11β-HSD1 reduces metabolic efficacy and adiponectin synthesis in hypertrophic adipocytes

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Abstract

Mitochondrial dysfunction in hypertrophic adipocytes can reduce adiponectin synthesis. We investigated whether 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) expression is increased in hypertrophic adipocytes and whether this is responsible for mitochondrial dysfunction and reduced adiponectin synthesis. Differentiated 3T3L1 adipocytes were cultured for up to 21 days. The effect of AZD6925, a selective 11β-HSD1 inhibitor, on metabolism was examined. *db/db* mice were administered 600 mg/kg AZD6925 daily for 4 weeks via gastric lavage. Mitochondrial DNA (mtDNA) content, mRNA expression levels of *11β-Hsd1* and mitochondrial biogenesis factors, adiponectin synthesis, fatty acid oxidation (FAO), oxygen consumption rate and glycolysis were measured. Adipocyte hypertrophy in 3T3L1 cells exposed to a long duration of culture was associated with increased *11β-Hsd1* mRNA expression and reduced mtDNA content, mitochondrial biogenesis factor expression and adiponectin synthesis. These cells displayed reduced mitochondrial respiration and increased glycolysis. Treatment of these cells with AZD6925 increased adiponectin synthesis and mitochondrial respiration. Inhibition of FAO by etomoxir blocked the AZD6925-induced increase in adiponectin synthesis, indicating that 11β-HSD1-mediated reductions in FAO are responsible for the reduction in adiponectin synthesis. The expression level of *11β-Hsd1* was higher in adipose tissues of *db/db* mice. Administration of AZD6925 to *db/db* mice increased the plasma adiponectin level and adipose tissue FAO. In conclusion, increased 11β-HSD1 expression contributes to reduced mitochondrial respiration and adiponectin synthesis in hypertrophic adipocytes.

Key Words

- adiponectin
- mitochondria
- 11β-hydroxysteroid dehydrogenase type 1
- adipocyte hypertrophy
- glycolysis
- fatty acid oxidation

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Introduction

Adiponectin, the most abundant protein in adipocytes, has many favorable effects on metabolism, including improvement of insulin action and reduction of atherosclerotic processes (Ouchi *et al*. 2001, Yamauchi *et al*. 2003, Haluzík *et al*. 2004, Kim *et al*. 2007). Unlike other adipocytokines, plasma levels of adiponectin are

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paradoxically reduced in obese subjects (Kern *et al.* 2003). The underlying mechanisms for this phenomenon are not yet completely understood, but include adipose tissue hypoxia (Ye *et al.* 2007, Jiang *et al.* 2011), increased proinflammatory cytokine levels, and oxidative stress in dysfunctional adipocytes (Otani 2011). Another hypothesis that links adipocyte hypertrophy to reduced adiponectin synthesis is mitochondrial dysfunction (Koh *et al.* 2007, Kusminski & Scherer 2012). We and others have previously reported that mitochondrial function is necessary for adiponectin synthesis in adipocytes (Koh *et al.* 2007, Huh *et al.* 2012, Wang *et al.* 2013, Capllonch-Amer *et al.* 2014). Additionally, mitochondrial dysfunction in adipocytes could be an important cause of insulin resistance and inflammation in obesity (Kusminski & Scherer 2012, Medina-Gómez 2012, Ryu *et al.* 2013). Dysfunctional mitochondria can generate excessive reactive oxygen species (Chaturvedi & Flint Beal 2013) and, conversely, oxidative stress can induce mitochondrial dysfunction in adipocytes (Frohnert & Bernlohr 2013, Hahn *et al.* 2014).

Cushing's syndrome is a prototypic metabolic syndrome. Excessive glucocorticoid levels cause the development of central obesity, hypertension, dyslipidemia and insulin resistance. However, circulating cortisol levels are not consistently elevated in human idiopathic obesity (Walker *et al.* 2000). Rather, intracellular dysregulation of cortisol metabolism is considered to be important in the pathogenesis of insulin resistance and obesity. Two isozymes of 11β-hydroxysteroid dehydrogenase (11β-HSD) regulate interconversion of active and inactive glucocorticoids. 11β-HSD1 converts inactive glucocorticoids, cortisone and 11-dehydrocorticosterone into the active glucocorticoids, cortisol and corticosterone, and it is highly expressed in liver, lung, vasculature and adipose tissues (Seckl & Walker 2001, Chapman *et al.* 2013). 11β-HSD1 deficiency reportedly exacerbates acute inflammation (Chapman *et al.* 2013). However, in some inflammatory settings, such as obesity or diabetes, 11β-HSD1-deficiency is beneficial, as it acts to reduce inflammation (Chapman *et al.* 2013). Notably, 11β-HSD1 activity is increased in adipose tissues of leptin-resistant Zucker obese rats (Livingstone *et al.* 2000). Adipose-specific overexpression of 11β-HSD1 in transgenic mice produced typical features of metabolic syndrome (Masuzaki *et al.* 2001). Conversely, 11β-HSD1-deficient mice are resistant to diet-induced obesity and show higher expression levels of adiponectin in adipose tissues (Morton *et al.* 2004).

However, the mechanism whereby increased adipocyte 11β-HSD1 levels are related to obesity and other metabolic diseases is unclear. Results from a previous study indicated that increased endogenous 11β-HSD1 reduces endothelial nitric oxide synthase (eNOS) expression in endothelial cells (Liu *et al.* 2009). As eNOS plays an important role in mitochondrial biogenesis (Valerio *et al.* 2006) and adiponectin synthesis (Koh *et al.* 2010), we proposed the hypothesis that increased 11β-HSD1 expression could be responsible for mitochondrial dysfunction and reduced adiponectin synthesis in hypertrophic adipocytes.

Material and methods

Cell culture

3T3L1 preadipocytes (ATCC CL-173; Manassas, VA, USA) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) in an incubator with 5% CO_2 and 95% O_2 at 37 °C. These cells were differentiated into mature adipocytes by culturing them in DMEM medium with 10% FBS, insulin (1 μM), 3-isobutyl-1-methylxanthine (0.5 mM; Sigma–Aldrich) and dexamethasone (1 μM; Sigma–Aldrich) for 1 day. Then, cells were cultured in DMEM medium with 10% FBS and insulin (1 μM) for 21 days with or without a selective 11β-HSD1 inhibitor, AZD6925 (10 μM), which was kindly provided by AstraZeneca (Scott *et al.* 2012). The drug was administered when the culture medium was replaced every other day. For dexamethasone experiments, dexamethasone (50 μM) was added to media after media replacements were carried out.

