White adipose tissue contributes to UCP1-independent thermogenesis

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3-AR agonists elicit strong thermogenesis in vivo, and this effect has been largely attributed to activation of BAT, a thermogenic organ. The mechanism of brown fat thermogenesis involves fatty acid oxidation and uncoupling of oxidative phosphorylation through uncoupling protein (UCP)1. Thus 3-AR stimulation does not produce thermogenesis in brown adipocytes of mice lacking UCP1 (3, 7, 21, 22). Furthermore, 3-AR-mediated whole animal thermogenesis is sharply reduced in UCP1 knockout (KO) mice (8).

Although UCP1-dependent BAT thermogenesis clearly plays a dominant role in the overall metabolic response to acute effects of 3-AR agonists, various lines of evidence indicate that WAT plays a role as well. For example, restoring 3-AR expression in BAT of 3-AR KO mice does not fully restore thermogenesis to acute 3-AR agonist treatment, whereas restoration in both brown and white fat does (12, 17). Furthermore, mice lacking UCP1, and hence brown adipocyte thermogenesis, still exhibit 20–40% of wild-type thermogenesis in response to 3-AR stimulation (8). These observations suggest that WAT directly or indirectly participates in the overall thermogenic response to acute 3-AR stimulation and that a component of thermogenesis is UCP1 independent.

The above analysis concerns the effects of acute 3-AR activation. The anti-obesity and anti-diabetes effects of 3-AR agonists, however, require chronic drug exposure. Chronic exposure to 3-AR agonists elevates UCP1 expression in BAT, and it is thought that BAT mediates the augmented thermogenic capacity seen after chronic treatment (6, 14). However, because BAT cannot account fully for 3-AR-mediated thermogenesis to acute agonist stimulation, it is possible that other tissues contribute to the augmented thermogenic capacity seen after chronic exposure to 3-AR agonists. In this regard, recent work by Himms-Hagen et al. (15) is of interest. These investigators demonstrated that chronic exposure to 3-AR agonists induces mitochondrial biogenesis in WAT, suggesting that this tissue might be capable of oxidizing lipid in metabolic plasticity; obesity; diabetes; tissue remodeling

3-AR agonists have profound therapeutic effects in rodent models of obesity and diabetes (2, 9, 14). 3-AR in rodents are expressed almost exclusively in brown (BAT) and white adipose tissue (WAT), and the therapeutic effects of 3-AR agonists have been attributed to dramatic stimulation of adipose tissue triglyceride hydrolysis coupled to elevated fatty acid oxidation in BAT. However, the direct and indirect contribution of adipose tissue types to the therapeutic effects of these compounds is largely unknown.

3-AR agonists are nearly exclusively expressed in brown and white adipose tissues, and chronic activation of these receptors by selective agonists has profound anti-diabetes and anti-obesity effects. This study examined metabolic responses to acute and chronic 3-AR activation in wild-type C57Bl/6 mice and congenic mice lacking functional uncoupling protein (UCP)1, the molecular effector of brown adipose tissue (BAT) thermogenesis. Acute activation of 3-AR doubled metabolic rate in wild-type mice and sharply elevated body temperature and BAT blood flow, as determined by laser Doppler flowmetry. In contrast, 3-AR activation did not increase BAT blood flow in mice lacking UCP1 (UCP1 KO). Nonetheless, 3-AR activation significantly increased metabolic rate and body temperature in UCP1 KO mice, demonstrating the presence of UCP1-independent thermogenesis. Daily treatment with the 3-AR agonist CL-316243 (CL) for 6 days increased basal and CL-induced thermogenesis compared with naive mice. This expansion of basal and CL-induced metabolic rate did not require UCP1 expression. Chronic CL treatment of UCP1 KO mice increased basal and CL-stimulated metabolic rate of epididymal white adipose tissue (EWAT) fourfold but did not alter BAT thermogenesis. After chronic CL treatment, CL-stimulated thermogenesis of EWAT equalled that of interscapular BAT per tissue mass. The elevation of EWAT metabolism was accompanied by mitochondrial biogenesis and the induction of genes involved in lipid oxidation. These observations indicate that chronic 3-AR activation induces metabolic adaptation in WAT that contributes to 3-AR-mediated thermogenesis. This adaptation involves lipid oxidation in situ and does not require UCP1 expression.

