caloric restriction in other models. Its mammalian homolog SIRT1 has been implicated in stress tolerance and several metabolic pathways (Guarente, 2006). UCP skeletal muscle SIRT enzyme activity was 2-fold higher than WT in young mice and 4-fold higher in old mice (Figure 2B, left panel). To confirm this finding, PGC-1α acetylation was assayed in young and old mice (Figure 2B, right panel; Figure S3). As expected, less acetylated PGC-1α protein was detected in UCP skeletal muscle. SIRT induction was not systemic since there were no differences in SIRT enzyme activity between WT and UCP mice in liver, brain, heart, or spleen (Figure S4).

A number of proteins may coordinate metabolism, stress responses, and disease susceptibility. Two such proteins, AMPK and mTOR, were reciprocally affected in UCP skeletal muscle, with higher levels of phosphorylated AMPK and lower levels of phosphorylated mTOR as compared to WT (Figure 2C). This effect was not systemic since these proteins were similarly activated in liver and spleen of UCP and WT mice (data not shown). Activated levels of another such protein, p53, were lower in UCP as compared to WT animals in liver (Figure 2C), a site remote from muscle, but were unaffected by genotype in muscle itself or spleen (data not shown). We were unable to detect differences between WT and UCP mice in tissue levels of FoxO1/3a, HSP70, or HSP90 (data not shown).

As an unbiased approach to identify potential mediators of the delayed death phenotype of UCP mice, plasma proteins were subjected to proteomic analysis (Figure 2D). Multiple spots contained fibrinogen isoforms that were less abundant in young and old UCP mice as compared to WT mice. Complement C3 (row 13 in Figure 2D) also appeared to be present at lower levels in the plasma of young and old UCP mice. Both of these differences were confirmed in separate cohorts of mice by ELISA. Fibrinogen levels were ~10% lower in UCP mice, consistent with human epidemiologic studies identifying fibrinogen as a risk factor for nonvascular as well as vascular causes of mortality (Danesh et al., 2005). UCP mice had ~40% lower levels of C3, which is involved in many inflammatory disorders and is highly correlated with both fibrinogen and body mass index in humans (Capuano et al., 2006).

Serum levels of IGF-1, adiponectin, and leptin were also different in UCP mice (Figure 2F). Each may be related to decreased adiposity. Interruption of IGF-1 signaling increases life span in some animals (Holzenberger et al., 2003), and IGF-1 signaling may predispose to malignancy in humans and mice (LeRoith and Roberts, 2003). Concentrations of IGF-1 are decreased in humans with low body mass (Gram et al., 2006), and IGF-1 levels were also lower in UCP mice (Figure 2F). Adiponectin is higher and leptin lower with decreased adiposity (Faraj et al., 2003), the pattern seen in UCP mice (Figure 2F). Elevated adiponectin has been associated with increased longevity in other mouse studies (Wang et al., 2006).

Delayed Atherosclerosis in Uncoupled Mice

To determine whether skeletal muscle respiratory uncoupling has beneficial effects on atherosclerosis, UCP mice were bred with apolipoprotein E-deficient ($apoE^{-/-}$) mice. When fed a "western-type" high-fat (HF) diet, these animals develop hyperlipidemia and atherosclerotic lesions resembling those seen in early human vascular disease. After 6 weeks of HF diet, en face assays of the aorta showed less atherosclerosis in UCP $apoE^{-/-}$ mice as compared to WT $apoE^{-/-}$ littermates (Figure 3A). After 12 weeks on the same HF diet, lesion extent in UCP mice had increased to the same level as WT littermates (data not shown), indicating that the presence of the UCP transgene delayed but did not abolish disease. UCP $apoE^{-/-}$ and WT $apoE^{-/-}$ mice had similar levels of fasting cholesterol on a chow diet (Figure 3B, 0 weeks) that increased to the same degree in both genotypes after HF feeding (Figure 3B, 3 and 6 weeks), and there was no genotype effect on cholesterol-containing lipoproteins (Figure 3C), suggesting that changes in circulating cholesterol, a major mediator of vascular injury, are not responsible for the delayed development of atherosclerosis in UCP mice.