Cell viability assay

Cells were harvested and plated in 96-well plates at 1×10^3 cells/well and maintained at 37 °C in a humidified incubator. Ten microliters of CCK-8 solution (Dojindo Molecular Tech, Baltimore, MD, USA) was added into each of three wells and the cells were incubated in this solution for 1 h. Absorbance was measured at 450 nm using a SpectraMax 450 PC (Molecular Devices, Sunnyvale, CA, USA) to estimate the number of viable cells in each well.

Oil red O staining

Cells were washed twice with PBS, fixed in 3.7% formaldehyde for 1 h and stained for 30 min with 0.2% (w/v) oil red O solution in 60% (v/v) isopropanol. They were then washed several times with water, and excess water was evaporated by placing the stained cultures at approximately 32 °C.

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Real-time PCR analysis

mRNA expression levels were quantified by real-time PCR using an ABI PRISM 7000 sequence detection system (Applied Biosystems) with a SYBR Green PCR kit (Applied Biosystems). Two micrograms of total RNA was reverse transcribed with oligo (dT) using M-MuLV reverse transcriptase (Roche Diagnostics). In the wells of 96-well optical plates, 12.5 μl SYBR Green master mix was added to 12.5 μl cDNA (corresponding to 50 ng of total RNA input) and 200 nM of forward and reverse primers in water. The plates were heated for 10 min at 95 °C followed by 40 PCR cycles of 15 s at 95 °C and 60 s at 60 °C. The amplification of T-box was used as an internal control. Ratios of target gene to T-box expression levels were calculated by subtracting the threshold cycle (*C*t) of the target gene from the *C*t of T-box and raising 2 to the power of this difference. The entire process of calculating *C*t, preparing a standard curve and determining the starting copy number for unknowns was performed by the software of the 7700 system. The primers were designed on the basis of nucleotide sequences in the GenBank database. The relative amounts of mRNA were calculated using the relative cycle threshold method (PerkinElmer Wallace, Wellesley, MA, USA). Total RNA was isolated using TRIzol reagent (Invitrogen). One microgram of each sample was reverse transcribed with random primers using the Reverse Aid M-MuLV reverse transcription kit (Fermentas, Hanover, MD, USA). Target primer sequences are listed in Supplementary Table 1, see section on supplementary data given at the end of this article.

Quantification of mitochondrial DNA content

Mitochondrial DNA (mtDNA) content was quantified by real-time PCR. Mouse nuclear 18S rRNA was used as the internal control. The ratio of the expression of the target gene to that of 18S rRNA was calculated. The primers for detecting the cytochrome *b* gene (14 146–15 289) of the murine mitochondrial genome were from the GenBank nucleotide sequences. The primer sequences were: forward, 5′-CCA CTT CAT CTT ACC ATT TA-3′; reverse, 5′-ATC TGC ATC TGA GTT TAA TC-3′ (GenBank AB042432.1, *Mus musculus domesticus* mitochondrion).

Measurement of adiponectin

Total adiponectinin culture media and mouse plasma was measured using RIAs (Linco Research, St Charles, MO, USA).

Analysis of high-molecular-weight adiponectin

Plasma (1 μl) was diluted with a non-reducing sample buffer and subjected to 6% SDS–PAGE under non-reducing and non-heat-denaturing conditions (Wang *et al*. 2006). The samples were then blotted on nitrocellulose membranes and immunostained with anti-mouse adiponectin antibody (Adipogen, Seoul, Korea).

Animals

Eight-week-old male *db/db* and their control (*db*/+) mice (SLC, Shizuoka, Japan) were used in the experiments. Animal experiments were approved by the Institutional Animal Care and Use Committee of the Asan Institute for Life Sciences, Seoul, Korea. By using a small number of animals ($n=3$ each), we first examined the effects of various doses of AZD6925 on plasma adiponectin levels, finding that administration of 600 mg/kg per day of AZD6925 for 4 weeks significantly increased plasma adiponectin levels in *db/db* mice (Supplementary Figure 1, see section on supplementary data given at the end of this article). AZD6925 was dissolved in a 1:1 mixture of DMSO. AZD6925 (600 mg/kg per day dissolved in 200 μl of vehicle (DMSO, Tween 80, and 0.9% saline (1:1:4, respectively))) or the same volume of vehicle was administered to the mice by gastric lavage. Plasma corticosterone levels of mice exhibit circadian variation and the maximum efficacy of the 11β-HSD1 inhibitor occurs when the drug is administered in the afternoon (Véniant *et al*. 2009). Therefore, the drug was administered at 1630 h daily for 4 weeks. The food intake and body weight of the animals were recorded every week. After 4 weeks, mice were fasted for 5 h in the morning and then killed. Blood samples were collected for biochemical analyses and the white adipose tissue (WAT) and liver were rapidly removed and frozen at −80 °C.

Histology

Epididymal adipose tissues and livers from *db/db* and control mice were fixed in 10% formalin, dehydrated, embedded in paraffin and sectioned for hematoxylin/eosin staining. Images were captured using an Olympus BX60 camera and processed in Adobe Photoshop (Adobe).

Measurement of plasma metabolic parameters

Plasma glucose and lactate concentrations were determined using a glucose and lactate analyzer (YSI

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2300; Yellow Springs Instruments, Yellow Springs, OH, USA). Plasma free fatty acid (FFA) and triglyceride concentrations were determined by enzymatic assays using kits from Wako Chemical (Osaka, Japan) and Sigma respectively. The plasma insulin level was determined by a RIA (Linco Research). The levels of plasma leptin and resistin were determined by the ELISA technique according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Measurements of cellular respiration and the rate of glycolysis

A XF24 Seahorse Bioscience instrument (North Billerica, MA, USA) was used to measure the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of 3T3L1 cells. A total of 4×10^4 cells were seeded per well. For the XF24 assay, DMEM growth media were replaced by unbuffered DMEM supplemented with 25 mM glucose, 1 mM pyruvate and 2 mM L-glutamine, and cells were incubated at 37 °C in a CO_2-free incubator for 1 h. Cells were then placed in the instrument and the basal OCR and ECAR were recorded for 24 min before 1 μg/ml oligomycin, 1 μM FCCP, and 1 μM rotenone +2 μM antimycin A (Sigma–Aldrich) were added consecutively according to the protocol provided by the manufacturer (Abe *et al*. 2010). The rate of glycolysis was estimated from the ECAR. All OCR and ECAR values were normalized based on the cell number. The cellular bioenergetic profiles observed for the OCR provide detailed information about the individual components of the respiratory chain (Abe *et al*. 2010). The key parameters (i.e. the basal OCR, ATP-linked OCR, proton leakage, maximal OCR, reserve capacity and non-mitochondrial OCR) were analyzed as described by Hill *et al*. (2012) (Supplementary Figure 2, see section on supplementary data given at the end of this article).

