

# UCP3 Regulates Cardiac Efficiency and Mitochondrial Coupling in High Fat–Fed Mice but Not in Leptin-Deficient Mice

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These studies investigate the role of uncoupling protein 3 (UCP3) in cardiac energy metabolism, cardiac O<sub>2</sub> consumption (MVO<sub>2</sub>), cardiac efficiency (CE), and mitochondrial uncoupling in high fat (HF)–fed or leptin-deficient mice. UCP3KO and wild-type (WT) mice were fed normal chow or HF diets for 10 weeks. Substrate utilization rates, MVO<sub>2</sub>, CE, and mitochondrial uncoupling were measured in perfused working hearts and saponin-permeabilized cardiac fibers, respectively. Similar analyses were performed in hearts of *ob/ob* mice lacking UCP3 (U3OB mice). HF increased cardiac UCP3 protein. However, fatty acid (FA) oxidation rates were similarly increased by HF diet in WT and UCP3KO mice. By contrast, MVO<sub>2</sub> increased in WT, but not in UCP3KO with HF, leading to increased CE in UCP3KO mice. Consistent with increased CE, mitochondrial coupling was increased in the hearts of HF-fed UCP3KO mice. Unexpectedly, UCP3 deletion in *ob/ob* mice reduced FA oxidation but had no effect on MVO<sub>2</sub> or CE. In addition, FA-induced mitochondrial uncoupling was similarly enhanced in U3OB compared with *ob/ob* hearts and was associated with elevated mitochondrial thioesterase-1 protein content. These studies show that although UCP3 may mediate mitochondrial uncoupling and reduced CE after HF feeding, it does not mediate uncoupling in leptin-deficient states. *Diabetes* 61:3260–3269, 2012

**O**besity impacts cardiac structure and function, hemodynamic load, and left ventricular remodeling and is an independent risk factor for heart failure (1,2). Obesity also increases the risk for hypertension, diabetes, and dyslipidemia, contributing to increased risk of myocardial ischemia and heart failure. Obesity is also associated with increased cardiac lipid accumulation (3,4), which correlates with diastolic dysfunction (5–7).

In addition, altered substrate metabolism and mitochondrial bioenergetics have also been implicated in the development of cardiac dysfunction in obesity. Both obese humans and animals exhibit increased myocardial fatty acid (FA) utilization, increased myocardial O<sub>2</sub> consumption (MVO<sub>2</sub>), and reduced cardiac efficiency (CE) (8–10). The maintenance of CE is essential for cardiac function under conditions of limited substrate or oxygen availability, particularly when the heart relies on a less efficient

substrate such as FAs. Thus, the diabetic heart, which uses predominantly FAs because glucose utilization is compromised, is less efficient, which compromises its tolerance to ischemia.

Several mechanisms have been proposed to explain this increase in MVO<sub>2</sub>, such as increased mitochondrial uncoupling (11) and increased O<sub>2</sub> cost for noncontractile functions of the heart such as basal metabolism and excitation-contraction coupling (10,12). Increased cardiac mitochondrial uncoupling has been confirmed in rodent models of genetic obesity (13,14) and diet-induced obesity (15) and is believed to be mediated by uncoupling proteins 2 and 3 (UCP2 and UCP3), mitochondrial thioesterase-1 (MTE-1), and the adenine nucleotide translocator (ANT). Indeed, induction of UCP3 and MTE-1 proteins correlates with increased circulating free FAs (FFAs) in patients undergoing coronary artery bypass surgery and in high fat (HF)–fed rats (15,16), which is consistent with UCP3 and MTE-1 being peroxisome proliferator–activated receptor- $\alpha$ –regulated targets. In contrast to HF feeding, UCP2 and UCP3 protein content is either unchanged or decreased in genetic models of obesity, such as *ob/ob* and *db/db* mice (11,14,17), despite increased mitochondrial uncoupling, suggesting the existence of other potential mechanisms that mediate this uncoupling. One possibility is increased reactive oxygen species (ROS)–mediated activation of these proteins (17) or the involvement of other proton-conducting proteins in the mitochondria.

Whereas these correlative studies have suggested a role for UCP3 in mitochondrial uncoupling, mediated by HF diet or obesity, direct evidence of its involvement has been missing. Thus, we used mice with whole-body deletion of UCP3 to investigate its role in substrate metabolism, O<sub>2</sub> consumption, CE, and mitochondrial uncoupling after HF feeding or in *ob/ob* mice. We show that UCP3 deletion in the heart of HF-fed mice reduced MVO<sub>2</sub> and increased CE without influencing FAs or glucose oxidation rates. Consistent with increased CE, mitochondrial coupling, as evidenced by increased ATP/oxygen consumption (O) ratios, was preserved in HF-fed UCP3KO mice. By contrast, mitochondrial uncoupling persisted in UCP3-deficient *ob/ob* mice and was associated with reduced CE and enhanced MTE-1 protein expression. These results confirm a role for UCP3 in HF-induced mitochondrial uncoupling and exclude its involvement in the regulation of CE in leptin-deficient mice.

## RESEARCH DESIGN AND METHODS

**Experimental animals and diets.** The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication no. 85-23, revised 1996) and was approved by the Institutional Animal Care and Use Committee of the University of Utah. Male

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Received 17 January 2012 and accepted 21 May 2012.

DOI: 10.2337/db12-0063

This article contains Supplementary Data online at <http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db12-0063/-/DC1>.

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UCP3 knockout mice (a gift from Bradford B. Lowell, Beth Israel Deaconess Medical Center, Boston, MA) and wild-type (WT) mice on a mixed background were fed an HF or normal chow (NC) diet for 10 weeks starting at 10 weeks of age. The HF diet contained 45% fat (Research Diets, Inc., New Brunswick, NJ) and the NC contained 4.5% fat (Purina Laboratories, Framingham, MA). A detailed description of the composition of the diets used in this study can be found in Supplementary Table 1.

