ABCC1 confers tissue-specific sensitivity to cortisol versus corticosterone: a rationale for safer glucocorticoid replacement therapy

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Title: ABCC1 confers tissue-specific sensitivity to cortisol versus corticosterone: a rationale for safer glucocorticoid replacement therapy

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One Sentence Summary: Corticosterone is excluded from adipose tissue by the trans-membrane transporter ABCC1 and is as effective as cortisol for ACTH suppression, but lacks metabolic adverse effects.

27 **Abstract**

28 The aim of treatment in congenital adrenal hyperplasia is to suppress excess adrenal androgens
29 while achieving physiological glucocorticoid replacement. However, current glucocorticoid
30 replacement regimes are inadequate, because doses sufficient to suppress excess androgens
31 almost invariably induce adverse metabolic effects. Although both cortisol and corticosterone are
32 glucocorticoids that circulate in human plasma, any physiological role for corticosterone has
33 been neglected. In the brain, the ATP-binding cassette transporter ABCB1 exports cortisol but
34 not corticosterone. Conversely, ABCC1 exports corticosterone but not cortisol. We show that
35 *ABCC1* but not *ABCB1* is expressed in human adipose, and that ABCC1 inhibition increases
36 intracellular corticosterone but not cortisol and induces glucocorticoid-responsive gene
37 transcription, in human adipocytes. Both C57Bl/6 mice treated with the ABCC1 inhibitor
38 probenecid and FVB mice with deletion of *Abcc1* accumulated more corticosterone than cortisol
39 in adipose after adrenalectomy and corticosteroid infusion. This accumulation was sufficient to
40 increase glucocorticoid-responsive adipose transcript expression. In human adipose tissue, tissue
41 corticosterone concentrations were consistently low, and *ABCC1* mRNA was upregulated in
42 obesity. To test the hypothesis that corticosterone effectively suppresses ACTH without the
43 metabolic adverse effects of cortisol, we infused cortisol or corticosterone in patients with
44 Addison's disease. ACTH suppression was similar, but subcutaneous adipose transcripts of
45 glucocorticoid-responsive genes was higher after cortisol than corticosterone. These data indicate
46 that corticosterone may be a metabolically favorable alternative to cortisol for glucocorticoid

47 replacement therapy when ACTH suppression is desirable, as in congenital adrenal hyperplasia,
48 and justify development of a pharmaceutical preparation.

Introduction

Congenital adrenal hyperplasia (CAH) is characterized by impaired adrenal steroidogenesis, with glucocorticoid deficiency resulting in reduced suppression of adrenocorticotrophic hormone (ACTH), and hence ACTH-dependent adrenal androgen excess. Current treatment guidelines recommend cortisol (hydrocortisone) as the treatment of choice, but recent studies have found that the doses required to suppress adrenal androgen production are usually associated with adverse effects (*1, 2*), notably in adipose tissue where chronic glucocorticoid excess promotes obesity and associated metabolic dysfunction (*3*). The ideal treatment would have higher potency in suppressing ACTH and lower potency as a glucocorticoid acting in peripheral tissues. Here, we provide a rationale and proof of concept for using corticosterone therapy as an alternative to hydrocortisone (cortisol).

A neglected characteristic of the hypothalamus-pituitary-adrenal (HPA) axis is that many species produce two glucocorticoids, cortisol and corticosterone, unlike rats and mice that lack 17-hydroxylase (CYP17) in their adrenals and therefore secrete only corticosterone. Although results of human glucocorticoid receptor binding studies are inconsistent (*4, 5*), both glucocorticoids appear to have similar affinities for mineralocorticoid receptors (*6*) and are subject to similar metabolism (*7*). However, there is emerging evidence for tissue-specific responses to cortisol and corticosterone, mediated by differential susceptibility to transmembrane transport by ATP-binding cassette (ABC) transporters (*8-12*). In rodents, ABCB1 (also known as multidrug resistance protein 1, MDR1, or p-glycoprotein) exports cortisol but not corticosterone across the blood-brain barrier (*10, 12*). Disproportionately high corticosterone concentrations in cerebrospinal fluid and brain tissue suggest that the same

mechanism operates in humans (*10, 13*). Another transporter, ABCC1 (or multidrug resistance-related protein 1, MRP1), exports corticosterone but not cortisol in vitro *(11)*. We show expression of ABCC1 but not ABCB1 in human adipose tissue, report in vitro and in vivo data supporting the hypothesis that adipose tissue excludes corticosterone and is preferentially sensitive to cortisol, and provide proof of concept that corticosterone may be a useful therapy especially when suppression of ACTH is desirable, as in CAH.

Results

ABCC1 but not ABCB1 is expressed in human adipose

In a transcriptomic analysis of ABC transporters in healthy men, *ABCC1* but not *ABCB1* was highly expressed in subcutaneous adipose tissue (Figure 1A and figure S1). This pattern was confirmed by conventional reverse transcriptase polymerase chain reaction amplification in human adipose tissue and in differentiated SGBS human adipocytes (Figure 1B) (*14*), in which we also confirmed that ABCC1 protein and mRNA are regulated in parallel, for example when we induced ABCC1 by incubation with lipopolysaccharide (figure S2). However, in murine adipose tissue, both ABCB1 and ABCC1 were expressed (Figure 1C). Publicly available datasets do not provide sufficient data to test for patterns of ABCB1 and ABCC1 expression in multiple species, but we also found selective adipose expression of ABCC1 but not ABCB1 in horse, another species in which both cortisol and corticosterone are in the circulation (Figure 1D).

ABCC1 preferentially exports corticosterone and confers sensitivity to cortisol in human adipocytes in vitro

After incubation of SGBS adipocytes with cortisol or corticosterone for 24 hours, removal of extracellular steroid resulted in lower intracellular concentrations of corticosterone compared to those of cortisol over the subsequent 24 hours (Figure 2A), suggesting active preferential export of corticosterone. This was confirmed and attributed to ABCC1 using the inhibitors MK571 (*15*) and probenecid (*11*). MK571 concentrations (10 μM) sufficient to inhibit ABCC1-dependent export of calcein by ~40% in SGBS cells (figure S3A) (*16*) increased accumulation of

intracellular deuterated corticosterone assessed qualitatively using coherent anti-Stokes Raman scattering (CARS) microscopy (Figure 2B). Similarly, both MK571 and probenecid increased intracellular tritiated corticosterone much more so than tritiated cortisol (Figure 2C). Effects of MK571 and probenecid on corticosterone retention were time-dependent (figure S3B) and dose-dependent (figure S3C,D).

To test the consequences of ABCC1-mediated steroid transport for glucocorticoid receptor (GR) activation, we used A549 human epithelial cells (which express ABCC1 and GR) transfected with a glucocorticoid-responsive MMTV-luciferase reporter (*17*). The response was greater to cortisol than corticosterone without inhibition of ABCC1, and MK571 potentiated luciferase induction by corticosterone but not cortisol (figure S4A). In differentiated SGBS adipocytes, we tested a panel of known GR-responsive gene transcripts to determine a suitable candidate for assessing acute cortisol versus corticosterone sensitivity (Figure 2D). Of these, only *PER1* was induced acutely (2 hours) by glucocorticoids, consistent with its rapid response (*18*) and high sensitivity (*19*) in other systems. We showed greater sensitivity to cortisol than corticosterone for induction of *PER1* transcription (Figure 2D). MK571 directly increased the amount of this transcript (figure S4B), so probenecid was used alone as an ABCC1 inhibitor, reversing the differential sensitivity to cortisol over corticosterone (Figure 2D). Similar results were obtained after 24 hour incubations (figure S4C). Finally, to confirm whether adipocytes are functionally more sensitive to cortisol than corticosterone, SGBS preadipocytes were differentiated in the presence of glucocorticoid; cortisol but not corticosterone induced marked triglyceride accumulation over 21 days (Figure 2E).

Genetic disruption or pharmacological inhibition of Abcc1 in mice results in preferential accumulation of corticosterone in adipose tissue

Male control mice (*Abcc1*$^{+/+}$) and mice deficient in Abcc1 (*Abcc1*$^{-/-}$) on a FVB genetic background were adrenalectomized and infused with corticosterone and cortisol by subcutaneous osmotic minipump for 7 days, followed by measurement of steroids in plasma and tissue by liquid chromatography tandem mass spectrometry (LC-MS/MS). Plasma analysis (Figure 3A) indicated impaired clearance of glucocorticoids in *Abcc1*$^{-/-}$ mice, with elevated steady state concentrations of corticosterone and a trend to elevated cortisol compared with control mice, but there was no difference in the circulating corticosterone:cortisol ratio. In the brain (Figure 3B), corticosterone concentrations were higher in *Abcc1*$^{-/-}$ mice than controls, consistent with increased plasma concentrations, and cortisol was low in both groups, consistent with exclusion of cortisol by ABCB1 (*10*). However, there was no effect of ABCC1 deficiency on brain corticosterone:cortisol ratio. In contrast, in subcutaneous adipose tissue (Figure 3C), *Abcc1* deficiency resulted in an elevated corticosterone:cortisol ratio, with a marked increase in corticosterone but not cortisol concentrations.

To assess if pharmacological inhibition of ABCC1 mimicked the effects of genetic deletion on adipose glucocorticoid concentrations, we treated mice with probenecid. Male C57Bl/6 mice underwent the same adrenalectomy and steroid infusion protocol as *Abcc1*$^{+/+}$ and *Abcc1*$^{-/-}$ mice, and were treated with probenecid (150 mg/kg/day) or vehicle (saline). Probenecid did not alter corticosterone or cortisol concentrations in the plasma (Figure 3D) or in the brain (Figure 3E); however, consistent with the results from *Abcc1*$^{-/-}$ mice, probenecid increased corticosterone but not cortisol concentrations in subcutaneous adipose tissue (Figure 3F).

To test the metabolic consequences of the increase in intra-adipose corticosterone concentrations with ABCC1 inhibition, we repeated the experiment with probenecid, adding an additional control group with no glucocorticoid infusion, and assessed adipose tissue for glucocorticoid-responsive gene transcripts (Figure 4). Plasma corticosterone and cortisol concentrations were unaffected by probenecid in corticosteroid-infused groups, as before (Figure 4A), and below the lower limit of detection (0.5 ng/ml) in vehicle-treated adrenalectomized animals. Corticosteroid infusion reduced weight gain during the 7-day treatment (Figure 4B) and induced adipose expression of *Per1, Atgl, Hsl,* and *Lpl* (Figure 4C); the effects on body weight and adipose *Per1*, *Atgl,* and *Lpl* were potentiated by probenecid (Figure 4B,C).

Corticosterone concentrations are low in human adipose, and not increased in obesity

To compare endogenous cortisol and corticosterone concentrations in adipose tissue, and to test whether downregulation of ABCC1 might enhance corticosterone action in adipose tissue in obesity, we collected subcutaneous and visceral adipose biopsies during elective abdominal surgical procedures from 6 lean and 6 obese patients, with characteristics shown in table S1. *ABCC1* mRNA transcripts were increased in obese subjects in both subcutaneous and visceral adipose (Figure 5A). Adipose tissue cortisol was readily measured in both adipose depots, as previously described (*20*), and not different between lean and obese subjects (Figure 5B). Corticosterone concentrations in lean and obese subjects were below or near the limit of detection by LC/MS-MS in both visceral and subcutaneous adipose tissue (Figure 5B).

Human adipose is less sensitive to corticosterone than cortisol

Having established that selective expression of ABCC1 protects human adipose tissue from corticosterone, we then tested whether corticosterone is less potent than cortisol at inducing metabolic effects in adipose. This was achieved using doses of cortisol and corticosterone

equipotent for ACTH suppression. We performed a randomized single-blind crossover study in patients with Addison's disease using sequentially increasing steady state infusions of deuterated glucocorticoids, D8-corticosterone and D4-cortisol, which can be distinguished from residual endogenous glucocorticoids by mass spectrometry (*21*). Characteristics of study participants are summarized in table S2. Circulating concentrations of cortisol (sum of cortisol, D4-cortisol, and its active metabolite D3-cortisol) and corticosterone (D8-corticosterone alone; endogenous corticosterone was undetectable) indicated that serially increasing steady state concentrations were achieved as intended (Figure 6A). Total steady state concentrations tended to be higher for cortisol than corticosterone, although the differences were not statistically significant and there were no differences in free cortisol and corticosterone measured after equilibrium dialysis (in samples pooled between 260 and 330 min of infusion, free cortisol = 38.7 ± 8.2 nM and free corticosterone = 37.2 ± 4.7 nM). Neither ACTH concentrations at baseline nor their suppression during glucocorticoid infusion were different between cortisol and corticosterone (Figure 6B,C; Table 1). Amongst biochemical markers of glucocorticoid action – glucose, insulin, glycerol, and free fatty acids – only free fatty acids rose during steroid infusion (Table 1), perhaps because the infusions were short and the glucocorticoid concentrations achieved were within the physiological range (*22*). Insulin concentrations fell, paradoxically, during steroid infusions. None of these plasma biochemical markers differed between D8-corticosterone and D4-cortisol infusion (Table 1). However, in subcutaneous adipose biopsies obtained at the end of infusions, the acutely responsive glucocorticoid-responsive transcript *PER1* was substantially higher and *LPL* also modestly higher after D4-cortisol than D8-corticosterone infusion (Figure 6D).