As with C57BL/6 UCP mice in the survival study, body weight (Figure 3D) was lower in chow-fed UCP $apoE^{-/-}$ animals (Figure 3D, 0 weeks). Weight remained lower with HF feeding (Figure 3D, 1.5, 3, and 6 weeks) in UCP $apoE^{-/-}$ mice, likely because of their increased metabolic rate (determined in a metabolic chamber; Figure S5). There was no difference in fasting glucose between

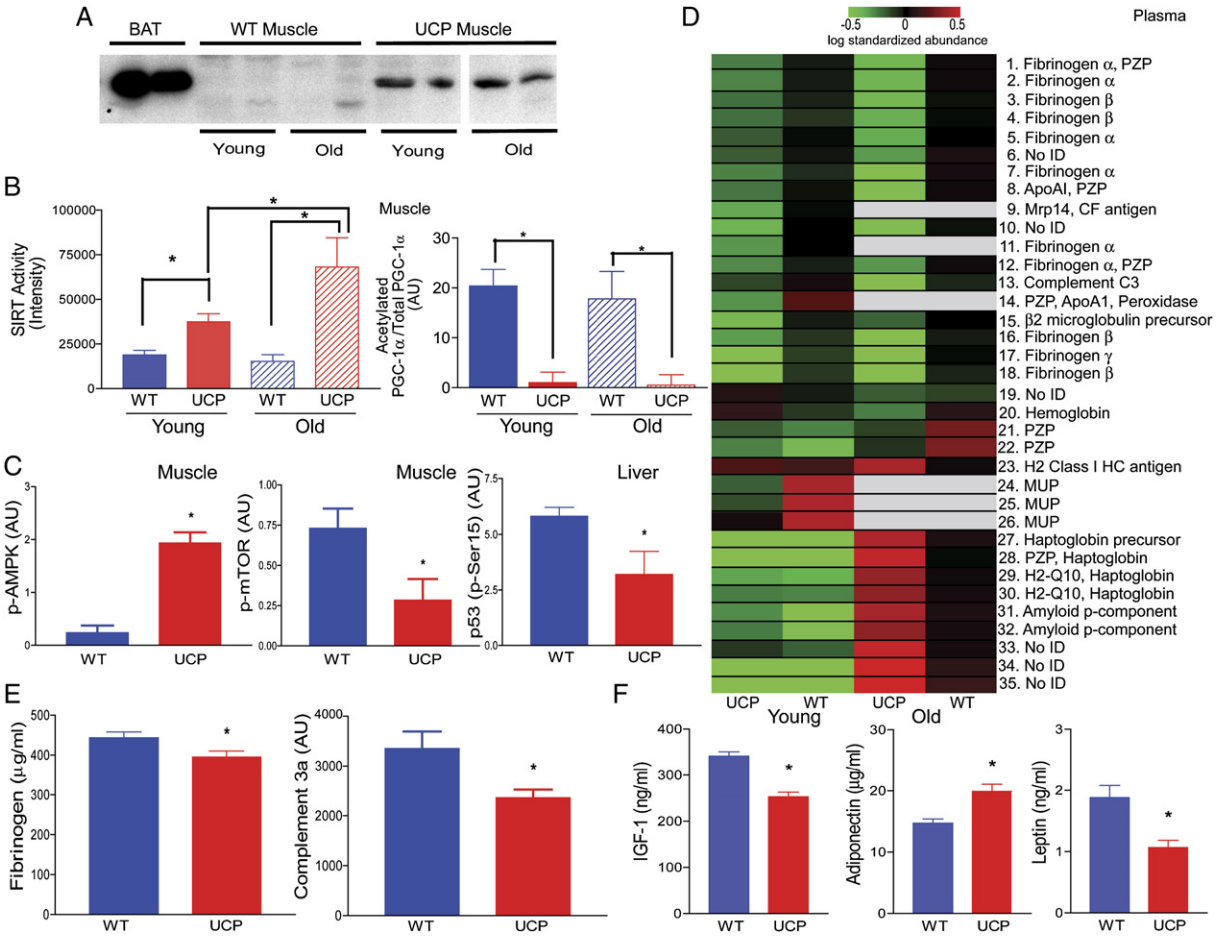

Figure 2. Biochemical Characteristics Associated with Increased Survival in Uncoupled Mice

