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Article

Human Brown Adipocyte Thermogenesis Is Driven by β_2 -AR Stimulation

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SUMMARY

Stimulation of brown adipose tissue (BAT) thermogenesis in humans has emerged as an attractive target to improve metabolic health. Pharmacological stimulations targeting the β_3 -adrenergic receptor (β_3 -AR), the adrenergic receptor believed to mediate BAT thermogenesis, have historically performed poorly in human clinical trials. Here we report that, in contrast to rodents, human BAT thermogenesis is not mediated by the stimulation of β_3 -AR. Oral administration of the β_3 -AR agonist mirabegron only elicited increases in BAT thermogenesis when ingested at the maximal allowable dose. This led to off-target binding to β_1 -AR and β_2 -AR, thereby increasing cardiovascular responses and white adipose tissue lipolysis, respectively. *ADRB2* was co-expressed with *UCP1* in human brown adipocytes. Pharmacological stimulation and inhibition of the β_2 -AR as well as knockdown of *ADRB1*, *ADRB2*, or *ADRB3* in human brown adipocytes all confirmed that BAT lipolysis and thermogenesis occur through β_2 -AR signaling in humans (ClinicalTrials.gov NCT02811289).

INTRODUCTION

Promoting the recruitment of energy dissipating mechanisms can exert significant metabolic benefits by protecting from the

end-organ complications that often result from a chronic imbalance between energy intake relative to energy demands. In rodents, the stimulation of brown adipose tissue (BAT), through β -adrenergic receptor (β -AR) signaling elicited by increased

Context and Significance

Mirabegron, a drug that stimulates the β_3 -adrenoceptor and was previously prescribed for individuals with overactive bladders, has recently been repurposed to improve metabolic health by stimulating brown adipose tissue (BAT) in humans. Here, Blondin et al. report that, in contrast to previous investigations, mirabegron does not activate human BAT when given at the commonly prescribed dose. Further examination of BAT biopsies from participants showed that the lack of effect may be explained by the absence of the β_3 -adrenoceptor. Rather, human BAT expresses the β_2 -adrenoceptor, which is responsible for its metabolic activation. These findings identify a new target to stimulate human BAT and provide an explanation as to why medications targeting the β_3 -adrenoceptor have historically performed poorly in human clinical trials.



sympathetic nervous system activity or selective pharmacological targeting of the β_3 -adrenergic receptor (β_3 -AR), has been one of the most effective means of dissipating excess energy and thereby improving metabolic health (Berbée et al., 2015; Feldmann et al., 2009; Stanford et al., 2013; Trayhurn, 1979; Zaror-Behrens and Himms-Hagen, 1983). In humans, mild cold exposure has been the most potent and robust stimulus to activate BAT, whereas the outcomes resulting from the use of pharmacological sympathomimetics have been conflicting. Mild cold exposure increases BAT oxidative metabolism (Blondin et al., 2014; Muzik et al., 2012; Ouellet et al., 2012; U Din et al., 2016) and the rate of glucose (Ouellet et al., 2012; Virtanen et al., 2009), endogenous fatty acid (Ouellet et al., 2012; U Din et al., 2016), and dietary fatty acid BAT uptake (Blondin et al., 2017b). In contrast, the use of pharmacological sympathomimetics such as ephedrine (Astrup et al., 1985; Carey et al., 2013; Cypess et al., 2012) or β -AR agonists (Baskin et al., 2018; Cypess et al., 2015; Finlin et al., 2018; Lee et al., 2013; Vosselman et al., 2012) to stimulate BAT has led to wide-ranging outcomes and often unwanted cardiovascular effects. Yet given the clear evidence of β_3 -AR-mediated thermogenesis in rodents, it has been assumed that this receptor was also mediating the activation of BAT in humans. Recently, mirabegron, a β_3 -AR agonist with efficacy at the human receptor and approved for the treatment of overactive bladder, has been used to target the β_3 -AR in human BAT (Baskin et al., 2018; Cypess et al., 2015; Finlin et al., 2018, 2020; O'Mara et al., 2020). Whereas oral ingestion of the maximal allowable dose of mirabegron (200 mg) increased whole-body energy expenditure and the accumulation of [^{18} F] fluorodeoxyglucose (^{18} FDG, glucose PET analog) in BAT, it also stimulated some unwanted cardiovascular responses (Baskin et al., 2018; Cypess et al., 2015; O'Mara et al., 2020). One explanation for these responses could be that the effects are in fact not mediated directly by the β_3 -AR, but rather reflects a generalized β -AR stimulation. Indeed, an increase in whole-body energy expenditure and/or BAT glucose uptake can occur independently of direct stimulation of BAT thermogenesis (Blondin et al., 2017a). The increased heart rate and systolic blood pressure induced with mirabegron, aside from being undesirable off-target secondary effects, also implied that such high doses were not selective to the β_3 -AR (Baskin et al., 2018), but were rather a generalized β -AR stimulation. Whether the β_3 -AR agonist mirabegron when provided in a lower dose can definitively target human BAT to increase thermogenesis, without activating cardiovascular responses, remains unknown.

Here, we therefore aimed to determine whether β_3 -AR stimulation specifically activates BAT thermogenesis in humans. Using *in vivo* and *in vitro* models, from three independent laboratories, we found that human BAT from the deep neck region is activated through β_2 -AR signaling rather than the β_3 -AR signaling pathway that is well established in rodents.

RESULTS

Mirabegron Mediates a Suboptimal Metabolic Response in BAT

The primary purpose of this study was to directly compare BAT oxidative metabolism under cold compared to β_3 -AR agonist stimulation in lean healthy individuals. We hypothesized that

the acute oral administration of a lower dose of mirabegron (50 mg) would result in an increase in BAT oxidative metabolism and whole-body energy expenditure, to a similar extent as cold exposure, without influencing the cardiovascular responses previously seen with the higher dose of mirabegron (200 mg). The primary outcome measures of this clinical study as entered in clinicaltrials.gov (NCT02811289) included BAT oxidative metabolism, BAT net glucose uptake, BAT volume of metabolic activity, and whole-body glucose partitioning and are all reported in the manuscript, with the exception of BAT volume. We opted to stop analyzing the BAT volume of metabolic activity over the course of the study, as we began to recognize the limitations in quantifying BAT volume using ^{18} FDG PET (described in detail in Carpentier et al., 2018). Secondary endpoints can be found at <https://clinicaltrials.gov/ct2/show/NCT02811289>.

We first examined the pharmacokinetics and effects of a therapeutic dose (50 mg) and the maximal allowable dose (200 mg) of mirabegron on whole-body energy expenditure and BAT metabolism compared to a mild cold exposure (18°C) in healthy lean men (Table S1 for participant characteristics). Using liquid chromatography-tandem mass spectrometry, we determined that the time to maximal plasma mirabegron concentration occurred approximately 240 min (median C_{max} : 22 nM) and 180 min (C_{max} : 157 nM) after oral administration of the 50 and 200 mg dose, respectively, which coincided with our dynamic list-mode PET acquisition (Figure 1A; STAR Methods). Oral administration of the maximal allowable dose of mirabegron increased mean skin temperature, while mild cold exposure led to a fall in mean skin temperature that was consistent with our previous investigations (Blondin et al., 2014, 2015, 2017a, 2017b; Ouellet et al., 2012) (Figure 1B). Compared to pre-treatment, oral administration of either the 50 or 200 mg dose of mirabegron increased energy expenditure (Figure 1C; Table 1). We were initially interested in determining whether the therapeutic dose, previously shown to selectively stimulate β_3 -AR in the bladder, could increase BAT oxidative metabolism. Unexpectedly, we found that the 50 mg dose did not elicit an increase in BAT oxidative metabolism (Figure 1D) or BAT blood flow (Figure 1E), as determined from PET imaging-acquired ^{11} C-acetate tissue kinetics, or elicit a change in CT-derived tissue radiodensity (marker of change in intracellular triglyceride content) (Figure 1F). Neither was glucose uptake affected by this dose, as the rate of BAT glucose uptake was consistent with what is commonly reported under unstimulated/room temperature conditions (Orava et al., 2011) (Figure 1G).

The absence of a metabolic response of BAT to 50 mg of mirabegron led us to consider whether the higher 200 mg dose could stimulate BAT oxidative metabolism or only glucose uptake, as was previously reported (Baskin et al., 2018; Cypess et al., 2015; O'Mara et al., 2020). This higher dose increased BAT oxidative metabolism on average 2.1-fold (from 0.003 s^{-1} [95% CI: 0.003–0.004] at room temperature to 0.008 s^{-1} [95% CI: 0.003–0.013] with 200 mg mirabegron) (Figure 1D), accompanied by an increase in BAT blood flow (0.91 SUV_{mean} [95% CI: 0.72–1.09] at room temperature versus 1.19 SUV_{mean} [95% CI: 1.03–1.35] with 200 mg mirabegron) (Figure 1E) and glucose uptake (from $9.4 \text{ nmol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$ [95% CI: 3.0–15.8] with 50 mg mirabegron to $22.1 \text{ nmol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$ [95% CI: 12.8–31.4] with 200 mg mirabegron) (Figure 1G). However, these rates remained

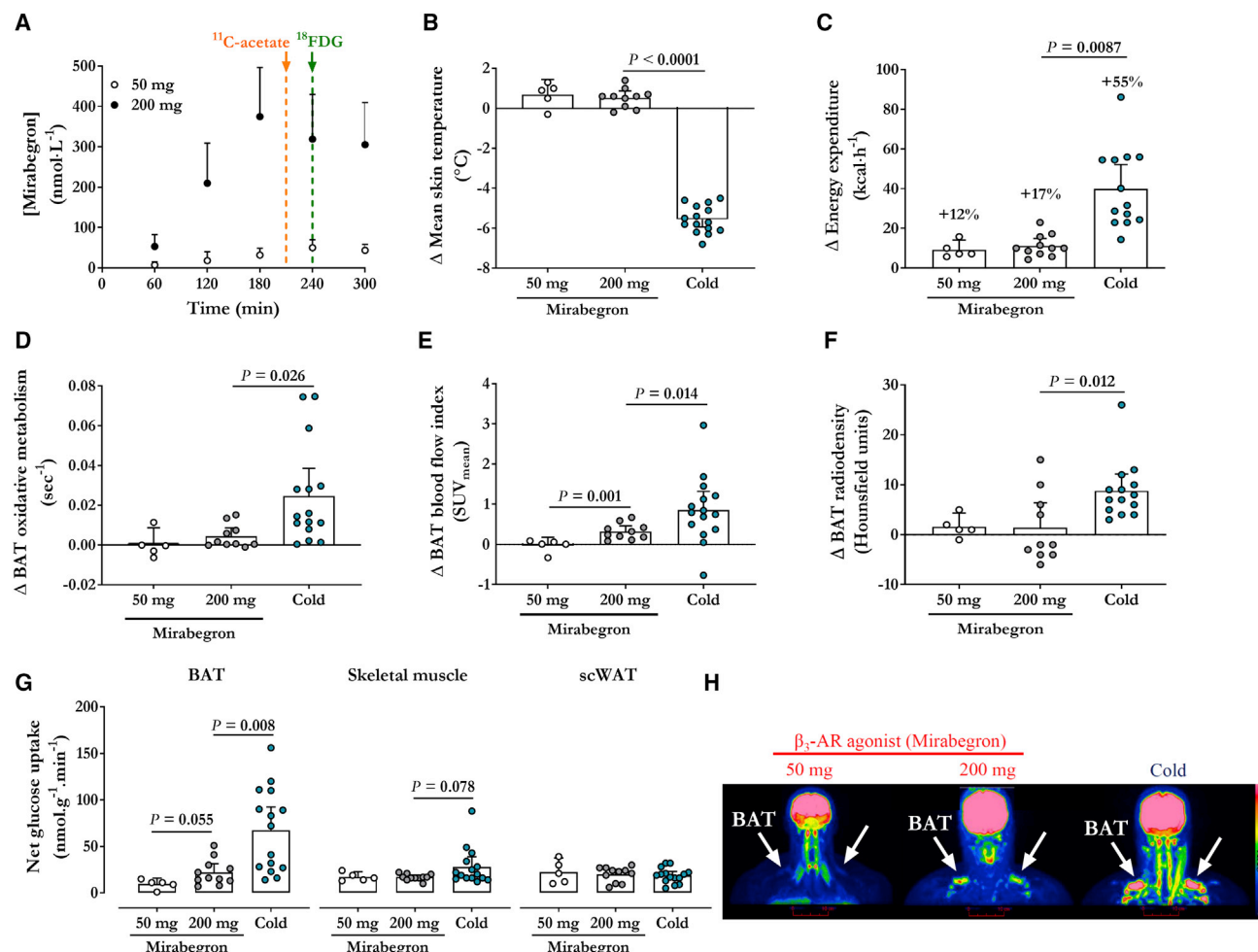


Figure 1. Mirabegron Increases BAT Blood Flow and Glucose Uptake

(A) Circulating mirabegron levels following oral administration of 50 mg (n = 5) or 200 mg (n = 11) of the β_3 -AR agonist mirabegron in humans. ¹¹C-acetate (orange arrow) and [¹⁸F]fluorodeoxyglucose (¹⁸FDG, green arrow) radiotracers were injected 210 and 240 min after oral administration of mirabegron, respectively, followed by a dynamic list-mode PET acquisition.