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Elevated insulin is considered to be the major cause of obesity and diabetes. Elevation of body fat mass is generally associated with increased insulin concentrations, and as insulin levels increase, the rate of hepatic glucose production is decreased, whereas the rate of peripheral glucose uptake is increased. Therefore, increased insulin sensitivity in peripheral tissues is the primary mechanism of insulin resistance in type 2 diabetes.

There is increasing evidence that insulin resistance is associated with the development of obesity and diabetes, and with the development of the metabolic syndrome (1). It is not clear whether increased insulin sensitivity is the cause or the consequence of the development of obesity and diabetes. In the present study, we examined the effect of insulin resistance on the development of obesity and diabetes in C57BL/6 mice. The results indicate that insulin resistance is a primary cause of the development of obesity and diabetes.

MATERIALS AND METHODS

Animals. Wild-type C57Bl/6 (WT) and congenic UCP1 KO mice were obtained from L. Kozak of Pennington Research Institute (Baton Rouge, LA), bred at Wayne State University, and used when 2–6 mo old. For all experiments, animals were matched for age, and only male mice were used. Mice were maintained at 23 °C in a 12:12-h light-dark cycle and had continuous access to food and water. Testing was performed during the light phase.

Experiment 1. WT and UCP1 KO mice were anesthetized with pentobarbital sodium (50 mg/kg) and immediately placed on a warm surface to stabilize body temperature. Interscapular BAT (IBAT) was carefully exposed through a 2-cm incision, a 21-gauge laser Doppler probe (Transonic, Ithaca, NY) was placed into the tissue, and a temperature probe (Physitemp, Clifton, NJ) was placed into the peritoneal cavity. After stabilization of temperature and tissue blood perfusion, mice were injected intraperitoneally with norepinephrine (100 nmol) or BRL-37344 (50 nmol; RBI, Natick, MA). Temperature and blood flow data were recorded at 2-min intervals. Whole body oxygen consumption (VO2) was determined using an open-circuit system (Accuscan Oxyplot, Columbus, OH). After 10 min of stable baseline data had been collected, animals were injected with adrenergic agonists, as indicated in Figs. 1–4, and VO2 was measured over the following 20–30 min.

Figure 1. Norepinephrine (NE)-induced thermogenesis in wild-type (WT) and uncoupling protein (UCP1) knockout (KO) mice. Pentobarbital sodium-anesthetized mice were injected ip with 100 nmol of NE at time 0 (arrow). NE significantly elevated metabolic rate (oxygen consumption [VO2]) in WT and UCP1 KO mice relative to baseline (P < 0.01), and WT mice were significantly more responsive to NE than were UCP1 KO mice (P < 0.01). Values are means ± SE; n = 4.

Results

Experiment 1. Injection of norepinephrine (NE; 100 nmol) approximately doubled the metabolic rate of WT mice, as expected (Fig. 1). The effect of NE on metabolic rate was severely reduced in UCP1 KO mice. Nonetheless, NE significantly elevated metabolic rate in UCP1 (700 µl volume) for measurement of VO2 with a Clark-style electrode (Quibit Systems, Kingston, ON, Canada) at 35 °C under constant stirring. Respiration rates were determined by linear regression of the O2 concentration traces (Vernier Software, Beaverton, OR).

Quantitative RT/PCR analysis. Total RNA from EWAT was isolated, and cDNA was synthesized, as previously described (11). Synthesized cDNA was subjected to quantitative (q)RT-PCR (iCycler, Bio-Rad) in duplicate, with SYBR-green (FAM) dye. The following primers were used: PPIA ()+ GTG-GTCTTTTGGGAAAGGTGAA, (−) TTACAGGACATTGGCAG; pyruvate dehydrogenase kinase 4 (PDK4) (+) GCC-CATCCATGTAGGAGAGA, (−) GAGGGAGACCACAGAAGA; long-chain acyl-CoA dehydrogenase (Acad1) (+) CTCATGCAAGAGCTCACA, (−) CCACAAAAAGCTCTG-GTGACA; cytochrome c oxidase subunit 8b (Cox8b) (+) TGCG-GAAGTTCACAGTGTTTC, (−) CTCAGGGATGTCGAAC-TTCA.