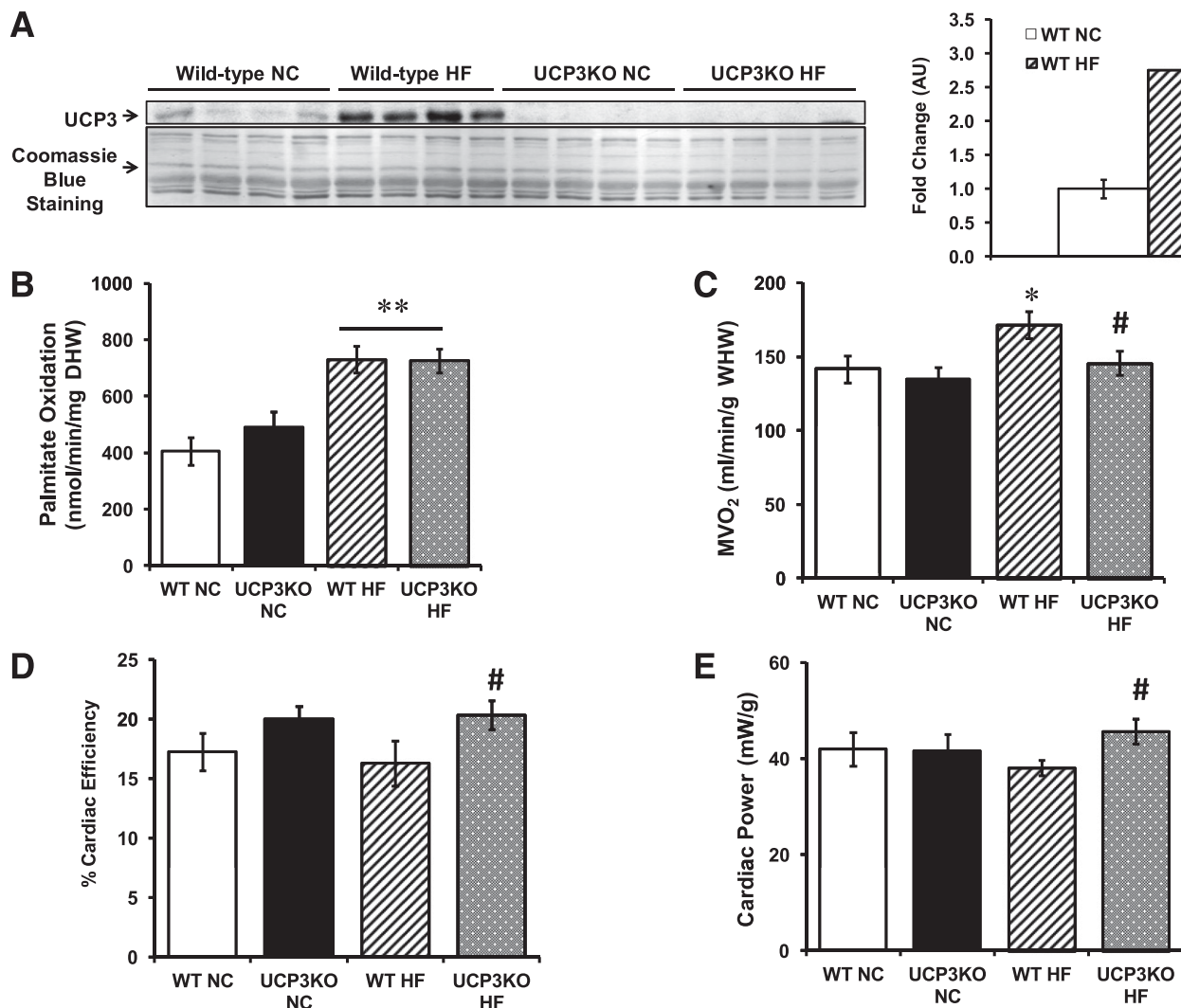
UCP3KO mice were back-crossed to C57BL/6J background for 10 generations before they were crossed with B6.*ob/ob*<sup>+</sup> mice that were purchased from The Jackson Laboratory (Bar Harbor, ME). Double-mutant *ob/ob.UCP3<sup>-/-</sup>* mice (referred to as U3OB) were generated at the University of Utah from an *ob/ob.UCP3<sup>-/-</sup>* intercross. Because of the low efficiency of breeding, both males and females were used for the U3OB studies.

**Determination of cardiac substrate utilization and MVO<sub>2</sub>.** Palmitate oxidation, glucose oxidation, glycolysis, cardiac power, MVO<sub>2</sub>, and CE were determined using isolated working heart preparations as described before (9). Glycolytic flux was determined by measuring the amount of <sup>3</sup>H<sub>2</sub>O released from the metabolism of exogenous [<sup>5-<sup>3</sup>H</sup>]glucose, glucose oxidation was determined by trapping and measuring <sup>14</sup>CO<sub>2</sub> released by the metabolism of [U-<sup>14</sup>C]glucose, and palmitate oxidation was determined in a separate set of perfused hearts by measuring the amount of <sup>3</sup>H<sub>2</sub>O released from [9,10-<sup>3</sup>H] palmitate. MVO<sub>2</sub> was measured at 20-min intervals in hearts that were used for

the determination of palmitate oxidation using a fiber-optic oxygen sensor (Ocean Optics, Orlando, FL).

**Mitochondrial respiration measurements.** Mitochondrial function was studied using the saponin-permeabilized cardiac fiber technique as previously described (18). In brief, V<sub>o</sub> (state 2) respiration was measured after addition of either 20 μmol/L palmitoyl-carnitine with 2 mmol/L malate or 5 mmol/L glutamate and 2 mmol/L malate. V<sub>ADP</sub> (state 3) respiration was assessed by adding 1 mmol/L ADP, and V<sub>oligo</sub> (state 4) respiration was measured after addition of 1 μg/mL oligomycin (ATP synthase inhibitor). Mitochondrial ATP measurements were performed by bioluminescence assay using the ENLITEN ATP assay kit (Promega, Madison, WI). ATP/O ratio was calculated as ATP/state 3 respiration. All the mitochondrial respirations and ATP determinations were performed at 25°C.

**Western blot analysis.** Total proteins were extracted from frozen heart muscle, and protein concentrations were determined by using Micro BCA reagents (Pierce, Rockford, IL). Proteins were resolved by SDS-PAGE and electrotransferred onto a polyvinylidene fluoride membrane (Millipore Corp., Bedford, MA). The following antibodies were used: rabbit anti-UCP3 (1:500; Affinity Bioreagents, Golden, CO), rabbit anti-4-HNE-Michael adducts (1:10,000; EMD Chemicals Inc., Gibbstown, NJ), polyclonal rabbit anti-MTE-1 (1:2,000; a gift of Dr. Stefan Alexson, Karolinska Institute, Stockholm, Sweden), mouse anti-MnSOD (1:2,000; BD Biosciences, San Jose, CA), rabbit



**FIG. 1.** UCP3 deletion does not alter cardiac FA oxidation but increases CE during HF feeding. **A:** Representative Western blot of cardiac UCP3 protein content and the corresponding densitometry normalized to Coomassie Blue staining in WT and UCP3 knockout mice fed NC or HF diet for 10 weeks.  $n = 4$  per genotype and per feeding condition. **B:** Palmitate oxidation. **C:** MVO<sub>2</sub>. **D:** Percent CE. **E:** Cardiac power in the hearts of WT and UCP3 knockout mice fed NC or HF for 10 weeks.  $n = 6$  per genotype for NC and  $n = 7-8$  per genotype for HF. Data are means  $\pm$  SEM. An unpaired, two-tailed Student *t* test was used to compare the groups in **A**. **B–E:** A two-way ANOVA was performed to analyze differences by diet and by genotype, including Bonferroni post hoc test, when significant interaction occurred. \*\* $P < 0.005$  vs. diet (NC vs. HF); # $P < 0.05$  UCP3KOHF vs. WT HF. AU, arbitrary units; WHW, wet heart weight; DHW, dry heart weight. \* $P < 0.05$  vs. diet (NC vs. HF).

polyclonal anti-ANT1 serum (1:2,000; provided by Douglas Wallace, University of California, Irvine, CA), and mouse anti-complex I (the 39-kDa protein), anti-complex II (the 30-kDa subunit), anti-complex III (Core I), anti-complex IV (COXIV), and anti-complex V ( $\alpha$ -subunit) (1:2,000; Invitrogen, Carlsbad, CA). For loading controls, mouse anti- $\alpha$ -tubulin (1:1,000; Sigma-Aldrich, St. Louis, MO) or Coomassie Blue R-250 (Bio-Rad, Hercules, CA) staining were used. Protein detection was carried out with the appropriate horseradish peroxidase-conjugated secondary antibody and ECL or ECL Plus detection systems (Amersham Biosciences, Piscataway, NJ).