(B and C) Change in mean skin temperature (B) and whole-body energy expenditure (C) from basal levels in response to oral administration of 50 mg (n = 5) or 200 mg (n = 11) of mirabegron and mild cold exposure (18°C, n = 15).

(D) Change in supraclavicular BAT oxidative metabolism from basal levels measured at room temperature relative to 210 min after oral administration of 50 mg (n = 5) or 200 mg (n = 10) and 120 min into a mild cold exposure (18°C, n = 15).

(E) Change in supraclavicular BAT blood flow index from basal levels measured at room temperature relative to 210 min after oral administration of 50 mg (n = 5) or 200 mg (n = 10) and 120 min into a mild cold exposure (18°C, n = 15).

(F) Change in supraclavicular BAT radiodensity from basal levels measured at room temperature relative to 210 min after oral administration of 50 mg (n = 5) or 200 mg (n = 10) and 120 min into a mild cold exposure (18°C, n = 15).

(G) Supraclavicular BAT (left panel), skeletal muscle (center panel), and WAT (right panel) net glucose uptake in response to oral administration of 50 mg (n = 5) or 200 mg (n = 11) of mirabegron and mild cold exposure (18°C, n = 15). Skeletal muscle glucose uptake represents mean uptake of regions of interest drawn from *m. pectoralis major*, *m. trapezius*, *m. deltoideus*, and *m. sternocleidomastoid*.

(H) Cervico-thoracic PET images of ¹⁸F-FDG uptake in response to oral administration of 50 mg (left image) or 200 mg (center image) and mild cold exposure (right image). Same participant is presented for 200 mg mirabegron and cold exposure. White arrows represent supraclavicular BAT depot.

Data are reported as mean with 95% CI. Difference between 50 and 200 mg mirabegron determined using Mann-Whitney test. Difference between 200 mg mirabegron and mild cold exposure determined using paired-sample t test. See also Table S1 for participant characteristics.

significantly lower than what is observed during mild cold exposure (Figures 1D–1H). The greater whole-body thermogenic stimulation derived from mild cold exposure (Table 1) resulted in a greater increase in tissue radiodensity, an indication of greater depletion in BAT intracellular triglycerides (Figure 1F), and a greater skeletal muscle glucose uptake (Figure 1G).

High-Dose Mirabegron Leads to Nonselective β -AR Activation

To examine the specificity of mirabegron as a β_3 -AR agonist, we examined cardiovascular and white adipose tissue (WAT) lipolysis outcomes, which are primarily driven by β_1 -AR and β_2/β_1 -AR signaling (Lafontan and Berlan, 1993), respectively. Using

Table 1. Hormone and Metabolite Concentrations at Room Temperature and with the Ingestion of 50 or 200 mg of Mirabegron or Mild Cold Exposure in Human *In Vivo* Physiological Study

	50 mg		200 mg		Cold Exposure	
	Basal	Intervention	Basal	Intervention	Basal	Intervention
EE (kcal/h)	76.6 (62.4–90.7)	85.5 (69.7–101.2) ^b	72.0 (64.9–79.0)	83.3 (76.9–89.7) ^{a,e}	74.0 (68.7–79.3)	113.6 (101.9–125.3) ^d
Ra _{Glucose} (μmol/min)	1,822 (1,418–2,227)	1,266 (939–593) ^d	2,093 (1,783–2,404)	1,306 (1,129–1,482) ^d	1,887 (1,651–2,123)	1,318 (1,139–1,497) ^d
Glucose (mmol/L)	5.0 (4.6–5.4)	4.8 (4.5–5.1)	4.8 (4.5–5.0)	4.4 (4.2–4.6) ^b	4.9 (4.7–5.1)	4.4 (4.3–4.6) ^d
Insulin (pmol/L)	92.9 (29.8–156.1)	91.1 (36.7–145.5)	79.4 (29.5–129.3)	77.8 (30.1–125.5) ^e	82.3 (56.7–107.8)	63.7 (33.7–93.6) ^b
Glucagon (pg/mL)	51.3 [10.7–188.8]	45.2 [17.4–203.1]	50.5 (28.9–72.0)	49.1 (30.2–68.0)	51.6 (37.1–66.1)	48.2 (32.1–64.3)
TG (mmol/L)	0.9 (0.4–1.5)	0.8 (0.4–1.2)	0.8 (0.6–1.0)	0.7 (0.6–0.9)	0.8 (0.6–1.0)	0.8 (0.6–1.0)
NEFA (μmol/L)	355 (59–651)	670 (367–973) ^a	345 (262–428)	834 (655–1013) ^{d,e}	343 (261–425)	625 (513–737) ^b
TSH (IU/L)	1.8 (1.4–2.1)	1.3 (1.0–1.6) ^b	1.6 (1.2–2.0)	1.3 (0.9–1.6) ^b	1.6 (1.3–1.9)	1.4 (1.1–1.7)
Free T3 (pmol/L)	5.5 (5.3–5.8)	5.3 (5.1–5.5)	5.3 (4.9–5.7)	5.0 (4.5–5.4) ^{c,f}	5.6 (5.3–6.0)	5.5 (5.3–5.8)
Free T4 (pmol/L)	17.5 (14.7–20.2)	17.5 (15.9–19.1)	16.6 (15.6–17.6)	17.5 (16.4–18.7) ^c	16.6 (15.6–17.7)	17.8 (16.6–19.1) ^d
Cortisol (nmol/L)	243 (158–328)	151 (102–200) ^c	235 (200–270)	152 (115–189) ^{d,e}	229 (205–252)	237 (187–287)
Leptin (ng/mL)	1.1 [0.2–5.1]	1.2 [0.1–3.9]	1.3 (0.5–2.0)	0.9 (0.4–1.4) ^a	1.2 (0.6–1.8)	0.7 (0.3–1.2) ^a
Total ghrelin (pg/mL)	610 (404–816)	607 (406–808)	647 (537–757)	588 (516–660) ^{c,f}	654 (579–728)	664 (580–749)
Acylated ghrelin (pg/mL)	28.9 [10–140]	21 [12–213]	57.2 (30.6–83.8)	48.0 (20.4–75.5)	43.5 (20.4–66.5)	53.3 (29.9–76.7)
HOMA-IR	1.71 (0.57–2.86)	1.66 (0.69–2.64)	1.44 (0.57–2.30)	1.37 (0.56–2.18)	1.51 (1.06–1.96)	1.13 (0.62–1.65) ^b

Values are means with 95% confidence interval (CI) in parentheses for normally distributed data and median [interquartile range] for nonparametric data. Two-way ANOVA for repeated-measures with Bonferroni post hoc test.

^aEffect of intervention, $p \leq 0.05$

^b $p < 0.01$

^c $p < 0.001$

^d $p < 0.0001$

^eDifferent than cold exposure, $p < 0.01$

^f $p < 0.001$

the 50 mg dose, systolic and diastolic blood pressure did not change from pre-treatment levels (Figures 2A and 2B), while heart rate did trend toward increasing, on average 6 beats·min⁻¹ above pre-treatment levels (95% CI: 1–12, $p = 0.06$) (Figure 2C). This increase in heart rate is consistent with the average increases reported in FDA briefing documents (FDA, 2012). In contrast, the 200 mg dose increased systolic blood pressure and heart rate, on average by 10 mmHg (95% CI: 7–13, $p < 0.0001$) and 11 beats·min⁻¹ (95% CI: 7–15, $p < 0.0001$) (Figures 2A and 2C). The latter is consistent with and of the same magnitude as previously reported secondary effects (Baskin et al., 2018; Cypess et al., 2015; Malik et al., 2012) and among the dose-dependent treatment emergent adverse events previously documented from the mirabegron clinical trials for the treatment of overactive bladder (Sacco and Bientinesi, 2012). Indeed, a single 200 mg dose of mirabegron was previously shown to increase heart rate by up to 12 beats·min⁻¹ and systolic blood pressure up to 10 mmHg, which was attenuated when co-ingested with propranolol (β_1/β_2 -AR antagonist) or bisoprolol (a β_1 -AR antagonist), suggesting β_1 -AR-mediated stimulation of the heart (van Gelde-reen et al., 2017). To examine the effects of mirabegron on WAT lipolysis, we used continuous infusion of [²H]glycerol and [U-¹³C]palmitate to determine the rate of systemic appearance of glycerol (indicator of whole-body WAT lipolysis) and NEFA. While the 50 mg dose of mirabegron did not increase the lipolytic rate or fatty acid oxidation, the 200 mg dose increased both

whole-body lipolysis (Figure 2D) and fatty acid oxidation (Figure 2F). The increased systemic rate of appearance of glycerol (Figure 2D) and NEFA (Figure 2E) at the maximal allowable dose is consistent with off-target binding to the β_2/β_1 -AR in WAT, resulting in the stimulation of lipolysis. In steady-state conditions, the difference between the total fatty acids released upon intracellular hydrolysis of triglyceride ($3 \times \text{Ra}_{\text{glycerol}}$) and the rate of total fatty acid oxidation (calculated from indirect respiratory calorimetry) will provide the total rate of fatty acid re-esterification, as this is primarily its alternative metabolic fate. The marked increase in whole-body lipolytic rate in response to the 200 mg dose of mirabegron exceeded the whole-body fatty acid oxidation rate, suggestive of a significant increase in the re-esterification of these fatty acids (Figure 2G). Since only a fraction of the total fatty acids released following intracellular lipolysis appeared in circulation, this suggests that a large proportion of fatty acids were retained *in situ* and ultimately re-esterified at the primary site of lipolysis (intracellular cycling), rather than after their transit in circulation where they can be taken up by the liver and incorporated into very low-density lipoproteins (VLDLs) or intracellular fat droplets (extracellular cycling). We estimate that this futile triglyceride-fatty acid cycle accounted for approximately 55% of the increase in energy expenditure in response to the 200 mg dose of mirabegron, compared to 28% during cold exposure (Figure 2H). Assuming that the thermogenic contribution of the heart represents 20% of whole-body energy expenditure at rest (Kudomi

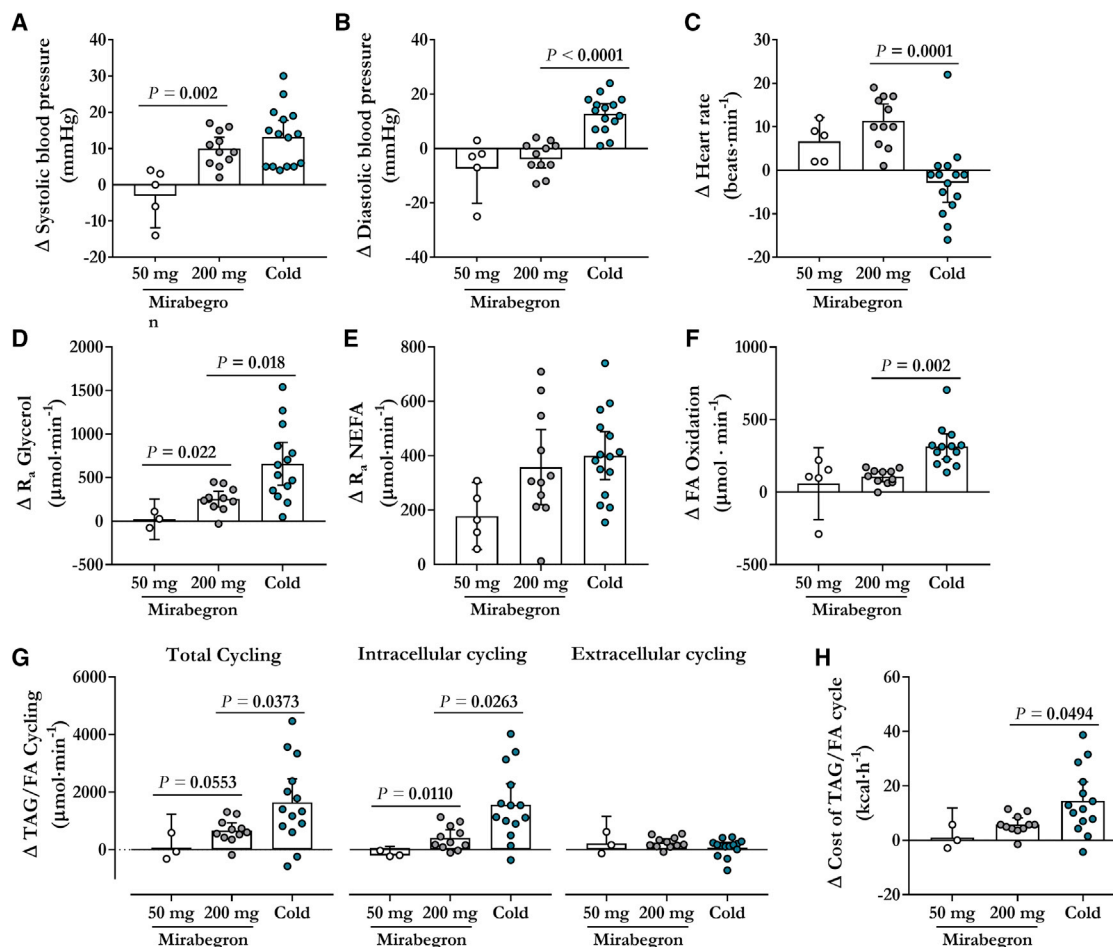


Figure 2. High-Dose Mirabegron Leads to Concomitant β -AR Activation

(A–C) Change in systolic blood pressure (A), diastolic blood pressure (B), and heart rate (C) from basal levels in response to oral administration of 50 mg (n = 5) or 200 mg (n = 11) of mirabegron and mild cold exposure (18°C, n = 15).