Discussion

Our findings shed light on tissue-specific responsiveness to cortisol and the often-neglected 'second' glucocorticoid corticosterone, mediated by local expression of steroid-selective ABC transporters. In combination with tissue-specific expression of either mineralocorticoid or glucocorticoid receptors and tissue-specific intracellular metabolism of corticosteroids, the availability of alternate endogenous glucocorticoids that are differentially transported out of target cells provides a mechanism for subtle control of the otherwise highly conserved pathway of glucocorticoid action. Moreover, as summarized in the schematic in Figure 7, our data show how this insight can be exploited to develop corticosterone as a potentially safer alternative to cortisol for glucocorticoid replacement therapy when ACTH suppression is desirable.

For in vitro studies, we used SGBS adipocytes, an established model of human adipocytes (*14*), to show that in the absence of ABCB1, endogenous ABCC1 expression is sufficient to reduce intracellular corticosterone but not cortisol concentrations. An equilibrium in intracellular cortisol and corticosterone concentrations was reached after approximately 4 hours in adipocytes. Our CARS microscopy data suggest that glucocorticoids co-localize with lipid droplets within adipocytes, potentially resulting in their intracellular retention and explaining the slow turnover of cortisol in adipose tissue in vivo *(20)*. The magnitude of the effect of ABCC1 on steroid export appears to be substantial, with a 3-4 fold increase in intracellular corticosterone when ABCC1 is inhibited in SGBS cells, and a 2-fold increase in adipose corticosterone in vivo in mice. In a physiological context, if the plasma glucocorticoid pool is comprised of 90-95%

cortisol and 5-10% corticosterone, as reported in previous studies (*10, 13, 23-32*) (table S3), the total (cortisol plus corticosterone) intra-adipose glucocorticoid pool may be reduced by as much as ~7% as a result of ABCC1 activity. In a pathological context, we hypothesized that reduction of adipose ABCC1 in obesity might contribute to metabolic dysregulation through increased intra-adipose corticosterone. However, *ABCC1* expression was increased in obesity, suggesting a potential protective mechanism to limit adipose exposure to glucocorticoids, which is considered to be beneficial in obesity (*3*). Indeed, adipose glucocorticoid concentrations were consistent with ABCC1 acting as constitutive barrier to maintain very low corticosterone concentrations in human adipose tissue, even in obese individuals. In a pharmacological context, such as if corticosterone were used as glucocorticoid replacement therapy, the consequences of ABCC1 activity for the intra-adipose glucocorticoid pool could be much greater. We tested this in vivo in mice and humans.

Our findings in vivo support our cell-based work, with both genetic deletion and pharmacological inhibition of ABCC1 resulting in preferential accumulation of corticosterone over cortisol in the adipose tissue. Because 17α-hydroxylase, which is necessary to produce cortisol, is not expressed in mouse adrenals, we studied this in adrenalectomized mice infused with equal concentrations of both glucocorticoids. Plasma steroid concentrations achieved during infusion were similar within strains, but slightly higher in C57Bl6 than FVB mice, potentially reflecting strain-specific differences in glucocorticoid clearance. *Abcc1*$^{-/-}$ mice, but not mice treated with probenecid, showed higher steady-state plasma concentrations of cortisol and corticosterone compared to wild type controls, implicating ABCC1 in total glucocorticoid clearance; this effect, however, appears to require total loss of *ABCC1,* as it was not observed with a competitive inhibitor, probenecid. Of note, the only spontaneous phenotype reported in

Abcc1$^{-/-}$ mice is a reduced susceptibility to inflammation (*33*), which may be mediated by systemic or intracellular corticosterone excess, but this has not been studied. Using probenecid, we showed that the increase in intra-adipose corticosterone concentrations with ABCC1 inhibition is sufficient to potentiate the induction of key lipolytic genes (*Atgl* and *Hsl)* and prevent weight gain.

To investigate the physiological and therapeutic implications of these findings, we performed a study in patients with Addison's disease, who lack endogenous glucocorticoids. We selected patients with Addison's disease rather than with CAH to avoid confounding effects of high androgen concentrations in the adipose tissue. Corticosterone is only available as an experimental tool for infusion and not as a licensed pharmaceutical, therefore only short-term manipulation was possible. At similar concentrations of free cortisol and corticosterone, we found equipotent suppression of ACTH, but substantially greater gene transcript induction in adipose tissue by cortisol. In adipose, cortisol induced greater transcript expression than corticosterone not only for *PER1*, which is known to be rapidly induced by glucocorticoids (*18*) and sensitive at low concentrations which do not alter other glucocorticoid-responsive genes (*19*), but also for *LPL,* a key enzyme involved in adipose triglyceride uptake. The lack of differences in circulating metabolic markers is likely attributable to the short duration of infusions and the glucocorticoid concentrations within the physiological range (*22*); previous studies have that demonstrated plasma glucose, insulin, and glycerol show responses only to several hours of 'physiological' glucocorticoid infusion (*34, 35*). It remains possible that there are intrinsic differences in transcriptional response to cortisol and corticosterone in human adipose tissue, even when the same concentrations of steroid are present, but this seems unlikely given our findings in SGBS adipocytes that cortisol is no more potent than corticosterone when

ABCC1 is inhibited. Despite our confirmation that cortisol concentrations are disproportionately low in the mouse brain, and earlier observations of relatively low cortisol in human brain and cerebral spinal fluid (*10, 13*), we did not find that corticosterone was more effective than cortisol in suppressing ACTH. This may reflect the major feedback signal being mediated in the pituitary rather than higher centers within the brain, under non-stressed conditions. Nonetheless, the discrepancy between suppression of ACTH and induction of adipose *PER1* and *LPL* by cortisol versus corticosterone supports our interpretation that adipose tissue is physiologically more responsive to cortisol than corticosterone, and that this can be exploited therapeutically.

Synthetic glucocorticoids in common use as glucocorticoid therapy, specifically prednisolone and dexamethasone, are not transported by ABCC1 (*11*) and so are likely to access adipose tissue similarly to cortisol. To validate and exploit these findings, it will therefore be important to develop a suitable pharmaceutical preparation of corticosterone. With longer term therapy, corticosterone and cortisol effects can be compared in conditions requiring effective ACTH suppression such as CAH, Nelson's syndrome, and glucocorticoid-suppressible hyperaldosteronism. Although we have focused here on adipose tissue, differential expression of ABCB1 and ABCC1 may determine tissue-specific responses to corticosterone and cortisol in other tissues as well. Adverse effects will need to be compared not only for metabolism and obesity, but also for immune suppression and osteoporosis. Nevertheless, our findings suggest that the substantial investment required to develop a corticosterone-based drug product is worthwhile.

Materials and Methods

Study design

To investigate the role of adipose ABCC1 regulating cortisol versus corticosterone action, we conducted studies in cells, mice, and humans, as detailed below. In vitro experiments were performed in triplicate with the number of experiments and outcomes defined in figure legends. For in vivo studies in mice, experiments were approved by the institutional ethical committee and conducted under UK Home Office license in male mice aged 10-12 weeks at the start of the experiment. Sample sizes were chosen for 80% power to detect magnitudes of difference inferred from in vitro experiments at $P<0.05$, with the number of mice and outcomes defined in figure legends. Mice of each genotype were randomly assigned to interventions within each experiment. The studies in humans were conducted with approval from the South East Scotland Research Ethics Committee and NHS Lothian Research and Development (13/SS/0210 for Addison's disease study and 10/S1102/39 for surgical adipose tissue collection study), and with written informed consent of participants. The surgical adipose tissue collection study was a case-control design comparing adipose tissue mRNA amounts in lean and obese participants randomly selected from those undergoing surgery. An initial exploratory study was conducted in n=6 per group and showed results contradicting the hypothesis that *ABCC1* transcripts are decreased in obesity, so the study was not expanded. For the Addison's disease study, eligible patients participated in a single-blind randomized crossover study comparing infusions of deuterated corticosterone and deuterated cortisol for effects on ACTH, adipose tissue mRNA, and circulating metabolic indices. Blinding of the clinical investigator was impractical because of the

requirements for administration of different loading and infusion doses of cortisol and corticosterone, but samples were processed in the laboratory with blinded codes and only decoded for statistical analysis; patients were blinded to treatment. Sample size was calculated for 90% power to detect 20% difference in ACTH at $p<0.05$. Inclusion/exclusion criteria for each study are detailed below. There were no dropouts, and no outliers were excluded from any studies.

Human adipose tissue microarray

Affymetrix microarray data were obtained from subcutaneous adipose biopsies of 9 healthy men, aged 36.6 ± 2.1 years with body mass indices 29.2 ± 2.1 kg/m^2 (*36*). Probes for transcripts encoding ABC transporters were selected and ranked by transcript intensity ($\log^2$ transformed).

Polymerase chain reaction

Human tissue biobank cDNA (Primerdesign Ltd) was used as a template for the identification of ABC transporters in human tissue of interest. Equine peri-renal adipose tissue collection from clinical cases euthanized for reasons other than endocrine disease/systemic inflammation was approved by the University of Edinburgh Veterinary ethics and research committee. Where applicable, total RNA extraction was carried out by centrifugation in Qiazol lysis reagent using an RNeasy mini kit (Qiagen), according to the manufacturer's instructions. RNA integrity was checked on a 1% agarose gel, after which cDNA (500 ng RNA/reaction) was synthesized using a high capacity cDNA kit (Invitrogen), according to the manufacturer's instructions. cDNA was used as a template for specific primers (Invitrogen; table S4) for RT-PCR and amplified using GoTaq DNA polymerase (Promega) as per the manufacturer's instructions, on a Techne thermocycler (95 °C for 5 min; 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s; 72 °C for 5 min). Products were subjected to gel electrophoresis on a 2% agarose gel in TAE buffer

316 (50x stock buffer: 2 M Tris Base, 1 M glacial acetic acid, 100 mM disodium EDTA) containing
317 GelRed (Cambridge Bioscience). Gels were imaged using a Gel-Doc system (Uvitec). Product
318 size was confirmed against a 100 bp DNA ladder (Invitrogen).

319 **In vitro studies**

320 *SGBS cell culture*

321 Human preadipocyte Simpson-Golabi-Behmel syndrome (SGBS) cells were a kind gift from
322 Martin Wabitsch, University of Ulm, Germany (*14*). Cells were maintained in high-glucose
323 DMEM-F12 (Lonza), supplemented with FBS (10% v/v), penicillin (100 IU/ml), streptomycin
324 (100 IU/ml), biotin (33 μM), and pantothenic acid (17 μM) at 37°C in 5% CO_2. Differentiation
325 was induced as previously described (*37*). Cells were cultured in steroid-free medium for 24
326 hours before experimentation.

327 *Primary cell culture*

328 Subcutaneous adipose tissue samples were obtained from patients undergoing elective abdominal
329 surgery at the Royal Infirmary of Edinburgh. Upon tissue collection, adipose was digested, and
330 the stromal vascular fraction was isolated and differentiated as previously described (*38*). In
331 brief, after removal of connective tissue and blood vessels, adipose tissue was digested in
332 collagenase type I (615 U/g tissue, 90 min, 37 °C). After overnight plating in high-glucose
333 DMEM-F12 supplemented with FBS (10% v/v), penicillin (100 IU/ml), streptomycin (100
334 IU/ml), biotin (33 μM), and pantothenic acid (17 μM), cells were differentiated for 3 days using
335 the above medium without serum, but with addition of triiodothyronine (1 nM), transferrin (10
336 μg/ml), insulin (66 nM), IBMX (500 μM), dexamethasone (1 μM), and rosiglitazone (10 μM).
337 From day 4 onwards, cells were maintained in this differentiation medium but without IBMX,
338 dexamethasone, or rosiglitazone.

Tritiated glucocorticoid retention assays

To measure uptake and retention of steroids, SGBS adipocytes were incubated in the presence of either corticosterone or cortisol (20 nM 1,2,6,7-$^{3}H_4$-steroid, 480 nM unlabeled steroid) for 1, 4, 8, and 24 hours before being washed in PBS, lysed in cellular lysis buffer (0.5% SDS), mixed with Prosafe FC+ liquid scintillation cocktail (Meridan Biotechnologies), and read for 1 min on a β-scintillation counter. A separate batch of SGBS adipocytes were incubated with steroids for 24 hours as described above, then washed in PBS before fresh steroid-free medium was added to each well and the decline in intracellular ^{3}H-steroid content measured at 1, 4, 8 and 24 hours.

To assess the effect of ABCC1 inhibition, SGBS adipocytes were pre-incubated for 1 hour in the presence of the indicated concentrations of MK-571 (Cayman Chemical), probenecid (Invitrogen), or vehicle (DMSO), before being incubated for a further 24 hours in the presence of inhibitor or vehicle and either corticosterone or cortisol (20 nM ^{3}H-steroid, 480 nM unlabeled steroid). Cells were then washed in PBS, lysed in cellular lysis buffer, mixed with liquid scintillation cocktail, and read for 5 min on a β-scintillation counter.