Statistical analysis. Data are presented as means ± SE. Data were evaluated by ANOVA. Post hoc analysis was performed with the Neuman-Keuls test for multiple comparisons among means.

Fig. 1. Norepinephrine (NE)-induced thermogenesis in wild-type (WT) and uncoupling protein (UCP1) knockout (KO) mice. Pentobarbital sodium-anesthetized mice were injected ip with 100 nmol of NE at time 0 (arrow). NE significantly elevated metabolic rate (oxygen consumption [VO2]) in WT and UCP1 KO mice relative to baseline (P < 0.01), and WT mice were significantly more responsive to NE than were UCP1 KO mice (P < 0.01). Values are means ± SE; n = 4.
KO mice by 25%, indicating that a component of the NE-dependent thermogenesis is independent of UCP1.

NE activates several AR subtypes in numerous tissues. In contrast, β₃-AR are expressed nearly exclusively in BAT and WAT, and expression in these tissues is necessary and sufficient to account for the full thermogenic effects of β₃-AR agonists (1, 9, 12). We therefore examined the effects of the selective β₃-AR agonist BRL-37344 (BRL; 50 nmol) on thermogenesis, body temperature, and BAT blood flow in WT and UCP1 KO mice.

Acute injection of BRL sharply elevated metabolic rate (Fig. 2A) and body temperature (Fig. 2B) in WT mice. The elevation of metabolic rate was greatly blunted, but not eliminated, in UCP1 KO mice. Interestingly, the elevation in body temperature was largely intact in UCP1 KO mice, suggesting that BRL might affect body temperature independently of metabolic rate. BAT blood flow is sharply elevated by increased local tissue respiration and is a sensitive physiological measure of BAT metabolism in situ (4, 24). Injection of BRL produced an immediate and sustained elevation of BAT blood flow in WT mice, as determined by laser

Fig. 2. Effects of the selective β₃-adrenergic receptor (AR) agonist BRL-37344 on metabolic rate (VO₂), body temperature, and brown adipose tissue (BAT) blood flow in WT, heterozygous, and UCP1 KO mice. Pentobarbital sodium-anesthetized WT (+/+), heterozygous (+/-), and UCP1 KO (-/-) mice were injected ip with BRL-37344 (BRL; arrow). Metabolic rate (A), change in core body temperature (B), and BAT blood flow (C) were determined as detailed in MATERIALS AND METHODS. TPU, tissue perfusion units. Values are means ± SE; n = 5–11.

Fig. 3. Effects of chronic CL-316243 (CL) treatment on CL-induced thermogenesis in WT and UCP1 KO mice. Mice were treated with saline (Sal) or CL daily for 6 days (CL-6d). On the 7th day, mice were anesthetized with Avertin, and metabolic rate (VO₂) was determined under basal conditions (B) and after stimulation (S) by ip injection of CL (30 nmol). Basal values are averages of the 5-min period before injection, and stimulated values are averages of the 5-min period 20–25 min after injection. Two-way ANOVA indicated significant effects of drug treatment and genotype (P < 0.0001) but no significant interaction. Values are means ± SE; n = 7–9.

Fig. 4. Time course of CL-induced thermogenesis (shown by increase in VO₂) in naive and chronically-treated UCP1 KO mice. Values are means ± SE; n = 4–5.
Doppler flowmetry (Fig. 2C). In contrast, BRL had no effect on tissue perfusion in UCP1 KO mice, indicating that the tissue is thermogenically unresponsive to $\beta_3$-AR activation.

Experiment 2. The therapeutic effects of $\beta_3$-AR agonists on diabetes and obesity require chronic exposure to the compounds. We therefore compared the acute and chronic effects of $\beta_3$-AR activation on metabolic rate and WAT function, gene expression, and histology. WT and UCP1 KO mice were injected daily for 6 days with saline or the highly selective $\beta_3$-AR agonist CL (30 nmol). On the 7th day, metabolic rate was determined under basal conditions and after stimulation by a supramaximal dose of CL (20 nmol; Fig. 3). As with NE and BRL, CL challenge strongly elevated metabolic rate in naive (i.e., saline-treated) WT mice, and this effect was significantly reduced, but not eliminated, in UCP1 KO mice. A 6-day course of CL treatment elevated both basal and stimulated $V_{\text{O}_2}$. Importantly, ANOVA indicated significant effects of drug treatment and genotype, but no interaction among these variables. In other words, the enhancement of thermogenesis by chronic CL treatment was independent of UCP1.