Fatty acid oxidation

The fatty acid oxidation (FAO) rate was measured based on $^{14}CO_2$ generation from [^{14}C] palmitate (NEN Life Sciences, Boston, MA, USA). Briefly, 50 μl tissue homogenates or 4×10^4 cells were added to reaction medium containing 0.1 mM palmitate (1-^{14}C palmitate at 0.5 μCi/ml) and incubated for 30 min at 30 °C. The reactions were stopped by adding 50 μl 4 N (2 M) sulfuric acid, and the CO_2 produced was trapped with 200 μl 1 N (1 M) sodium hydroxide. The trapped $^{14}CO_2$ and ^{14}C-labeled acid soluble products were measured by liquid scintillation counting, and the relative FAO rates were normalized to the protein contents of each tissue sample.

ATP measurement

Intracellular ATP levels were measured with LC–MS/MS analysis. Cells were harvested in 1.4 ml cold methanol/H_2O (80/20, v/v) after sequential washing with PBS and H_2O. They were lysed by vigorous vortexing, and 50 μl of 50 nM surrogate internal standard (Gln-d_4) was added. ATP was extracted along with other polar metabolites by liquid–liquid extraction after adding chloroform. The aqueous phase was dried in a vacuum centrifuge, and the sample was reconstituted with 50 μl of 50% methanol. All standards including surrogate internal standard and solvents were purchased from Sigma–Aldrich and JT Baker (Center Valley, PA, USA). The LC–MS/MS system was equipped with an Agilent 1290 HPLC (Agilent, Santa Clara, CA, USA) and Qtrap 5500 (ABSciex, Framingham, MA, USA), and a Synergi fusion column (Synergi 4u-fusion RP 80 A, 50×2.0 mm) was used. Five millimolar ammonium acetate in H_2O and 5 mM ammonium acetate in methanol were used as mobile phases A and B respectively. The separation procedure was as follows: hold at 0% of B for 5 min with 70 μl/min, 0–90% of B and 70–140 μl/min for 2 min, hold at 90% of B for 8 min with 140 μl/min, 90–0% of B and 140–70 μl/min for 1 min, then hold at 0% of B for 9 min with 70 μl/min to re-equilibrate the column. Column temperature was kept at 23 °C. Multiple reaction monitoring was used in negative ion mode and the peak area of the extracted ion chromatogram corresponding to the specific transition for ATP and the surrogate internal standard were used for quantification. The peak area ratios of ATP/surrogate internal standard, after normalization for protein per sample, were used for comparisons.

Triglyceride content

The triglyceride content of livers was determined in duplicate using a Sigma triglyceride (GPO-Trinder) kit.

Statistical analyses

All values are presented as means ± S.E.M. Differences between two groups were assessed using an unpaired two-tailed *t*-test. Data from more than two groups were assessed by ANOVA followed by a *post-hoc* least significant difference test. Statistical analyses were performed using SPSS-PC15 software.

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Results

11β-Hsd1 mRNA expression is increased in 3T3L1 adipocytes cultured for a prolonged period of time

After differentiation, 3T3L1 adipocytes were cultured in DMEM media for up to 21 days. Individual adipocytes appeared to be larger and cell viability had decreased significantly by 21 days of culture (Fig. 1A, Supplementary Figure 3A, see section on supplementary data given at the end of this article). The mRNA expression levels of *11β-Hsd1* and adiponectin significantly increased and decreased, respectively, in the cells at 21 days of culture (Fig. 1B and C). Total adiponectin levels in the media, mtDNA content and mRNA expression levels of mitochondrial biogenesis factors (*eNos*, peroxisome proliferator-activated receptor gamma coactivator-1α (*Pgc-1α*), and mitochondrial transcription factor A (*mtTfa*)) in adipocytes were also significantly reduced at 14 and 21 days (Fig. 1D, E, and F).

Dexamethasone potentiates, and inhibition of 11β-HSD1 reverses prolonged-culture-associated changes in cultured adipocytes

We next tested the effects of the synthetic glucocorticoid dexamethasone on prolonged-culture-associated changes in cultured adipocytes. Dexamethasone treatment increased fat accumulation in adipocytes (Fig. 2A), and many cells treated with dexamethasone were not viable at 21 days of culture (Supplementary Figure 3B). Dexamethasone treatment significantly reduced adiponectin levels in supernatants, mtDNA content, and the expression of mitochondrial biogenesis factors in adipocytes compared to control cells (Fig. 2B, C, and D). On the other hand, treatment of 3T3L1 adipocytes with AZD6925 significantly increased the mRNA expression of adiponectin and mitochondrial biogenesis factors at 21 days of culture (Fig. 2E).