Quantification of glucocorticoid-sensitive mRNA transcripts

In accordance with the time course of the clinical study, we performed an acute (2 hour) treatment in SGBS adipocytes and an extended (24 hour) treatment with either vehicle or cortisol (500 nM) to identify acutely upregulated glucocorticoid-responsive transcripts. To determine the effects of ABCC1 inhibition on glucocorticoid-induced transcripts, SGBS adipocytes were pre-incubated for 1 hour in the presence of probenecid (50 μM) or vehicle (DMSO), before a further 2 or 24 hours incubation in the presence of probenecid or vehicle and either corticosterone or cortisol (500 nM). Total RNA was extracted from adipocytes in Qiazol lysis reagent using a

RNeasy Mini Kit according to the manufacturer's instructions. RNA (250 ng) was reverse transcribed with random primers using the Applied Biosystems High-Capacity cDNA Reverse Transcription kit. Real-time PCR was performed using the Roche LightCycler 480 (Roche Applied Science). Primers (Invitrogen) were designed for use with intron-spanning probes within the Roche Universal Probe Library (UPL). Primer sequences and UPL probe numbers are shown in table S5. Results were corrected for abundance of 18S, which was not affected by treatment. All samples were analyszd in triplicate and amplification curves plotted (y axis fluorescence, x axis cycle number). Triplicates were deemed acceptable if the standard deviation of the crossing point was < 0.5 cycles. A standard curve (y axis crossing point, x axis log concentration) for each gene was generated by serial dilution of cDNAs pooled from different samples, fitted with a straight line, and deemed acceptable if reaction efficiency was between 1.7 and 2.1.

CARS microscopy

Coherent Anti-Stokes Raman Scattering (CARS) microscopy is a non-invasive, label-free imaging technique based on Raman spectroscopy. The experimental setup has been described previously (*39*). Briefly, a pump and tuneable Stokes laser (PicoTrain, High-Q laser and Levante Emerald Optical Parametric Oscillator) provided a specific vibrational coherence resulting in detectable photons which, when combined with a confocal laser scanning inverted microscope (C1 Eclipse, Nikon BV), provided a spatial image. For CARS imaging, the pump and Stokes laser beams were tuned such that the frequency difference would correspond to a specific Raman vibration.

SGBS adipocytes were incubated for 24 hours in glass-bottomed dishes with 2,2,4,6,6,17α,21,21-$^{2}H_{8}$-corticosterone (D8-corticosterone, 10 μM; Cambridge Isotopes) in the presence or absence of MK-571 (10 μM). For CARS imaging, the pump laser was tuned to 816.8

nm (12243 cm^{-1}) and Stokes laser to 1064 nm (9398.5 cm^{-1}) to obtain a CARS signal at 663 nm (2845 cm^{-1}) corresponding to the vibration of CH_2 in lipids (*39*). After initial documentation of Raman spectra to identify optimal wavelength (figure S5), excitation of the Raman vibration of the C-D bond in D8-corticosterone was achieved by adjusting the pump laser to 868.4 nm (11524.5 cm^{-1}), resulting in a CARS signal at 733 nm (2126 cm^{-1}), which was in a region of the Raman spectrum with low background signal. Images were processed using Nikon EZ-C1 3.4 software.

Protein analysis

Total protein extracts from cells were prepared in RIPA buffer (Santa Cruz). Samples were sonicated and centrifuged (13,000 x g, 15 min, 4 °C) before performing the BCA assay, adding 6X Laemmli sample buffer (LSB), boiling, and SDS-PAGE resolution. ABCC1 (Enzo Life Sciences, ALX-801-007) and HSP90 (Santa Cruz, sc-7947) were detected on Western blots using commercial antibodies.

Oil Red O staining and lipid quantification

To assess potential differing effects of cortisol and corticosterone on adipocyte differentiation, SGBS adipocytes were stimulated to undergo differentiation with or without the substitution of cortisol in the differentiation cocktail (*37*) for corticosterone. Accumulation of lipid during adipogenesis was visualized by Oil Red O (Sigma-Aldrich). Cells were washed twice with PBS and fixed with formalin (10% v/v) for 60 min, followed by a wash with isopropanol (60% v/v). Working Oil Red O solution was added to cells for 10 min, followed by 4 washes with H_2O. Cells were air-dried and dye extracted with isopropanol (100%). Absorbance of extracts were measured at 500 nm wavelength in an OPTImax microplate reader (Molecular Devices).

Calcein-AM Assay

Calcein–acetoxymethyl ester (AM) is a non-fluorescent, hydrophilic, cell membrane permeable molecule which is converted to fluorescent, hydrophilic calcein by intracellular esterases (*16*). Both calcein-AM and calcein are substrates for ABCC1, thus intracellular fluorescence is inversely proportional to ABCC1 activity. SGBS adipocytes were washed with PBS and pre-incubated for 1 hour in the presence of MK-571 (10 μM) or vehicle (DMSO), before being incubated for a further 5 hours in the presence of inhibitor or vehicle and calcein-AM (1 μM; Invitrogen). Cells were washed three times in PBS, then excitation absorbance was measured at 494 nm and emission absorbance was measured at 517 nm in an Infinite M1000 plate reader (Tecan). Data are expressed as percentage fluorescence of untreated control.

Luciferase reporter assay

Human epithelial A549 cells were grown in DMEM (Lonza), supplemented with FBS (10% v/v), penicillin (100 IU/ml), and streptomycin (100 IU/ml). Cells were seeded at $2x10^5$/ 35 mm well. After overnight incubation, medium was replaced with Opti-MEM (Lonza), and cells were transfected using Lipofectamine 2000 (Invitrogen) with a total of 2 μg DNA comprising 1 μg pMMTV-LTR-Luc (*40*) and 1 μg pKC275 (encoding β-galactosidase as internal control). After overnight incubation, medium was replaced with steroid-free medium, and cells were treated for 1 hour with MK-571 (10 μM) before a 24-hour incubation with inhibitor and either corticosterone or cortisol (500 nM). Luciferase and β-galactosidase activities were measured in cell lysates as described previously (*41*). β-Galactosidase activity was assayed using a Tropix Galacto Light Plus kit (Applied Biosystems). All transfections were carried out in triplicate, and the mean ratio of luciferase/ β-galactosidase activities was calculated.

Animal studies

Animals

Male *Abcc1* knockout mice (*Abcc1*$^{-/-}$) and FVB controls (*Abcc1*$^{+/+}$) were purchased from Taconic. Male C57Bl/J mice were purchased from Charles River. Mice were bilaterally adrenalectomized under fluorothane anesthesia. After surgery, drinking water was replaced with 0.9% NaCl, and animals were allowed to recover for 7 days. An osmotic mini-pump (Alzet Model 2001; 1 µl/hr) delivering 250 µg/day of corticosterone and 250 µg/day cortisol or vehicle (DMSO: propylene glycol; 50:50 v/v) was inserted subcutaneously under anesthesia and left in place for 7 days. *Abcc1*$^{+/+}$ and *Abcc1*$^{-/-}$ mice received no further treatment. C57Bl/6 mice were given daily s.c. injections of probenecid (150 mg/kg) or vehicle (saline). This dose was previously reported to inhibit transporter activity in vivo (*42*). 7 days after osmotic pump implantation, animals were culled by decapitation. Plasma was extracted from trunk blood and stored at -20 °C. Tissue was extracted and stored at -80 °C.

Plasma and tissue steroid extraction and LC-MS/MS quantification

Steroids were extracted from plasma by liquid-liquid extraction (chloroform, 10:1). Briefly, plasma (100 µl) was enriched with internal standard (D4-cortisol and epi-corticosterone, 25 ng each). 1 ml chloroform was added and vortexed. Supernatant was reduced to dryness under oxygen-free nitrogen (OFN) at 60°C and reconstituted in mobile phase [70 µl water/acetonitrile (70:30, v/v)]. Steroids were extracted from brain and adipose as previously described (*20*), with the substitution of D4-cortisol and D8-corticosterone as internal standards. Steroids were extracted from brain by homogenizing whole brain in methanol:acetic acid (100:1 v/v; 10 ml) and centrifuging (5000x g, 10 min, 4°C). The supernatant was reduced to dryness under OFN at

60°C, reconstituted in methanol:dichloromethane:water (7:2:1, v/v; 3 ml), and enriched with internal standard (D4-cortisol and D8-corticosterone, 25 ng each). Samples were passed through a diethylaminohydroxypropyl Sephadex LH-20 anion exchange column (GE Healthcare). Columns were washed with methanol:dichloromethane:water (7:2:1, v/v; 2 ml) and methanol:dichloromethane:water (2:2:1, v/v; 1 ml). All flow-through/wash was collected and reduced to dryness under OFN at 60°C. Samples were reconstituted in methanol:acetic acid (100:1 v/v; 2 ml), and water (2 ml) was added. Samples were passed through pre-conditioned C-18 Bond-Elut columns (Agilent Technologies). Columns were washed with water (2 ml), methanol:water (50:50 v/v; 3 ml), and hexane:ethyl acetate (5:1 v/v; 2 ml). Samples were eluted in ethyl acetate (2 ml), dried under OFN at 60°C and reconstituted in mobile phase [60 µl water/acetonitrile (70:30, v/v)].

Quantitative analysis of steroids was carried out by liquid chromatography tandem mass spectrometry (LC-MS/MS). Chromatographic separation was achieved using a Waters Acquity UPLC system, with detection on an ABSciex QTRAP 5500 mass spectrometer operated with Analyst software version 1.6.1. The mass spectrometer was operated using the Turbospray Ion source, with nitrogen as the source, curtain and collision gas (40 and 60 psi, medium), source temperature of 550ºC, spray voltage of 4.5 kV, and an entrance potential of 10 V. Compound-specific tuning was performed using methanolic solutions of steroids and isotopically labeled internal standards. The protonated molecular ions were subjected to collision-induced dissociation, and the most abundant precursor-product transitions were selected; *m/z* 347.2 → 91.1, 121.1 at 69 V, *m/z* 355.1 → 337.0 at 19 V, *m/z* 363.2 → 121.0, 77.0 at 29 and 101 V, and *m/z* 367.0 → 121.1 at 29 and 101 V collision energy for corticosterone, D8-corticosterone, cortisol, and D4-cortisol, respectively. Analytes were eluted on a Waters Sunfire C18 column

(150 x 2.1 mm; 3.5 μm) at 30ºC (injection volume; 30 μL) protected by a Kinetex KrudKatcher at a flow rate of 0.5 mL/min, starting at 30:70 and rising linearly to 90:10 [water+0.1% formic acid (FA):acetonitrile+0.1% FA] by 6 minutes and a total run time, including re-equilibration, of 9 minutes. The peak areas were integrated using Xcalibur software (Thermo Electron) and quantified against a calibration curve. Steroid concentrations are presented corrected for total tissue weight or plasma volume.

Quantification of glucocorticoid-sensitive mRNA transcripts

RNA analysis performed was as described above. Primer sequences and UPL probe numbers are shown in table S6. Results were corrected for abundance of the mean combination of *18S* and *Tbp*, which was not affected by treatment.

Adipose tissue steroid concentrations and *ABCC1* mRNA in lean and obese humans

We recruited 12 subjects who were at the Royal Infirmary of Edinburgh for elective abdominal surgery for non-malignant disease, and who did not have systemic or local active inflammation. Paired adipose tissue samples were obtained intra-operatively from the subcutaneous and visceral depots, stored on dry ice then at -80C, and extracted for steroid quantification as previously described (*20*) and for RNA analysis as described above.

Cortisol versus corticosterone infusion in patients with Addison's disease

Participants

Patients with Addison's disease were identified from the clinic database of the Edinburgh Centre for Endocrinology and invited to attend a screening visit, where written informed consent was obtained and eligibility assessed by a medical questionnaire, physical examination, and routine

499 blood tests. Inclusion criteria were: a diagnosis of autoimmune Addison's disease and age >18 y.
500 Exclusion criteria were: alcohol intake >28 units/week; abnormal screening blood results (full
501 blood count; renal, liver, and thyroid function tests); pituitary disease; pregnant or breastfeeding;
502 anti-inflammatory glucocorticoid therapy by any route in the preceding 3 months; cardiac, renal
503 or liver failure; uncontrolled hypertension (systolic BP >160 mmHg or diastolic >100 mmHg);
504 blood donation in preceding 3 months (to avoid anemia); or research study participation in
505 preceding 6 weeks.

506 *Study protocol*

507 Participants attended for study visits on two occasions, separated by at least one week.
508 Participants withheld hydrocortisone from 14:00h on the day before each study visit, and omitted
509 fludrocortisone (when prescribed) the morning before the study visit and on the morning of the
510 study visit. They attended the clinical research facility at 08:00h after an overnight fast from
511 22:00h. At t = -15 minutes, intravenous cannulae (18G) were inserted in each antecubital fossa.
512 At t = 0, saline infusion (0.9 %, 125 ml/h) was commenced through the cannula in the left arm,
513 and blood samples collected from the cannula in the right arm. At t = 60 min, infusion of
514 deuterated glucocorticoid (D8-corticosterone or D4-cortisol) was commenced. Order of steroid
515 infusion was allocated randomly, and study participants were blinded to the order of infusate.
516 9,11,12,12-$[^2H]_4$-cortisol (D4-cortisol) and 2,2,4,6,6,17,21,21-$[^2H]_8$-corticosterone (D8-
517 corticosterone) were obtained from Cambridge Isotope Laboratories, dissolved in pharmaceutical
518 grade ethanol/water (90:10, v/v) and filtered to form sterile stock solutions, stored (-40 °C) for a
519 maximum of 8 weeks. On study days, D8-corticosterone (4.18 mg/ml) or D4-cortisol (2.5
520 mg/ml) stock solution (5 ml) was dissolved in sodium chloride 0.9% *w/v* (495 ml). At t = 60 min,
521 a priming dose (0.65 μmol D8-corticosterone; 0.23 μmol D4-cortisol) was administered over 4

minutes, followed by steady state infusion (27.6 nmol/min D8-corticosterone; 3.7 nmol/min D4-cortisol) for 86 minutes. Further priming doses were administered at t = 150 min (1.95 μmol D8-corticosterone; 0.65 μmol D4-cortisol) followed by constant infusion (111.2 nmol/min D8-corticosterone; 17.2 nmol/min D4-cortisol) until t = 240 min, and at 240 min (3.89 μmol D8-corticosterone; 1.55 μmol D4-cortisol) followed by constant infusion (277.8 nmol/min D8-corticosterone; 51.5 nmol/min D4-cortisol) until t = 330 min.