(A) Levels of UCP1 protein as detected by western blotting. The first two lanes on the left were loaded with protein extracted from brown adipose tissue (BAT). The WT muscle and UCP muscle lanes were loaded with equal amounts of protein extracted from skeletal muscle of two different young (3 months old) and old (>12 months old) mice.

(B) Left panel: SIRT enzyme activity in skeletal muscle from 12 young WT, 12 young UCP, 5 old WT, and 7 old UCP mice. Right panel: acetylated PCG-1α levels normalized to total PCG-1α in 6 young WT, 6 young UCP, 6 old WT, and 6 old UCP mice.

(C) Levels of phosphorylated signaling molecules in skeletal muscle of young WT and UCP mice. Phosphorylated AMPK, mTOR, and p53 levels were determined by western blotting, normalized to total levels of the same proteins, and quantified by densitometry.

(D) Proteomic analysis of plasma from young and old WT and UCP mice. Age- and UCP-induced changes in plasma proteins were identified by two-dimensional difference gel electrophoresis (2D-DIGE) image and multivariate analysis. Proteins were identified using tandem mass spectrometry after in situ gel digestions with trypsin. PZP, pregnancy zone protein; MUP, major urinary protein.

(E) Confirmation of differences for two proteins identified by proteomic analysis. Fibrinogen was assayed in 8 WT and 11 UCP mice (left panel), and complement 3a was assayed in 4 WT and 4 UCP mice (right panel). AU, arbitrary units.

(F) Adiposity-related serum measurements in WT and UCP mice. IGF-1 was assayed in 25 WT and 28 UCP mice (left panel), adiponectin was measured in 25 WT and 30 UCP mice (middle panel), and leptin was assayed in 14 WT and 18 UCP mice (right panel).

Data are presented as mean ± SEM. *$p < 0.05$.

genotypes on a chow diet (Figure 3E, 0 weeks), but fasting glucose was lower (glycemic excursions in glucose tolerance tests were also lower; data not shown) in UCP *apoE*$^{-/-}$ animals fed a HF diet (Figure 3E, 3 and 6 weeks). These metabolically beneficial differences in weight and glucose are associated with decreased insulin resistance, but whether these variables directly impact vascular disease is unknown. Other findings in UCP mice might account for delayed vascular disease. Like the animals in the survival study, UCP *apoE*$^{-/-}$ mice had lower levels of IGF-1 (Figure 3F) and higher levels of adiponectin (Figure 3G), differences that persisted over 6 weeks of HF feeding. IGF-1 levels may predict vascular lesions in humans (Hietaniemi et al., 2005), and transgenic expression of adiponectin decreases atherosclerosis in *apoE*$^{-/-}$ mice (Yamauchi et al., 2003).

As an unbiased approach to identify potential mediators of the delayed vascular disease phenotype of UCP mice, proteomic multivariate analysis was used to identify differentiating plasma proteins in WT *apoE*$^{-/-}$ and UCP *apoE*$^{-/-}$

