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# Recycling Between Cortisol and Cortisone in Human Splanchnic, Subcutaneous Adipose, and Skeletal Muscle Tissues In Vivo

Katherine A. Hughes,<sup>1</sup> Konstantinos N. Manolopoulos,<sup>2</sup> Javaid Iqbal,<sup>1</sup> Nicholas L. Cruden,<sup>1</sup> Roland H. Stimson,<sup>1</sup> Rebecca M. Reynolds,<sup>1</sup> David E. Newby,<sup>1</sup> Ruth Andrew,<sup>1</sup> Fredrik Karpe,<sup>2</sup> and Brian R. Walker<sup>1</sup>

11 $\beta$ -Hydroxysteroid dehydrogenase type 1 (11 $\beta$ HSD1) is a therapeutic target in metabolic syndrome because it catalyses reductase regeneration of cortisol from cortisone in adipose and liver. 11 $\beta$ HSD1 can also catalyze the reverse dehydrogenase reaction in vitro (e.g., if cofactor is limited). We used stable isotope tracers to test the hypothesis that both 11 $\beta$ HSD1-reductase and -dehydrogenase activities occur in human metabolic tissues in vivo. 1,2-[<sup>2</sup>H]<sub>2</sub>-Cortisone (d2-cortisone) was validated as a tracer for 11 $\beta$ -dehydrogenase activity and its inhibition by licorice. d2-Cortisone and 9,11,12,12-[<sup>2</sup>H]<sub>4</sub>-cortisol (d4-cortisol) (to measure 11 $\beta$ -reductase activity) were coinjected and venous samples obtained from skeletal muscle, subcutaneous adipose ( $n = 6$ ), and liver ( $n = 4$ ). Steroids were measured by liquid chromatography-tandem mass spectrometry and arteriovenous differences adjusted for blood flow. Data are means  $\pm$  SEM. 11 $\beta$ -Reductase and -dehydrogenase activities were detected in muscle (cortisol release  $19.7 \pm 4.1$  pmol/100 mL/min, d3-cortisol  $5.9 \pm 1.8$  pmol/100 mL/min, and cortisone  $15.2 \pm 5.8$  pmol/100 mL/min) and splanchnic (cortisol  $64.0 \pm 11.4$  nmol/min, d3-cortisol  $12.9 \pm 2.1$  nmol/min, and cortisone  $19.5 \pm 2.8$  nmol/min) circulations. In adipose, dehydrogenase was more readily detected than reductase (cortisone release  $38.7 \pm 5.8$  pmol/100 g/min). Active recycling between cortisol and cortisone in metabolic tissues in vivo may facilitate dynamic control of intracellular cortisol but makes consequences of dysregulation of 11 $\beta$ HSD1 transcription in obesity and diabetes unpredictable. Disappointing efficacy of 11 $\beta$ HSD1 inhibitors in phase II studies could be explained by lack of selectivity for 11 $\beta$ -reductase. *Diabetes* 61:1357–1364, 2012

**G**lucocorticoids are key regulators of fuel metabolism. In recent years, it has become clear that in addition to tight control of circulating cortisol by the hypothalamic-pituitary-adrenal axis, intracellular cortisol levels are controlled by local metabolism (1). For example, recent studies using a stable isotope tracer, 9,11,12,12-[<sup>2</sup>H]<sub>4</sub>-cortisol (d4-cortisol) (2), have demonstrated that in vivo hepatic and subcutaneous adipose 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ HSD1) regenerates cortisol from inert cortisone, releasing  $\sim 900$  and  $15$  pmol of cortisol/100 g tissue/min, respectively (3,4). This 11 $\beta$ HSD1-reductase activity is driven

by NADPH cofactor derived from hexose-6-phosphate dehydrogenase (H6PDH), colocalized with 11 $\beta$ HSD1 in the endoplasmic reticulum lumen (5). Altered cortisol generation in tissues such as adipose and liver has been implicated in the metabolic complications of obesity (6,7), and 11 $\beta$ HSD1 inhibition is a potential therapeutic target to lower intracellular cortisol levels in type 2 diabetes (8,9). However, phase 2 trials with small-molecule inhibitors have delivered mixed results (10,11).