Figure 4 illustrates the time course of thermogenesis induced by CL in UCP1 KO mice previously treated with saline or CL. CL appeared to increase metabolic rate in two temporally distinct phases. The rapid phase occurred during the first 6 min after injection, with the sustained elevation occurring thereafter. Although both phases appeared to be augmented by chronic CL...
treatment, the first phase was affected more dramatically.

Conditions that produce sustained activation of WAT lipolysis, such as cold exposure and β3-AR agonist treatment, induce mitochondrial biogenesis in WAT and the appearance of multilocular cells, some of which express UCP1 (15). These observations suggest that chronic β3-AR activation induces metabolic adaptation in WAT, which might contribute significantly to the thermogenic response. Therefore, the above experiment was repeated, and the thermogenic responses of EWAT and IBAT were determined in vitro under basal conditions and after systemic challenge by CL.

As shown in Fig. 5, metabolic rate was low in EWAT of naive mice, and acute CL treatment produced a slight, but nonsignificant, increase in tissue respiration. Chronic treatment with CL dramatically increased basal metabolic rate in EWAT (P < 0.001). CL challenge significantly elevated thermogenesis in EWAT of chronically-treated mice (P < 0.01), and this elevation was fourfold greater (P < 0.001) than that observed in naive mice.

In control UCP1 KO animals, IBAT tissue thermogenesis was 7–8 times greater than that of EWAT (Fig. 5) and ~30% lower than that of WT mice (not shown). IBAT tissue thermogenesis was not significantly affected by either acute or chronic treatment with CL. Importantly, EWAT thermogenesis equaled that of IBAT (relative to tissue weight) after chronic CL treatment and CL challenge.

The elevated metabolic capacity of EWAT was reflected in the histological appearance and gene expression pattern of the tissue. As shown in Fig. 6, 6 days of CL treatment resulted in the fragmentation of the single large lipid droplet characteristic of white adipocytes into multiple lipid droplets. These lipid droplets were completely surrounded by mitochondria in numerous cells, as demonstrated by confocal imaging of MitoTracker staining. The magnitude of the MitoTracker staining was highly similar in WT and UCP1 KO mice.

WAT mRNA from naive mice and mice treated chronically with CL was subjected to qRT-PCR analysis, with focus on genes that are involved in substrate selection and fatty acid oxidation. Chronic CL treatment induced PDK4 expression similarly in WT and UCP1 KO mice (Fig. 7A). Similar results were obtained for expression of LCAD (Fig. 7B), a key enzyme in mitochondrial β-oxidation. Finally, chronic CL treatment strongly (>20-fold) induced expression of Cox8b (Fig. 7C).

**DISCUSSION**

Recent work with transgenic and KO mouse models strongly indicates that thermogenic responses to β3-AR agonists require activation of both BAT and WAT (5, 12). BAT clearly contributes importantly to the acute effects of β3-AR stimulation. BAT thermogenesis induced by β3-AR activation appears to be entirely dependent on UCP1, as indicated by the absence of elevated blood flow in the tissue in UCP1 KO mice. These observations confirm results of in vitro studies and extend them to the functioning of the intact tissue in situ (3, 7, 21, 22).

The dependence of BAT thermogenesis on UCP1 indicates that UCP1 KO mice represent a model with which to explore mechanisms of thermogenesis that are largely independent of BAT. In naive mice, UCP1-independent thermogenesis constitutes 20–40% of the total thermogenic response to various β-AR agonists. As mentioned above, the therapeutic effects of β3-AR activation require chronic treatment. The present results indicate that chronic CL treatment significantly elevates both basal and stimulated thermogenesis. Importantly, the present data indicate that this effect does not require UCP1.