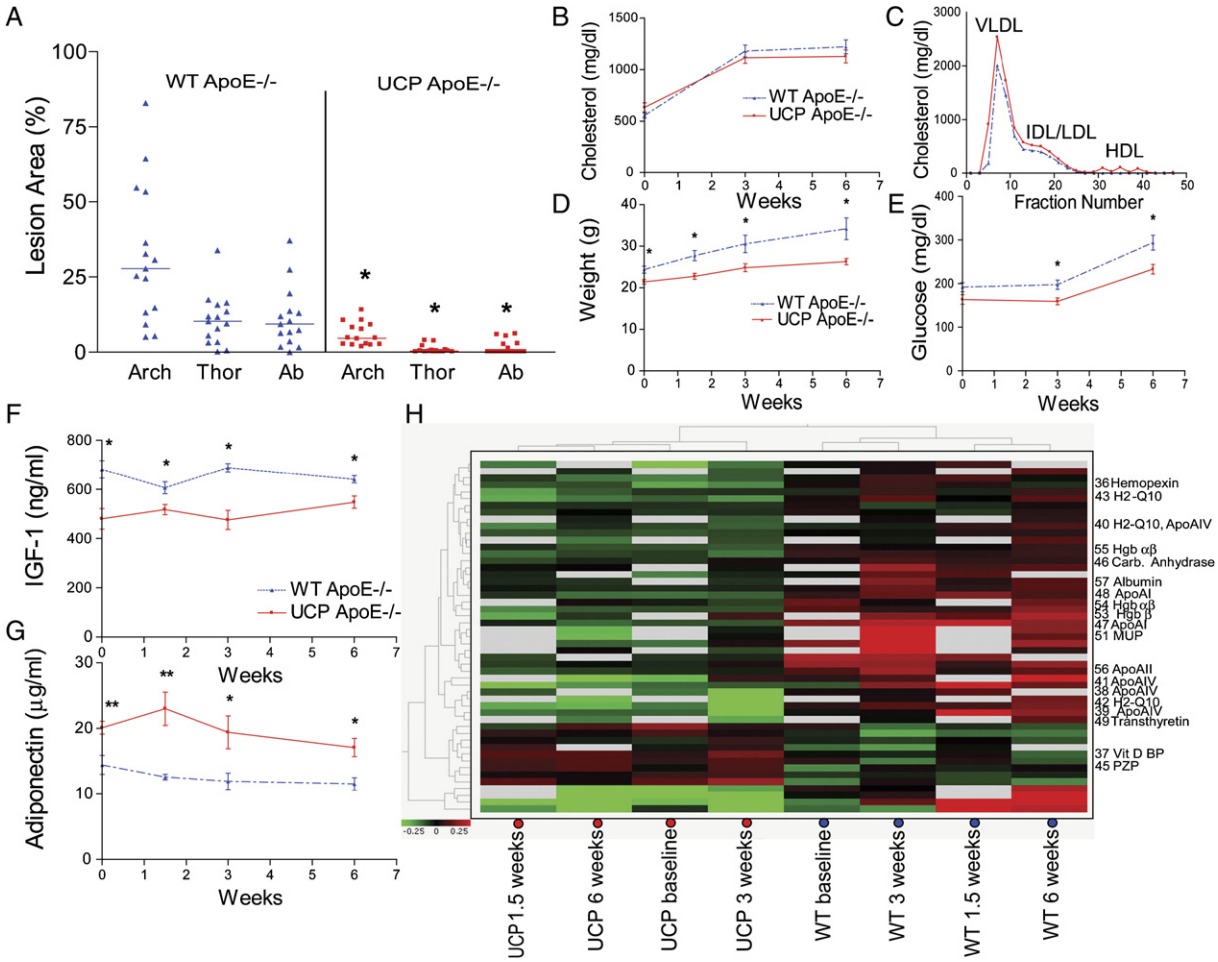

Figure 3. Delayed Diet-Induced Atherosclerosis Is Associated with Respiratory Uncoupling in *apoE* Null Mice

(A) UCP mice on the *apoE* null background were fed a "western-type" high-fat (HF) diet for 6 weeks, and atherosclerotic lesion extent was then determined by en face analysis of the percent atherosclerotic involvement of the intimal surface of the aortic arch, thoracic aorta, and abdominal aorta for 15 WT and 15 UCP littermates.

(B and C) Fasting cholesterol levels over time (B) and lipoprotein profiles at sacrifice (C) for the mice in (A).