One factor that may contribute to unpredictable efficacy of 11 $\beta$ HSD1 inhibitors is the potential reversibility of reactions catalyzed by 11 $\beta$ HSD1. In states of NADPH deficiency, 11 $\beta$ HSD1 can switch direction and exhibit predominant dehydrogenase activity, e.g., when 11 $\beta$ HSD1 is liberated from the intracellular environment or when H6PDH is disrupted by gene targeting in mice (12). Adipose 11 $\beta$ -dehydrogenase activity, inactivating cortisol to cortisone, has been demonstrated in primary cell cultures and during intra-adipose microdialysis in vivo (13,14). As adipose 11 $\beta$ HSD2 expression is negligible and 11 $\beta$ HSD1 expression is abundant (15), this dehydrogenase activity may be attributed to 11 $\beta$ HSD1 and be metabolically protective (16). However, whether it is of sufficient magnitude to influence intracellular cortisol concentrations has not been determined.

11 $\beta$ HSD1 is also expressed in liver (3) and skeletal muscle (17)—both important sites of insulin resistance in type 2 diabetes. d4-Cortisol infusion confirms the liver as a major source of extra-adrenal cortisol production by 11 $\beta$ -reductase (3,4,18). A decline in cortisone concentration between arterial and portal vein samples (4) suggests that there is also 11 $\beta$ -dehydrogenase in the splanchnic circulation. This is most likely attributable to 11 $\beta$ HSD2 in the gut; whether there is 11 $\beta$ -dehydrogenase activity in the liver is unknown. In rodents, skeletal muscle 11 $\beta$ HSD1 activity is low compared with adipose and liver (19), while in humans evidence for in vivo skeletal muscle 11 $\beta$ HSD1 activity is equivocal, as no gradients in cortisol or cortisone concentrations were found across forearm muscle (20) but a small amount of reductase activity was detected in the leg using d4-cortisol (18,21). Ex vivo studies have demonstrated skeletal muscle 11 $\beta$ HSD1-reductase activity and a small amount of 11 $\beta$ HSD1-dehydrogenase activity in tissue homogenates (22). Since 11 $\beta$ HSD1 expression in skeletal muscle is higher in diabetes (23), it is important to clarify its function.

d4-Cortisol allows quantification of 11 $\beta$ -reductase because the deuterium in the 11 $\alpha$  position is removed by 11 $\beta$ -dehydrogenase to form d3-cortisone, which in turn is regenerated to d3-cortisol by incorporation of a proton;

From the <sup>1</sup>University of Edinburgh/British Heart Foundation Centre for Cardiovascular Science, Queen's Medical Research Institute, Edinburgh, U.K.; and the <sup>2</sup>Nuffield Department of Clinical Medicine, Oxford Centre for Diabetes, Endocrinology and Metabolism, University of Oxford, Oxford, U.K. Corresponding author: Katherine A. Hughes, katherine.hughes@ed.ac.uk. Received 24 September 2011 and accepted 4 February 2012.

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dilution of d4-cortisol by d3-cortisol exclusively reflects 11 $\beta$ -reductase activity (2). It is not possible to quantify 11 $\beta$ -dehydrogenase during steady-state infusion of d4-cortisol because the rate of appearance ( $R_a$ ) of d3-cortisone cannot be quantified without infusing a tracer into the cortisone pool. For this purpose, we have developed a novel tracer method with 1,2- $^2\text{H}$ -cortisone (d2-cortisone) and used it in combination with d4-cortisol to measure simultaneous 11 $\beta$ -dehydrogenase and reductase activities in vivo. We used these isotopes in conjunction with arteriovenous sampling to test the hypothesis that there is interconversion of cortisol and cortisone in subcutaneous adipose, splanchnic, and skeletal muscle tissues.

## RESEARCH DESIGN AND METHODS

**Chemicals and reagents.** Reagents were obtained from Sigma (Poole, U.K.), Steraloids (Newport, RI), or VWR (Lutterworth, U.K.) unless otherwise specified. Solvents were high-performance liquid chromatography grade from Fisher Scientific (Loughborough, U.K.). d2-Cortisone, 1,2- $^2\text{H}$ -cortisol (d2-cortisol), and d4-cortisol were obtained from Cambridge Isotope Laboratories (Andover, MA), and unlabeled cortisol was obtained from Calbiochem (Nottingham, U.K.), indocyanine green (ICG) from Pulsion Medical (Middlesex, U.K.), and 1,2- $^3\text{H}$ -cortisone from GE Healthcare UK (Little Chalfont, U.K.).

**In vitro validation of d2-cortisone tracer.** To establish that d2-cortisone is a substrate for human 11 $\beta$ HSD1, human embryonic kidney (HEK)293 cells ( $2 \times 10^5$ ) stably transfected with human 11 $\beta$ HSD1 (24) were incubated (1–24 h,  $n = 6$ ) with d2-cortisone or cortisone (2  $\mu\text{mol/L}$ ). Steroids were extracted and analyzed by liquid chromatography–tandem mass spectrometry.