(D) Change in systemic rate of appearance of glycerol as index of whole-body lipolytic rate from basal levels in response to oral administration of 50 mg (n = 3) or 200 mg (n = 11) of mirabegron and mild cold exposure (18°C, n = 14).

(E) Change in systemic rate of appearance of NEFA, from basal levels in response to oral administration of 50 mg (n = 5) or 200 mg (n = 11) of mirabegron and mild cold exposure (18°C, n = 15).

(F) Change in whole-body fatty acid oxidation determined by indirect calorimetry, from basal levels in response to oral administration of 50 mg (n = 5) or 200 mg (n = 11) of mirabegron and mild cold exposure (18°C, n = 13).

(G) Change in total, intracellular, and extracellular triacylglycerol/fatty acid (TAG/FA) cycling from basal levels in response to oral administration of 50 mg (n = 3) or 200 mg (n = 11) of mirabegron and mild cold exposure (18°C, n = 14).

(H) Change in the energetic cost of TAG/FA cycle.

Data are reported as mean with 95% CI. Difference between 50 and 200 mg mirabegron determined using Mann-Whitney test. Difference between 200 mg mirabegron and mild cold exposure determined using paired-sample t test.

et al., 2019; Müller et al., 2013), we estimate that the increase in heart rate observed with the 200 mg mirabegron could account for 31% of the increase in energy expenditure. Thus, this dose of mirabegron seemed sufficiently high to induce non-specific adrenergic responses in various human tissues. Together, upward of 86% of the increase in energy expenditure upon treatment with a high dose of mirabegron can be accounted for by off-target effects, unrelated to BAT activation. Hence, we were unable to activate BAT thermogenesis in humans when using the β_3 -AR agonist at a therapeutically relevant dose, and when using the maximal dose, β_1 -/ β_2 -AR-dependent responses were stimulated in the heart and WAT.

Human Brown Adipocyte Thermogenesis Is Driven by β_2 -AR Stimulation

The observations from our human *in vivo* studies led us to question whether the β_3 -AR subtype is truly required to stimulate BAT thermogenesis in humans. We accessed RNA sequencing data from a previous study analyzing human BAT biopsies obtained from the deep neck region (Jespersen et al., 2020) (Figure 3A). Similarly, RNA sequencing analysis of *in vitro* differentiated brown adipocytes revealed that gene expression for the β_3 -AR (*ADRB3*) is nearly undetectable in human brown adipocytes (Tran et al., 2020) (Figure 3B). This analysis demonstrated that most of the β -AR mRNAs in human brown adipocytes are

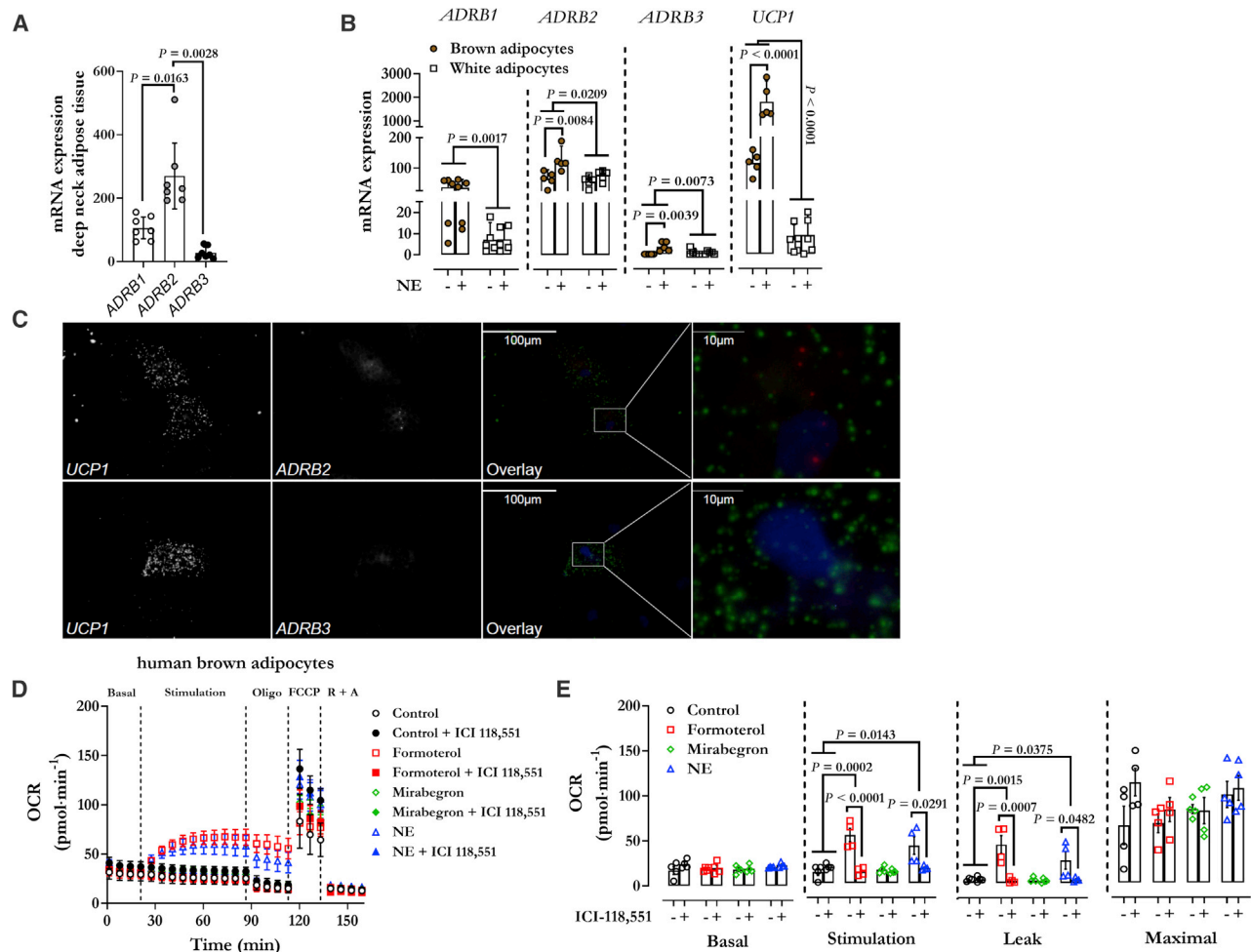


Figure 3. Human Brown Adipocyte Thermogenesis Driven by β_2 -AR Stimulation

(A) Relative gene expression, determined by RNA sequencing analysis, of the three β -AR subtypes from brown adipose tissue biopsies derived from the deep neck depot. Data are mean with 95% CI of $n = 7$ biologically independent samples.

(B) Relative gene expression, determined by RNA sequencing analysis, of the three β -AR subtypes and *UCP1* from *in vitro* differentiated brown and white preadipocytes, differentiated without and with norepinephrine (NE) (1 μ M) stimulation. Data are mean with 95% CI of $n = 5$ biologically independent samples.

(C) *UCP1*, *ADRB2*, and *ADRB3* expression by fluorescent *in situ* hybridization. *UCP1* and *ADRB2* or *ADRB3* detection in *in vitro* differentiated human brown adipocytes. In far right colored images, DAPI staining in blue, *UCP1* staining in green, and β -AR subtype staining in red.

(D) Oxygen consumption rate (OCR) of brown adipocytes stimulated with the β_2 -AR agonist formoterol (5.5 ng/mL), β_3 -AR agonist mirabegron (40 μ g/mL), and NE (1 μ M) pre-incubated without and with the β_2 -AR antagonist ICI 118,551 (1 μ g/mL). Oligo, oligomycin; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenyl-hydrazone; R + A, rotenone + antimycin A. Data are mean $\pm$ SEM of $n = 4$ biologically independent samples.

(E) Effect of addition of formoterol, mirabegron, and NE on brown adipocyte OCR (Stimulation), leak respiration, and chemically uncoupled respiration (Maximal) pre-incubated without and with the β_2 -AR antagonist ICI 118,551.

Data are mean $\pm$ SEM of $n = 4$ biologically independent samples. Paired-sample *t* test (A), two-way ANOVA with Tukey post hoc test (B and E). See also Table S2 (A) and Table S3 (C–F) for donor characteristics.

represented by the β_2 -AR (*ADRB2*) subtype followed by β_1 -AR (*ADRB1*). On average, $66\% \pm 23\%$ of the β -AR mRNA expression consists of *ADRB2* when differentiated in an unstimulated state, which increases to $77\% \pm 11\%$ when acutely stimulated with norepinephrine (Figure 3B). The *ADRB1*, *ADRB2*, and *ADRB3* were all more abundant in the brown compared to white adipocytes, and both *ADRB2* and *ADRB3* expression in the brown adipocytes increased in response to norepinephrine stimulation. As expected (Lafontan and Berlan, 1993), in white adipocytes 87%–91% of β -AR mRNA consists of *ADRB2* (Figure 3B). These findings are consistent with results previously reported in brown

adipocytes obtained from the perirenal depot of human newborns (Deng et al., 1996) and epicardial adipose tissue (Chechi et al., 2017). Other investigators have reported the presence of *ADRB3* in BAT from the supraclavicular (Jespersen et al., 2019; Virtanen et al., 2009), deep neck (Cypess et al., 2013), and perirenal depots (Jespersen et al., 2019; Krief et al., 1993). Of these, only two also reported the presence of *ADRB1* and *ADRB2* subtypes, with one showing a higher relative abundance of the *ADRB2* compared to *ADRB1* and *ADRB3* in the perirenal depot (Krief et al., 1993), and the other a similar abundance between the *ADRB2* and *ADRB3* (Jespersen et al., 2019). Hence,

ADRB2 is undoubtedly expressed in human BAT. However, human BAT is heterogeneous, consisting of a mixture of multilocular adipocytes interspersed among unilocular, more white-like adipocytes (Cypess et al., 2013; Jespersen et al., 2013, 2019; Perdikari et al., 2018). To assess whether *ADRB2* was expressed in active brown adipocytes, we stimulated *in vitro* differentiated human brown adipocytes acutely with norepinephrine and co-stained cells for *UCP1* and *ADRB2* using RNAscope *in situ* hybridization assays. Convincingly, we demonstrate that *ADRB2* is co-expressed with *UCP1* in norepinephrine-stimulated human brown adipocytes, whereas *ADRB3* remained undetected (Figure 3C).

To investigate whether the adrenergic response in human BAT is mediated by β_2 -AR or β_3 -AR, we stimulated *in vitro* differentiated primary human brown adipocytes with selective β_2 -AR or β_3 -AR agonists or a β_2 -AR antagonist and examined the coupled and uncoupled respiration in response to norepinephrine or to saline, as positive and negative controls, respectively. Importantly, norepinephrine has affinity to all three β -AR subtypes. *In vitro* differentiated human brown adipocytes respond to norepinephrine stimulation with a pronounced increase in uncoupled respiration (Jespersen et al., 2019, 2020), as would be expected for mature brown adipocytes. Consistent with the *in vivo* results, stimulation of human brown adipocytes with the β_3 -AR agonist mirabegron did not stimulate coupled or uncoupled respiration (Figures 3D and 3E). In contrast, stimulation with the highly selective β_2 -AR agonist formoterol (646 times higher affinity for human β_2 -AR over β_3 -AR) (Baker, 2010) markedly increased leak respiration, to a similar extent as with norepinephrine stimulation (Figures 3D and 3E). Consistently, pre-exposing the human brown adipocytes to the β_2 -AR antagonist ICI-118,551 completely abolished both the formoterol- and the norepinephrine-stimulated leak respiration (Figures 3D and 3E), strongly supporting our hypothesis that the brown adipocyte respiration was induced through β_2 -AR and not through the β_3 -AR.