At t = 330 min, a needle aspiration biopsy of subcutaneous abdominal adipose tissue was obtained as previously described (*36*) and stored at -80 ºC.

Blood samples were obtained at 10-20 min intervals in potassium EDTA tubes (2.7 ml) pre-chilled on wet-ice and serum gel tubes (9 ml; both Monovette, Sarstedt). Potassium EDTA tubes were centrifuged at 4 ºC within 30 minutes of sampling; serum gel samples were left at room temperature for 30-45 minutes before centrifugation. Serum and plasma was stored at -80 ºC.

Laboratory analyses

In plasma, ACTH was quantified by ELISA (IBL International) within 6 weeks of sampling, and non-esterified fatty acids (NEFAs)(Zen-Bio) and glycerol (Sigma-Aldrich) quantified by colorimetric assays. In serum, glucocorticoids were quantified by LC-MS/MS, insulin by ELISA (DRG Diagnostics), and glucose by colorimetric assay (Cayman Chemical).

LC-MS/MS analysis was undertaken as described for animal samples above with the exception that 11α-epimers of corticosterone (epi-corticosterone; *m/z* 347.2 → 91.1, 121.1 at 69 V) and cortisol (epi-cortisol; (*m/z* 363.2 → 121.0, 77.0 at 29 and 101 V) were used as internal standards instead of D8-corticosterone and D4-cortisol.

To account for any differences in protein binding between cortisol and corticosterone, cortisol and corticosterone isotopologs were also measured by LC-MS/MS after equilibrium dialysis of

plasma. To achieve the necessary sensitivity, samples were pooled for the final steady state period from each infusion (260-330 min), and 4 x 1 mL aliquots of plasma were dialyzed into 1.5 mL phosphate buffered saline across 12-14 kD dialysis membrane (Medicell) for 16 hours at 37 ^{o}C, as previously described (*43*), before LC-MS/MS analysis of the pooled dialysate as above. Real-time qPCR was carried out in adipose tissue as with SGBS cells above.

Statistical analysis

For cell and mouse-based studies, comparisons were performed using two-way ANOVA with Bonferroni post-hoc tests or unpaired Student's t tests as outlined in each figure legend. For the Addison's disease study, data from each steady state period (80-140 min; 160-240 min; 260-330 min) were averaged, and comparisons between D4-cortisol and D8-corticosterone and the interaction with changes over time were performed by two-way ANOVA. Adipose tissue data were compared with paired Student's t tests. P values for statistically significant differences are presented in table S7.

Supplementary Materials

Supplementary figures

Fig. S1. Whole gel PCR images of ABC transporter expression in tissues and cells.

Fig. S2. Correlation of *ABCC1* mRNA and protein levels in human adipocytes.

Fig. S3. ABCC1 inhibition in SGBS adipocytes.

Fig. S4. Effects of ABCC1 inhibition on GR-mediated transcription.

Fig. S5. Optimisation of CARS microscopy for detection of intracellular D8-corticosterone.

Supplementary tables

Table S1. Characteristics of lean and obese study participants providing adipose biopsy samples during surgery.

Table S2. Characteristics of Addison's study participants.

Table S3. Summary of studies describing plasma corticosterone and cortisol concentrations in healthy subjects.

Table S4. Primer sequences for PCR and corresponding expected product size.

Table S5. Human primer sequences for qPCR and corresponding probe number from Roche Universal Probe Library (UPL).

Table S6. Murine primer sequences for qPCR and corresponding probe number from Roche Universal Probe Library (UPL).

Table S7. Summary table of exact *P* values (provided as an Excel file).

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Figures:

Fig. 1. *ABCC1*, but not *ABCB1*, is expressed in human adipose tissue. **(A)** Transcript microarray data for members of the ABC transporter family, ranked by intensity, in subcutaneous adipose tissue from 12 healthy men. *ABCC1* but not *ABCB1* is highly expressed in human adipose. Reverse transcriptase polymerase chain reaction amplification (RT-PCR) showing expression of *ABCB1* and *ABCC1* in **(B)** human tissues and fully differentiated SGBS human adipocytes, **(C)** murine adipose, and **(D)** horse adipose. MNC = mononuclear cells, Sk. Mus = skeletal muscle.

Fig. 2. ABCC1 preferentially exports corticosterone from adipocytes. **(A)** Export of ^{3}H-cortisol and ^{3}H-corticosterone from SGBS adipocytes after steroid loading for the previous 24 hours (480 nM unlabeled steroid + 20 nM tritiated steroid). Data are expressed as a percentage of intracellular steroid counts per minute (CPM) after 24 hour loading (n = 3, *P<0.05, **P<0.01, two-way ANOVA with Bonferroni post-hoc test). **(B)** Coherent anti-Stokes Raman Scattering (CARS) images of SGBS cells treated with D8-corticosterone (500 nM) with or without ABCC1 inhibition (MK571, 10 µM, 24 hours). Resonance of the C-H bonds abundant in lipid is represented in red; C-D resonance from D8-corticosterone is represented in green, and is more abundant with MK571 inhibition, notably in a distribution around the intracellular lipid droplets (scale bar = 20 µm). **(C)** Effect of ABCC1 inhibition with either MK571 (10 µM) or probenecid (PBN, 50 µM) on ^{3}H-cortisol and ^{3}H-corticosterone retention in SGBS adipocytes after 24 hour incubation. (n = 3, ***P<0.001 vs Vehicle, ##P<0.01, ###P<0.001 vs cortisol, two-way ANOVA with Bonferroni post-hoc test). **(D)** SGBS adipocytes incubated with corticosterone or

cortisol (500 nM, 2 hours) in the presence or absence of probenecid (PBN, 50 μM). Transcripts measured are period circadian clock (*PER1),* adiponectin (*ADIPOQ*), adipose triglyceride lipase (*ATGL*), and hormone sensitive lipase (*HSL*). (n = 3, **P*<0.05 vs Control, #*P*<0.05 vs – PBN, two-way ANOVA with Bonferroni post-hoc test. **(E)** SGBS pre-adipocytes show greater triglyceride accumulation after incubation with cortisol than corticosterone (100 nM), quantified by Oil Red O staining (n = 3, ****P*<0.001 vs corticosterone, two-way ANOVA with Bonferroni post-hoc test). All data are mean ± SEM, exact *P* values are given in table S7.

Fig. 3. *Abcc1*$^{-/-}$ deletion or pharmacological inhibition in mice results in preferential accumulation of corticosterone in adipose tissue. **(A - C)** Adrenalectomized male wild type (*Abcc1*$^{+/+}$) or ABCC1 knockout (*Abcc1* $^{-/-}$) FVB mice were infused with corticosterone and cortisol for 7 days. **(D - E)** Adrenalectomized male C57Bl/6 mice were infused with corticosterone and cortisol, together with either probenecid or vehicle (saline), for 7 days. Plasma (A,D), brain (B,E), and subcutaneous adipose (C,F) corticosterone (C'one) and cortisol were quantified by LC/MS-MS and are presented as concentrations and as corticosterone:cortisol ratios (Ratio). All data are mean ± SEM, n = 6-8 per group, (A - C) **P*<0.05 vs *Abcc1*$^{+/+}$, (D - E) **P* <0.05 vs vehicle, Student's t test, exact *P* values are given in table S7.

Fig. 4. Pharmacological inhibition of ABCC1 potentiates adipose GC-responsive transcript expression. **(A)** Plasma corticosterone (C'one) and cortisol concentrations, and their ratio, were not altered between mice receiving 7 days treatment with corticosteroid (corticosterone and cortisol 250 μg/day) in the presence or absence of probenecid (150 mg/kg/day). Adrenalectomized mice not receiving corticosteroid infusion had corticosterone or cortisol concentrations below the detectable limit (0.5 ng/ml). **(B)** Change in body weight of

adrenalectomized mice after 7 days of treatment with corticosteroid (steroid) in the presence or absence of probenecid (PBN), or control (no corticosteroid). **(C)** Subcutaneous adipose transcript expression of the glucocorticoid-responsive genes period circadian clock 1 (*Per1*), adipose triglyceride lipase (*Atgl*), hormone sensitive lipase (*Hsl*), fatty acid synthase *(Fas)*, diacylglycerol O-Acyltransferase 1 *(Dgat1),* and lipoprotein lipase *(Lpl).* All data are mean ± SEM (n = 7-11 per group, *P<0.05 vs control, #P<0.05 vs steroid + vehicle, two-way ANOVA with Bonferroni post-hoc test), exact P values are given in table S7.

Fig. 5. Corticosterone concentrations are low in human adipose tissue. Adipose biopsies were obtained during elective abdominal surgery from 6 lean and 6 obese patients. **(A)** *ABCC1* mRNA concentrations were upregulated in obese subcutaneous (SC) and visceral (Visc) adipose tissue. (n = 6, *P <0.05, **P<0.01 vs lean, unpaired Student's t tests). **(B)** Adipose tissue cortisol concentrations were readily detectable, but corticosterone concentrations were low or below the limit of detection (LOD) in SC and visceral adipose [samples below limit of detection are assigned a value of 0.86 pmoles/g (LOD)]. Neither cortisol nor corticosterone differed between lean and obese participants. All data are mean ± SEM, exact P values are given in table S7.

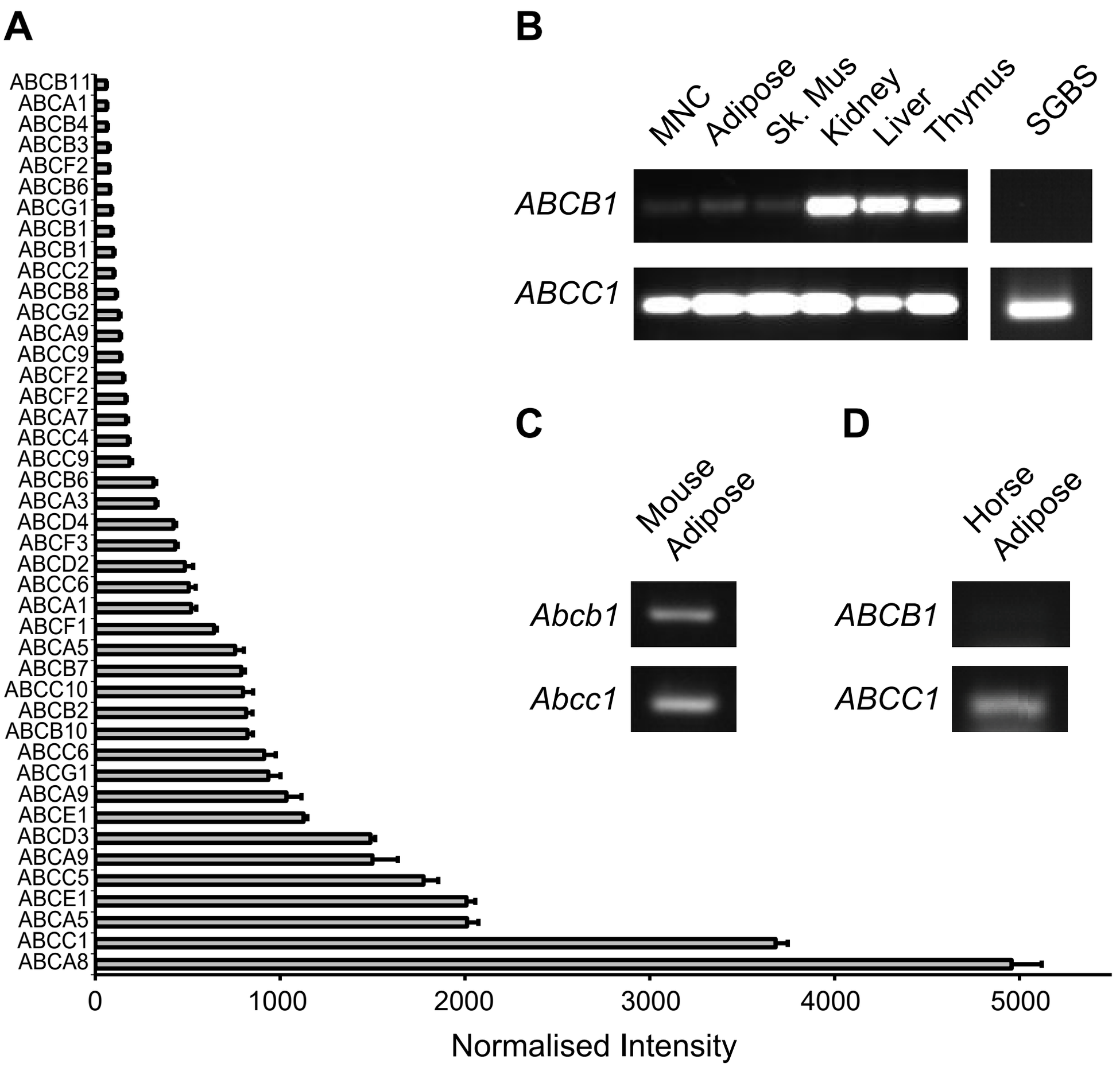
Fig. 6. Human adipose tissue is more sensitive to cortisol than corticosterone. A randomized single blind crossover study with ramped infusion of deuterated cortisol (D4-Cortisol) or deuterated corticosterone (D8-Corticosterone) was conducted in 9 patients with Addison's disease. Plasma concentrations of total cortisol and corticosterone **(A)** or ACTH **(B)** were not significantly different throughout the study. **(C)** Suppression of ACTH was similar during high-dose infusion of cortisol or corticosterone. **(D)** Cortisol induced a greater rise in *PER1* and *LPL*