**Oxidative stress markers.** ROS levels were measured in heart homogenates by the conversion of nonfluorescent 2',7'-dichlorodihydrofluorescein diacetate to the fluorescent 2',7'-dichlorofluorescein in the presence of cellular esterases and endogenous ROS with modifications as described previously (19).

**Glucose tolerance tests, serum metabolites, and insulin levels.** Glucose tolerance tests were performed after a 6-h fast as previously described (20). Blood glucose was determined using a glucometer (Glucometer Elite; Bayer, Tarrytown, NY). Insulin concentrations were determined using the sensitive rat insulin RIA kit (Linco Research Inc., St. Charles, MO). FFA concentrations were determined using the 1/2-micro FA test kit (Roche Diagnostics, Mannheim, Germany), and triglyceride (TG) concentrations were determined using the L-type TG H kit (Wako, Richmond, VA).

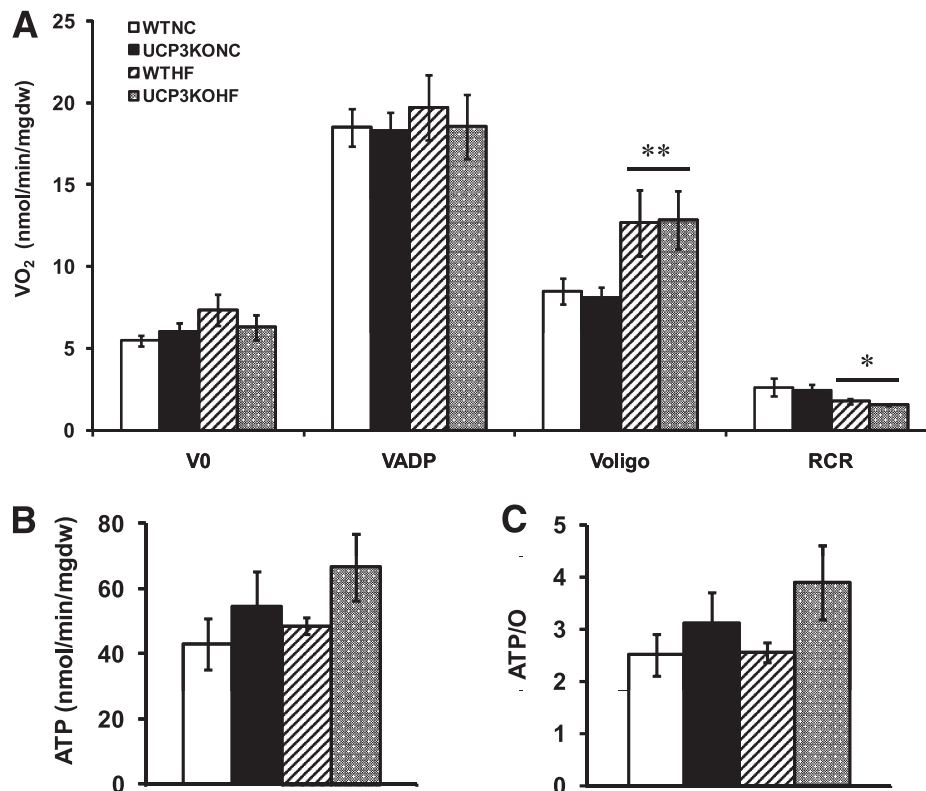
**Statistical analysis.** All data are expressed as means  $\pm$  SEM. The significance of differences was determined by the use of an unpaired, two-tailed Student *t* test when only two groups were compared, a two-way ANOVA followed by a Bonferroni post hoc analysis when significant interaction occurred (performed for the HF study since two variables were considered: genotype and diet), or a one-way ANOVA followed by a Student *t* test when four independent genotypes were compared (performed for the U3OB study).

## RESULTS

**The effect of UCP3 deficiency on body weights, heart weights, and glucose and insulin levels.** Body weights increased similarly in WT and UCP3KO on HF diet

(Supplementary Fig. 1A). Heart weights slightly but significantly increased with HF feeding in WT and UCP3KO (Supplementary Fig. 1B). Heart weight/body weight or ratios were similarly reduced in WT and UCP3KO on HF (Supplementary Fig. 1C), whereas no differences were observed for ratios of heart weight to tibia length between the groups (Supplementary Fig. 1D). Furthermore, WT and UCP3KO were glucose intolerant after 10 weeks of HF feeding. Their glucose levels were indistinguishable under NC conditions and increased similarly after HF feeding (21). In addition, insulin levels for WT and UCP3KO on HF were similar (Supplementary Fig. 2), suggesting no obvious alterations in glucose tolerance and insulin levels resulting from ubiquitous UCP3 deletion.

**UCP3 deficiency reduces O<sub>2</sub> consumption and increases CE during HF feeding without changing FA oxidation.** To investigate the role of UCP3 in the regulation of cardiac FA oxidation, O<sub>2</sub> consumption, and CE after HF feeding, male WT and UCP3KO mice (on a mixed background) were fed an HF diet (45% calories from fat) for 10 weeks, after which these parameters were examined in isolated working hearts. As illustrated in Fig. 1A, and consistent with previous reports (22), HF feeding resulted in a significant 2.5-fold increase in cardiac UCP3 protein in WT hearts, whereas no UCP3 expression was detected in UCP3KO hearts. Furthermore, HF feeding resulted in a significant ( $P < 0.005$ ) increase in palmitate oxidation that persisted in UCP3-deficient hearts (Fig. 1B). Increased cardiac palmitate oxidation in HF-fed WT mice was associated with a significant increase in MVO<sub>2</sub>, which