To further validate the thermogenic responses of the human brown adipocytes, we investigated the maximal lipolytic response of human immortalized brown adipocytes incubated with mirabegron, formoterol, or norepinephrine in the presence or absence of ICI-118,551. These results demonstrate that a higher concentration of mirabegron is required, relative to formoterol, to elicit the maximal lipolytic response and this response can be suppressed with the β_2 -AR antagonist ICI-118,551 (Figures 4A, 4C, 4D, and 4F). It is important to note that receptor-binding studies using membrane fractions from Chinese hamster ovary cells expressing all three human β -AR subtypes have shown that the affinity of mirabegron for β_3 -AR is ~ 40 nM (Takasu et al., 2007), which represents two orders of magnitude lower than what was required to elicit a lipolytic response in the human brown adipocytes of the present study. In contrast, nanomolar (10^{-9} M) concentrations of the β_2 -AR agonist formoterol can already elicit an increase in brown adipocyte lipolysis, which can also be suppressed in the presence of ICI-118,551 (1 μ g/mL) (Figures 4B and 4E). Upon determining the appropriate dose response of brown adipocytes to mirabegron and formoterol, we then extended these determinations to brown adipocytes immortalized and differentiated from three different patients. Lipolysis increased in response to mirabegron (1 μ M)

in the cells derived from some patients (Figures 4H and 4I), but not others (Figures 4G and 4J). In contrast, formoterol (100 nM) elicited a robust lipolytic response in all three patients (Figures 4H–4J). Importantly, in the presence of ICI-118,551 (3 μ g/mL), mirabegron-, formoterol-, and norepinephrine-stimulated lipolysis was completely inhibited.

The assumption that β_3 -AR signaling is essential to stimulate the intracellular lipolysis required to activate UCP-1-dependent BAT thermogenesis is built upon extensive literature from BAT studies performed in rodent models (Cannon and Nedergaard, 2004). To compare our findings with a model where β_3 -AR was known to be the active receptor, we therefore examined the rodent β -AR distribution within the whole tissue, isolated stromal vascular fraction, and isolated adipocytes of interscapular BAT harvested from mice housed at room temperature ($\sim 22^\circ\text{C}$). Consistent with the long-established literature, we found that β_3 -AR was the predominant β -AR subtype in mouse BAT (mRNA and protein) (Figures 5A and 5B). In contrast to the human brown adipocytes, the intracellular lipolysis of mouse-derived brown adipocytes increased upon stimulation with mirabegron, as well as with formoterol and norepinephrine (Figures 5C and 5D). The stimulated intracellular lipolysis was only partially suppressed in the presence of ICI-118,551 (Figures 5E–5H), consistent with the reliance on other β -AR subtypes, primarily the β_3 -AR, to initiate lipolysis in mouse brown adipocytes. This is consistent with what has previously been reported in isolated brown adipocytes derived from mice lacking β_3 -AR (Preitner et al., 1998). These findings show that our protocol is consistent with established literature on β -AR signaling in rodent BAT, hence underscoring the validity of the data from our human models.

Brown Adipocyte β_2 -AR Knockdown Impairs Thermogenesis

To validate that the β_2 -AR is essential for human brown adipocyte thermogenesis, we performed a transient small interfering RNA (siRNA) knockdown of *ADRB2* in brown adipocytes obtained from four different donors (Figure 6A). *ADRB2* mRNA levels were effectively knocked down in both unstimulated and stimulated (norepinephrine and formoterol) conditions (Figure 6A). This led to a minor *ADRB3* compensatory response (Figure 6B), which was insufficient to maintain *UCP1* and *PPARGC1A* gene expression upon *ADRB2* knockdown. Both *UCP1* and *PPARGC1A* mRNA fell to levels seen under the unstimulated control condition (Figures 6C and 6D). To examine the effect of the *ADRB2* knockdown on brown adipocyte thermogenesis, we measured norepinephrine- and formoterol-stimulated respiration of differentiated human brown adipocytes (Figure 6E). Exposure to norepinephrine and formoterol stimulated leak respiration in control cells but was significantly suppressed following the siRNA knockdown of *ADRB2* (Figure 6F). The targeted knockdown of *ADRB2* reduced norepinephrine- and formoterol-stimulated respiration by $27\% \pm 11\%$ and $36\% \pm 6\%$, respectively (Figure 6G). Leak respiration was particularly reduced by *ADRB2* knockdown in response to norepinephrine and formoterol, falling by $50\% \pm 9\%$ and $55\% \pm 7\%$, respectively. We next performed transient knockdown of the *ADRB1*, *ADRB2*, and *ADRB3* in parallel in human brown adipocytes from different donors (Figure 6H). Cell strains without

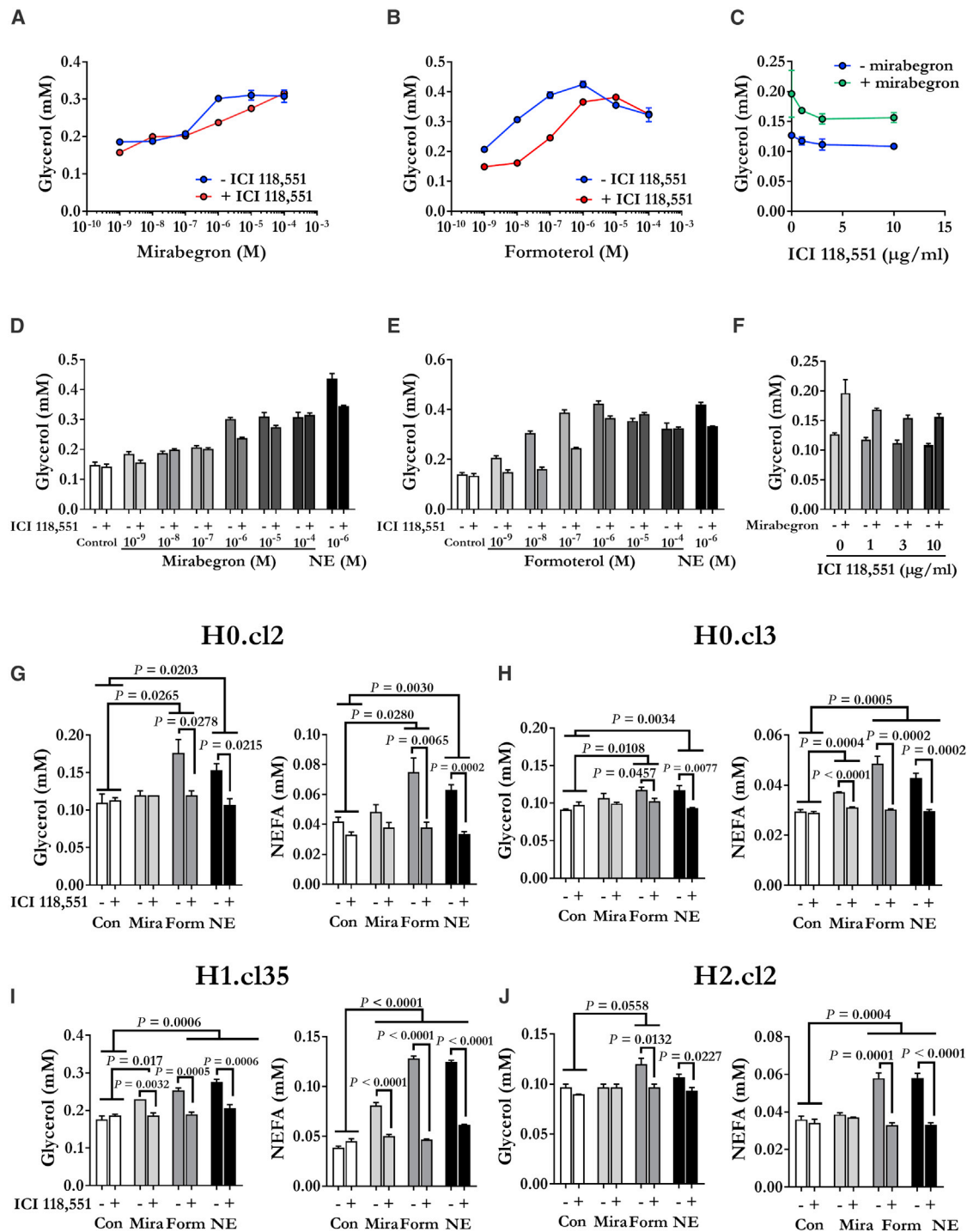


Figure 4. Human Brown Adipocyte Lipolysis Driven by β_2 -AR Stimulation

(A–F) Dose-response curves of glycerol release, as index of intracellular lipolysis, from immortalized and cloned brown adipocytes stimulated with varying doses of mirabegron or formoterol and norepinephrine (NE) (1 μ M) pre-incubated without and with the β_2 -AR antagonist ICI 118,551 (1 μ g/mL). Data are mean $\pm$ SEM of three technical replicates.

(G–J) Glycerol release, as index of intracellular lipolysis, and NEFA release from brown adipocytes immortalized and cloned from three patients, stimulated with mirabegron (Mira, 1 μ M), formoterol (Form, 100 nM), and NE (1 μ M) pre-incubated without and with the β_2 -AR antagonist ICI 118,551 (3 μ g/mL).

Data are mean $\pm$ SEM of three independent experiments (n = 3 technical replicates). Two-way ANOVA with Tukey post hoc test. See also Table S4 for donor characteristics.

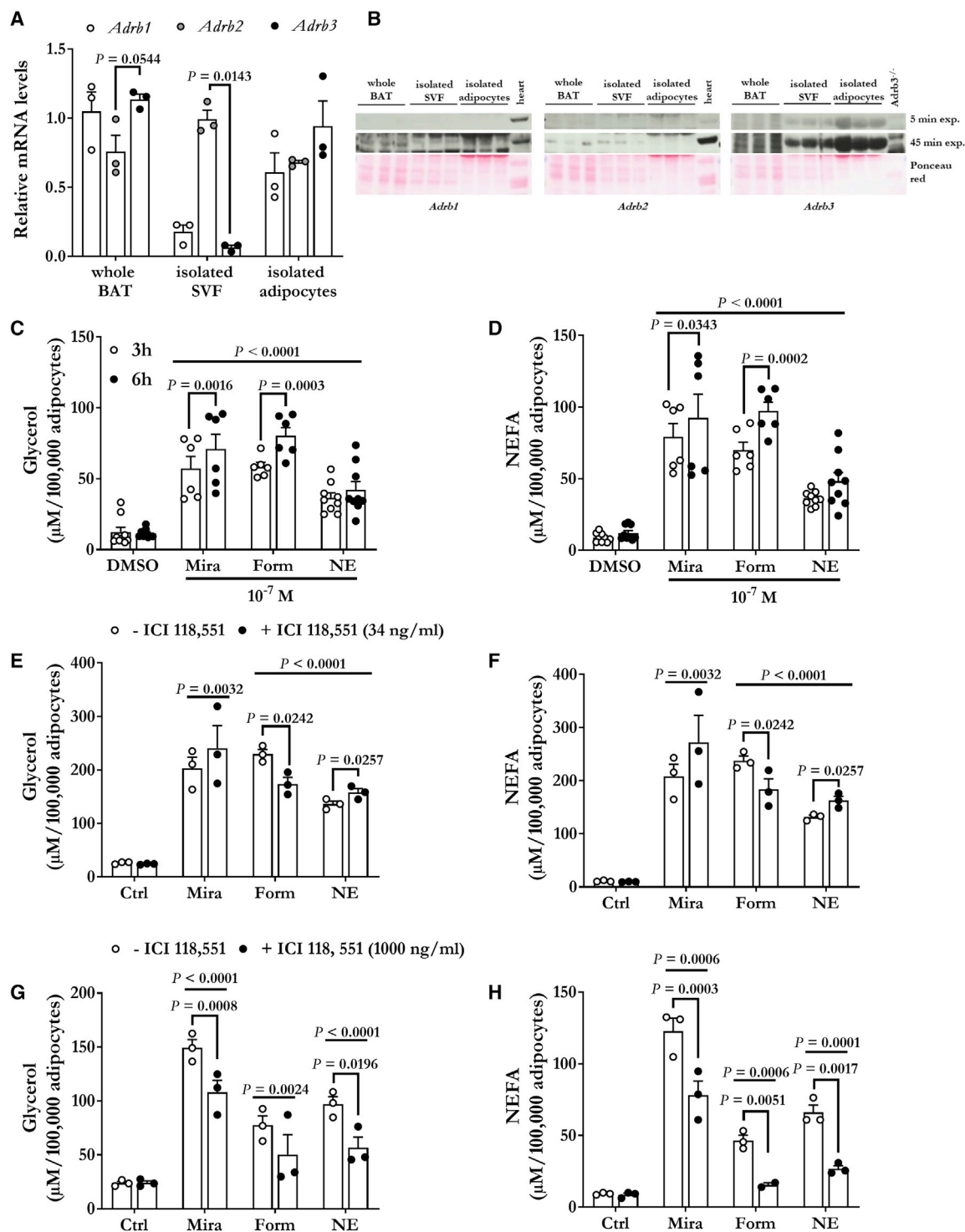


Figure 5. Mouse Brown Adipocyte Lipolysis in Response to β -AR Agonist Stimulation

(A) Relative gene expression of the β -AR in whole tissue, isolated stromal vascular fractions (SVFs), and isolated adipocytes of interscapular BAT harvested from 10-week-old C57BL/6J mice housed at room temperature ($\sim 22^\circ\text{C}$). Data are mean $\pm$ SEM of $n = 3$ biologically independent samples.

(B) Western blot and ponceau staining of β_1 -AR, β_2 -AR, and β_3 -AR in whole tissue, isolated SVFs, and isolated adipocytes of interscapular BAT harvested from 10-week-old C57BL/6J mice housed at room temperature ($\sim 22^\circ\text{C}$). $n = 3$ biologically independent samples.