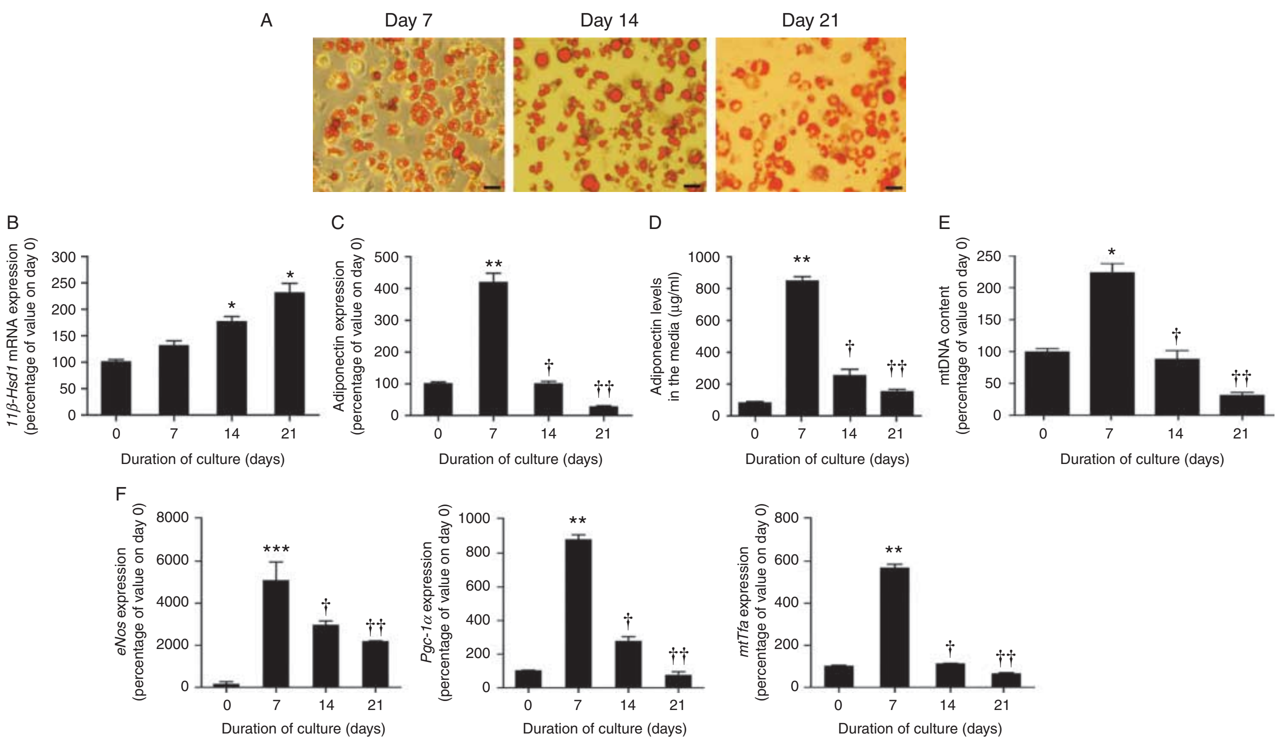

Figure 1
Increased levels of *11β-Hsd1* mRNA expression during prolonged culture are associated with reductions in mitochondrial biogenesis and adiponectin synthesis in cultured 3T3L1 adipocytes. (A) Oil red O staining in the cultured cells. Bars 100 μm. (B) The mRNA levels of *11β-Hsd1*. (C) Adiponectin expression levels measured using real-time PCR. (D) Total adiponectin levels in culture supernatants measured by ELISA. (E) Levels of mtDNA. (F) Levels of mRNA transcripts that encode the mitochondrial biogenesis markers *eNos*, *Pgc-1α* and *mtTfa*. $*P<0.05$, $**P<0.01$, $***P<0.001$ versus cells at day 0; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ versus cells at day 7. Results represent the mean ± S.E.M. of five independent experiments, each performed in triplicate, with day 0 defined as 100%.

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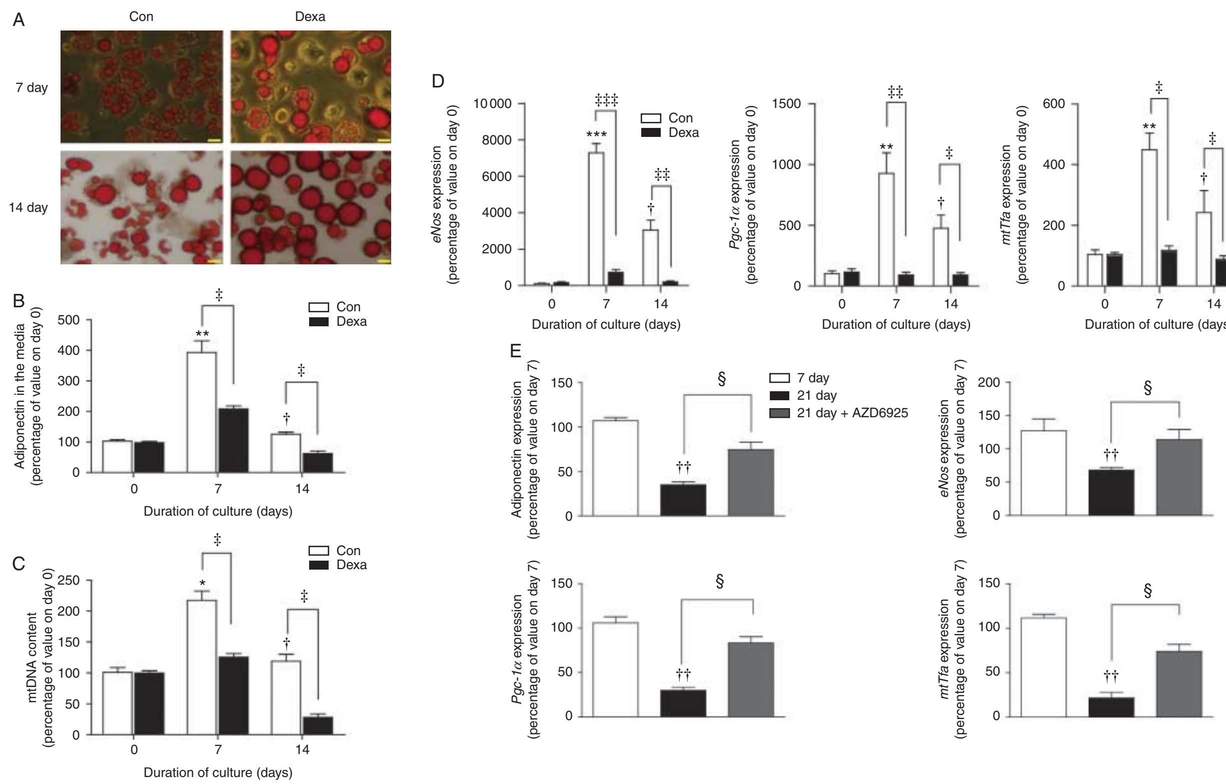

Figure 2

Dexamethasone reduces adiponectin synthesis and mitochondrial biogenesis. Dexamethasone (Dexa, 50 μM) was added every 2 days when the media was changed. Cells were harvested at days 7 and 14. (A) Oil red O staining in cultured cells. Bars 50 μm. (B) Adiponectin levels in culture supernatants. (C) Levels of mtDNA measured by real-time PCR. (D) Levels of mRNA transcripts for mitochondrial biogenesis factor genes. (E) Effects of AZD6925 on the expression of adiponectin and mitochondrial biogenesis markers in adipocytes that underwent 21 days of culture. Culture media with AZD6925 (10 μM) were replaced every other day. Data are presented as means ± S.E.M. ($n=5$ except for Fig. 2E ($n=3$)), each performed in triplicate, with day 0 set to 100%. *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus cells at day 0; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ versus cells at day 7; $^{\ddagger}P<0.05$, $^{\ddagger\ddagger}P<0.01$, $^{\ddagger\ddagger\ddagger}P<0.001$ versus untreated cells; $^{\S}P<0.05$ versus cells at day 21.