In addition to elevating the maximal level of thermogenesis, chronic CL treatment also accelerated the rate...
at which that maximum was achieved. The slow elevation of thermogenesis in naive UCP1 KO mice contrasts with the rapid induction of $V_{O_2}$ seen in WT mice in which fatty acids are rapidly oxidized by BAT in situ. The relatively slow activation of thermogenesis in naive KO mice suggests that UCP1-independent thermogenesis might involve oxidation of mobilized lipid by nonadipose tissues like liver and muscle. The time course of CL-induced thermogenesis in UCP1 KO mice chronically treated with CL resembled that of WT mice and suggests that this augmentation of thermogenesis might involve the direct effects of CL on adipocytes. In vitro analysis of EWAT from UCP1 KO mice demonstrated that chronic CL treatment dramatically increased basal and CL-stimulated thermogenesis. In contrast, neither acute nor chronic CL treatment affected thermogenesis in IBAT from UCP1 KO mice. Importantly, the mass-specific metabolic rate of EWAT equaled that of BAT after chronic CL with acute CL challenge. Although the quantitative significance of the elevated WAT metabolism to total in vivo thermogenesis is difficult to extrapolate from in vitro data, it is likely that WAT contributes more than BAT in UCP1 KO mice given the much greater mass of WAT in these animals. The present study addressed the molecular profile and metabolic activity of adipose tissue after chronic treatment with CL. The results demonstrate that chronic activation of WAT $\beta_3$-AR induces genes involved in substrate selection, fatty acid oxidation, and mitochondrial biogenesis. PDK4 phosphorylates the pyruvate dehydrogenase complex, thereby suppressing glucose utilization in favor of lipid oxidation (23); Acad1 (LCAD) is a key step in mitochondrial $\beta$-oxidation and has been shown to be essential for nonshivering thermogenesis (13); and Cox8b is a subunit of cytochrome c oxidase that is induced during mitochondrial biogenesis. The strong upregulation of these genes, along with the pronounced induction of mitochondrial biogenesis, indicates that CL induces a novel plasticity in WAT that shifts the metabolic profile from lipid storage to lipid oxidation in situ. Kozak’s laboratory (Hofmann et al. (16) and Liu et al. (20)) has provided compelling evidence for the presence of UCP1-independent thermoregulatory thermogenesis. For example, Liu et al. reported that mildly cold-stressed UCP1 KO mice exhibited lower levels of plasma free fatty acids, higher levels of plasma ketones, and a lower cumulative respiratory quotient, all indicative of greater lipid oxidation. Similar effects occur during chronic treatment with $\beta_3$-AR agonists (2, 14, 18, 24). Furthermore, mild cold stress produces morphological changes in inguinal fat of UCP1 KO mice, similar to those produced by CL treatment in epididymal fat. These observations raise the possibility that CL and mild cold stress engage similar thermogenic mechanisms in UCP1 KO mice and that one component of that thermogenesis is likely to involve activation of white fat thermogenesis. The quantitative significance of WAT to overall thermogenesis is presently unclear and is likely to depend on the nature and magnitude of the stimulus, as well as the thermogenic mechanisms available to the animal. For example, chronic CL treatment targets adipose tissue and produces more extensive WAT remodeling than does mild cold stress. In contrast, cold stress might be expected to elevate sympathetic activity and thereby activate thermogenesis in liver and muscle, which are important extra-adipose sites of lipid oxidation/thermogenesis. Finally, it is important to recognize that, although chronic CL treatment increases the thermogenic capacity of WT and UCP1 KO mice similarly, the mechanisms involved could differ, with WT mice relying mainly on BAT and UCP1 KO mice engaging WAT, muscle, and liver. Clearly, an important goal of future work will be to identify the tissues that contribute to UCP1-independent thermogenesis. $\beta_3$-AR agonists are highly effective therapeutics in rodent models of obesity and diabetes (9, 10, 19). Nonetheless, the mechanisms and sites of $\beta_3$-AR action remain poorly understood. The dominant role of UCP1-dependent thermogenesis in the acute effects of $\beta_3$-AR agonists has cast doubt as to whether adipokinetic drugs will be useful as human therapeutics, given the paucity of BAT in adult humans. The present results, however, indicate that WAT is capable of pronounced thermogenic activity in response to sustained lipolytic stimulation. Clearly, the abundance of the tissue target is not limiting in human obesity and type 2 diabetes. Understanding the mechanisms controlling WAT metabolic plasticity could lead to the identification of novel points of therapeutic intervention for treatment of obesity and diabetes in humans.

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DISCLOSURES

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