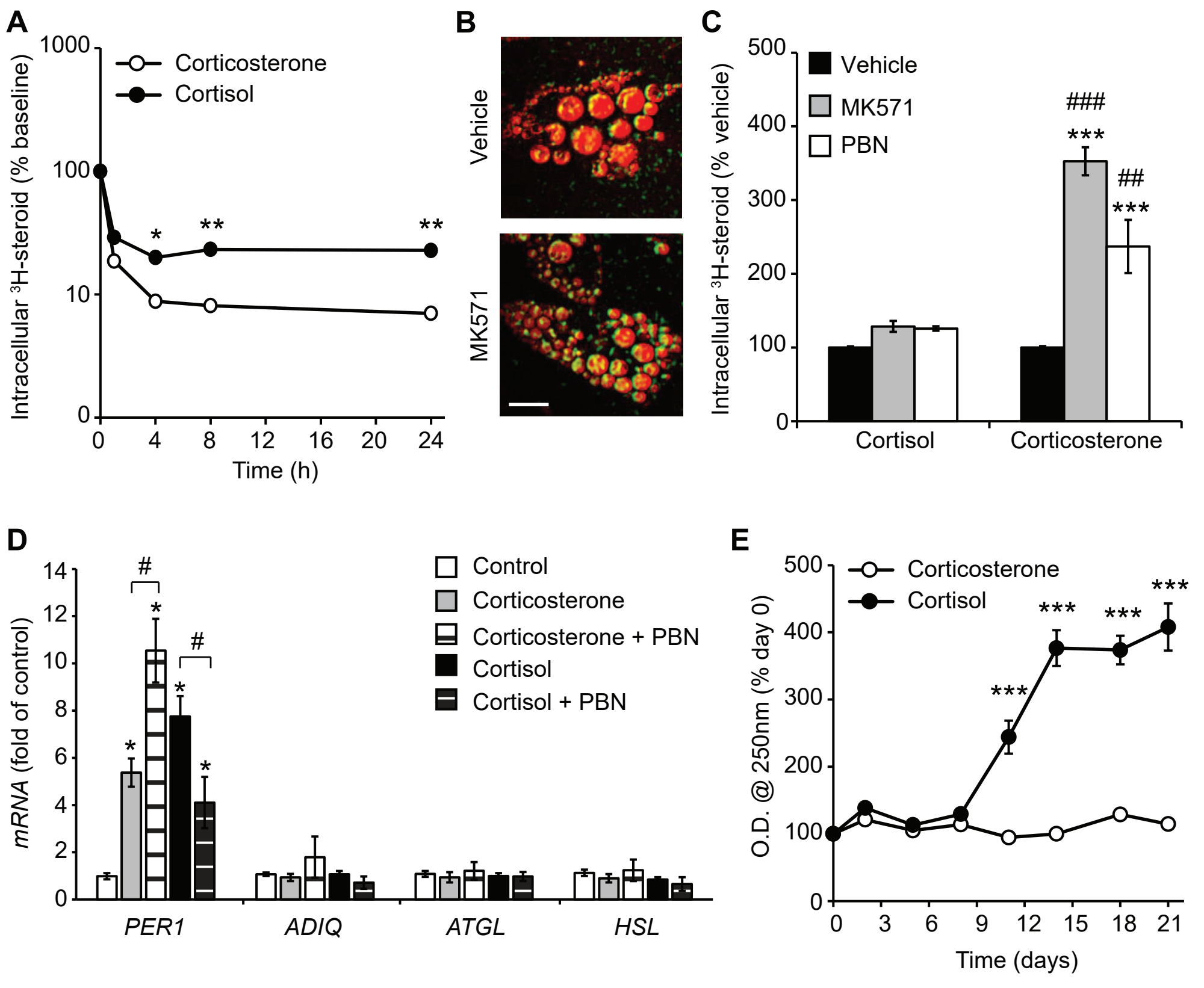
mRNA concentrations in subcutaneous adipose tissue (*P<0.05 vs corticosterone, paired Student's t-tests). All data are mean ± SEM, n=9, exact P values are given in table S7.

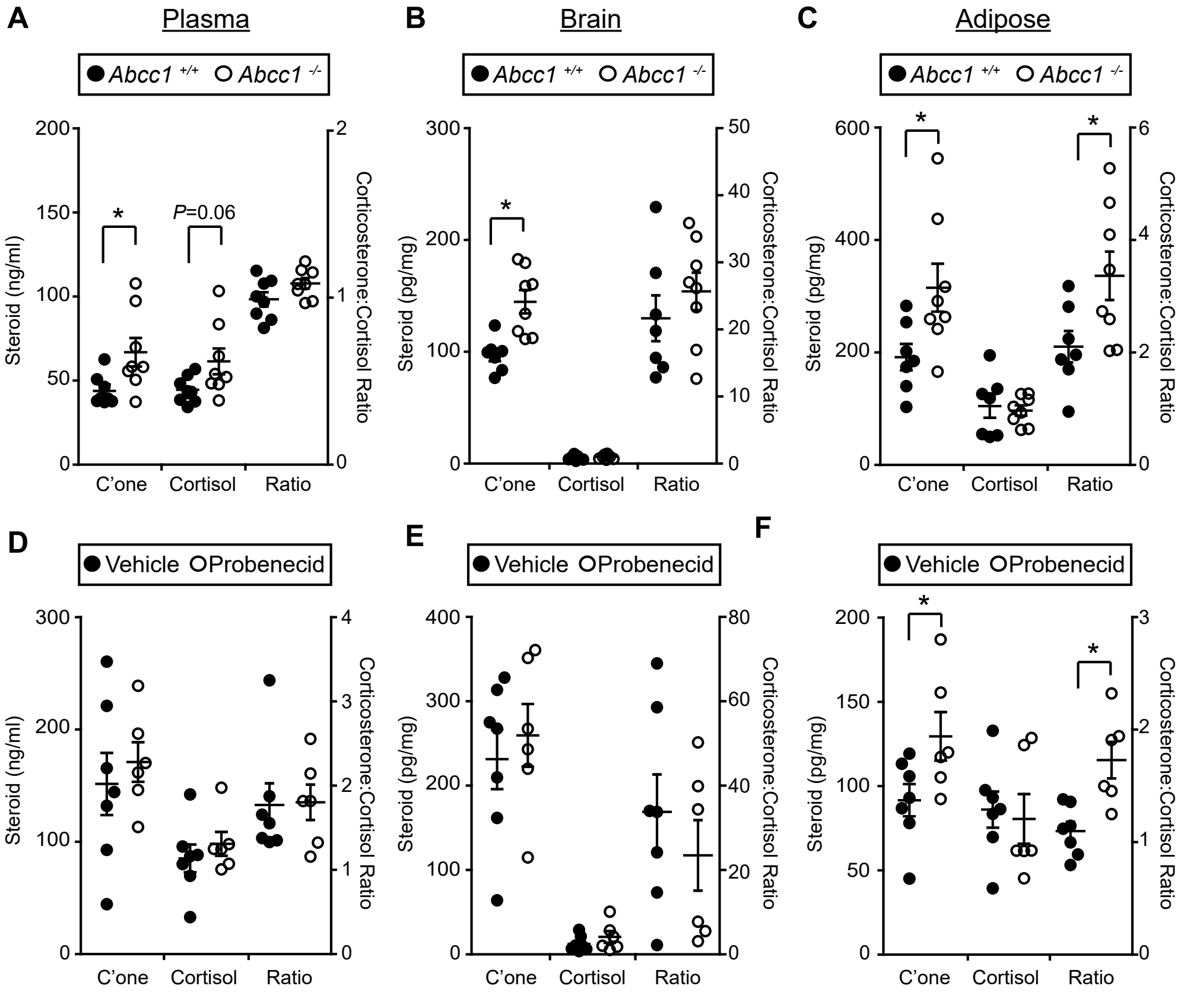
Fig. 7. Differential effects of cortisol and corticosterone in brain versus adipose are conferred by tissue-specific expression of ABC transporters. In conventional glucocorticoid replacement therapy, cortisol (hydrocortisone) action in the brain is limited by export through ABCB1, but the absence of ABCB1 in human adipose tissue allows potent effects of cortisol on peripheral metabolism. With corticosterone therapy, the lack of transport by ABCB1 in the brain allows corticosterone to exert a potent effect to suppress ACTH, but in adipose tissue, corticosterone action is limited by ABCC1, protecting against adverse effects of corticosterone on peripheral metabolism.

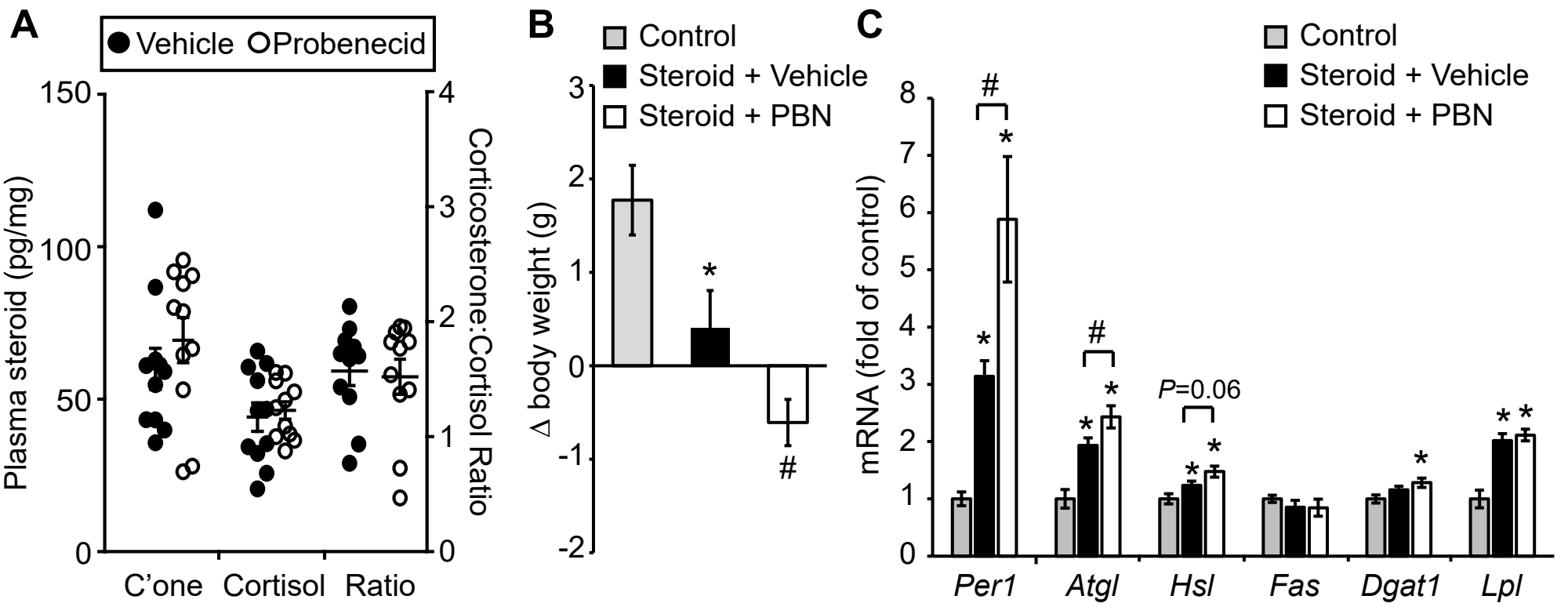
Table 1. Plasma biochemistry during infusion of either deuterated cortisol or corticosterone in patients with Addison's disease. Data are mean ± SEM of within-subject averages from samples obtained during each steady state infusion period. $N = 9$. Comparisons were by two-way repeated measures ANOVA: only ACTH ($P<0.001$), non-esterified fatty acids (NEFA, ($P=0.018$), and insulin ($P=0.042$) changed with duration of steroid infusion, but there were no differences between corticosterone or cortisol infusions and no significant interactions between steroid and duration.