Mitochondrial respiration is reduced in adipocytes following prolonged culture

We next measured the OCR using a Seahorse instrument. The basal OCR, proton leakage, ATP-linked OCR and maximal OCR were significantly reduced in adipocytes cultured for 14 and 21 days compared to cells on day 7 of culture (Fig. 3A and B, Supplementary Figure 4A, see section on supplementary data given at the end of this article). The ECAR at the basal state and after treatment with various mitochondrial respiration inhibitors progressively increased with prolonged culture (Fig. 3C, Supplementary Figure 4B). Prolonged culture reduced ATP concentration to a significantly lower level by 21 days (Fig. 3D). Similarly, FAO, as measured by ^{14}C-palmitate oxidation, declined progressively after prolonged periods of culture (Fig. 3E). Administration of AZD6925 for 21 days reversed changes in the OCR and ECAR in the cells that underwent prolonged culture (Fig. 3F, G, and H, Supplementary Figure 4C and D).

AZD6925 increases FAO to increase adiponectin synthesis in cultured adipocytes

In a separate experiment, differentiated 3T3L1 adipocytes at day 5 of culture were serum starved for 6 h to eliminate the influence of endogenous glucocorticoids that are present in FBS (Garbrecht *et al.* 2006). Cells were then incubated with serum-free medium containing cortisone (250 nM) with or without AZD6925 (10 μM) for 24 h (Supplementary Figure 5A, see section on supplementary data given at the end of this article). Treatment of cortisone-treated cells with AZD6925 increased adiponectin secretion, mtDNA content, and the expression of mitochondrial biogenesis factors (Fig. 4A, B, and C).

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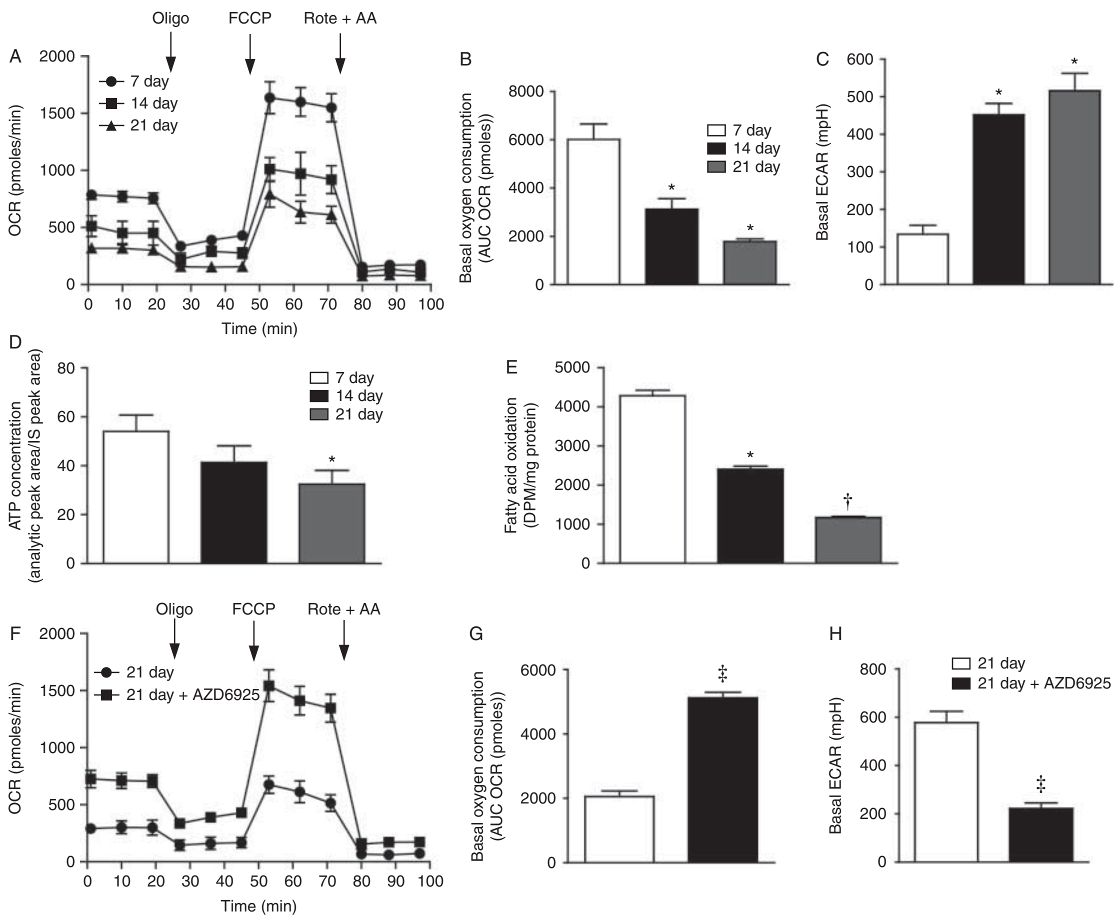

Figure 3

Adipocytes that underwent prolonged culture exhibit reduced mitochondrial respiration. (A, B, and C) Changes in mitochondrial bioenergetics were observed using the Seahorse XF-24 system throughout a 21-day culture period with or without AZD6925. The basal OCRs of cells were recorded for 24 min, and 1 μg/ml oligomycin (Oligo), 1 μM FCCP and 1 μM rotenone (Rote) +2 μM antimycin A (AA) were added sequentially. (A) Real-time measurements of OCR. (B) The area under the curve (AUC) of basal oxygen consumption. (C) The basal ECAR (D) ATP concentrations measured by LC–MS/MS. (E) Changes in FAO. The FAO was measured as the $^{14}CO_2$ generated from [^{14}C] palmitate. (F, G, and H) Effect of AZD6925 on real-time OCR (F), basal oxygen consumption (G) and basal ECAR (H) at 21 days of culture. Data are presented as means ± s.e.m. ($n=5$ except for Fig. 3F, G, and H ($n=3$)) with each experiment performed in triplicate. *$P<0.05$ versus cells at day 7; †$P<0.05$ versus cells at day 14; ‡$P<0.05$ versus cells at day 21.

Similar to the results found in fat cells under conditions of prolonged culture, treatment with cortisone significantly reduced the basal OCR, proton leakage, ATP-linked OCR and maximum OCR. AZD6925 significantly increased the basal, ATP-linked, and maximum OCR compared with cortisone-treated cells (Fig. 4D, Supplementary Figure 5B and C). Cortisone and AZD6925 increased and decreased, respectively, the ECAR (Supplementary Figure 5D). Cortisone treatment also significantly decreased FAO, as measured by ^{14}C-palmitate oxidation. AZD6925 significantly increased FAO (Fig. 4E). Co-administration of the FAO inhibitor etomoxir nearly completely reversed the AZD6925-induced increase in supernatant adiponectin levels (Fig. 4F), indicating that 11β-HSD1-mediated reductions of FAO are responsible for the reduction in adiponectin synthesis.