(D and E) Body weight (D) and fasting glucose levels (E) over time for the mice in (A).

(F and G) IGF-1 (F) and adiponectin (G) levels for WT *apoE* null and UCP *apoE* null mice on a chow diet (time 0) and over 6 weeks of HF diet.

(H) Multivariate analysis of multiplex 2D-DIGE plasma protein images from WT *apoE* null and UCP *apoE* null mice on a chow diet (baseline) and after 1.5, 3, and 6 weeks of HF diet. Gel features that changed in pairwise comparisons of WT and UCP samples were analyzed using an unsupervised hierarchical clustering algorithm in the Extended Data Analysis (EDA) module of DeCyder (v6.5). Clustering of plasma samples is shown in the top tree, and clusters from the individual proteins are shown on the left.

Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$.

mice at baseline on a chow diet as well as at various time points with HF feeding. An unsupervised hierarchical cluster analysis of the proteins that showed significant differences (in relative abundance; p values are provided in Table S1) for all pairwise comparisons between genotypes is presented in Figure 3H. The majority of these proteins were expressed at lower levels in UCP $apoE^{-/-}$ mice as compared to WT $apoE^{-/-}$ mice, and these differences were amplified with HF feeding. This is best seen by comparing the predominantly red profile (indicating increased protein levels) of WT mice after 6 weeks on HF diet (far right column) with the predominantly green profile (indicating lower protein levels) of UCP mice after the same intervention (second column from the left). Eight of the gel features identified by mass spectrometry in Figure 3H represent components of high-density lipoprotein (HDL) (apoAI, apoAII, apoAIV, and hemopexin), and all were increased in WT as compared to UCP mice. It is difficult to extrapolate cholesterol data from mice to humans, given the striking differences in lipid processing between these species and the fact that the data are being derived from essentially herbivorous animals fed a HF diet. However, HDL-associated proteins have been reported to be increased in humans with vascular disease and present in human atherosclerotic lesions (Vaisar et al., 2007), raising the possibility that certain conditions (such as the chronic inflammation of HF feeding)