For assessment of any primary isotope effect, HEK293/11 $\beta$ HSD1 cells ( $2 \times 10^5$ ) were incubated (6–24 h,  $n = 6$ ) with  $^3\text{H}$ -cortisone (5 nmol/L) and cortisone or d2-cortisone (0–4,955 nmol/L). Media was extracted and  $^3\text{H}$ -steroids

quantified by HPLC with online  $\beta$ -scintillation counting (Berthold LB509 detector; Berthold Technologies, Harpenden, U.K.).

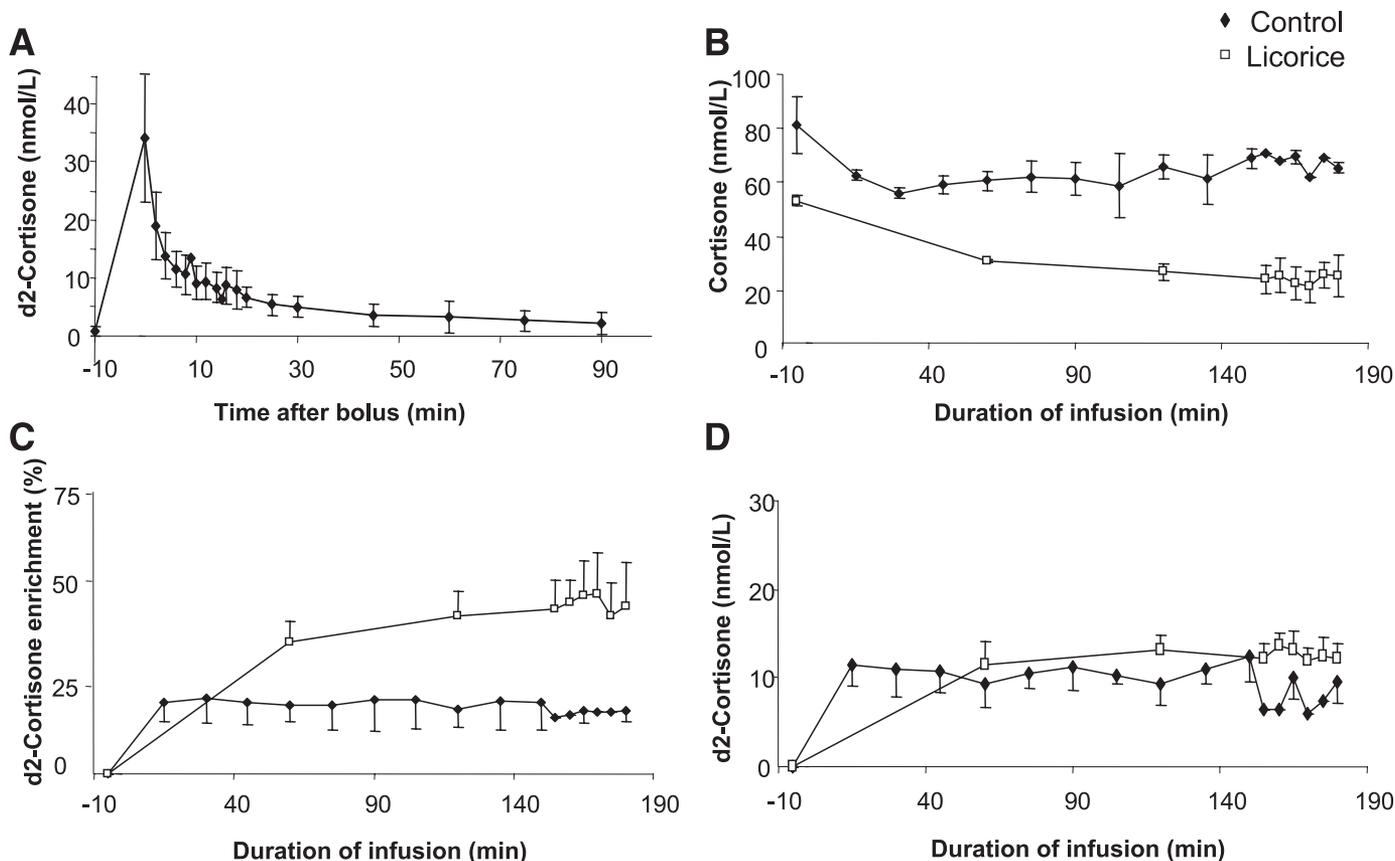
To compare metabolism by other enzymes, human liver cytosol (25.21 mg/mL protein) was incubated ( $n = 6$ , 37°C, 8 h) with  $^3\text{H}$ -cortisone (10 nmol/L) and either cortisone or d2-cortisone (10  $\mu\text{mol/L}$ ), in the presence of a cofactor-generating system (2 mmol/L NADPH), glucose-6-phosphate (5 mmol/L), and glucose-6-phosphate dehydrogenase (0.5 units/250  $\mu\text{L}$ ). The reaction was terminated with ethyl acetate (10 vol), and the solvent extracts were analyzed by HPLC, with products identified by comparison of retention times with unlabeled standards detected by absorbance at  $\lambda$  244 nm for cortisone and 195 nm for reduced metabolites.