Administration of AZD6925 increases plasma adiponectin levels and FAO in adipose tissues

AZD6925 treatment did not affect body weight or food intake (Supplementary Figure 6, see section on supplementary data given at the end of this article) but resulted in significant reductions in fasting plasma glucose and insulin levels (Table 1). AZD6925 also decreased plasma lactate, FFA and glycerol levels (Table 1). Adipose tissue *11β-Hsd1* expression levels were significantly higher in *db/db* mice than in control mice (Livingstone *et al*. 2009, Fig. 5A).

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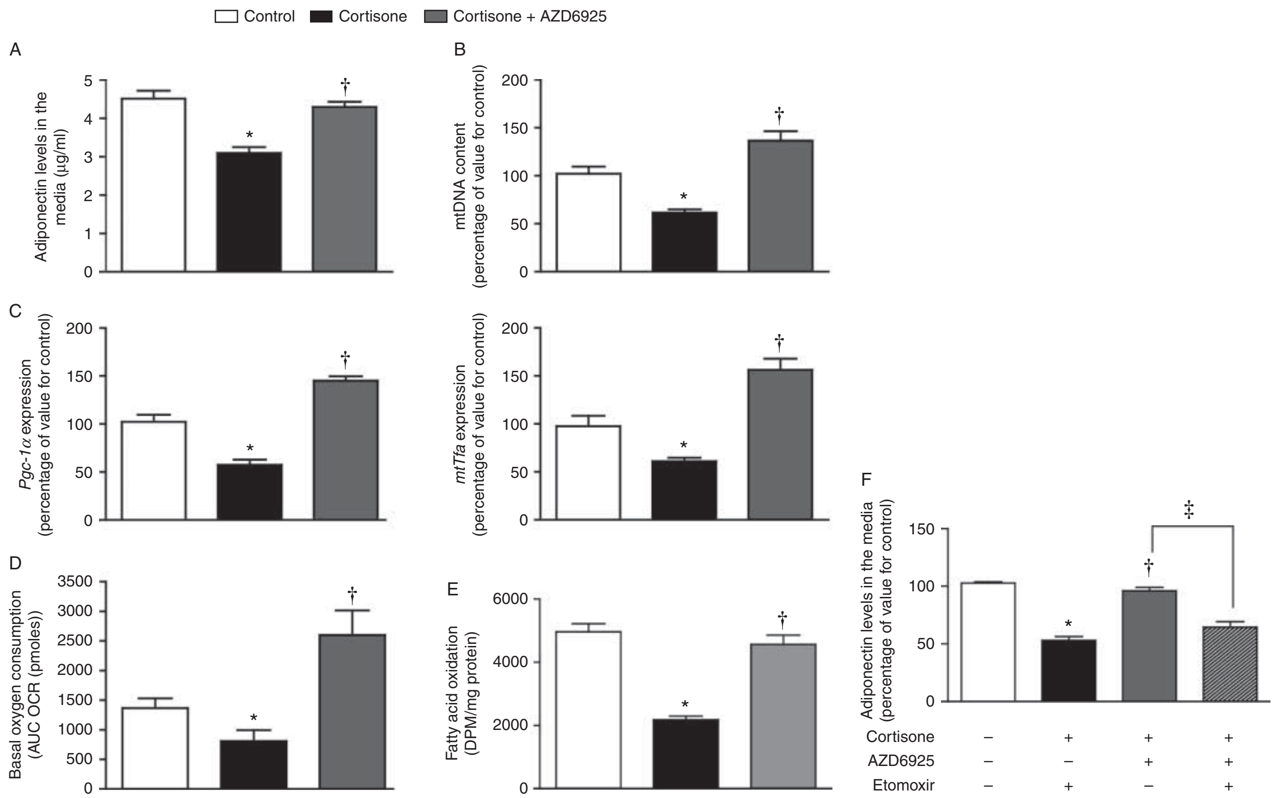

Figure 4

Inhibition of 11β-HSD1 rescues cortisone-induced reductions in adiponectin synthesis and mitochondrial FAO. Differentiated adipocytes were treated with cortisone (250 nM) with or without AZD6925 (10 µM) for 24 h. (A) Adiponectin levels in culture supernatants. (B) Levels of mtDNA measured by real-time PCR. (C) Levels of mRNA transcripts that encode mitochondrial biogenesis markers. (D) The AUC of the basal OCR. (E) FAO. (F) The effect of pharmacological inhibition of FAO with etomoxir on adiponectin secretion. Results represent the mean ± s.e.m. of five independent experiments, each performed in triplicate. *$P<0.05$ versus DMSO-treated control cells; †$P<0.05$ versus cortisone-treated cells; ‡$P<0.05$ versus cotreatment with cortisone and AZD6925.

AZD6925 did not significantly increase the plasma adiponectin level in the control mice. On the other hand, plasma total and high-molecular-weight adiponectin levels were lower in *db/db* mice and increased in response to AZD6925 treatment (Fig. 5B and C). Unlike plasma adiponectin, plasma leptin and resistin levels were not affected by AZD6925 treatment (Supplementary Figure 7, see section on supplementary data given at the end of this article).

Individual adipocytes appeared to be larger in *db/db* mice and to be smaller in AZD6925-treated *db/db* mice (Fig. 5D). AZD6925 treatment did not increase the expression levels of mitochondrial biogenesis factors in the adipose tissues of *db/db* mice (data not shown). However, AZD6925 treatment significantly increased FAO (Fig. 5E) and the expression of molecules involved in FAO, such as carnitine palmitoyl transferase-1b (*Cpt-1b*), pyruvate dehydrogenase kinase 4 (*Pdk4*) and peroxisome proliferator-activated receptor α (*Pparα*) (Fig. 5F).

Administration of AZD6925 prevents hepatic steatosis, increases mitochondrial biogenesis, and changes macrophage polarization in the liver

Knockdown of 11β-HSD1 has been shown to protect mice from hepatic steatosis and dyslipidemia (Li *et al*. 2011). In accordance with this, AZD6925 prevented hepatic steatosis in *db/db* mice (Fig. 6A) and reduced liver triglyceride levels (Fig. 6B). Additionally, AZD6925 increased mtDNA content, as well as FAO and FAO-related genes in the liver (Fig. 6C, D, and E). Unlike in WAT, AZD6925 treatment increased the expression levels of mitochondrial biogenesis factors in the liver (Fig. 6F).