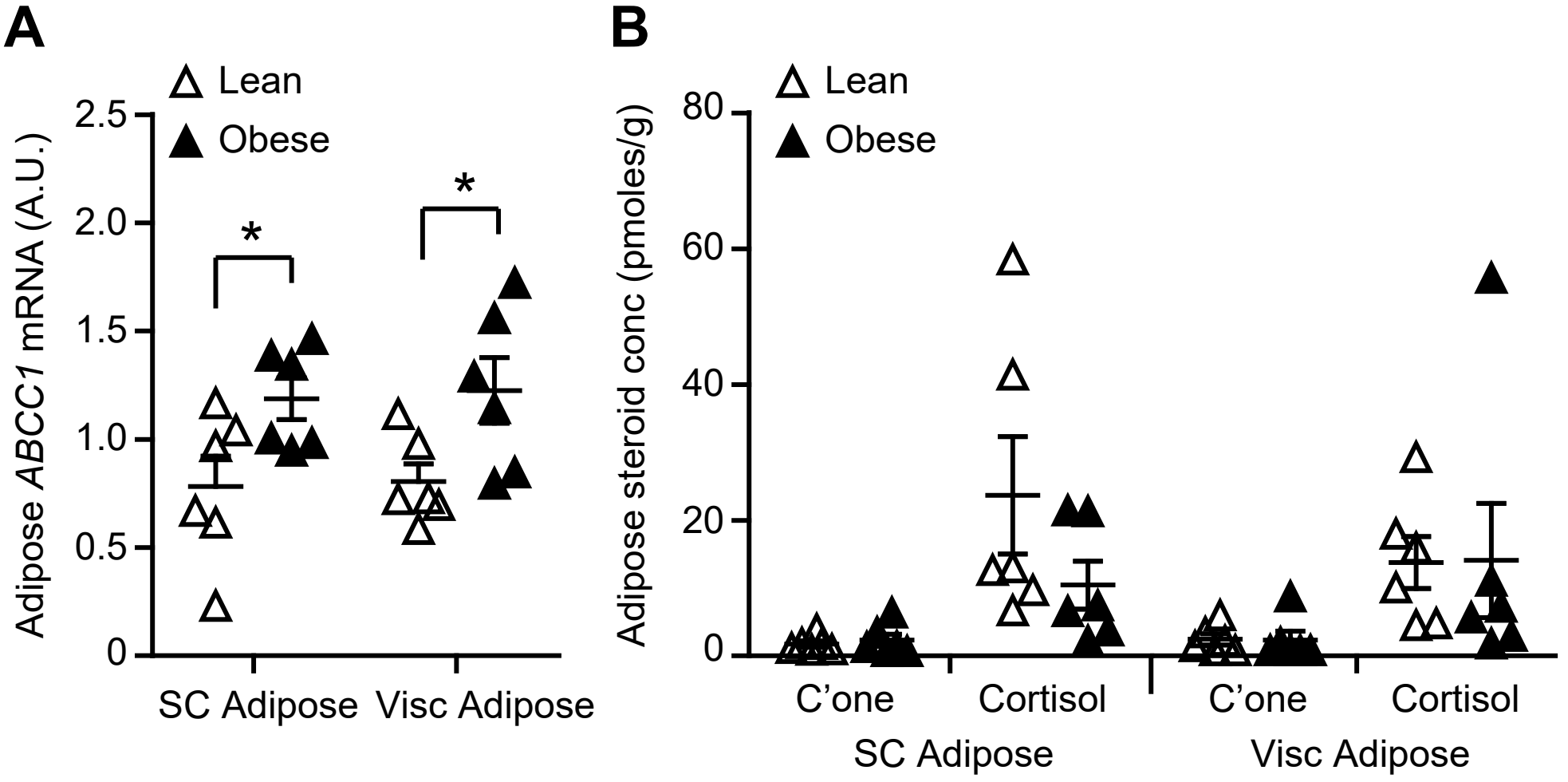
	Cortisol				**Corticosterone**			
Duration of infusion (min)	***0-60***	***80-140***	***160-240***	***260-330***	***0-60***	***80-140***	***160-240***	***260-330***
Total deuterated steroid (nM)	0	53 ± 11	190 ± 37	419 ± 74	0	34 ± 4	156 ± 15	340 ± 26
Endogenous cortisol (nM)	18 ± 4	12 ± 2	8 ± 2	5 ± 1	22 ± 7	16 ± 5	11 ± 3	7 ± 2
ACTH (pM)	42 ± 5	40 ± 5	37 ± 5	30 ± 5	42 ± 4	43 ± 4	38 ± 5	33 ± 5
Glucose (mM)	4.5 ± 0.1	4.3 ± 0.1	4.4 ± 0.2	4.6 ± 0.2	4.7 ± 0.2	4.8 ± 0.2	4.7 ± 0.2	4.8 ± 0.2
Insulin (pM)	44 ± 11	39 ± 7	38 ± 7	35 ± 6	43 ± 8	39 ± 6	37 ± 6	33 ± 5
NEFA (μM)	333 ± 54	415 ± 59	474 ± 63	477 ± 60	338 ± 47	391 ± 50	420 ± 66	412 ± 58

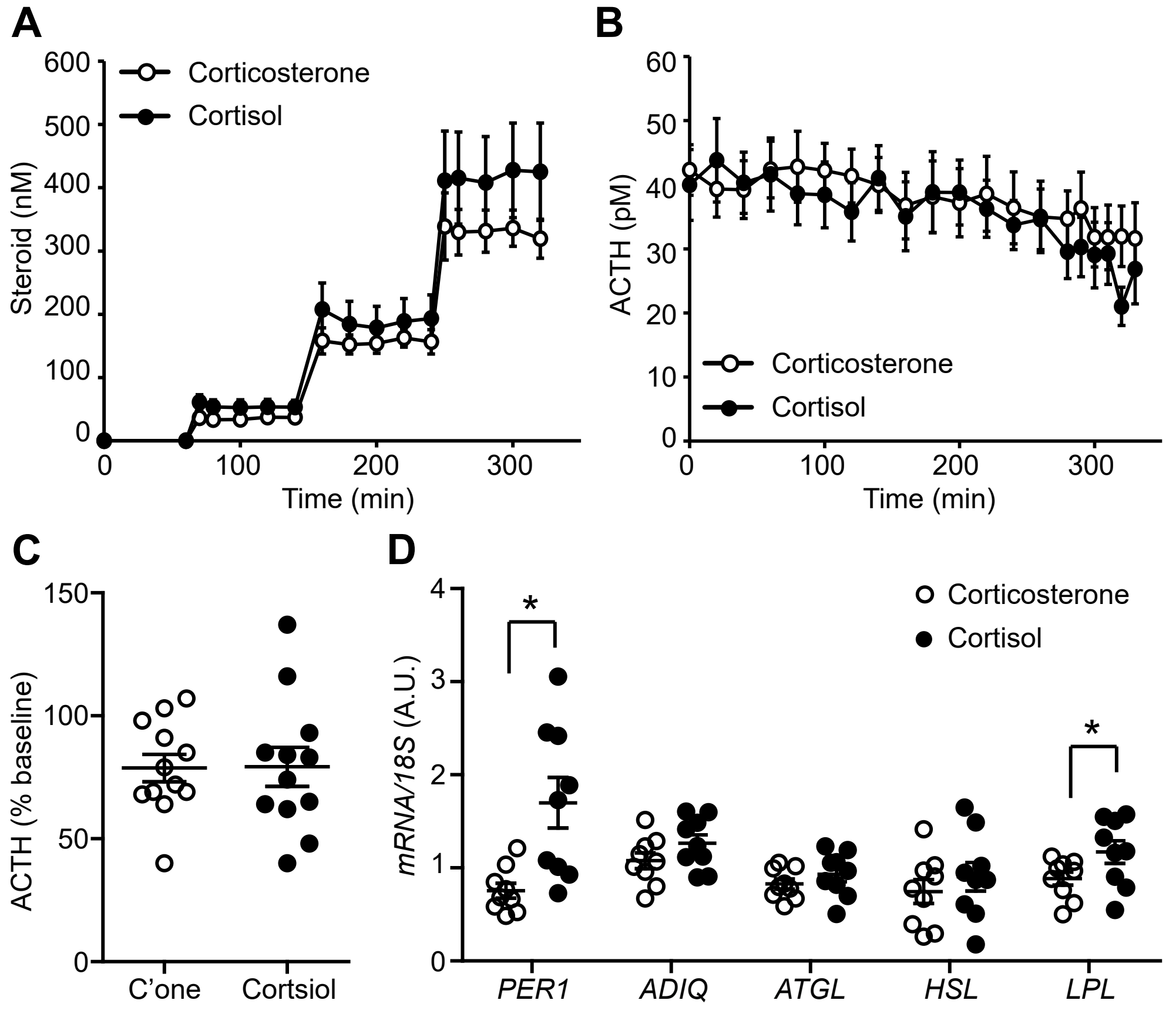

A
ABCB11
ABCA1
ABCB4
ABCB3
ABCF2
ABCB6
ABCG1
ABCB1
ABCB1
ABCC2
ABCB8
ABCG2
ABCA9
ABCC9
ABCF2
ABCF2
ABCA7
ABCC4
ABCC9
ABCB6
ABCA3
ABCD4
ABCF3
ABCD2
ABCC6
ABCA1
ABCF1
ABCA5
ABCB7
ABCC10
ABCB2
ABCB10
ABCC6
ABCG1
ABCA9
ABCE1
ABCD3
ABCA9
ABCC5
ABCE1
ABCA5
ABCC1
ABCA8
0
1000
2000
3000
4000
5000
Normalised Intensity
B
MNC
Adipose
Sk. Mus
Kidney
Liver
Thymus
SGBS
ABCB1
ABCC1
C
Mouse Adipose
Abcb1
Abcc1
D
Horse Adipose
ABCB1
ABCC1

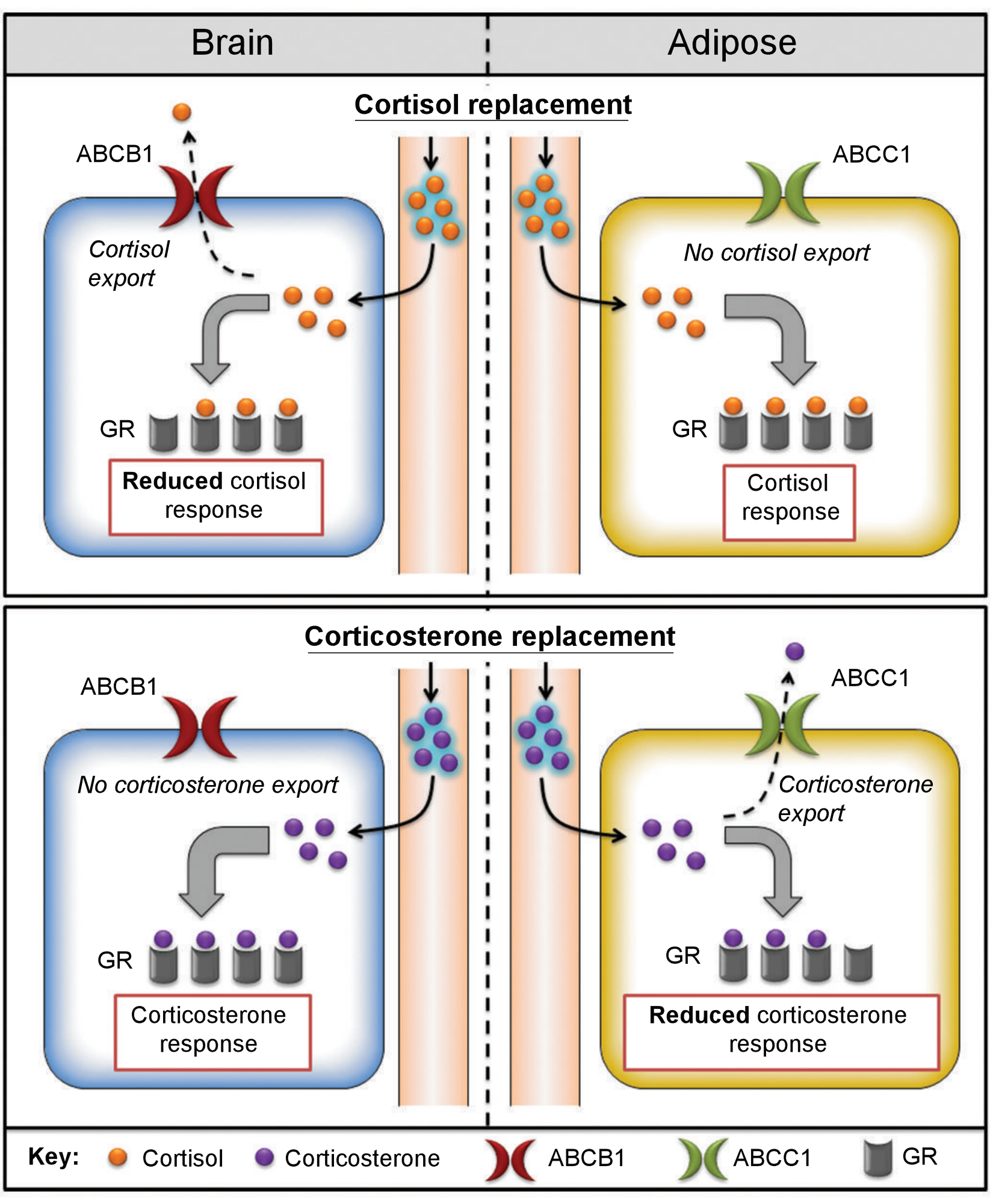

A
Intracellular ³H-steroid (% baseline)
1000
100
10
0
Corticosterone
Cortisol
*
**
**
0
4
8
12
16
20
24
Time (h)
B
Vehicle
MK571
C
Intracellular ³H-steroid (% vehicle)
500
400
300
200
100
0
Vehicle
MK571
PBN
###
***
##
***
Cortisol
Corticosterone
D
mRNA (fold of control)
14
12
10
8
6
4
2
0
#
*
#
*
*
*
Control
Corticosterone
Corticosterone + PBN
Cortisol
Cortisol + PBN
PER1
ADIQ
ATGL
HSL
E
O.D. @ 250nm (% day 0)
500
400
300
200
100
0
Corticosterone
Cortisol
***
***
***
***
0
3
6
9
12
15
18
21
Time (days)