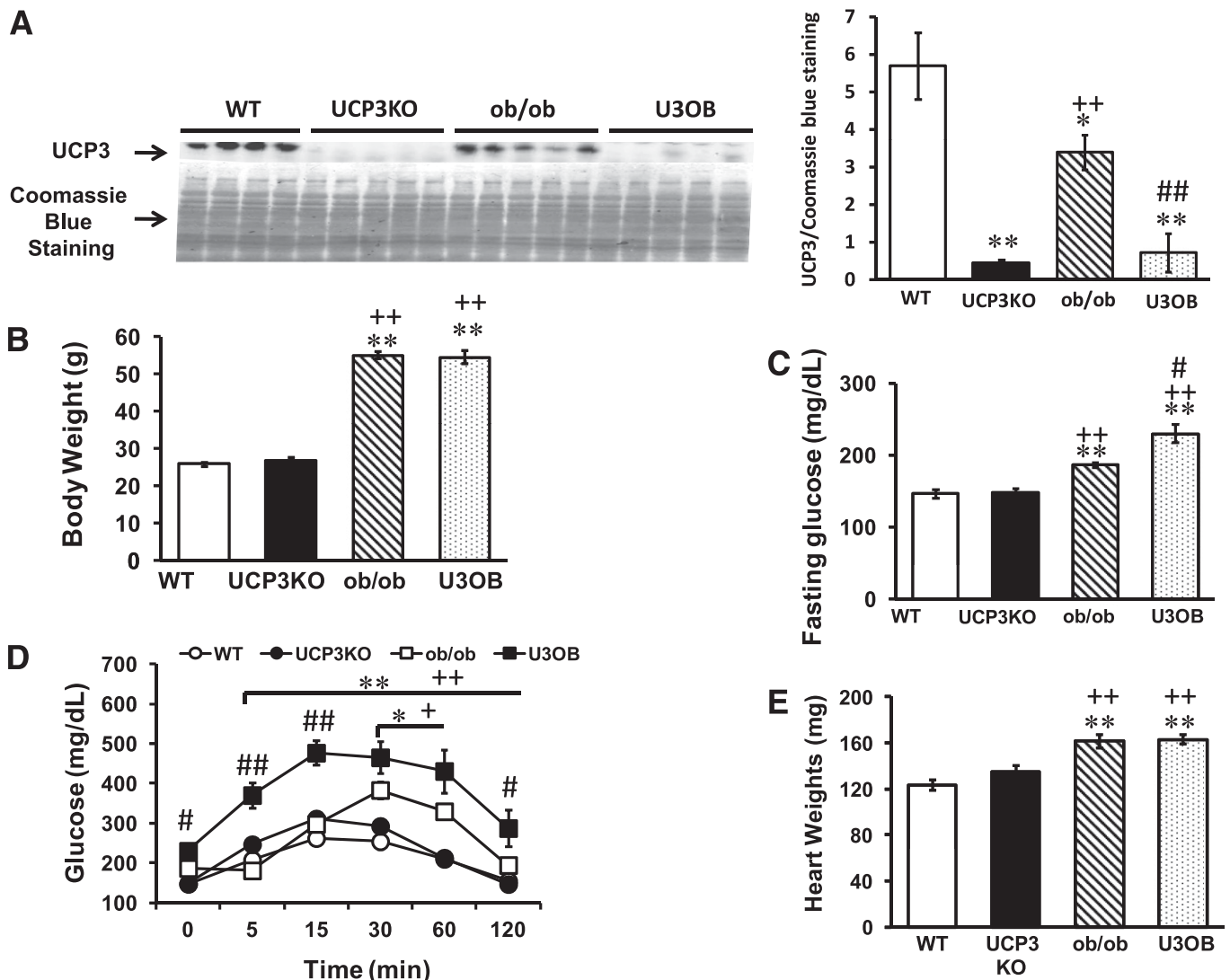
**FIG. 2.** UCP3 deletion in the heart increases mitochondrial ATP/O ratios upon HF feeding. **A:** Mitochondrial respiration. **B:** ATP synthesis rate. **C:** ATP/O ratios in saponin-permeabilized cardiac fibers using palmitoyl-carnitine as substrate. Mice were fed NC or HF diet for 10 weeks, and hearts were not preperfused.  $n = 5$  per genotype for NC and  $n = 5-6$  per genotype for HF. Data are means  $\pm$  SEM. Two-way ANOVA was performed to analyze differences between diet and genotype, followed by Bonferroni post hoc test when significant interaction occurred. \* $P < 0.05$ ; \*\* $P < 0.005$  for diet (NC vs. HF). mgdw, milligrams dry weight.

was not present in UCP3KO mice on HF (Fig. 1C), leading to a significant increase in CE in UCP3KO mice on HF (Fig. 1D). Cardiac power was significantly enhanced ( $P < 0.05$ ) in UCP3KO on HF when compared with WT on HF (Fig. 1E), suggesting that UCP3KO mice on HF are more efficient in using the energy derived from the higher FA oxidation when compared with WT mice fed the same diet.

**Increased mitochondrial coupling despite higher  $V_{\text{oligo}}$  respiration and reduced respiratory control ratios in UCP3KO on HF.** We then investigated if increased CE in UCP3KO mice was associated with increased mitochondrial coupling. In saponin-permeabilized cardiac fibers using palmitoyl-carnitine as substrate, ADP-stimulated maximal respiration ( $V_{\text{ADP}}$ ) was unchanged in all groups (Fig. 2A) but ATP synthesis and ATP/O ratios trended higher in UCP3KO mice on HF (Fig. 2B and C). Interestingly, the maintenance of higher ATP and ATP/O ratios in UCP3KO on HF occurred despite increased state 4 respiration ( $V_{\text{oligo}}$ ) and diminished RCR (Fig. 2A). In fact,

HF diet induced similar increases in  $V_{\text{oligo}}$  and reduction in respiratory control ratio (RCR) in both WT and UCP3KO. To investigate the cause for increased  $V_{\text{oligo}}$  in WT and UCP3KO mice on HF, we measured UCP2 mRNA expression. The level of UCP2 mRNA was the same between the groups (data not shown). In addition and because of their involvement in proton and FA cycling, we measured the protein content of ANT and MTE-1 in WT and UCP3KO mice fed NC or HF diet for 10 weeks. As shown in Supplementary Fig. 3, ANT expression was similarly diminished in WT and UCP3KO by HF feeding, whereas MTE-1 protein content was increased by HF feeding in both genotypes (Supplementary Fig. 3). It is worth noting that MTE-1 levels were significantly reduced in UCP3KO on NC when compared with WT fed the same diet.

**UCP3 deletion in *ob/ob* mice did not affect body weights but exacerbated glucose intolerance.** To investigate the role of UCP3 in leptin-deficient mice, we crossed UCP3KO to *ob/ob* mice to obtain U3OB mice



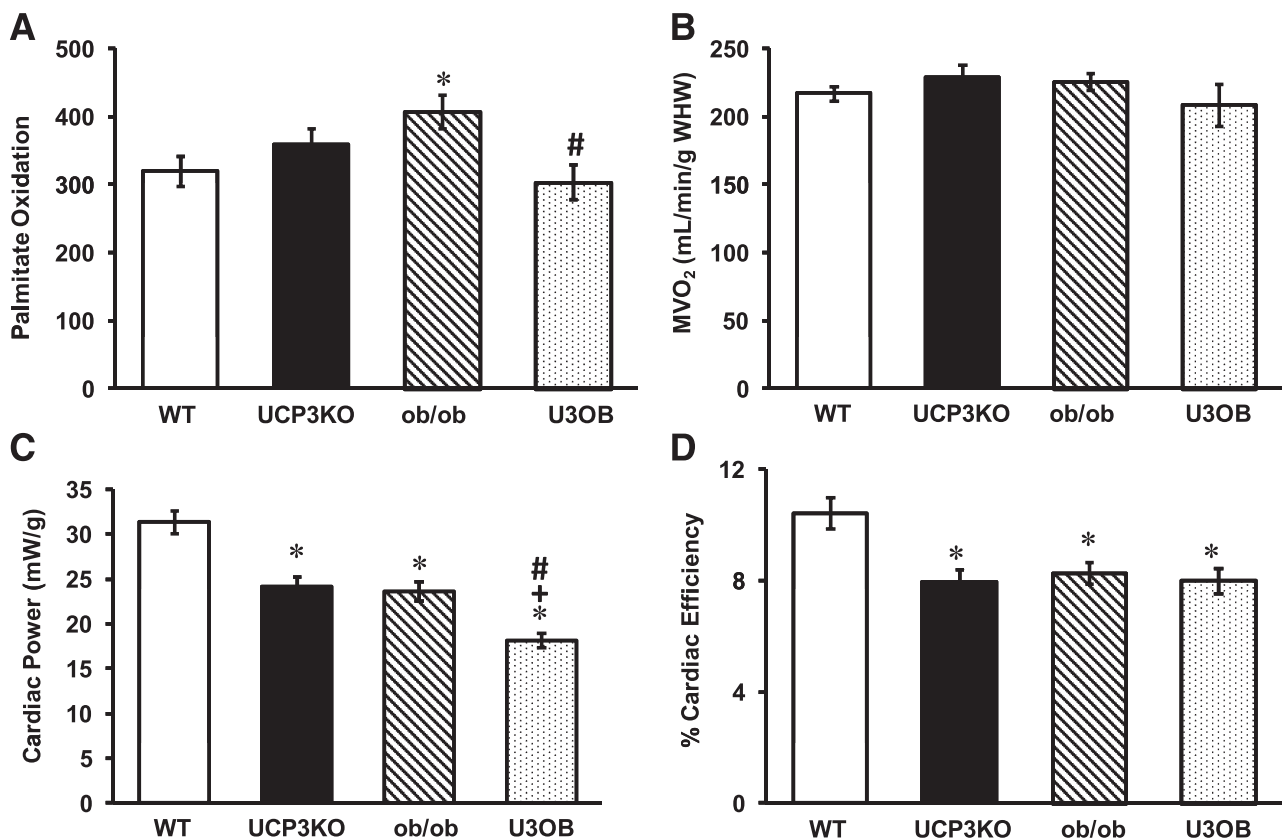
**FIG. 3.** Impact of UCP3 deletion in *ob/ob* mice on body weight, heart weight, and glucose homeostasis. **A:** Representative immunoblot and the corresponding densitometry of UCP3 protein normalized to Coomassie Blue staining. **B:** Body weights. **C:** Fasting blood glucose levels. **D:** Glucose tolerance tests. **E:** Heart weights of WT, UCP3KO, *ob/ob*, and U3OB mice at 20 weeks of age.  $n = 4-5$  per group for Western blots.  $n = 8$  for WT,  $n = 13$  for UCP3KO,  $n = 6-9$  for *ob/ob*, and  $n = 6-8$  for U3OB for all other measurements. \* $P < 0.05$ ; \*\* $P < 0.005$  vs. WT; + $P < 0.05$ ; ++ $P < 0.005$  vs. UCP3KO; # $P < 0.05$ ; ## $P < 0.005$  vs. *ob/ob*.