Results of previous studies have indicated that 11β-HSD1 is involved in the regulation of the immune system (Kipari *et al*. 2013). We thus examined the expression of several markers of M1 (TNFα, iNOS, IL6) and M2 (YM1, Arg1) macrophages (Lumeng *et al*. 2007). Expression of

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Table 1 Metabolic parameters of *db/db* mice versus control mice. Data are presented as means ± S.E.M. ($n=5$)

Parameters	Control mice	*db/db*	*db/db* + AZD6925
Final body weight (g)	29.1±1.6	41.2±2.5*	39.61±2.7
Epididymal white adipose tissue weight (g)	0.4±0.09	1.5±0.2*	1.4±0.3
Insulin (pmol/l)	163.5±18.9	1633.1±189.9*	356.7±56.7†
Glucose (mmol/l)	11.3±0.5	44.6±4.7*	25.6±3.1†
Lactate (mmol/l)	3.8±0.9	7.4±1.1*	3.1±0.7†
FFA (mmol/l)	3.6±0.7	6.6±1.1*	3.2±0.5†
Glycerol (mmol/l)	3.3±0.7	7.1±1.2*	4.2±0.9†

*$P<0.05$ versus control mice; †$P<0.05$ versus untreated *db/db* mice.

Tnfα was significantly increased in adipose tissue, whereas *Il6* expression was significantly higher in the liver in *db/db* mice than in control mice. Administration of AZD6925 to *db/db* mice significantly decreased *Il6* expression in the liver. Expression of *Ym1* and *Arg1* was significantly higher in both adipose tissue and the liver in *db/db* mice. Interestingly, Administration of AZD6925 to *db/db* mice further increased the levels of these markers in the liver (Supplementary Figure 8A and B, see section on supplementary data given at the end of this article).

Discussion

In our present study, we found that increased 11β-HSD1 expression levels are associated with reductions in

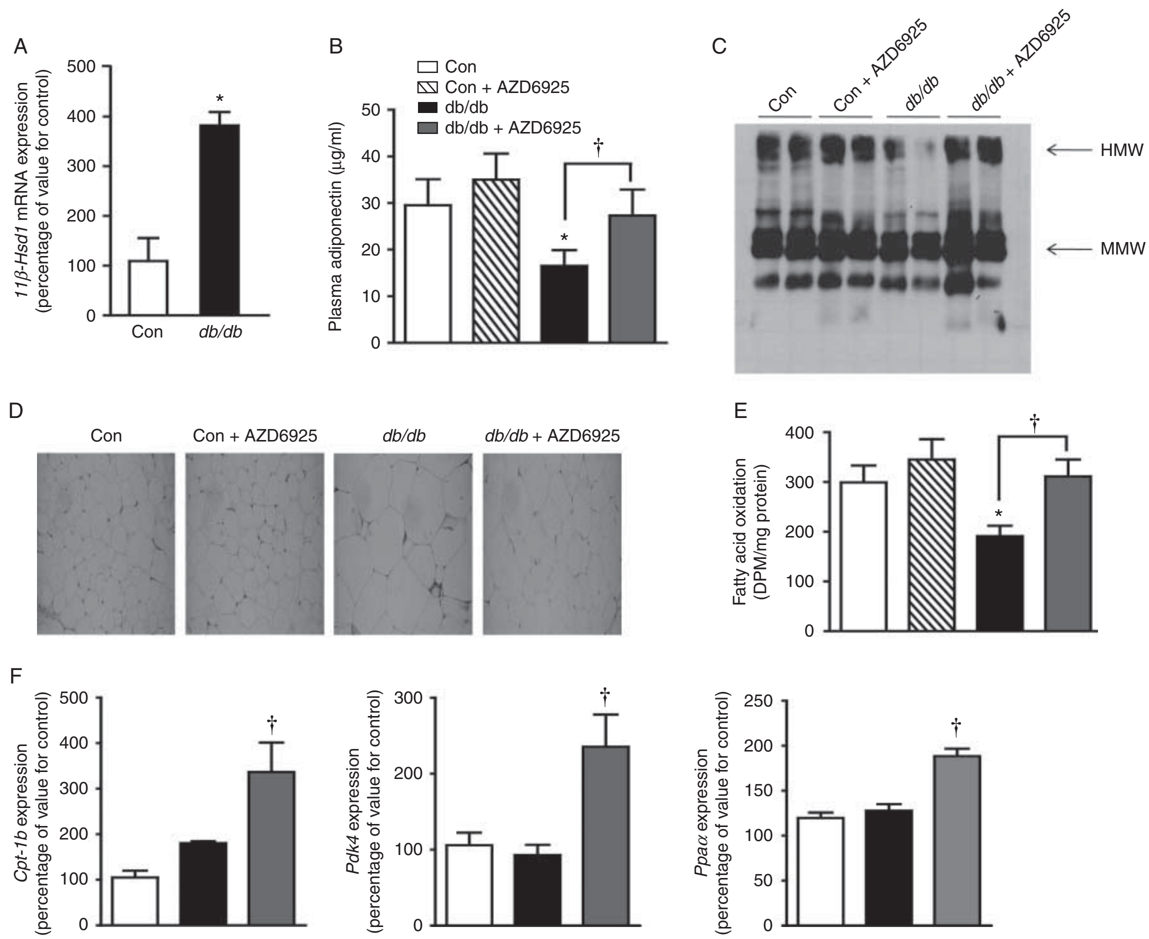

Figure 5
The effect of AZD6925 on adiponectin synthesis and mitochondrial function in *db/db* mice. Eight-week-old male *db/db* mice were treated for 4 weeks with 600 mg/kg AZD6925 daily by oral gavage. (A) Levels of *11β-Hsd1* in adipose tissue, as measured by real-time PCR analysis. (B and C) Plasma levels of total adiponectin measured by RIA (B) and western blotting of high-molecular-weight adiponectin (C). (D) Histological examination of adipose tissues. Original magnification, 200×. (E) FAO in the adipose tissue. (F) The mRNA expression levels of FAO-related genes, as measured by real-time PCR analysis. Data are presented as means ± S.E.M. ($n=5$); *$P<0.05$ versus control mice; †$P<0.05$ versus untreated *db/db* mice.