(C and D) Glycerol release, as index of intracellular lipolysis, and NEFA release from isolated brown adipocytes, stimulated with $1 \mu\text{M}$ mirabegron (Mira, $n = 6$), formoterol (Form, $n = 6$), or norepinephrine (NE) ($n = 9$), measured 3 and 6 h following stimulation. Data are mean $\pm$ SEM of independent experiments. Two-way ANOVA with Tukey post hoc test.

(legend continued on next page)

knockdown were excluded to assess the effects of reduced receptor expression on *UCP1* mRNA levels. *ADRB3* mRNA levels were undetectable regardless of treatment. Transient siRNA knockdown of the *ADRB1* and *ADRB2* (Figure 6I) had differential effects on *UCP1* expression, with knockdown of the *ADRB2* suppressing *UCP1* expression by $48\% \pm 16\%$. *UCP1* mRNA levels were also measured in samples transfected with siRNAs targeting *ADRB3*, which, as expected, did not reduce *UCP1* (Figure 6I). Further, norepinephrine stimulation robustly increased *UCP1* expression, whereas stimulation with the β_3 -AR agonist CL-316,243, similarly to mirabegron, had no effect (Figure 6J). In further support of the role of the β_2 -AR in human brown adipocyte thermogenesis, transient siRNA knockdown of *ADRB1* and *ADRB3* had no effect on norepinephrine-stimulated respiration, whereas leak respiration was reduced under norepinephrine stimulation upon *ADRB2* knockdown only, falling by $54\% \pm 15\%$ (Figure 6K).

DISCUSSION

Recent reports have emphasized the use of the β_3 -AR agonist mirabegron as a pharmacological strategy to target human BAT (Baskin et al., 2018; Cypess et al., 2015; Finlin et al., 2018, 2020; Loh et al., 2019; O'Mara et al., 2020), whereas the application of β_3 -AR agonists has historically performed poorly in human clinical trials (Arch, 2011; Larsen et al., 2002; Redman et al., 2007) and stimulates cardiovascular responses upon both acute (Baskin et al., 2018; Cypess et al., 2015) and daily administration (O'Mara et al., 2020). Several hypotheses have been proposed to explain these discrepancies, including an absence of BAT in humans, poor expression of β_3 -AR, or poor efficacy of the agonists (Arch, 2011). With the presence of BAT thermogenesis in humans now unequivocal, our data propose an alternative explanation, namely that human BAT thermogenesis is driven primarily by β_2 -AR-mediated signaling.

We provide several lines of evidence supporting the premise that human BAT thermogenesis is mediated by the stimulation of the β_2 -AR. Our *in vivo* experiments demonstrate that only a maximal dose of mirabegron, which elicits a cross-activation with all three β -AR subtypes leading to the stimulation of cardiovascular responses (β_1 -AR mediated) and WAT lipolysis (primarily β_2 -AR mediated), could stimulate BAT oxidative metabolism. Such a cross-activation at high doses of mirabegron is consistent with data previously reported in FDA advisory committee briefing documents (FDA, 2012). These documents report that despite the 105-fold higher affinity for the β_3 -AR over the β_1 -AR and 33-fold higher affinity over the β_2 -AR, mirabegron also shows poor specificity between β -AR subtypes at high exposure levels. To further support the potential role of β_2 -AR in BAT thermogenesis, *in vitro* experiments independently conducted by two laboratories clearly demonstrated that both *in vitro* differentiated brown adipocytes from the deep neck depot and immor-

talized brown adipocytes obtained from the isthmus region of the thyroid gland could increase uncoupled respiration and lipolysis, respectively, upon β_2 -AR, but not β_3 -AR, agonist stimulation. This β_2 -AR-mediated response was suppressed when brown adipocytes were pre-exposed to a β_2 -AR antagonist. The preferential role of β_2 -AR in human brown adipocytes was further emphasized by our knockdown experiments in which only the ablation of this receptor resulted in reduced response to norepinephrine-stimulated respiration and *UCP1* mRNA induction, whereas knockdown of *ADRB1* or *ADRB3* had no reducing impact on norepinephrine response. Interestingly, the role of β_3 -AR in human brown adipocytes was recently challenged elsewhere (Riis-Vestergaard et al., 2020). However, in that study, β_1 -AR was found to be the receptor of choice for brown adipocyte signaling, in favor of β_3 -AR, whereas β_2 -AR was not assessed. Importantly, the study was based on experiments in one immortalized cell line, whereas we here utilized both immortalized and non-immortalized cells derived from several different human donors. Previous investigations have administered formoterol orally (Lee et al., 2013) or salbutamol (a less specific β_2 -AR agonist) intravenously (Hoeks et al., 2003) in humans, which stimulated thermogenesis and fatty acid oxidation to a similar magnitude to what was observed here with the maximal dose of mirabegron, without the off-target cardiovascular responses. However, the source of this increased thermogenesis remains to be determined.

Our discovery that β_2 -AR stimulation is essential to human BAT lipolysis and thermogenesis now presents a novel pharmacological target to stimulate human BAT. These results also provoke further questions regarding the intrinsic differences between human and rodent brown adipocytes and the implications for translational BAT research. The question remains as to whether the proportion of the different β -AR subtypes and even the intrinsic thermogenic activity differ between depots in adult humans and rodents and whether certain depots in rodents more closely resemble human BAT. Recent evidence suggests that differences in the expression of genes responsible for thermogenesis and the *in vivo* adipocyte morphology between rodent and human BAT may reflect their contrasting thermal and nutritional conditions (de Jong et al., 2019). In the present study, we observe clear differences in the expression of β -AR subtypes and lipolytic/thermogenic responses to different β -AR agonists in cells isolated from humans and mice, which argues for intrinsic species-specific differences rather than differences in activity or recruitment state due to environmental exposures. Our findings also lead to further questions regarding the commonalities between WAT and BAT in humans and whether a generalized dysregulation in adipose tissues can impair both energy storage and dissipation leading to an imbalance in whole-body energy homeostasis. For example, the blunted lipolytic action of catecholamines in WAT observed in insulin resistance and type 2 diabetes has been attributed to a reduction in β_2 -AR density in

(E and F) Glycerol (E) and NEFA (F) release from isolated brown adipocytes, stimulated with 1 μ M mirabegron (Mira), formoterol (Form), or NE pre-incubated without and with the β_2 -AR antagonist ICI 118,551 (34 ng/mL). Data are mean $\pm$ SEM of three independent experiments. Two-way ANOVA with Tukey post hoc test.

(G and H) Glycerol (G) and NEFA (H) release from isolated brown adipocytes, stimulated with 1 μ M mirabegron (Mira), formoterol (Form), or NE pre-incubated without and with the β_2 -AR antagonist ICI 118,551 (1,000 ng/mL). Data are mean $\pm$ SEM of three independent experiments. Two-way ANOVA with Tukey post hoc test.

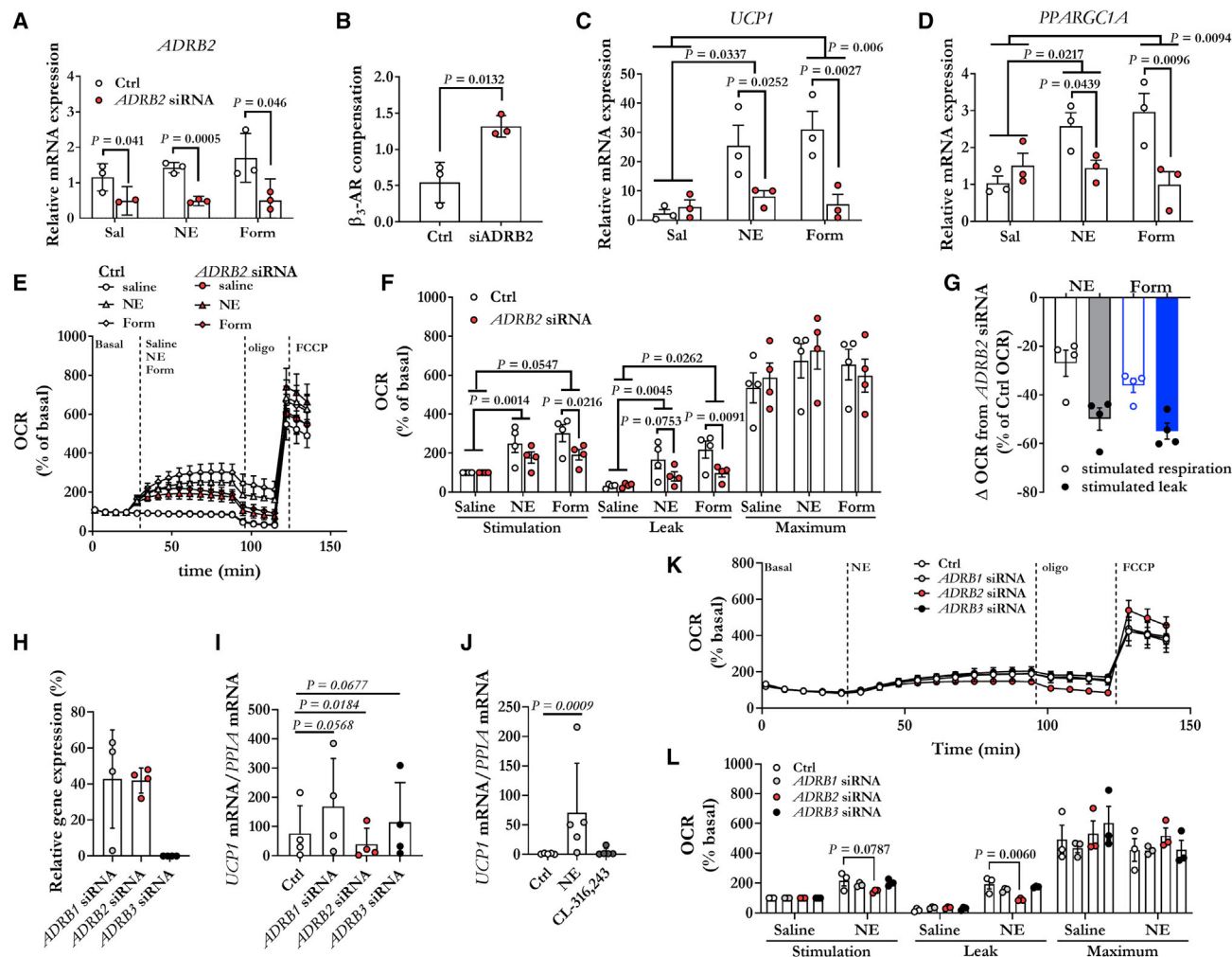


Figure 6. Brown Adipocyte β_2 -AR Knockdown Impairs Thermogenesis

(A) Relative gene expression of the β_2 -AR from brown adipocytes treated without (Ctrl) and with $ADRB2$ siRNA to knock down β_2 -AR mRNA in adipocytes under norepinephrine (NE) (1 μ M) and formoterol (Form, 5.5 ng/mL) stimulation. Data are mean $\pm$ SEM of $n = 3$ biologically independent samples.

(B) Effect of transfecting brown adipocytes without (Ctrl) and with $ADRB2$ siRNA on β_3 -AR gene expression. Data are mean $\pm$ SEM of $n = 3$ biologically independent samples.

(C and D) Effect of transfecting brown adipocytes without (Ctrl) and with $ADRB2$ siRNA on $UCP1$ (C) and $PPARGC1A$ (D) expression following NE (1 μ M) or formoterol (Form, 5.5 ng/mL) stimulation. Data are mean $\pm$ SEM of $n = 3$ biologically independent samples.

(E) Oxygen consumption rate (OCR) of brown adipocytes treated without (Ctrl) and with $ADRB2$ siRNA and stimulated with NE (1 μ M) or formoterol (5.5 ng/mL). Oligo, oligomycin; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; R + A, rotenone + antimycin A. Data are mean $\pm$ SEM of $n = 4$ biologically independent samples.

(F) Effect of treating brown adipocytes without (Ctrl) and with $ADRB2$ siRNA on NE- or formoterol-stimulated OCR (Stimulation), leak respiration, and chemically uncoupled respiration (Maximum). Data are mean $\pm$ SEM of $n = 4$ biologically independent samples.

(G) Change in NE- or formoterol (Form)-stimulated OCR of brown adipocytes following $ADRB2$ siRNA. Data are mean $\pm$ SEM of $n = 4$ biologically independent samples.

(H and I) Relative gene expression of $ADRB1$, $ADRB2$, and $UCP1$ of brown adipocytes treated without (Ctrl) and with $ADRB1$, $ADRB2$, or $ADRB3$ siRNA to knock down β_1 -, β_2 -, or β_3 -AR mRNA in adipocytes under NE stimulation. Data are mean $\pm$ SEM of $n = 4$ biologically independent samples.

(J) Relative gene expression of $UCP1$ of brown adipocytes treated without (Ctrl) and with NE or the β_3 -AR agonist, CL-316,243 (10 nM). Data are mean $\pm$ SEM of $n = 5$ biologically independent samples.

(K) OCR of brown adipocytes treated without (Ctrl) and with $ADRB1$, $ADRB2$, or $ADRB3$ siRNA and stimulated with NE (1 μ M). Data are mean $\pm$ SEM of $n = 4$ biologically independent samples.