**Clinical protocols.** All studies were approved by the local research ethics committee, and written informed consent was obtained. Participants had normal blood indices (hemoglobin, renal function, and glucose) and had not received glucocorticoid treatment by any route for 3 months prior to the studies.

**In vivo pharmacokinetics of d2-cortisone.** For calculation of pharmacokinetics and estimation of a steady-state infusion rate, three healthy lean men received an intravenous bolus of d2-cortisone (141  $\mu\text{g}$  in 0.9% saline, wt/vol 50 mL) over 5 min with venous blood sampled at intervals for 90 min from the contralateral arm.

For measurement of endogenous cortisone production rate at steady state and for testing the effect of inhibition of 11 $\beta$ -dehydrogenase with licorice, three healthy lean men attended on two occasions, once before and a second time after eating 200 g black licorice (Panda licorice chews, 3.8% licorice extract; Panda, Vaajakoski, Finland) daily for 2 days. A priming dose (76  $\mu\text{g}$  in 0.9% saline, wt/vol) and a 3-h infusion of d2-cortisone (105.3  $\mu\text{g/h}$ , 0.9% saline, wt/vol) were administered. Venous blood was sampled at intervals (Fig. 1).

**Cortisol/cortisone interconversion in subcutaneous adipose and skeletal muscle.** Six healthy lean men attended after an overnight fast in a quiet temperature-controlled (24°C) room. A cannula (18G) was placed anterogradely into a right antecubital fossa vein for infusions. Retrograde 20G cannulae were placed as follows: 1) in a superficial vein on the anterior abdominal wall, under ultrasonic guidance, to sample from subcutaneous adipose (25); 2) in a deep branch of the median cubital vein in the left antecubital fossa



**FIG. 1.** In vivo d2-cortisone administration. **A:** Plasma d2-cortisone concentrations after an intravenous bolus of d2-cortisone without licorice. **B:** Plasma cortisone concentrations after infusion of tracer with and without prior licorice administration. **C:** Plasma enrichment of cortisone with d2-cortisone following infusion of tracer with and without prior licorice administration. **D:** Plasma d2-cortisone concentrations following infusion of tracer with and without prior licorice administration. All data are means  $\pm$  SEM for  $n = 3$ . For statistical comparisons, see Table 1.

to sample from forearm skeletal muscle (26); and 3) in a dorsal vein of the right hand, with the hand warmed to 60°C to sample arterialized blood (27). For confirmation of correct placement of cannulae, O<sub>2</sub> saturation (GEM OPL; Instrumentation Laboratory, Bedford, MA) was confirmed to be >85, <40, and >98% in the adipose, skeletal muscle, and arterialized samples, respectively.

d2-Cortisone and d4-cortisol (40% in cortisol) were administered in 0.9% saline, wt/vol, as intravenous boluses of 76.0 µg and 3.5 mg followed by continuous intravenous infusions of 105.3 µg/h and 1.74 mg/h, respectively. Arterialized samples were obtained preinfusion and at hourly intervals for 3 h. From 3 h, four sets of blood samples were obtained simultaneously from all sites at 10-min intervals. Prior to taking of samples from the deep forearm vein, a wrist cuff was inflated to 200 mmHg for 2 min to remove blood flow from the hand. Forearm blood flow was measured by venous occlusion plethysmography (28) (Hokanson, Bellevue, WA) immediately after sampling using the mean of three readings during intermittent occlusion of the upper arm with a cuff inflated to 40 mmHg. Adipose tissue blood flow was measured continuously during the study using a Mediscint γ-counter probe after a subcutaneous injection of 1–2 MBq <sup>133</sup>Xe lateral to the umbilicus (29).