(*ob/ob.UCP3<sup>-/-</sup>*). We confirmed the absence of UCP3 protein in U3OB hearts by Western blot as shown in Fig. 3A. At 20 weeks of age, body weights were significantly elevated in *ob/ob* and U3OB mice (Fig. 3B). In contrast, fasting glucose levels, which were elevated in *ob/ob* mice compared with WT and UCP3KO mice, were further increased in U3OB mice (Fig. 3C). Furthermore, U3OB mice were more glucose intolerant when compared with all other groups (Fig. 3D). Consistent with the U3OB mice being more glucose intolerant, these mice also exhibited impaired insulin secretion in response to glucose, as shown by lower insulin levels 30 min after glucose injection when compared with *ob/ob* mice, despite similar baseline insulin levels (Supplementary Fig. 4A). Lastly, serum FFAs were increased similarly in *ob/ob* and U3OB mice (Supplementary Fig. 4B), but serum TGs were the same across all genotypes (Supplementary Fig. 4C).

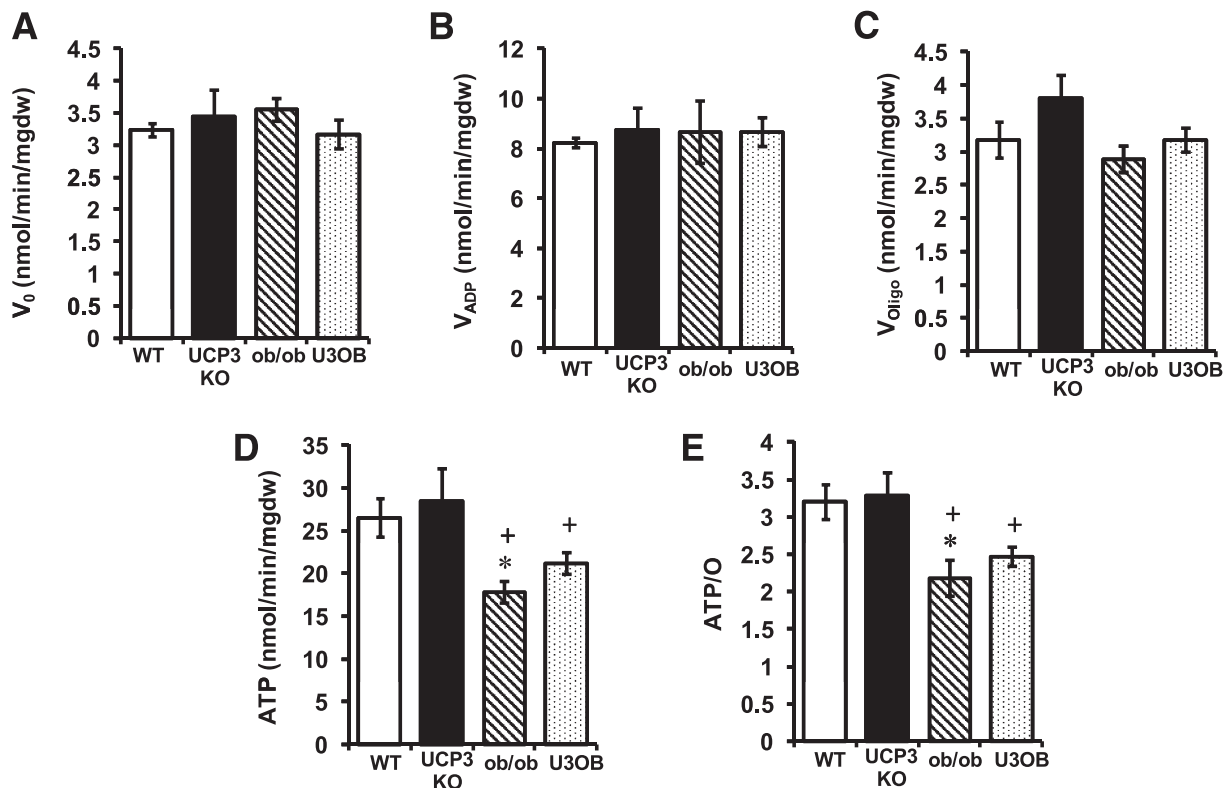
**UCP3 deletion in *ob/ob* mice reduced palmitate oxidation without changing MVO<sub>2</sub> or CE.** In accordance with a previous report (14), we observed a significant increase in heart weights in *ob/ob* and U3OB mice compared with WT and UCP3KO mice (Fig. 3E). Because we previously showed that FA-induced mitochondrial uncoupling was in part responsible for reduced CE in *ob/ob* and *db/db* mice (11,17), we hypothesized that deletion of UCP3 protein in *ob/ob* hearts would reduce cardiac O<sub>2</sub> consumption and enhance CE. Palmitate oxidation, which was higher in *ob/ob* hearts, was significantly reduced ( $P < 0.05$ ) in U3OB hearts (Fig. 4A). However, no differences were observed between groups for MVO<sub>2</sub> at 20 weeks of

age (Fig. 4B). Because cardiac power was reduced in all groups relative to WT (Fig. 4C), CE was significantly lower in UCP3KO, *ob/ob*, and U3OB relative to WT mice (Fig. 4D).