(L) Effect of treating brown adipocytes without (Ctrl) and with $ADRB1$, $ADRB2$, or $ADRB3$ siRNA on NE-stimulated OCR (Stimulation), leak respiration, and chemically uncoupled respiration (Maximum). Data are mean $\pm$ SEM of $n = 4$ biologically independent samples.

Paired-sample t test (A, B, and H–J), two-way ANOVA with Tukey post hoc test (C, D, F, and L).

isolated white adipocytes (Reynisdottir et al., 1994). While this does not appear to impair BAT thermogenesis under cold-stimulated conditions in individuals with type 2 diabetes (Blondin et al., 2015), this may be a result of the greater sympathetic innervation and/or local norepinephrine turnover in BAT relative to WAT. Alternatively, a reduction in β_2 -AR density in BAT may impact its thermogenic activity differentially from its recruitment, explaining the apparently lower BAT volume in these individuals.

Clearly, further work is required to clarify the discrepancies between species and adipose tissue depots, but the present findings provide a novel perspective for which to target human BAT. Our data combined with these previous findings support further investigations into the regulation of human BAT thermogenesis and recruitment, both in a healthy and dysregulated metabolic state, to provide further insight into the efficacy of recruiting futile metabolic cycles to dissipate excess energy. In addition, that BAT and whole-body thermogenesis can both increase in the cold while heart rate decreases significantly suggests that there is still much to be learned about pharmacologically mimicking the effects of cold exposure for developing BAT-activating agents in humans. One important consideration is the distinct difference in adrenergic activation and therefore cardiovascular and BAT responses resulting from the systemic delivery of β -AR agonists versus the local release of norepinephrine from sympathetic nerve terminals that elicit an integrated α - and β -adrenergic response.

Limitations of Study

The primary aim of this study was to investigate BAT thermogenesis following the oral administration of a single therapeutic or maximal allowable dose of the β_3 -AR agonist mirabegron. Follow-up *in vitro* experiments revealed that the oxygen consumption rate of human brown adipocytes is greatest under β_2 -AR agonism and abolished upon β_2 -AR antagonism. It remains unclear, however, whether β_2 -AR agonism can stimulate human BAT thermogenesis *in vivo*. Whether administration of a β_2 -AR agonist is more effective in stimulating human BAT thermogenesis *in vivo* and with fewer off-target effects than mirabegron is of considerable interest. While it was beyond the scope of the current clinical study, future investigations are required to examine the *in vivo* effects of β_2 -AR agonism on BAT thermogenesis. Careful consideration was given to the selection of formoterol as the β_2 -AR agonist for the *in vitro* experiments, as it is a prescribed pharmaceutical approved by the FDA and Health Canada for the treatment of asthma and chronic obstructive pulmonary disease and therefore most amenable for such a follow-up clinical trial. Although the *in vitro* evidence provides a strong basis to pursue the use of a β_2 -AR agonist to examine BAT thermogenesis *in vivo*, it is important to recognize that there may be certain differences in gene expression between *in vitro* differentiated brown adipocytes and brown adipocytes examined directly from biopsied tissue. As the cell cultures are grown two-dimensionally in a dish, it is reasonable to assume that they do not completely translate to the *in vivo* setting. Importantly, our data show that the proportional gene expression of the β -AR subtypes and *UCP1* from the *in vitro* differentiated brown adipocytes are in a similar range as the biopsied BAT (Figures 3A and 3B). Finally, while the present investigation provides evidence

that stimulation of the β_2 -AR is required to stimulate thermogenesis in brown adipocytes, radiolabeled binding studies are critical to further characterize the presence and density of β -AR in human BAT.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.cmet.2020.07.005>.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.C.C., D.P.B., C.S., S.N., M.C.S., V.H.J., P.C.N.R., E.N.K., F.P., S.M., F.H., D.R., E.E.T., and B.G.; Methodology, A.C.C., D.P.B., C.S., S.N., M.C.S., V.H.J., P.C.N.R., E.N.K., F.P., S.M., F.H., D.R., E.E.T., and B.G.; Investigation, A.C.C., D.P.B., C.S., S.N., M.C.S., V.H.J., P.C.N.R., E.N.K., F.P., S.M., F.H., D.R., E.E.T., and B.G.; Writing – Original Draft, D.P.B., A.C.C., and C.S.; Writing – Review & Editing, D.P.B., A.C.C., C.S., P.C.N.R., S.N., M.C.S., E.N.K., V.H.J., S.M., N.Z.J., S.K., M.R.B., F.P., D.R., E.E.T., F.H., and B.G.; Visualization, D.P.B.; Funding Acquisition, A.C.C., C.S., P.C.N.R., and F.P.

DECLARATION OF INTERESTS

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Beta-1 adrenergic receptor	Abcam	Cat# ab3442; RRID: AB_10890808
Beta-2 adrenergic receptor	Abcam	Cat# ab137494
Beta-3 adrenergic receptor	Abcam	Cat# ab94506; RRID: AB_10863818
anti-rabbit immunoglobulin G	Cedarlane	Cat# NA934V
Biological Samples		
Human brown adipose tissue (deep neck depot)	Jespersen et al., 2020	N/A
Human supraclavicular brown adipocytes and subcutaneous white adipocytes.	Jespersen et al., 2013	N/A
Human plasma samples	This paper	N/A
Chemicals, Peptides, and Recombinant Proteins		
Myrbetriq (Mirabegron)	Astellas Pharma	N/A
Insulin	Sigma-Aldrich, Millipore	Cat# RAB0327, Cat# HDIAB-34K-PMX5
Glucagon	Millipore	Cat# HDIAB-34K-PMX5
Leptin	Millipore	Cat# HDIAB-34K-PMX5
Adiponectin	Alpco	Cat# 80-ADPHU-E01
Ghrelin	Alpco	Cat# 32-5106
Seahorse XF base medium	Agilent	Cat# 103335-100
Norepinephrine	Amgros I/S	Cat# 745661
Formoterol	Sigma-Aldrich	Cat# 1286107
ICI 118,551	Sigma-Aldrich	Cat# I127
Mirabegron (<i>in vitro</i> experiments)	Sigma-Aldrich, MedChemExpress	Cat# SML2480, Cat# 14773
Oligomycin	Sigma-Aldrich	Cat# O4876
FCCP	Sigma-Aldrich	Cat# C2920
Antimycin	Sigma-Aldrich	Cat# A8674
Rotenone	Sigma-Aldrich	Cat# R8875
Lipofectamine RNAiMAX Transfection Reagent	Thermo Fisher Scientific	Cat# 13778150
DMEM/F-12, HEPES, no phenol red	Thermo Fisher Scientific	Cat# 11039-047
TaqMan Universal PCR Master Mix	Thermo Fisher Scientific	Cat# 4305719
PowerUp SYBR Green Master Mix	Thermo Fisher Scientific	Cat# A25777
Trizol	Roche, Life Technologies	Cat# 11667 165 001, Cat# 15596-018
Penicillin-Streptomycin	Thermo Fisher Scientific	Cat# BP295950
NucBlue Fixed Cell ReadyProbes Reagent	Thermo Fisher Scientific	Cat# R37606
Opti-MEM I Reduced Serum Medium	Thermo Fisher Scientific	Cat# 31985062
Critical Commercial Assays		
Seahorse XFe96 FluxPak	Agilent	Cat# 102416-100
Seahorse XF Cell Mito Stress Test Kit	Agilent	Cat# 103015-100
Direct-zol RNA miniprep kit	Zymo Research	Cat# R2063
High-Capacity cDNA Reverse Transcription kit	Thermo Fisher Scientific	Cat# 4374966
RNAscope Multiplex Fluorescent Reagent Kit v2	Advanced Cell Diagnostics	Cat# 323100
RNAscope Probe - Hs-UCP1-C (channel 2)	Advanced Cell Diagnostics	Cat# 2475821-C2

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
RNAscope Probe - Hs-ADRB2 (channel 1)	Advanced Cell Diagnostics	Cat# 450641
RNAscope Probe - Hs-ADRB3 (channel 1)	Advanced Cell Diagnostics	Made to order
Glycerol reagent set	Instruchemie, Sigma-Aldrich	Cat# 2913, Cat# F6428
NEFA C kit	Wako diagnostics, Randox laboratories	Cat# 434-91795, Cat# FA115
DC Protein Assay	Bio-Rad	Cat# 5000111
illustra RNAspin kit	GE Healthcare	Cat# 25050070
Advanced qPCR mastermix with supergreen dye	Wisent	Cat# 800-431
Experimental Models: Cell Lines		
Immortalized brown adipocytes	Liu et al., 2019	N/A
Experimental Models: Organisms/Strains		
Mouse: Male wild-type C57BL/6J	The Jackson Laboratory	JAX: 000664
Mouse: Male <i>Adrb3</i> ^{-/-}	The Jackson Laboratory	JAX: 006402
Oligonucleotides		
TaqMan primers	Life Technologies	Listed in Table S4
SybrGreen primers	N/A	Listed in Table S4
Short interfering RNAs	Dharmacon	Listed in Table S4
Software and Algorithms		
GraphPad Prism 8 for statistical analysis	GraphPad Software	RRID: SCR_002798
LabTEP	Centre d'imagerie moléculaire de Sherbrooke (CIMS)	https://www.crchus.ca/axes-de-recherche/imagerie-medicale/
Adobe Photoshop	Adobe	https://www.adobe.com/
ViiA7 software	Applied Biosystems	Applied Biosystems
Other		
[¹¹ C]acetate, radiotracer	CIMS	N/A
[¹⁸ F]FDG, radiotracer	CIMS	N/A
[3- ³ H]glucose, stable isotope tracer	PerkinElmer	Cat# NET331A005MC
[1,1,2,3,3- ² H]glycerol, stable isotope tracer	Cambridge Isotopes	Cat# DLM-1229
[U- ¹³ C]palmitate, stable isotope tracer	Cambridge Isotopes	Cat# CLM-3934
Gemini TF PET-CT scanner	Philips	N/A
Liquid-conditioned suit	Med-Eng	Cat# 1004340
Isotemp 6200R28 water bath	Fisher Scientific	Cat# 13-874-676
Vmax 29n (indirect calorimetry)	Sensormedics	N/A
Thermochron iButton	Maxim	Cat # DS1922H
Jonah temperature capsule	Mini-Mitter	N/A
Myomonitor IV EMG system	Delsys	https://delsys.com/

RESOURCE AVAILABILITY**Lead Contact**

Further information and requests for resources and reagents should be directed to the Lead Contact, André Carpentier (andree.carpentier@usherbrooke.ca).

Materials Availability

This study did not generate new unique reagents. We hereby disclose that the availability of biological material including the human cell cultures and tissue is dependent on specific permission from the Danish Data Protection Agency and on the researcher's adherence to the specifications of this permission.

Data and Code Availability

This study did not generate new code. Complete data for BAT RNA sequencing in the paper is available in [Jespersen et al. \(2020\)](#) and [Tran et al. \(2020\)](#).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

In Vivo Study Participants

A total of 22 men were enrolled in the study (Figure S1). However, six participants withdrew from the study before undertaking any experimental procedures. Of the remaining 16 participants, one completed the 50 mg mirabegron arm without completing the mild cold exposure and another did not receive the ^{11}C -acetate tracer injection following the oral administration of 200 mg of mirabegron due to problems with the production of the radiotracer. Characteristics of participants can be found in Table S1. Informed consent was obtained from all participants in accordance with the Declaration of Helsinki, the protocol received approval from the Human Ethics Committee of the Centre de recherche du Centre hospitalier universitaire de Sherbrooke and Health Canada provided regulatory approval for the off-label use of mirabegron. The experimental *in vivo* study was registered on [ClinicalTrials.gov](https://clinicaltrials.gov) (NCT02811289). The inclusion criteria were as follows: (i) male; (ii) age between 18 and 45 years; (iii) BMI < 30 kg/m²; (iv) normal glucose tolerance (2 h post 75 g OGTT glucose concentration < 7.8 mmol/L); (v) HbA1c < 5.8%. Exclusion criteria included: (i) overt cardiovascular disease as assessed by medical history, physical exam, and abnormal ECG; (ii) treatment with any drug known to affect lipid or carbohydrate metabolism; (iii) presence of liver or renal disease, uncontrolled thyroid disorder, previous pancreatitis, bleeding disorder, or other major illness; (iv) smoking (> 1 cigarette/day) and/or consumption of > 2 alcoholic beverages per day; (v) prior history or current fasting plasma cholesterol level > 7 mmol/L or fasting TG > 6 mmol/L.

Human Tissues and Cells

The human brown adipose tissue samples (N = 7, data presented in Figure 3A only) represent a normal weight sub-group of a cohort consisting of 26 women and 7 men undergoing thyroidectomy for a euthyroid goiter (Jespersen et al., 2020). Subject details can be found in Table S2. In accordance with the reported prevalence of hypoparathyroidism (Jørgensen et al., 2020), the number of men was small, hence we do not have the power necessary to assess the effect of sex on the reported outcomes. Biopsies were immediately snap frozen in liquid nitrogen and stored at -80°C until analyses. The study was approved by the Scientific-Ethics-Committee of the Capital Region of Denmark, journal number: H-1-2014-015 and was conducted in accordance with the Helsinki declaration. All participants provided informed consent prior to participation in the study.