A
Plasma
Abcc1 +/+
Abcc1 -/-
Steroid (ng/ml)
Corticosterone:Cortisol Ratio
*
P=0.06
C'one
Cortisol
Ratio
B
Brain
Steroid (pg/mg)
C
Adipose
D
Vehicle
Probenecid
E
F


A
Vehicle
Probenecid
Plasma steroid (pg/mg)
Corticosterone:Cortisol Ratio
150
100
50
0
4
3
2
1
0
C'one
Cortisol
Ratio
B
Control
Steroid + Vehicle
Steroid + PBN
Δ body weight (g)
3
2
1
0
-1
-2
*
#
C
Control
Steroid + Vehicle
Steroid + PBN
mRNA (fold of control)
8
7
6
5
4
3
2
1
0
#
*
P=0.06
Per1
Atgl
Hsl
Fas
Dgat1
Lpl


A
Lean
Obese
Adipose ABCC1 mRNA (A.U.)
2.5
2.0
1.5
1.0
0.5
0
*
*
SC Adipose
Visc Adipose
B
Lean
Obese
Adipose steroid conc (pmoles/g)
80
60
40
20
0
C'one
Cortisol
C'one
Cortisol
SC Adipose
Visc Adipose


A
Corticosterone
Cortisol
Steroid (nM)
600
500
400
300
200
100
0
0
100
200
300
Time (min)
B
ACTH (pM)
60
50
40
30
20
10
0
Corticosterone
Cortisol
0
100
200
300
Time (min)
C
ACTH (% baseline)
150
100
50
0
C'one
Cortsiol
D
mRNA/18S (A.U.)
4
3
2
1
0
*
*
Corticosterone
Cortisol
PER1
ADIQ
ATGL
HSL
LPL


Brain
Adipose
Cortisol replacement
ABCB1
Cortisol export
GR
Reduced cortisol response
ABCC1
No cortisol export
GR
Cortisol response
Corticosterone replacement
ABCB1
No corticosterone export
GR
Corticosterone response
ABCC1
Corticosterone export
GR
Reduced corticosterone response
Key:
Cortisol
Corticosterone
ABCB1
ABCC1
GR

Supplementary Materials:

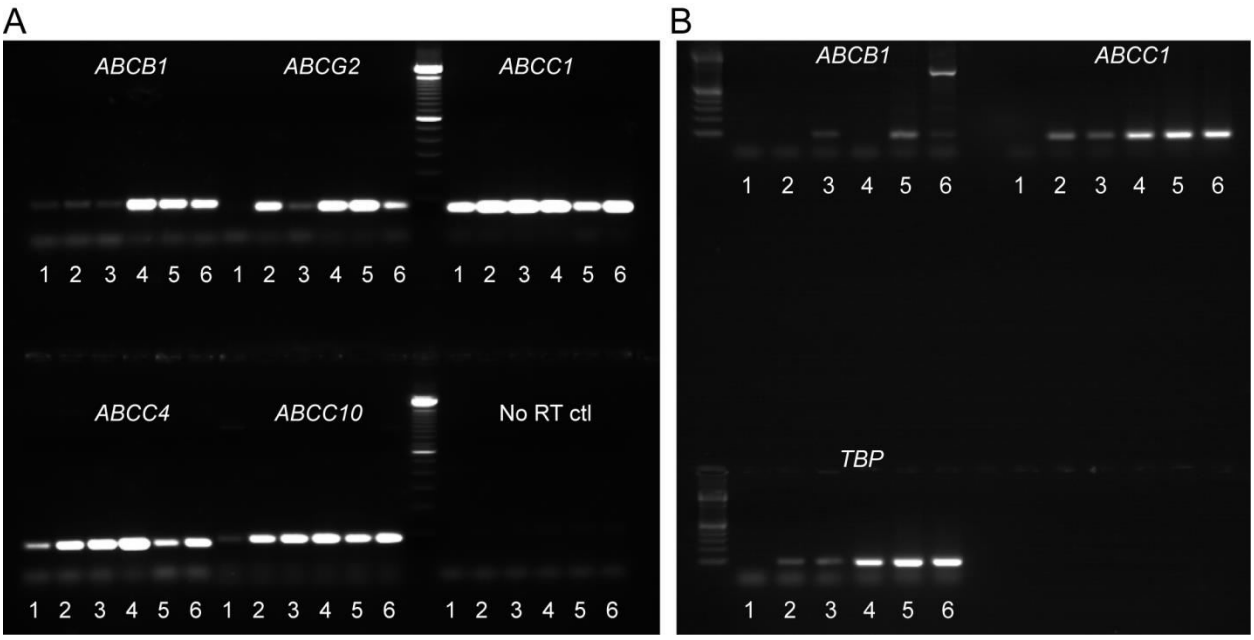


Fig. S1. Whole gel PCR image of ABC transporter expression in tissues and cells. **(A)** PCR gel of ABC transporter expression from human tissues. Lanes are denoted as follows: 1 = mononuclear cells, 2 = adipose, 3 = skeletal muscle, 4 = kidney, 5 = liver, 6 = thymus. Genes are indicated above bands. No RT ctl = negative control (without reverse transcriptase enzyme) for each sample. **(B)** Whole gel PCR image of *ABCB1, ABCC1,* and *TBP* in various cell lines. Lanes are denoted as follows: 1 = negative control, 2 = A549, 3 = HepG2, 4 = SGBS, 5 = HeLa, 6 = MCF-7.

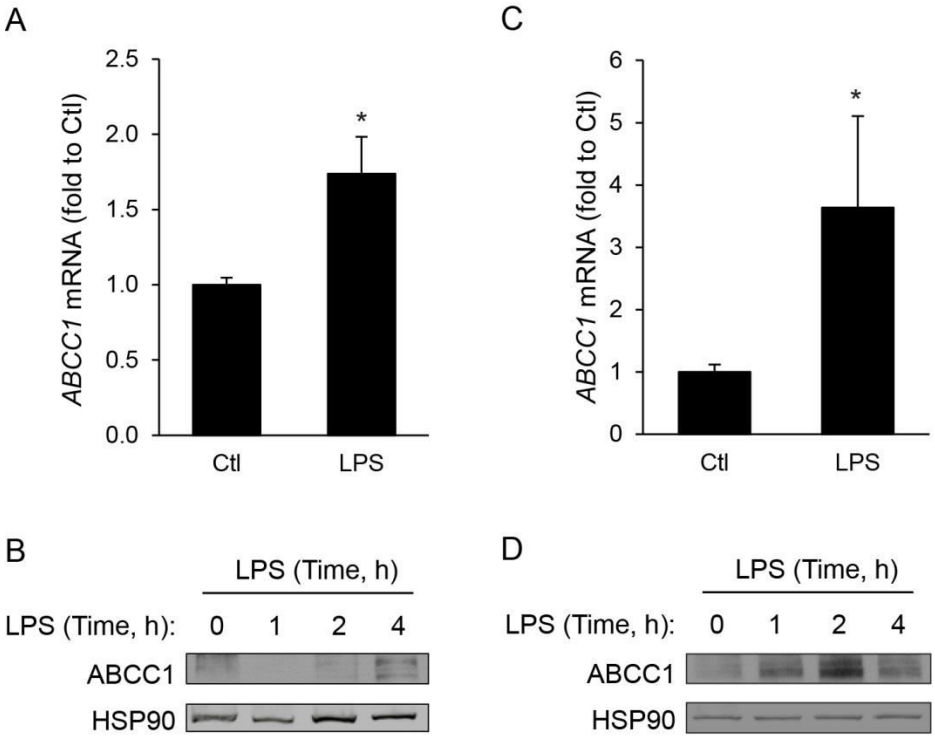


Fig. S2. Correlation of *ABCC1* mRNA and protein concentrations in human adipocytes. (**A** and **B**) SGBS adipocytes and (**C** and **D**) primary human adipocytes treated with lipopolysaccharide (100 ng/ml) for the indicated times. mRNA concentrations after 2 hours of treatment (**A** and **C**) correlate with protein concentrations (**B** and **D**). Data for mRNA are mean ± SEM, $n = 3$, * $P<0.05$ vs control (Ctl).

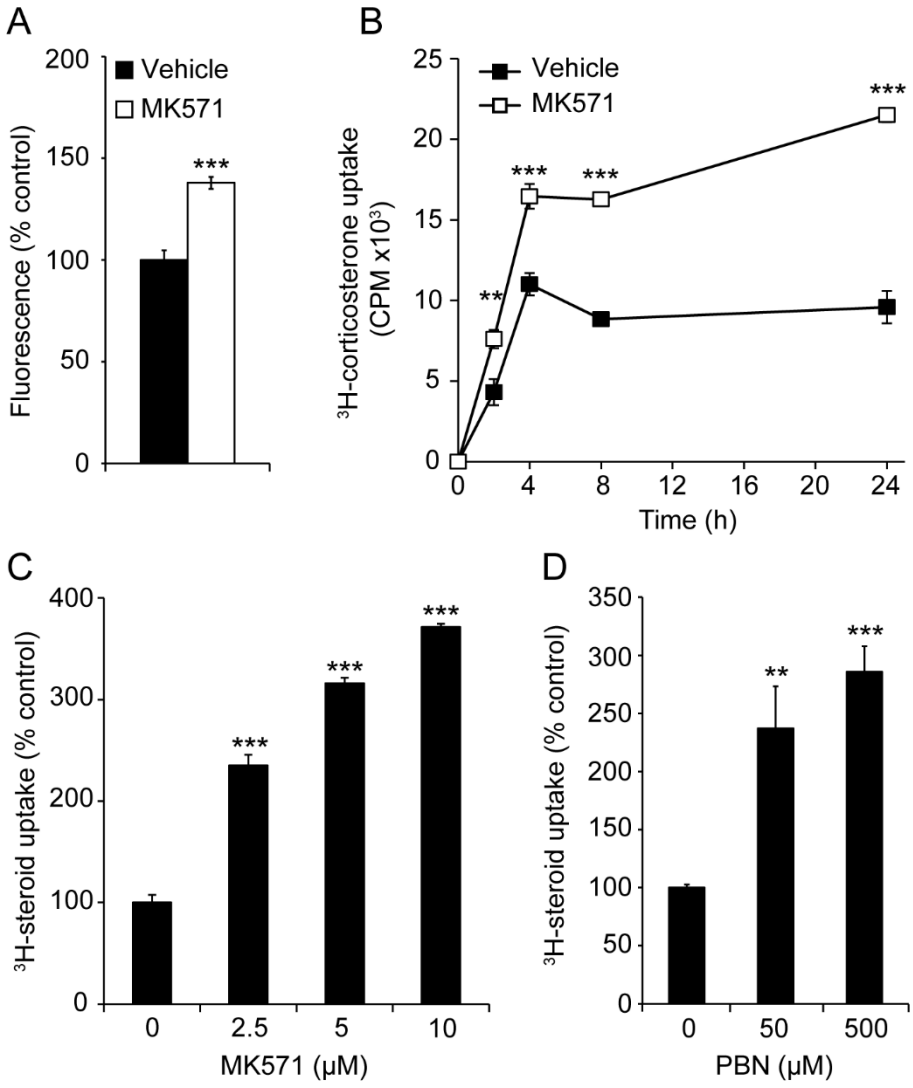


Fig. S3. ABCC1 inhibition in SGBS adipocytes. **(A)** SGBS adipocytes incubated with Calcein-AM for 90 min demonstrate the extent of ABCC1 inhibition by MK571 (10 μM; $n = 6$, Student's t-test: ***P<0.001 vs control). **(B)** Time course of ^{3}H-corticosterone accumulation with ABCC1 inhibition (MK571, 10 μM; $n = 3$, two-way ANOVA with Bonferroni post-hoc test: **P<0.01, ***P<0.001). Dose response of ^{3}H-corticosterone accumulation over 24 hours after ABCC1

inhibition with various concentrations of **(C)** MK571, or **(D)** probenecid (PBN; $n = 3$, one-way ANOVA: $^{**}P<0.01$, $^{***}P<0.001$ vs 0). All data are mean ± SEM.