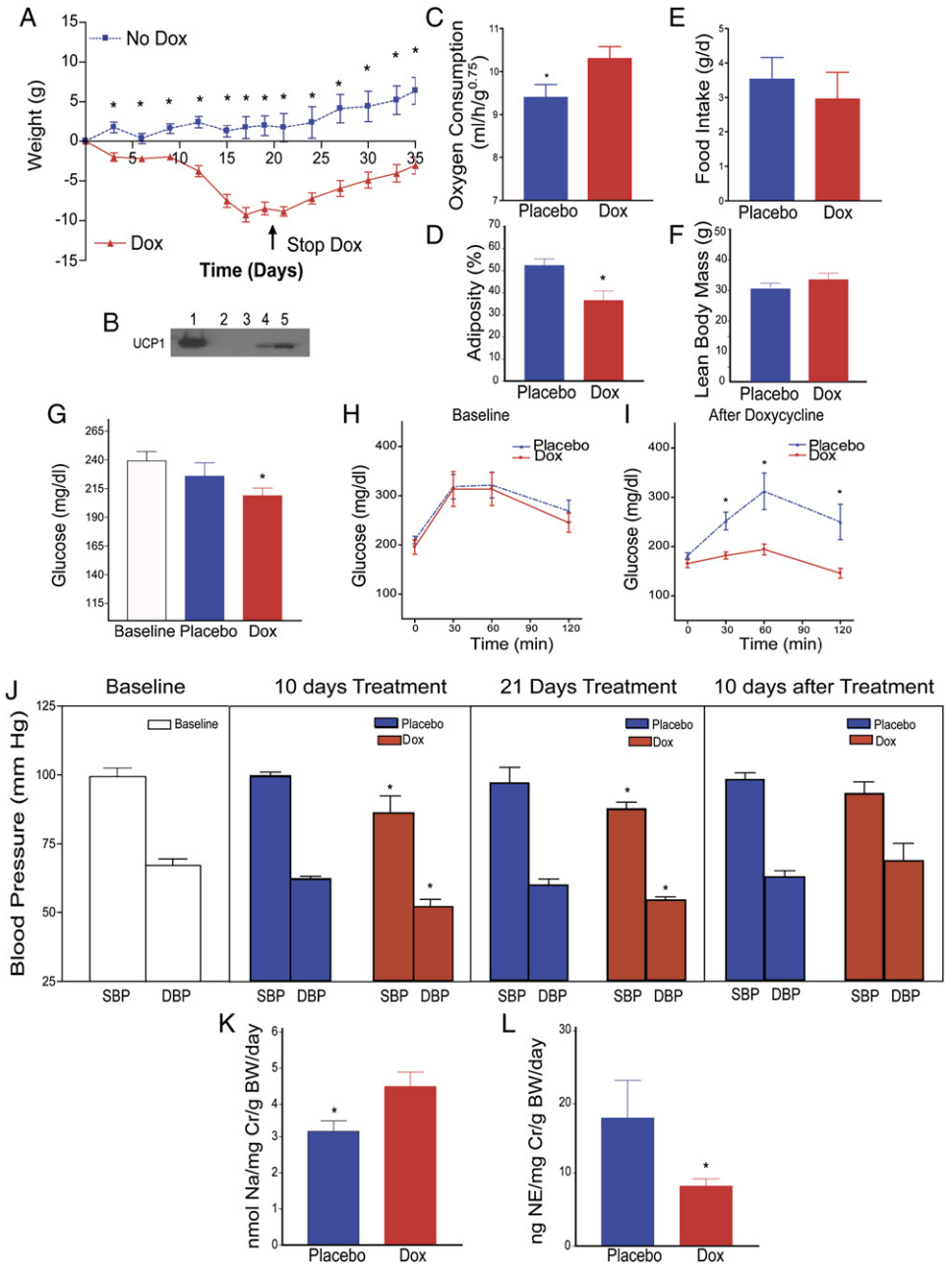

Figure 4. Reversal of Increased Adiposity with Inducible Skeletal Muscle Uncoupling in Agouti Yellow Mice Lowers Blood Glucose and Blood Pressure

(A) A^{y}/a genetically obese mice carrying two transgenes, a *TRE-UCP1* minigene and a cassette consisting of the reverse tetracycline transactivator driven by a modified creatine kinase promoter (*MCK-rtTA*), were implanted with placebo pellets (No Dox) or pellets designed to release doxycycline (Dox) over a period of 21 days. The mean weight of animals before randomization was 56.9 g (time 0). Data are presented as changes in weight relative to baseline weight over time for 26 mice treated with Dox and 14 mice treated with placebo.

(B) Western blot of UCP1 protein in BAT (positive control, lane 1) and skeletal muscle 1 week after implantation of pellets containing placebo (tissues from two separate animals in lanes 2 and 3) or Dox (tissues from two separate animals in lanes 4 and 5).

(C) Metabolic rate following induction of UCP expression in skeletal muscle. *MCK-rtTA-TRE-UCP1* A^{y}/a animals were treated with placebo (7 males) or doxycycline (8 males) using implanted pellets, and oxygen consumption was determined by indirect calorimetry.

(D) Adiposity for 10 Dox and 9 placebo animals at day 18.

(E) Food intake for 4 animals in each group at day 15.

(F) Lean body mass for 10 Dox and 9 placebo animals at day 18.

(G) Fasting plasma glucose at baseline (40 animals) and at days 18–21 for Dox-treated animals (26) and placebo-treated animals (14).

(H and I) Glucose tolerance tests at baseline (5 mice for Dox, 7 for placebo) (H) and at day 17 of treatment (6 mice per condition) (I), with results presented as blood glucose concentrations.

render HDL atherogenic. Our results suggest that respiratory uncoupling in skeletal muscle antagonizes these effects.