Primary brown preadipocytes were also obtained from the deep neck fat depot located in the supraclavicular region, whereas primary white adipocytes were obtained from subcutaneous abdominal adipose tissue (Jespersen et al., 2013; Tran et al., 2020) (ethical approval number: H-A-2009-020). These cells represent a small subset of two previously published cohorts (Jespersen et al., 2013; Tran et al., 2020): (1) N = 5, presented in Figure 3B are derived from Tran et al. (2020) where a full description of subjects can be found, and; (2) N = 6, presented in Figures 3C–3E and 6 are derived from Jespersen et al. (2013) and subject details can be found in Table S3. Given the uneven distribution of male and female patients and low sample size, there is insufficient power to assess sex-related differences on the reported outcomes. Cells from different donors were subcultured and differentiated *in vitro*, as previously described (Jespersen et al., 2013; Larsen et al., 2019). Briefly, frozen aliquots of isolated adipogenic progenitors (Jespersen et al., 2013) were thawed in DMEM/F12 (Life technologies) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) (Life technologies). Cells were cultured at 37°C , 5% CO₂. Following expansion, cells were seeded, grown into 100% confluence, and allowed two days of post-confluence incubation before differentiation media I was added. This consisted of DMEM/F12 containing 1% Penicillin-Streptomycin, 0.1 μM dexamethasone (Sigma-Aldrich), 100 nM insulin (Actrapid, Novo Nordisk or Humulin, Eli Lilly), 200 nM rosiglitazone (Sigma-Aldrich), 540 μM isobutylmethylxanthine (IBMX) (Sigma-Aldrich), 2 nM T3 (Sigma-Aldrich) and 10 $\mu\text{g}/\text{mL}$ transferrin (Sigma-Aldrich). Media was refreshed every third day. IBMX was removed from the differentiation media at day three and rosiglitazone was removed at day six of differentiation. Cells were harvested at day 12 of differentiation. Adrenergically induced coupled and uncoupled respiration was determined with a Seahorse XF96 Extracellular Flux analyzer. Human brown preadipocytes were expanded in cell culture dishes until reaching 100% confluency and then plated 1:1 on Seahorse cell culture plates for differentiation. For the comparison of different adrenergic agonists, cells were fully differentiated as previously described (Larsen et al., 2019). Media was changed to Seahorse assay medium (phenol-free Seahorse XF base medium with 2 mM glutamine, 1 mM pyruvate and 25 mM glucose) 1 h before the Seahorse analysis on day 12 of differentiation.

Immortalized brown adipocytes were derived from brown preadipocytes isolated from biopsies obtained from the area close to the isthmus region of the thyroid gland from three patients scheduled for surgery in the thyroid region and who gave written informed consent. The procedure was approved by the Medical Ethical Committee of the Oxford Centre for Diabetes and performed in accordance with the principles of the revised Declaration of Helsinki. Subject details can be found in Table S4 and includes data presented in Figure 4. Cells were reversibly immortalized by using a lentiviral vector conferring doxycycline-controlled expression of simian virus large T antigen and cultured at 37°C , 5% CO₂ in growth medium (DMEM/F12 GlutaMAX medium, containing 10% heat-inactivated fetal bovine serum, penicillin and streptomycin) supplemented with 100 ng/mL doxycycline (Sigma-Aldrich), as previously described (Liu et al., 2019).

Mouse Brown Adipocytes

C57BL/6J mice (all males, 10 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Interscapular brown adipose tissue (BAT) depots were harvested from 4 mice, pooled, and digested with collagenase II (0.8 mg/mL) in oxygenated Krebs

buffer for 30 min in a 37°C incubator at 180 rpm. Then, tissue lysates were shaken vigorously for 10 s, and fresh Krebs buffer containing collagenase II (2 mg/mL) added, for an additional 20 min digestion. Floating isolated brown adipocytes were then washed 2 times with Krebs Buffer before being used for lipolysis assay.

METHOD DETAILS

In Vivo Study Design

Participants took part in two experimental sessions, separated by at least 7 days and with the order randomly assigned, in which participants ingested either 50 mg or 200 mg of the β_3 -AR agonist mirabegron (Myrbetriq, Astellas Pharma Canada) and took part in a 3 h cold exposure protocol. None of the participants were diagnosed with diabetes, impaired fasting glucose or impaired glucose tolerance, based on medical history, repeated assessment of fasting glucose concentration, and a 75 g oral glucose tolerance test. None were taking any medication, had any current medical condition known to affect lipid levels or insulin sensitivity, or had known cardiovascular or other medical conditions.

Each experimental session consisted of a baseline period at ambient temperature ($\sim 22^\circ$), followed by the oral administration of mirabegron or a 180 min mild cold exposure, elicited using our standard cooling protocol designed to stimulate low-level shivering to ensure that the cold exposure is adequate to stimulate BAT thermogenesis (Ouellet et al., 2012). Experiments were conducted between 07.30 and 15.00 h, following a 12 h fast and 48 h without strenuous physical activity. Participants followed a 2-day standard isocaloric diet which was determined following a 3-day food record and accounting for their standard daily physical activity level, which was determined by wearing a portable arm band accelerometer for 7-days and filling out a validated physical activity questionnaire. Upon arriving to the laboratory, participants emptied their bladder, then were weighed, instrumented with autonomous wireless temperature sensors (Thermochron iButton model DS1921H, Maxim) placed on 12 sites to measure mean skin temperature and swallowed a telemetric thermometry capsule to measure core temperature (VitalSense monitor and Jonah temperature capsule, Mini-Mitter). On the cold exposure day, participants were also instrumented with surface electromyography sensors on the belly of eight muscles known to contribute significantly to shivering during cold exposure (Ouellet et al., 2012): *m. pectoralis major*, *m. deltoideus*, *m. trapezius*, *m. sternocleidomastoid*, *m. biceps brachii*, *m. rectus femoris*, *m. vastus medialis* and *m. vastus lateralis*. Participants were then fitted with the liquid conditioned suit and performed a series of isometric maximal voluntary contractions (MVC) for each of the eight muscles being recorded by surface electromyography.

Metabolic Heat Production, Fuel Selection, Body Temperature and Cardiovascular Measures

Whole-body metabolic heat production and fuel selection were determined by indirect respiratory calorimetry, corrected for protein oxidation (Ouellet et al., 2012), performed every 60 min for a duration of 20 min. The molar rate of fatty acid oxidation was determined by converting the rate of triglyceride oxidation (mg/min) to its molar equivalent ($\mu\text{mol/min}$), assuming the average molecular weight of human triglyceride is 860 g/mol, and multiplying the molar rate of triglyceride oxidation by three, as each mole of triglyceride contains three moles of fatty acids (Wolfe and Chinkes, 2005). Energy potentials of 16.3, 40.8 and 19.7 kJ/g were used to calculate the amount of heat produced from glucose, lipid and protein oxidation, respectively, and converted to a rate in kcal/h. Mean skin temperature was calculated using an area-weighted mean from temperatures measured continuously by means of autonomous wireless temperature sensors placed over the forehead, upper back, lower back, abdomen, forearm, quadriceps, hamstring, gastrocnemius, anterior tibialis, chest, bicep and hand. Blood pressure and heart rate were assessed every 60 min using an Omron blood pressure monitor (HEM-790IT, Omron Healthcare).

PET Imaging and Analysis

Participants remained supine in a PET and CT scanner (Phillips Gemini TF) for 30 min at ambient temperature, the final 90 min of the mirabegron treatment and throughout the duration of the 180 min cold exposure. Tissue-specific oxidative metabolism was determined by first performing a CT scan (40 mAs) centered at the cervico-thoracic region to correct for attenuation and to define PET regions of interest (ROI). At time -60 min (room temperature before treatment) and at time 210 min (i.e., 210 min following oral administration of mirabegron or 90 min after onset of cold exposure) a ~ 185 MBq bolus of ^{11}C -acetate was injected intravenously and was followed by a 30 min list-mode dynamic PET acquisition (frames: 24×10 s, 12×30 s, 4×300 s) centered at the cervico-thoracic junction (Ouellet et al., 2012). Tissue oxidative metabolism index (the rapid fractional tissue clearance of ^{11}C -acetate, k_{mono} , in s^{-1}) was estimated from tissue ^{11}C activity over time using monoexponential fit from the time of peak tissue activity. The peak BAT ^{11}C radioactivity determined from the activity curve was used as an index of tissue perfusion. Tissue-specific glucose uptake was determined by intravenously injecting a ~ 185 MBq bolus of ^{18}F FDG 30 min after ^{11}C -acetate injection at time 240 min (i.e., 240 min following oral administration of mirabegron or 120 min after onset of cold exposure), followed by a list-mode dynamic PET acquisition (frames: 12×10 s, 10×45 s, 7×90 s, 2×300 s) centered at the cervico-thoracic junction. The CT performed at time 210 min, prior to the ^{11}C -acetate injection, was used to correct for attenuation and define PET ROI for the ^{18}F FDG scans. Residual ^{11}C -acetate activity prior to the ^{18}F FDG scans were corrected by acquiring a 120 s frame prior to the injection of ^{18}F FDG and accounting for the disintegration rate and metabolic clearance of ^{11}C . Plasma and tissue time-radioactivity curves for ^{18}F FDG were analyzed graphically using the Patlak linearization method (Ouellet et al., 2012) with the image-derived arterial input function taken from the aortic arch. The slope of the plot in the graphical analysis is equal to glucose fractional uptake (K_i in min^{-1}). Net glucose uptake (K_m) was then calculated by multiplying K_i by circulating plasma glucose levels at the time of the PET image acquisition, which

assumes a lump constant value of 1.0 compared with endogenous plasma glucose. At time 300 min (i.e., 300 min after oral administration of mirabegron or 180 min of cold exposure), a whole-body CT scan (16 mAs) and whole-body static PET acquisition were performed to determine whole-body ^{18}F FDG organ distribution and tissue standard uptake value (SUV).

PET-CT image data was analyzed using the Sherbrooke LabTEP software. ROI were defined from the transaxial CT slices then copied to ^{11}C -acetate and ^{18}F FDG PET image sequences. For dynamic PET acquisitions, mean value of pixels (SUV_{mean}) for each time frame was recorded. ROI were drawn on the aortic arch for blood activity (input functions), the larger skeletal muscles in the field of view (e.g., *m. sternocleidomastoid*, *m. trapezius*, *m. pectoralis major*, *m. deltoideus*), on posterior cervical subcutaneous adipose tissue and on supraclavicular BAT.

Whole-Body Lipolysis and Triacylglycerol/Fatty Acid Cycling

Upon arriving to the laboratory, emptying their bladder and being instrumented with temperature sensors, indwelling catheters were placed in the antecubital vein in both arms for blood sampling and tracer infusions. A primed ($3.3 \times 10^6 \text{ dpm} \cdot \text{min}^{-1}$) continuous infusion ($0.33 \times 10^6 \text{ dpm} \cdot \text{min}^{-1}$) of $[3\text{-}^3\text{H}]\text{-glucose}$ was started 150 min before oral administration of mirabegron (time -150 min) or 270 min before the start of cold exposure to determine the plasma glucose appearance rate ($\text{Ra}_{\text{glucose}}$) (Ouellet et al., 2012). A continuous infusion of $[\text{U-}^{13}\text{C}]\text{-palmitate}$ ($0.01 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ in 100 mL 25% human serum albumin) and a primed ($1.6 \mu\text{mol} \cdot \text{kg}^{-1}$) continuous infusion ($0.08 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) of $[1,1,2,3,3\text{-}^2\text{H}]\text{-glycerol}$ were started 120 min before the oral administration of mirabegron (time -120 min) or 240 min before the start of cold exposure to determine plasma NEFA appearance rate (Ra_{NEFA}) and plasma glycerol appearance rate ($\text{Ra}_{\text{glycerol}}$) (Ouellet et al., 2012). Ra_{NEFA} was calculated by multiplying the plasma palmitate appearance rate by the fractional contribution of palmitate to total plasma NEFA concentrations.