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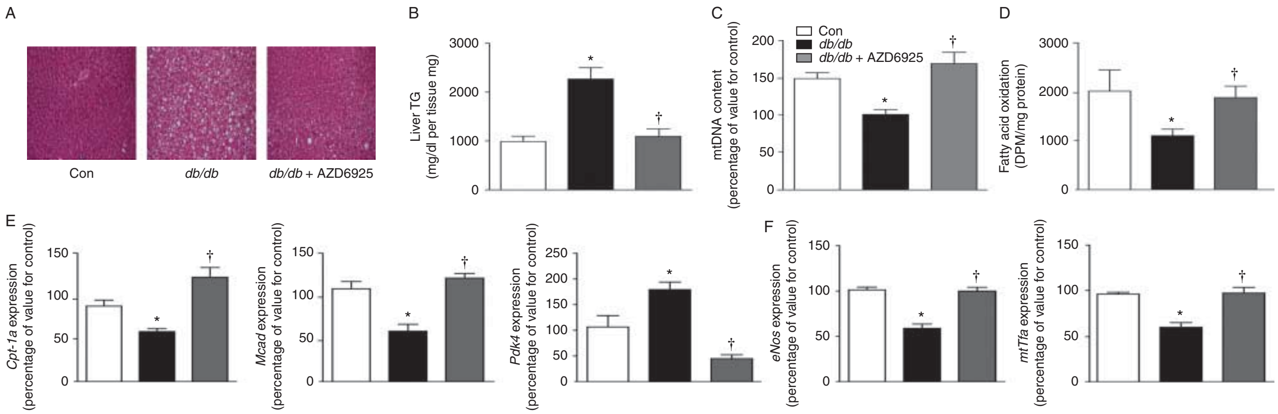

Figure 6
AZD6925 increases mitochondrial biogenesis in the liver and prevents hepatic steatosis. (A) Hematoxylin and eosin (original magnification, 200×) staining of representative liver samples from *db/db* mice with or without AZD6925 treatment. (B) Hepatic triglyceride levels. (C) mtDNA content. (D and E) FAO and expression levels of FAO-related genes. (F) Transcripts of mitochondrial biogenesis factors. Data are presented as means ± S.E.M. (n = 5 each). *P < 0.05 versus control mice; †P < 0.05 versus untreated *db/db* mice.

mitochondrial respiration and adiponectin synthesis in hypertrophic adipocytes. *11β-Hsd1* mRNA expression was significantly increased in the adipose tissues of *db/db* mice and adipocytes that were cultured for 3 weeks. In cultured adipocytes, mtDNA content, the expression of mitochondrial biogenesis factors, and adiponectin synthesis decreased with increased duration of culture. Dexamethasone accelerated prolonged-culture-associated fat accumulation in cultured adipocytes, and further reduced adiponectin synthesis and mitochondrial biogenesis. In contrast, the 11β-HSD1 inhibitor AZD6925 increased adiponectin synthesis and mitochondrial biogenesis. AZD6925 increased mitochondrial respiration and reduced glycolysis. Administration of AZD6925 to *db/db* mice reduced plasma glucose and lactate levels and increased FAO in adipose tissues.

Adipocytes exposed to prolonged culture appeared to be hypertrophic and exhibited decreased adiponectin synthesis. *11β-Hsd1* expression was significantly increased in these cells, and treatment with ADZ6925 increased adiponectin synthesis and mitochondrial respiration. FAO was decreased in hypertrophic adipocytes and etomoxir abrogated AZD6925-induced increases in adiponectin synthesis, indicating that fatty acid is the major 'fuel' for adiponectin synthesis in adipocytes. We also note that while glycolysis was increased in these cells, ATP levels were significantly lower than in cells at 7 days of culture. These results indicate that hypertrophic adipocytes cannot derive sufficient ATP from glycolysis to maintain viability, in contrast to cancer cells or activated macrophages (Galván-Peña & O'Neill 2014).

AZD6925 significantly increased the expression of mitochondrial biogenesis factors in cultured adipocytes. However, administration of AZD6925 did not increase the expression of mitochondrial biogenesis factors in adipose tissues. The reason for the discrepancy between these experiments is currently unknown. However, AZD6925 treatment significantly increased FAO in adipose tissues, indicating that this drug could improve mitochondrial function.

In contrast to adipose tissue, administration of AZD6925 to *db/db* mice significantly decreased triglyceride accumulation and increased FAO and the expression of mitochondrial biogenesis factors in the liver. This was associated with decreases in the fasting plasma levels of glucose, insulin and FFA. The accumulation of lipid metabolites in insulin-sensitive tissues is considered to be an important factor in the genesis of insulin resistance (Adams *et al*. 2004). Thus, it is suggested that increases in 11β-HSD1 and glucocorticoid signaling in the liver are responsible for decreased mitochondrial activity and insulin resistance. It should be noted, however, that the relationship between adiponectin and mitochondrial biogenesis may be different in different tissues. In contrast to adipocytes, where increased mitochondrial biogenesis increases adiponectin synthesis (Koh *et al*. 2007), adiponectin activates AMPK in the liver (Iwabu *et al*. 2010). Therefore, the beneficial effect of AZD6925 on mitochondrial function in the liver may be caused by increased plasma adiponectin.

Accumulating evidence has indicated that changes in metabolism play important roles in the regulation of

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inflammatory responses (O'Neill & Hardie 2013). Classically activated M1 macrophages are glycolytic, whereas M2 macrophages, which can act to restore homeostasis in the repair phase of inflammation, are more dependent on oxidative metabolism (Haschemi *et al.* 2012). 11β-HSD1 is induced in human monocytes upon differentiation to macrophages (Thieringer *et al.* 2001), and it has been proposed that increased 11β-HSD1 levels might be responsible for the glycolytic phenotype of M1 macrophages (Chinetti-Gbaguidi *et al.* 2012). In our present study, expression of *Il6*, a marker of M1 macrophages, was decreased, whereas the levels of *Ym1* and *Arg1*, markers of M2 macrophages, were increased in the livers of ADZ6925-treated *db/db* mice, indicating that the effect of ADZ6925 on liver metabolism is mediated, at least in part, by its effect on macrophages.

In conclusion, increased 11β-HSD1 expression in hypertrophic adipocytes is associated with reduced mitochondrial respiration and adiponectin synthesis. Administration of an 11β-HSD1 inhibitor increases mitochondrial respiration and adiponectin synthesis. These findings support and extend our previous finding that mitochondrial function is necessary for adiponectin synthesis and that mitochondrial dysfunction in adipocytes might explain the reduced plasma adiponectin levels in obesity (Koh *et al.* 2007).

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-15-0117.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

E H K designed the research and wrote the manuscript. H K and B J K performed the animal studies. A-R K, J H K, H-S P, M-O K, M S K and H-J K performed the *in vitro* studies. H J Y and S J K measured ATP concentrations. J S O, C-Y W, J E J and J L performed data analysis and interpretation. M H C reviewed the manuscript. K-U L takes responsibility for all research and reviewed and edited the manuscript. All authors assisted in editing the manuscript and approved the final version.

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