Inducible Uncoupling Reverses Established Metabolic Dysfunction

The beneficial effects of respiratory uncoupling on survival, cancer, and atherosclerosis (Figure 1, Figure 2, and Figure 3) were seen in mice engineered to express UCP1 in muscle throughout life. To determine whether it is possible to reverse, as opposed to prevent, age-related disease, we generated mice capable of tetracycline-inducible expression of UCP1 in skeletal muscle using the agouti yellow (A^y/a) model of genetic obesity. Mice in the A^y/a background expressing the reverse tetracycline transactivator (rtTA) driven by a modified muscle creatine kinase promoter were crossed with transgenic mice carrying a *TRE-UCP1* minigene. To induce skeletal muscle UCP1 expression, 5-month-old *MCK-rtTA-TRE-UCP1* A^y/a animals weighing over 50 g were implanted with either doxycycline (Dox) pellets releasing 65 mg of drug over 21 days or placebo pellets. We have previously shown that Dox treatment alone does not affect glucose metabolism or blood pressure in animals not engineered to express UCP (Bernal-Mizrachi et al., 2005). Body weights over the ensuing 35 days are shown in Figure 4A. Weight decreased in Dox-implanted mice but continued to increase in placebo-implanted mice. UCP1 protein was detected in the skeletal muscle of Dox-implanted mice (Figure 4B, lanes 4 and 5) and in brown adipose tissue as a positive control (Figure 4B, lane 1), but not in the skeletal muscle of placebo-implanted mice (Figure 4B, lanes 2 and 3). Induction of UCP1 in skeletal muscle increased metabolic rate (Figure 4C) and decreased adiposity (Figure 4D) but had no effect on food intake (Figure 4E) or lean body weight (Figure 4F). This reduction in adiposity was associated with and likely related to decreased fasting blood glucose (Figure 4G). Before treatment, both groups were glucose intolerant (Figure 4H), a condition that was reversed by Dox induction of UCP1 (Figure 4I).

UCP1 induction in skeletal muscle by Dox treatment also lowered blood pressure at two different time points (Figure 4J), an effect that was reversed when treatment was discontinued (Figure 4J, rightmost panel). Increased adiposity is associated with sympathetic nervous system activation and increased renal reabsorption of sodium leading to hypertension. UCP1 induction increased urinary sodium excretion (Figure 4K), likely by decreasing sympathetic nervous system activation (reflected by decreased excretion of norepinephrine; Figure 4L). These results in a genetic obesity model suggest that respiratory uncoupling in skeletal muscle can reverse, and not simply prevent, abnormal glucose metabolism and blood pressure.

Precedents for Beneficial Effects of Accelerating Metabolism in Humans

Vulnerability to age-related disease may be governed in part by pathways that respond to environmental deprivation such as caloric restriction. Respiratory uncoupling in muscle mimics nutritional deprivation as manifested by increased SIRT activity and activation of AMPK in muscle, decreased mTOR in muscle, decreased adipose tissue mass, reduced serum IGF-1, and increased serum adiponectin. Age-related diseases are characterized by chronic inflammation, and adipose tissue recruits inflammatory cells (Lumeng et al., 2007). In our mice, decreasing adiposity and inflammation by accelerating metabolism in skeletal muscle delayed death and diseases including malignancy, atherosclerosis, diabetes, and hypertension. Our survival data, showing an increase in average life span and no difference in maximal longevity, resemble the results of exercise studies by Holloszy and colleagues in male and female rats (Holloszy et al., 1985; Holloszy, 1993). Our results are also consistent with observational studies showing that increased energy expenditure, reflecting elevated skeletal muscle metabolism required for physical activity, is associated with decreased mortality in healthy older humans (Manini et al., 2006). Respiratory uncoupling specifically in skeletal muscle may be a particularly beneficial approach to increasing metabolism. Expression of UCP3, a UCP1 homolog found in skeletal muscle, predicts diabetes in humans (Gable et al., 2006; Schrauwen et al., 2006). Low levels of respiratory uncoupling, analogous to those studied in our mice, preserve mitochondrial function in aging human muscles (Amara et al., 2007).