Total, intracellular and extracellular triacylglycerol/fatty acid (TAG/FA) cycling was calculated using $\text{Ra}_{\text{glycerol}}$, Ra_{NEFA} and the rate of fatty acid oxidation. In brief, total TAG/FA cycling was calculated as the difference between the rate of fatty acid oxidation and the total amount of fatty acids made available by the hydrolysis of intracellular triglycerides ($3 \times \text{Ra}_{\text{glycerol}}$), which is derived primarily from WAT. Given the low activity of glycerol kinase in WAT, relative to other tissues, the glycerol produced following the complete hydrolysis of a triglyceride in WAT is rapidly excreted into circulation where it can serve as gluconeogenic substrate. Intracellular or primary TAG/FA cycling refers to the re-esterification of fatty acids within the cell where it was hydrolyzed. This is calculated from the difference between the systemic rate of appearance of NEFA (i.e., Ra_{NEFA}) and the total amount of fatty acids released following intracellular hydrolysis of triglycerides ($3 \times \text{Ra}_{\text{glycerol}}$). Extracellular or secondary TAG/FA cycling refers to the re-esterification of fatty acids after it has been released into the circulation, primarily packaged by the liver into triglyceride-rich lipoproteins (very-low density lipoproteins; VLDL) or intracellular lipid droplets. This is calculated from the difference between the total rate of fatty acid oxidation and the systemic appearance of fatty acids (i.e., Ra_{NEFA}). A detailed account of the assumptions and limitations associated with these calculations in humans have previously been discussed (Wolfe and Chinkes, 2005). The energy cost of TAG/FA cycling was calculated assuming that the re-esterification of each triglyceride requires ~ 8 ATP (or $\sim 144 \text{ kcal} \cdot \text{mol}^{-1}$ of triglyceride recycled), for the activation of each of the three fatty acids (2 ATP per fatty acid), and glycerogenesis (~ 2 ATP) providing the three-carbon backbone for triglyceride synthesis (Wolfe and Chinkes, 2005).

Biological Assays

Glucose, total NEFA TG, plasma cortisol, TSH, free T3 and free T4 were measured using specific radioimmunoassays and colorimetric assays (Ouellet et al., 2012). Plasma glucagon, insulin and leptin were measured using Luminex xMAP-based immunoassays (Millipore, Etobicoke, ON, Canada). Adiponectin, total and acylated ghrelin were measured by ELISA (Alpco, Salem, NH, USA). Individual plasma NEFA (palmitate, linoleate, oleate), $[\text{U-}^{13}\text{C}]\text{palmitate}$ enrichment and $[1,1,2,3,3\text{-}^2\text{H}]\text{glycerol}$ enrichment were measured by gas chromatography-mass spectrometry (Ouellet et al., 2012). $[3\text{-}^3\text{H}]\text{-glucose}$ specific activity was determined by liquid scintillation spectrometry (Ouellet et al., 2012).

Pharmacological Stimulation and Inhibition of Human $\beta\text{-2}$ and $\beta\text{-3}$ Adrenergic Receptors

The $\beta\text{-2}$ - or $\beta\text{-3}$ -AR agonists or antagonists included norepinephrine (Amgros I/S, Denmark) ($1 \mu\text{M}$), targeting primarily the $\beta\text{-3}$ -AR, but also has affinity to the $\beta\text{-2}$ -AR, formoterol (5.5 ng/mL), a $\beta\text{-2}$ -agonist, ICI 118,551 ($1 \mu\text{g/mL}$), a $\beta\text{-2}$ -antagonist, mirabegron, a $\beta\text{-3}$ -agonist ($40 \mu\text{g/mL}$) (all these compounds were from Sigma-Aldrich and were dissolved in DMSO according to the manufacturer's instructions). The control solution was saline (sodium chloride 9 mg/mL with the addition of the preservative included in the norepinephrine solution, sodium metabisulfite (Na_2SO_3) (Merck)).

Respirometry was performed by using a Seahorse XF96 Extracellular Flux analyzer with 4 measurements of basal respiration, followed by a compound injection (norepinephrine, formoterol, mirabegron, saline) and 10 measurements of stimulated respiration. Afterward successive injections of oligomycin ($2 \mu\text{M}$, Sigma O4876), FCCP ($0.75 \mu\text{M}$, Sigma C2920) and antimycin ($0.75 \mu\text{M}$, Sigma A8674)/rotenone ($0.75 \mu\text{M}$, Sigma R8875) allowed for the determination of leak respiration, respiratory capacity and non-mitochondrial respiration (3 measurements each). For the ICI 118,551, cells were preincubated for 60 min, prior to respirometry analysis.

siRNA Knockdown of ADRB1, ADRB2 and ADRB3

ADRB1, *ADRB2* and *ADRB3* were knocked down using the pre-designed ON-TARGETplus SMARTpool (Dharmacon, Horizon), consisting of a pool of four siRNAs targeting different sites on each of the mRNA transcripts of interest. An ON-TARGETplus Non-targeting Pool was utilized as a negative control. The final concentration of siRNAs in the media was 20 nM and Lipofectamine RNAiMAX

Transfection Reagent (Thermo Fisher Scientific) was used for the delivery, according to the manufacturer's recommendations. Cells from the four adult donors (Table S3) were transfected on day 9 of differentiation and were incubated with the siRNAs until the change to Seahorse assay medium on day 12 when cells were fully differentiated and displayed multilocular lipid droplets. Stimulation with norepinephrine (1 μ M), formoterol (5.5 ng/mL) or saline control was performed as described above.

Respirometry data were analyzed with Wave 2.4 software and non-mitochondrial respiration was subtracted from all other values to obtain mitochondrial respiration rates. All measurements were normalized internally to basal mitochondrial respiration and stimulated respiration was calculated as the average of the last two measurements after adrenergic stimulation, whereas stimulated leak respiration was defined as the average of the last two measurements after oligomycin injection in the presence of adrenergic stimulation.

RNA Isolation, cDNA Synthesis and qPCR

Cells were grown on 6-well plates and cultured and treated in parallel to the cells for respirometry analysis. Cells were transfected on day 9 and incubated with the siRNAs until day 12. Fully differentiated cells were changed into DMEM/F12 medium supplemented with 1% Pen/Strep and after 1.5 h stimulated with saline, 1 μ M norepinephrine or 5.5 ng/mL formoterol. After 4 h stimulation, cells were harvested with 375 μ L Directzol/ well and frozen for RNA isolation with the Micro Prep RNA isolation Kit (Zymo Research) according to the manufacturer's protocol.

cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Life Technologies), with an input of 250 ng RNA into a cDNA volume of 20 μ L. cDNA samples were diluted 1:10 and loaded in triplicates and quantitative real-time PCR (qPCR) was performed using the ViiA 7 platform (Applied Biosystems/Thermo Fisher Scientific). Relative gene expression was detected using either Pre-designed TaqMan gene expression assays or SYBRgreen (Applied Biosystems). The average target gene CT values for each triplicate were related to the average CT values for each triplicate of the housekeeping gene PPIA and relative gene expression was calculated using the ddCt method. Primer sequences are found in Table S5.

Fluorescent *In Situ* Hybridization (FISH)

UCP1, ADRB2 and ADRB3 were targeted in *in vitro* differentiated human brown adipocytes, using the RNAscope Multiplex Fluorescent Reagent Kit v2 (Cat. No. 323100). Cells were seeded and differentiated on two-chamber slides according to the same protocol as described above. At day 12 of differentiation, media was changed to DMEM/F12 (Invitrogen, Life technologies) for two h prior to addition of 10 μ M norepinephrine. Following four h of incubation, cells were fixed with 4% PFA for 15 min at room temperature and dehydrated with increasing concentrations of EtOH. Slides were stored in 100% EtOH at -20°C until the staining protocol was performed. Multiplex RNAscope staining was carried out according to manufacturer's protocol. RNAscope probes targeting UCP1, ADRB2 and ADRB3 mRNA transcripts were designed to allow for co-staining of DAPI, UCP1 and either of ADRB2 and ADRB3 (Key Resources Table). UCP1 probes were labeled using the FP1487001KT: Opal 520 Reagent Pack (PerkinElmer), whereas ADRB2 and ADRB3 probes were labeled using the FP1497001KT: Opal 690 Reagent Pack. The fluorophores were diluted 1:750 and preparations were blocked 30 min between the fluorophores. Finally, the slides were co-stained with DAPI NucBlue Fixed Cell ReadyProbes Reagent (Thermo Fisher). Fluorescent images were obtained using an EVOS FL Imaging System (Thermo Fisher). For optimizing image contrast and for producing black and white separate images and a colored overlay image, Adobe Photoshop was used. Briefly, images stained for either UCP1 or any of the ADRB receptors were transformed to black and white and auto adjustment for brightness/contrast, levels and curves were performed. Images were converted to smart object, flattened, and then overlaid in colors.

Immortalized Human Brown Adipocyte Lipolysis Stimulation with β -Adrenergic Receptor (ant)agonists

Cells were grown confluent in 2% gelatin coated wells and were then placed on growth medium without doxycycline for 2 days by which differentiation was initiated. Differentiation into brown adipocytes was induced by incubating cells with growth medium supplemented with 150 nM human insulin (Sigma-Aldrich), 1 μ M dexamethasone (Sigma-Aldrich), 1 μ M rosiglitazone (Sigma-Aldrich), 1 nM triiodothyronine (T3, Sigma-Aldrich), 500 μ M 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich) and 125 μ M indomethacin (Sigma-Aldrich) for four days. Thereafter, cells were further differentiated in growth medium supplemented with 150 nM insulin, 1 nM T3 and 1 μ M rosiglitazone for another 11–12 days. Throughout the proliferation and differentiation process, medium was replaced every other day. Fully differentiated brown adipocytes were pre-incubated with ICI 118,551 (Sigma-Aldrich) or vehicle (water) for 2 h. Subsequently, cells were incubated with mirabegron (MedChemExpress), formoterol (Sigma-Aldrich), norepinephrine (Sigma-Aldrich) or vehicle (DMSO; Sigma-Aldrich) in the presence or absence of ICI 118,551 for 6 h. Then, supernatant was collected for determination of glycerol and non-esterified fatty acid (NEFA) levels using free glycerol reagent set (Instruchemie) and NEFA C kit (Wako Diagnostics, Instruchemie), respectively.

Mouse Isolated Brown Adipocyte Lipolysis Assay

Equal number of isolated brown adipocytes were distributed in control, 10^{-7} M mirabegron and formoterol-containing microtubes. Adipocytes were then incubated in oxygenated Krebs buffer at 37°C under slight agitation for 6 h. For lipolysis assay with the β_2 -AR antagonist, brown adipocytes were pre-incubated with ICI 118,551 for 2 h before mirabegron and formoterol treatments for 4 h, totaling 6 h. After incubation, media was immediately used to perform NEFA and glycerol measurements by colorimetric assays (Randox Laboratories and Sigma-Aldrich respectively).

Mouse RNA Extraction and RT-qPCR

BAT depots from three animals were pooled and digested as described above. Floating isolated brown adipocytes and sedimented isolated stroma vascular fraction (SVF) were snap frozen for further quantification. Total RNA from isolated brown adipocytes and SVF were extracted using an illustra RNAspin kit (GE Healthcare). Quantitative PCR was carried using an ABI 7900 Fast (Life Technologies) and PCR products were detected by using the Wisent Advanced qPCR mastermix with supergreen dye (Wisent). Gene expression was determined by a standard curve and normalized to the expression of the 36B4 reference gene.

Mouse Protein Extraction and Western Blot

Pieces of BAT depots from three mice were pooled and minced. One part was then resuspended in ice-cold protein extraction buffer (50 mM HEPES-KOH pH 7.4, 40 mM NaCl, 2 mM EDTA, 1.5 mM NaVO₄, 50 mM NaF, 10 mM NaPyrophosphate, 10 mM NaBetaGlycerophosphate, 1% Triton x-100 and 1:1000 Protease Inhibitor Cocktail) and sonicated for 9 s three times at 40% of amplitude. Isolated brown adipocytes and stromal vascular fractions were resuspended in ice-cold protein extraction buffer, let on ice for 30 min, and then centrifuged for 10 min at 11 000 g at 4°C. Extracts were carefully transferred to a new tube to avoid contamination by fat. Proteins from the heart of wild-type C57BL/6J mice and from the BAT of *Adrb3*^{−/−} mice (Jackson # 006402) were also extracted as controls to validate antibody selectivity. Protein concentrations were determined using DC Protein Assay (Bio-Rad). Proteins were subjected to SDS-PAGE and transferred to PVDF membranes. Loading was evaluated by incubating membranes in red ponceau solution (0.1 g red ponceau, 5% acetic acid). Membranes were then incubated 1 h at room temperature in blocking buffer and overnight at 4°C with primary antibodies (*Adrb1*, *Adrb2* and *Adrb3*, Abcam, ab3442, ab137494 and ab94506 respectively) according to manufacturer's instructions. Membranes were then washed three times and incubated for 1 h with anti-rabbit immunoglobulin G conjugated to HRP (Cedarlane, NA934V) at room temperature. After three washes for 8 min, the immunoreactive bands were detected by chemiluminescence.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical Analyses

Statistical analysis was performed using GraphPad Prism 8.0. Data for *in vivo* human studies are expressed as mean with 95% CI. The Shapiro-Wilk test was performed to verify the normal distribution of data, when necessary. p values were calculated for the difference between 50 mg and 200 mg mirabegron using the non-parametric Mann-Whitney U test for unpaired samples. p values were also calculated for the difference between 200 mg mirabegron and mild cold exposure using a paired-sample t test. Data derived from *in vitro* determinations are expressed as mean ± SEM, except where noted. Two-way ANOVA with Tukey post hoc test was used for Seahorse measurements and lipolysis assay to compare with the control condition and for gene expression data to compare brown and white adipocytes in unstimulated and stimulated condition. All replicates are biologically independent replicates, except where noted. Statistical parameters and details can be found in the figures and figure legends.

ADDITIONAL RESOURCES

Clinicaltrials.gov identifier NCT02811289.