**Absence of UCP3 impairs cardiac function in leptin-deficient mice.** To assess the role of UCP3 in cardiac function of leptin-deficient *ob/ob* mice, we measured cardiac function ex vivo in perfused working hearts and in vivo using echocardiography. Cardiac power was similarly reduced in UCP3KO and *ob/ob* mice but further decreased in the U3OB mice (Fig. 4C). Similarly, a number of cardiac parameters were altered in U3OB when compared with *ob/ob*, such as reduced heart rate and left ventricular dilatation and cardiac hypertrophy (Supplementary Table 2). **UCP3 deletion in *ob/ob* hearts does not reverse FA-induced mitochondrial uncoupling.** We have previously shown that *ob/ob* and *db/db* mice exhibited an increase in FA-induced mitochondrial uncoupling partially via activation of UCPs (11,17). To definitively determine the role of UCP3 in this process, we measured mitochondrial respiration (Fig. 5A–C), ATP synthesis rates (Fig. 5D), and ATP/O ratios (Fig. 5E) in WT, UCP3KO, *ob/ob*, and U3OB hearts that were preperfused with 0.4 mmol/L palmitate and 5 mmol/L glucose. Consistent with our previously published data in 9-week-old *ob/ob* hearts, FA-induced mitochondrial uncoupling, as suggested by the significant reduction in ATP/O ratio, was present in *ob/ob* hearts at 20 weeks of age (Fig. 5E), and UCP3 deletion in *ob/ob* hearts had no effect on ATP synthesis rates or ATP/O ratios (Fig. 5D and E). The reduction in ATP/O ratio in *ob/ob* and U3OB was seen



**FIG. 4.** UCP3 deletion in *ob/ob* mice reduces myocardial palmitate oxidation, cardiac power, and CE. **A:** Palmitate oxidation. **B:** MVO<sub>2</sub>. **C:** Cardiac power. **D:** Percent CE in WT, UCP3KO, *ob/ob*, and U3OB mice at 20 weeks of age ( $n = 7$  per genotype). Data are means  $\pm$  SEM. \* $P < 0.05$  WT; + $P < 0.05$  UCP3KO; # $P < 0.05$  vs. *ob/ob*. WHW, wet heart weight.



**FIG. 5.** FA-induced mitochondrial uncoupling persists in U3OB hearts. *A*:  $V_0$  (no ADP). *B*:  $V_{ADP}$  (1 mmol/L exogenous ADP). *C*:  $V_{oligo}$  (1  $\mu$ g/mL of the ATP synthase inhibitor oligomycin). *D*: ATP synthesis rate. *E*: ATP/O ratios in saponin-permeabilized cardiac fibers using palmitoyl-carnitine as substrate. Hearts were preperfused in the Langendorff mode with 11 mmol/L glucose and 1 mmol/L palmitate for 30 min prior to mitochondrial respiration measurements ( $n = 8$  for WT,  $n = 7$  for UCP3KO,  $n = 5$  for *ob/ob*, and  $n = 9$  for U3OB). Data are means  $\pm$  SEM. \* $P < 0.05$  vs. WT; + $P < 0.05$  vs. UCP3KO. mgdw, milligrams dry weight.

only with palmitoyl-carnitine as substrate but not with glutamate (Supplementary Fig. 5). Furthermore, with glutamate there was a significant reduction in  $V_0$  in *ob/ob* and U3OB hearts that might suggest the existence of basal mitochondrial defects, which is further supported by reduced complex III protein content in UCP3KO, *ob/ob*, and U3OB hearts (Supplementary Fig. 6).

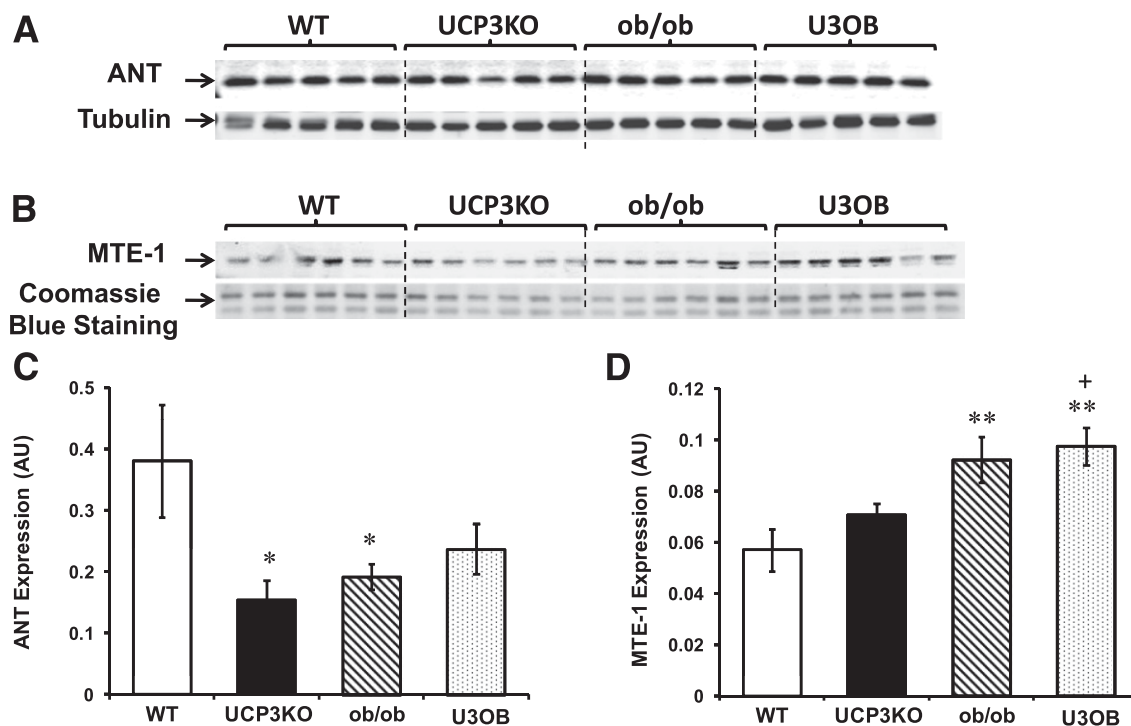
**Increased MTE-1 protein content in *ob/ob* and U3OB hearts.** To explore the mechanisms behind the increase in mitochondrial uncoupling in *ob/ob* hearts lacking UCP3, we measured ANT and MTE-1 protein expression. As shown in Fig. 6A and B, MTE-1 protein content was about 1.6-fold ( $P < 0.005$ ) higher in *ob/ob* and U3OB hearts compared with WT hearts. In contrast, ANT protein was significantly reduced in UCP3KO and *ob/ob* hearts and trended lower in the U3OB hearts.

**UCP3 deficiency exacerbates HF-induced oxidative stress in the heart.** In addition to its role in mitochondrial uncoupling, UCP3 has been implicated in preventing oxidative stress in skeletal muscle. Thus, higher  $H_2O_2$  emission was detected in muscle from fasted or exercise-trained UCP3KO (23,24). To evaluate this possibility, we measured total tissue ROS (dichlorodihydrofluorescein [DCF] fluorescence) and oxidative protein modifications (4-hydroxynonenal [4-HNE]). DCF fluorescence was increased with HF feeding and was further exacerbated in UCP3KO hearts (Fig. 7A). In the HF diet study (performed in mice on a mixed genetic background), despite an increase in total ROS, there was no increase in 4-HNE protein adducts after HF feeding (Fig. 7B). Unexpectedly, 4-HNE modifications were significantly lower in UCP3KO

hearts, but, in contrast to WT hearts, their levels increased with HF feeding (Fig. 7B). A different pattern was observed in the *ob/ob* study (in mice on the C57BL/6 background). In this case, deletion of UCP3 did not significantly change 4-HNE adduct concentrations. In *ob/ob* mice, there was a trend toward increased 4-HNE adduct levels that was not further exacerbated by UCP3 deficiency (Fig. 7C). In the HF study, MnSOD protein levels remained repressed in both genotypes on HF diet. In contrast, MnSOD protein levels in *ob/ob* and U3OB were similar to that of WT controls but higher than UCP3KO controls (Supplementary Fig. 7A and B).