The consequences of excess adiposity disproportionately affect older individuals. Excess adiposity can be treated through two simple approaches, decreasing energy intake or increasing energy consumption. Considerable effort is currently being devoted to the development of agents that decrease energy intake in hopes of decreasing adiposity and perhaps age-related disease. Our results indicate that increasing energy consumption in mice has beneficial effects on survival, vascular disease, elevated blood pressure, and diabetes. This intervention does not slow aging but may diminish susceptibility to pathology. Strategies to safely accelerate energy consumption specifically in skeletal muscle could decrease the impact of some common age-related diseases.

(J) Blood pressure at baseline (12 mice) and at varying time points during and after treatment (6 mice in each group). SBP, systolic blood pressure; DBP, diastolic blood pressure.
(K and L) Urinary sodium excretion (24 mice for Dox, 12 for placebo) (K) and urinary norepinephrine excretion (25 mice for Dox, 12 for placebo) (L). Data were derived from urine collections obtained over 3 days beginning at day 18 of treatment.
Data are presented as mean ± SEM. *$p < 0.05$.

EXPERIMENTAL PROCEDURES

Animals

Mice with ectopic expression of UCP1 limited to skeletal muscle were generated by injecting C57BL/6 × CBA hybrid embryos as described previously (Li et al., 2000). Transgenic animals with low-level protein expression (~1% of brown fat) were backcrossed with inbred C57BL/6 mice five times to yield animals used for the longevity experiments. The same strategy was followed with *apoE* null mice on the C57BL/6 background for the atherosclerosis experiments. Since the animals used for the longevity and atherosclerosis experiments were not on a homogeneous BL/6 background, all studies were performed with littermates to decrease potential confounding effects of genetic background. For the longevity studies, animals were weaned to mouse chow at 3 weeks of age and group housed as described in other survival studies (Holzenberger et al., 2003). For studies involving weight reduction, mice transgenic for a tetracycline-responsive element (*TRE*) driving *UCP1* gene expression (*TRE-UCP1*) (Bernal-Mizrachi et al., 2005) were bred with A^y/a mice. Mice expressing the reverse tetracycline transactivator (rtTA) controlled by a modified muscle creatine kinase promoter (*1256[3Emut]MCK*) (Grill et al., 2003) were also bred with A^y/a mice. Appropriate progeny from these two crosses were mated to generate doubly transgenic animals in the A^y/a model.

SIRT Activity and PGC-1α Acetylation Assays

Measurements were performed using a histone deacetylase assay kit (Upstate) according to the manufacturer's instructions. SIRT activity was determined as the NAD-dependent and nicotinamide-inhibitable ability of tissue extracts to deacetylate a fluorometric substrate. Released substrate was detected using a fluorescence plate reader. Each experiment included an appropriate standard curve, and all measurements were recorded within the linear response range of the assay.

PGC-1α acetylation status was determined as described previously (Rodgers et al., 2005). PGC-1α was immunoprecipitated from muscle protein lysate using H-300 antibody (Santa Cruz), and these immunoprecipitates were then western blotted using an anti-acetyl lysine antibody (Cell Signaling Technology) to detect acetylated PGC-1α. Total PGC-1α in separate aliquots of these lysates was detected by western blotting using H-300 antibody.

Protein Analyses

Samples for western blotting were resolved on 10%, 12%, or 4%–15% SDS-polyacrylamide gels and transferred to nitrocellulose. Tissue handling and ELISA details, antibody sources, and proteomics protocols, including two-dimensional difference gel electrophoresis (2D-DIGE) and gel feature multivariate analyses, are listed in Supplemental Experimental Procedures.

Atherosclerosis and Analytical Procedures

Atherosclerosis was measured using the en face technique (Semenkovich et al., 1998). Blood pressure was measured as described previously (Bernal-Mizrachi et al., 2005). Other analytical procedures are listed in Supplemental Experimental Procedures.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, five figures, and one table and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/6/6/497/DC1/.

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