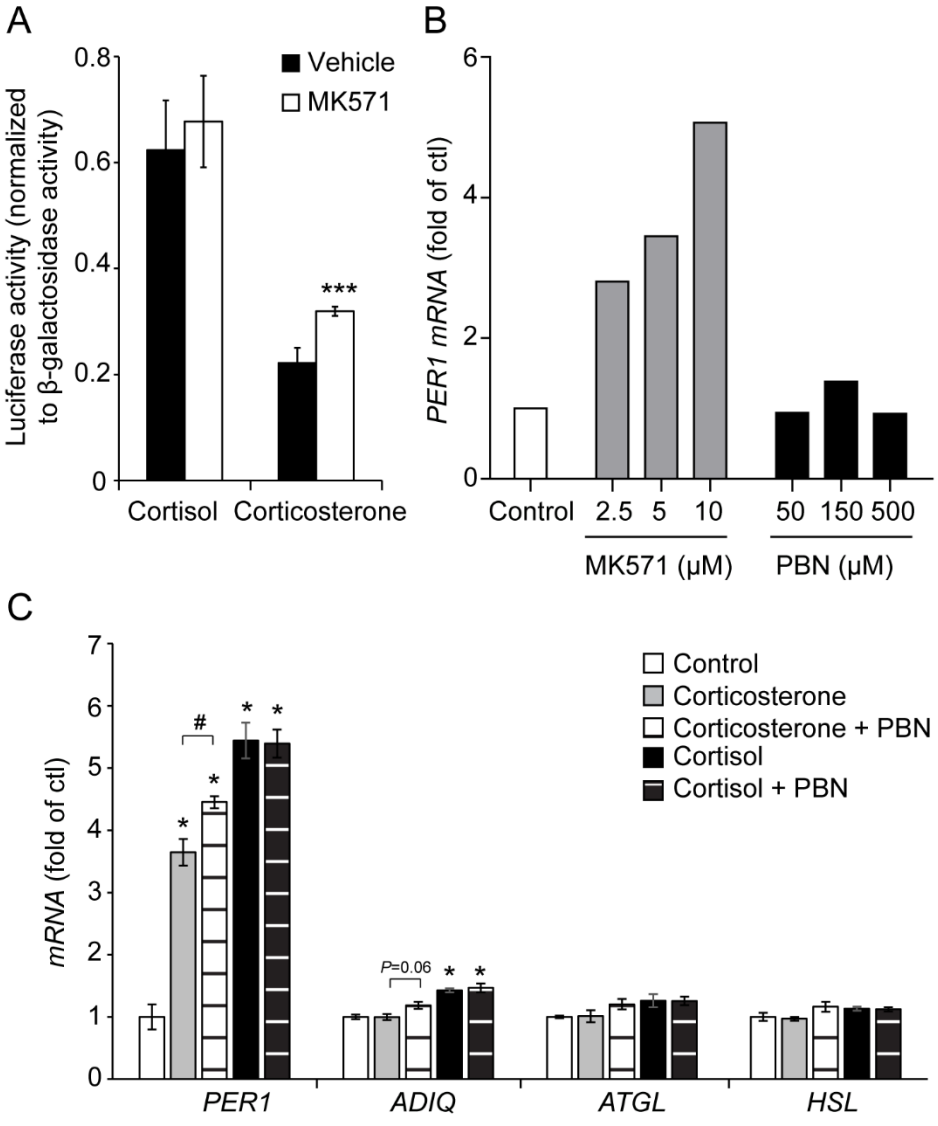


Fig. S4. Effects of ABCC1 inhibition on GR-mediated transcription. **(A)** A549 cells transfected with glucocorticoid-responsive MMTV-luciferase reporter and treated with corticosterone or cortisol (500 nM, 24 hours) show potentiation of response to corticosterone but not cortisol by ABCC1 inhibition (MK571, 10 μM; n = 3, two-way ANOVA with Bonferroni post-hoc test: ***P<0.001 vs vehicle). **(B)** Glucocorticoid-responsive period circadian clock 1 (*PER1*) gene expression in SGBS adipocytes treated with MK571 or probenecid (PBN) for 24 hours, n = 2.

(C) Glucocorticoid-responsive gene expression in SGBS adipocytes treated with corticosterone or cortisol (500 nM) in the presence of absence of probenecid (PBN, 50 μM) for 24 hours. Glucocorticoid-responsive genes are *PER1*, adiponectin (*ADIQ*), adipose triglyceride lipase (*ATGL*), and hormone sensitive lipase (*HSL*), (n = 3, Student's t-test: *P<0.05 vs control; #P<0.05 vs PBN). All data are mean ± SEM.

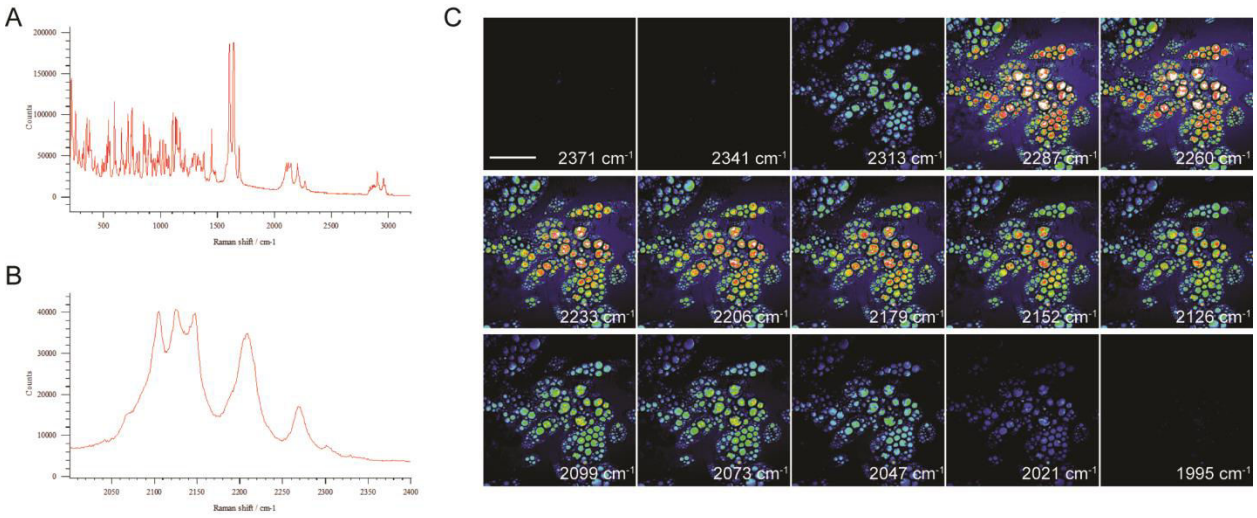


Fig. S5. Optimization of CARS microscopy for detection of intracellular D8-corticosterone. **(A)** Raman spectra of D8-corticosterone showing peaks representing C-D bonds between 2000 and 2300 cm^{-1}. **(B)** Magnified Raman spectrum of D8-corticosterone between 2000 and 2400 cm^{-1}. **(C)** Coherent Anti-stokes Raman Scattering (CARS) images of SGBS adipocytes treated with D8-corticosterone (10 µM, 24 hours) probed at inverse wavenumbers corresponding to the Raman spectrum and showing optimal detection at 2126 cm^{-1} (scale bar = 100 µm).

46 **Table S1.** Characteristics of lean and obese study participants providing adipose biopsy samples

47 during surgery.

48

	Lean	Obese
Age (years)	60 ± 4	59 ± 3
Gender (M/F)	2/4	2/4
Body mass index (kg/m^2)	23.9 ± 0.3	41.4 ± 1.9

49

Table S2. Characteristics of Addison's study participants.

	Mean ± SEM	Range
Age (years)	53 ± 5	20 - 65
Gender (M/F)	3/6	
Body mass index (kg/m^2)	24.7 ± 1.2	21.7 - 32.9
Years since diagnosis	19 ± 3	4 - 30
Daily hydrocortisone dose (mg)	21 ± 2	15 - 30
Daily fludrocortisone dose (µg)	76 ± 30	0 - 300

Table S3. Summary of studies describing plasma corticosterone and cortisol concentrations in healthy subjects.

Reference	Gender (M/F)	Age (years)	Corticosterone (B) nM	Cortisol (F) nM	B/F‡	n	Time	B assay Technique
Sweat, 1955 *(23)*	ns	ns	124.1 ± 14.4	298.0 ± 16.6	0.416	21	ns	Fluorometric method
Peterson, 1957 *(24)*	18/12	ns	31.7 ± 11.5	386.2 ± 33.1	0.082	30	ns	Isotope dilution/fluorescence
Ely et al, 1958 *(25)*	ns	ns	86.6 ± 5.8	300.7 ± 15.1	0.288	20	ns	Fluorometric method
Fraser et al, 1968 *(26)*	ns	ns	19.0 (3.8-66.4)	270.3 (85.5-557)	0.067	29	ns	Double isotope assay
Huther et al, 1970 *(27)*	M F	26.8 ± 2.6 31.3 ± 4.4	47.2 ± 6.0 49.7 ± 6.5	403.0 ± 37.5 422.4 ± 67.8	0.124 0.118	9† 10†	1045-1145*	Fluorometric method
Dluhy et al, 1972 *(28)*	8/2	21-34	26.3 ± 3.2	634.5 ± 55.2	0.041	10	0900	Radioimmunoassay
Newsome, Jr. et al 1972 *(29)*	ns	ns	11.5 ± 0.9	339.3 ± 22.1	0.034	8	ns	Competitive protein binding
Oddie et al, 1972 *(30)*	ns	ns	12.1 ± 2.6	383.4 ± 4.0	0.030	18	0900	Double isotope assay
West et al, 1973 *(31)*	M F[1] F[2]	19-50	11.4 ± 1.7 20.4 ± 2.0 16.7 ± 2.1	386 ± 36 386 ± 36 359 ± 21	0.030 0.053 0.047	15 9 9	0800	Radioimmunoassay
Nishida et al, 1977 *(32)*	ns	ns	23.0 ± 3.0	419.6 ± 28.7	0.055	10	0900	Radioimmunoassay
Karssen et al, 2001 *(10)*	M	57 ± 1.9	16.4 ± 3.1	350.0 ± 81.5	0.047	11	ns	LC-MS
Raubenheimer et al, 2006 *(13)*	M	39.2 (23-70)	58.4 ± 9.2	830.4 ± 68.4	0.069	16	0830-0900	Radioimmunoassay

Mean ± SEM or range (). ns = not specified

† = mean of 5 samples for each subject

* = approximate time inferred from clinical protocol

[1] follicular phase, [2] luteal phase

‡ mean [B] (nM)/mean [F] (nM)

Table S4. Primer sequences for PCR and corresponding expected product size.

Gene ID	Primer sequence 5' to 3'	Expected product size (bp)
ABCC1 Human	F: gcctattaccccagcatcg	69
	R: gatgcagttgcccacaca	
ABCB1 Human	F: aaggcatttacttcaaacttgtca	78
	R: tggattcatcagctgcatttt	
ABCG2 Human	F: tggcttagactcaagcacagc	67
	R: tcgtccctgcttagacatcc	
ABCC4 Human	F: cctggcgaattgttagctgt	68
	R: agcacggcacttaacagtga	
ABCC10 Human	F: agctcactgccaccaagg	76
	R: caagggaagttgttgagagga	
Abcc1 Mouse	F: ggaattttcggctgagtgtc	63
	R: agccaaatattgctgcacct	
Abcb1 Mouse	F: tgctttgtgggcaaaggta	106
	R: cacagttctgatggctgctaa	
ABCB1 Horse	F: tcaggtggccctggataa	157
	R: cgaactgtagacaagcgatga	
ABCC1 Horse	F: caaaatcatggctgccttaaa	89
	R: gaaagtgacatcgcgaaaca	

63 **Table S5.** Human primer sequences for qPCR and corresponding probe number from Roche

64 Universal Probe Library (UPL).

Gene ID	Primer sequence 5' to 3'	Roche UPL Probe Number
ABCC1 Human	F: gcctattaccccagcatcg	28
	R: gatgcagttgcccacaca	
ABCB1 Human	F: aaggcatttacttcaaacttgtca	18
	R: tggattcatcagctgcatttt	
PER1 Human	F: ctcttccacagctccctca	87
	R: ctttggatcggcagtggt	
LPL Human	F: atgtggcccggtttatca	25
	R: ctgtatcccaagagatggacatt	
ADIPONECTIN Human	F: ggtgagaagggtgagaaagga	85
	R: tttcaccgatgtctcccttag	
ATGL Human	F: ctccaccaacatccacgag	89
	R: ccctgcttgcacatctctc	
18S Human	F: cttccacaggaggcctacac	46
	R: cgcaaaatatgctggaacttt	

Table S6. Murine primer sequences for qPCR and corresponding probe number from Roche Universal Probe Library (UPL).

Gene ID	Primer sequence 5' to 3'	Roche UPL Probe Number
Abcc1 Murine	F: ggaattttcggctgagtgtc	105
	R: agccaaatattgctgcacct	
Abcb1 Murine	F: tgctttgtgggcaaaggta	78
	R: cacagttctgatggctgctaa	
Per1 Murine	F: gcttcgtggacttgacacct	71
	R: tgctttagatcggcagtggt	
Lpl Murine	F: ctcgctctcagatgccctac	95
	R: ggttgtgttgcttgccatt	
Atgl Murine	F: gagcttcgcgtcaccaac	89
	R: cacatctctcggaggacca	
Hsl Murine	F: gcgctggaggagtgttttt	3
	R: ccgctctccagttgaacc	
Fas Murine	F: ccaaatccaacatgggaca	34
	R: tgctccagggataacagca	
Dgat1 Murine	F: gcttctgcagtttggagacc	31
	R: tccagttctgccaaaagtaggt	
Tbp Murine	F: gggagaatcatggaccagaa	97
	R: gatgggaattccaggagtca	
18S Murine	F: ctcaacacgggaaacctcac	77
	R: cgctccaccaactaagaacg	