## DISCUSSION

The main findings of this study are that UCP3 deletion reduced  $MVO_2$  and increased CE and ATP/O ratios after HF feeding. In contrast, the absence of UCP3 in leptin-deficient mice failed to restore CE and mitochondrial uncoupling. Increased cardiac FA oxidation has been reported in mouse models of diet-induced obesity (25,26) and genetic obesity (9,27,28) and in obese humans (8). This higher FA utilization, which is mainly due to increased delivery of FFAs to the heart and reduced cardiac glucose uptake, is associated with a higher oxygen cost. Thus, CE, which takes into consideration the amount of work generated per molecule of oxygen consumed, is reduced in obese animals (9,26). The mechanisms for decreased CE are not well understood. Our laboratory and others have shown that FA-induced mitochondrial uncoupling plays an important role (11,13–15). In addition to mitochondrial



**FIG. 6.** Increased MTE-1 protein expression in *ob/ob* and U3OB hearts. **A** and **B**: Representative Western blots of ANT and the MTE-1 in whole-heart homogenates of WT, UCP3KO, *ob/ob*, and U3OB mice. **C** and **D**: Densitometry relative to tubulin and Coomassie Blue staining, respectively ( $n = 5$  per genotype for ANT blot and  $n = 6$  per genotype for MTE-1 blot). Data are means  $\pm$  SEM. \* $P < 0.05$ ; \*\* $P < 0.005$  vs. WT; + $P < 0.05$  vs. UCP3KO. AU, arbitrary units.

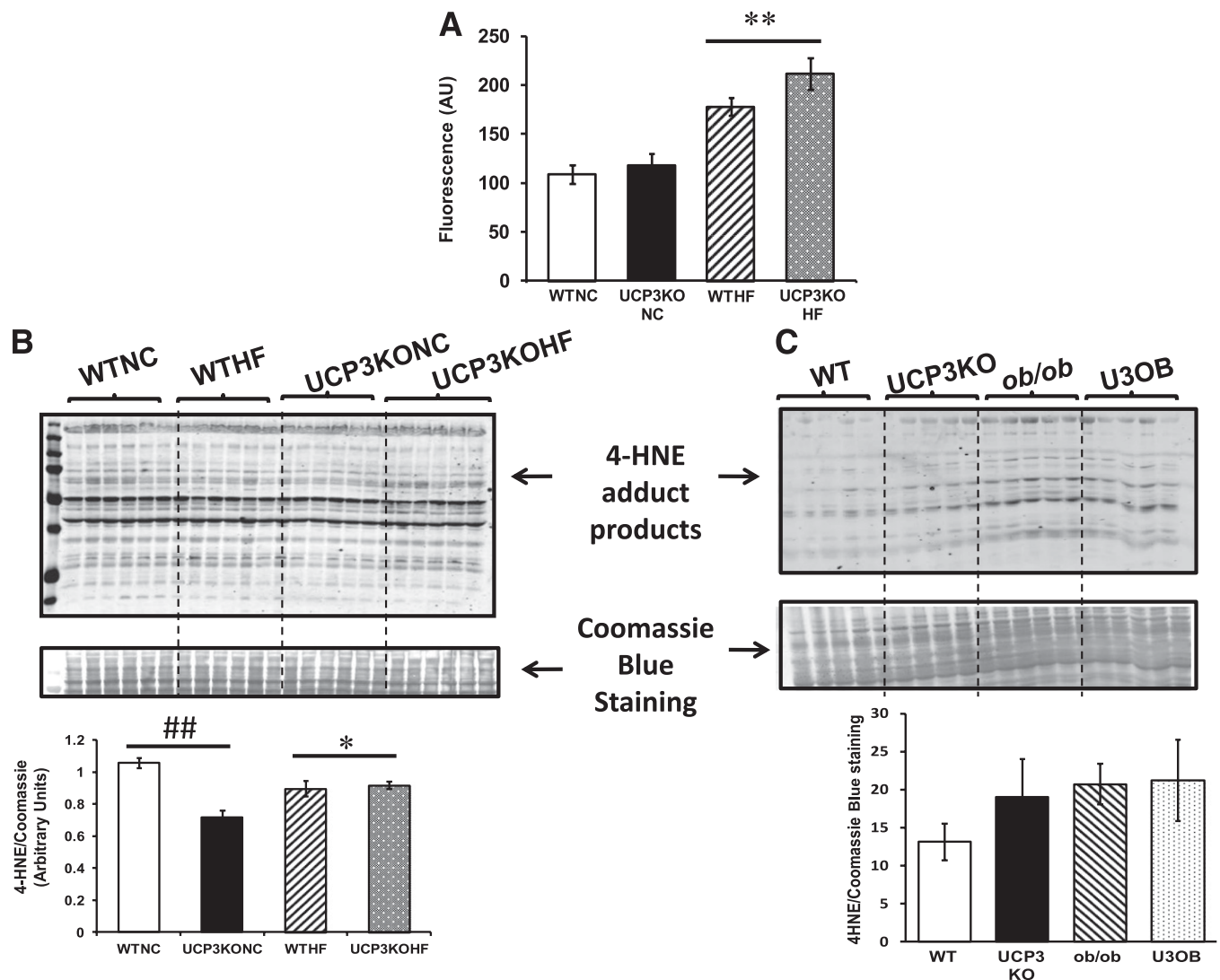
uncoupling, the existence of a higher oxygen cost for basal metabolism and excitation-contraction coupling was also proposed in *db/db* mice (12).

Mitochondrial uncoupling is mediated by several proteins, mainly the UCPs, ANT, and MTE-1. Of particular interest, UCP3 and MTE-1 expression in the heart is increased by HF feeding and is believed to be regulated by peroxisome proliferator-activated receptor- $\alpha$  (22). In addition, FA-induced mitochondrial uncoupling was only partially sensitive to UCP inhibition by guanosine diphosphate (GDP), suggesting the existence of additional mechanisms (15,17). To directly define the role of UCP3 in FA-induced mitochondrial uncoupling during HF feeding or in leptin-deficient obese mice, we first fed mice with absent UCP3 expression (on a mixed background) an HF diet for 10 weeks, and then we generated *ob/ob* mice lacking UCP3 expression by crossing UCP3KO mice (backcrossed to the C57BL/6 background) to B6.*ob*<sup>+/+</sup> mice. Consistent with previous reports, UCP3 protein expression in the heart was induced by HF diet (15). Our study demonstrates that UCP3 does not regulate cardiac FA oxidation. Although the involvement of UCP3 in the transport of FA anions to the cytosol can enhance the FA oxidation rate, this has never been proven. Furthermore, a study by Seifert et al. (24) clearly showed that the absence of UCP3 in skeletal muscle did not impair palmitate oxidation, even at elevated FA supply.

UCP3 deletion resulted in a decrease in  $MVO_2$  and an increase in CE after HF feeding. This result suggests a role for UCP3 in coupling substrate oxidation to oxygen consumption and was further supported by increased mitochondrial coupling in UCP3KO on HF. By using UCP3KO mice, this is the first study, to our knowledge, to show a direct role of UCP3 in myocardial mitochondrial

uncoupling during HF feeding. In accordance with our results, a recent study by Bugger et al. (29) showed that reduced UCP2 and UCP3 expression in the heart after doxorubicin treatment resulted in improved coupling of mitochondria. Despite increased mitochondrial coupling in UCP3KO mice, as measured by ATP/O ratios, we observed a higher  $V_{\text{oligo}}$  (state 4 respiration) in both WT and UCP3KO after HF feeding, leading to a reduction in the respiratory control ratio. One explanation could be that the extra oxygen cost is secondary to a futile cycle created by the re-entry of long-chain fatty acyl moieties into mitochondria or because of a higher FA anion export from the mitochondria that was previously shown to be independent of UCP3 in the heart (30). Consistent with this hypothesis, we observed a similar induction of MTE-1 by HF feeding in WT and UCP3KO that is independent of UCP3 and that could increase proton cycling, leading to enhanced  $V_{\text{oligo}}$ .

We then sought to investigate if UCP3 deletion in leptin-deficient *ob/ob* mice would also preserve CE and reverse the FA-induced mitochondrial uncoupling that has been previously reported in many studies (9,11,14). After backcrossing UCP3KO mice to the C57BL/6J strain for 10 generations, we crossed them with *ob*<sup>+/+</sup> mice to obtain *ob/ob.UCP3*<sup>-/-</sup> (U3OB) mice. Mice were born at a normal Mendelian ratio and were severely glucose intolerant at 20 weeks of age relative to *ob/ob* mice. The absence of UCP3 did not influence insulin levels after HF feeding (Supplementary Fig. 2), and both WT and UCP3KO became similarly glucose intolerant (21). These results suggest that ubiquitous UCP3 deletion did not affect the insulin resistance that develops after HF feeding. By contrast, when *ob/ob* mice were crossed to UCP3KO mice, fasting glucose levels increased and glucose intolerance worsened.



**FIG. 7.** ROS levels and oxidative modifications in the hearts of WT and UCP3KO mice on HF diet or in the *ob/ob* background. **A:** DCF fluorescence in heart homogenates from WT and UCP3KO mice fed NC or HF diet for 10 weeks ( $n = 6$  per group and per diet). **B:** Representative Western blot and the corresponding densitometry of 4-HNE adduct products normalized to Coomassie Blue staining. **C:** Representative Western blot and densitometry of 4-HNE abundance in heart homogenates from WT, UCP3KO, *ob/ob*, and U3OB mice ( $n = 5$  per genotype). Data are means  $\pm$  SEM. Two-way ANOVA was performed for **A** and **B** to analyze differences by diet and genotype, including Bonferroni post hoc test when significant interaction occurred. \* $P < 0.05$ ; \*\* $P < 0.005$  vs. NC of the same genotype; ## $P < 0.05$  for UCP3KONC vs. WTNC. AU, arbitrary units.

Possible explanations for these effects could be related to 1) the development of a  $\beta$ -cell dysfunction as evidenced by the failure of the U3OB mice to produce insulin in response to glucose challenge or 2) worsening of insulin resistance in muscle or liver of U3OB mice. Indeed, we observed a significant increase in muscle (data not shown) and liver (Supplementary Fig. 8D) TG content that could potentially correlate with decreased glucose uptake and insulin signaling in these tissues.

In contrast to HF feeding, the absence of UCP3 in leptin-deficient mice resulted in a reduction in palmitate oxidation when compared with *ob/ob* mice. Despite normalizing palmitate oxidation, the relative oxygen cost for contraction was still elevated in U3OB, as evidenced by the reduction in CE. Thus, FA-mediated mitochondrial uncoupling persisted in U3OB hearts despite the absence of UCP3. The unexpected reduction of FA oxidation in U3OB hearts could be due to reduced FA uptake or compromised oxidation, potentially as a result of mitochondrial dysfunction. Thus,

the reduction in CE in U3OB is driven largely by a reduction in cardiac performance coupled with persistent FA-induced mitochondrial uncoupling. Recent reports from our laboratory and others have shown that leptin treatment of *ob/ob* mice restored mitochondrial coupling (13,14), suggesting the existence of a leptin-dependent mechanism that regulates mitochondrial uncoupling in *ob/ob* hearts. Furthermore, caloric restriction in *ob/ob* mice was unable to restore mitochondrial coupling, which was only restored when leptin was repleted in the brain (13). Finally, one other possibility to explain the persistence of mitochondrial uncoupling in U3OB is the increase in MTE-1 protein, which plays a putative role in the cleavage of long-chain acyl-CoAs into FA anions and CoA when FA supply is high (31,32). Indeed, these FA anions can be channeled through UCP2 or ANT in the absence of UCP3.

In addition to identifying the direct involvement of UCP3 in diet-induced mitochondrial uncoupling in the heart, our findings confirm a role for UCP3 in ROS generation. Thus,



similar to skeletal muscle, UCP3 deletion in the heart exacerbates the ROS generation caused by HF feeding. In support of our results, Bugger et al. (19) showed a reduction of ROS in the hearts of type 1 diabetic AKITA mice that was associated with higher UCP3 protein expression. Despite elevated ROS generation in the hearts of HF-fed WT and UCP3KO mice, these hearts did not accumulate the lipid peroxidation product 4-HNE. One possible explanation for these results is that increased oxidation of FAs in WT and UCP3KO on HF might have reduced the availability of lipids to be oxidized. In support of increased cardiac FA oxidation in WT and UCP3KO on HF, these mice do not accumulate TGs or ceramide in their hearts (Supplementary Fig. 8A and B). Furthermore, the UCP3KO on NC had the lowest amount of 4-HNE, possibly due to more efficient FA oxidation.

Finally, our study has some limitations. For example, the genetic backgrounds of mice in the HF study are not identical to that of the *ob/ob* study, and for this reason, we have not directly compared mitochondrial and metabolic parameters between HF and *ob/ob* mice. Finally, although we show that UCP3 plays an important role in the regulation of CE after HF feeding, we cannot exclude a role for UCP2 in mediating mitochondrial uncoupling, particularly in the *ob/ob* study. In addition, whether UCP3 plays a role in CE in other models of diabetes, such as the streptozotocin-induced diabetes or the leptin receptor-deficient *db/db* mice, will need to be addressed in future investigations.

In conclusion, the current study provides evidence that UCP3 regulates MVO<sub>2</sub>, CE, and mitochondrial coupling in the hearts of HF-fed mice; however, other proteins, such as MTE-1 or leptin itself, may play an important role in regulating mitochondrial coupling in leptin-deficient models of obesity via UCP3-independent mechanisms.

#### ACKNOWLEDGMENTS

This work was supported by grants U01-HL-087947, R01-HL-73167, and P30-HL-101310 from the National Institutes of Health (NIH) to E.D.A., who is an Established Investigator of the American Heart Association (AHA). S.B. was supported by postdoctoral fellowships from the Juvenile Diabetes Research Foundation and the AHA and by Grant P30-HL-101310 from the NIH.

No potential conflicts of interest relevant to this article were reported.

S.B. researched data and wrote the manuscript. Y.H.H., T.J.T., B.H., J.T., C.O., and S.S. researched data and contributed to figure editing. S.P. researched data and contributed to figure editing. E.D.A. contributed to study design and reviewed and edited the manuscript. S.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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