**Cortisol/cortisone interconversion in the splanchnic circulation.** For facilitation of ethical hepatic vein cannulation, four patients undergoing elective coronary angiography for investigation of coronary artery disease were studied. Two venous cannulae (20G) were sited in peripheral veins. d2-Cortisone and d4-cortisol (20% in cortisol) were administered in 0.9% saline, wt/vol, as intravenous boluses of 75.6 µg and 3.5 mg followed by continuous intravenous infusions of 104.8 µg/h and 1.74 mg/h, respectively. A simultaneous infusion of intravenous ICG (30 mg/h constituted in water for injection) was used to estimate liver blood flow. After infusion for at least 3 h, femoral arterial and venous sheaths were inserted and participants underwent coronary angiography with follow-on percutaneous coronary intervention when indicated. Upon completion of the clinical procedure, cannulation of the hepatic vein was undertaken under fluoroscopic guidance via the venous sheath using a Swan-Ganz double lumen catheter (6F; Edward Life Sciences, Irvine, CA). A series of three paired blood samples was drawn simultaneously from hepatic vein and femoral artery.

**Laboratory analysis.** Blood samples were collected in lithium heparin and plasma stored at –80°C until analysis. Cortisol, d4-cortisol, d3-cortisol, d2-cortisol, cortisone, and d2-cortisone were extracted from plasma (1.5–2.0 mL) enriched with epicortisol (500 ng) using chloroform (15–20 mL) and analyzed using liquid chromatography–tandem mass spectrometry as previously described (4), except that chromatographic separation was optimized using a Restek Biphenyl Allure column (5 µm, 10 cm, 4.6 mm, 38°C, and 0.5 mL/min). The most abundant ions for analysis of the deuterated and nondeuterated steroids were selected by tuning on a TSQ Quantum Discovery Mass spectrometer, and conditions for multiple-reaction monitoring were optimized. The compounds subsequently underwent analysis on an LTQ Orbitrap FT-MS to confirm the isotopic composition of the product ions chosen. The mass transitions monitored during tandem mass spectrometry (positive electrospray mode, spray voltage 3.25 kV; source temperature 400°C; and collision gas pressure 1.5 mTorr) were 361→163 *m/z* for cortisone, 363→165 *m/z* for d2-cortisone, 363→121 *m/z* for cortisol and epi-cortisol (internal standard), 365→122 and 365→329 *m/z* for d2-cortisol, 366→121 *m/z* for d3-cortisol, and 367→121 *m/z* for d4-cortisol, with collision energies and tube lens voltages, respectively, of 22 and 168 (cortisone), 21 and 118 (d2-cortisone), 31 and 142 (cortisol), 23 and 13 with a tube lens 118 (d2-cortisol), 24 and 142 (d3-cortisol), and 28 and 142 (d4-cortisol). Concentrations of cortisol, cortisone, and d2-cortisone were determined using calibration curves. The peak areas of deuterated steroids were corrected for the abundances of naturally occurring isotopomers at baseline. In addition, the peak area of d4-cortisol was corrected for interference from the m+4 isotopomer of cortisol and the m+1 isotopomer of d3-cortisol. The peak area of d3-cortisol was corrected for interference from the m+3 isotopomer of cortisol and the m+1 isotopomer of d2-cortisol. Naturally occurring m+2 isotopomers of cortisone were detected at <1% and did not exceed background, so a correction was not applied.

Plasma d2-cortisol, d3-cortisol, and d4-cortisol concentrations were calculated by multiplying the concentrations of cortisol by their respective tracer-to-tracee ratios. Serum ICG was measured using HPLC as previously described (4). **Data analysis, kinetic calculations, and statistics.** Volume of distribution and half-life of d2-cortisone were calculated following bolus injection using Kinetica software (Thermo Scientific, Philadelphia, PA). Clearance was calculated at steady state (ss, 60–180 min) using Equation 1.

$$\text{Clearance (L/min)} = \frac{\text{rate of infusion (nmol/min)}}{\text{steady-state concentration (nmol/L)}} \quad (1)$$

Whole-body *R<sub>a</sub>* of cortisol, *R<sub>a</sub>* d3-cortisol, and net *R<sub>a</sub>* cortisone (nanomoles per minute) in arterial plasma were calculated by dividing the rate of tracer infusion (nanomoles per minute) by the respective tracer-to-tracee ratio:

$$R_a \text{ cortisol} = \frac{\text{rate of d4-cortisol infusion}}{(\text{d4-cortisol:cortisol})} - \text{rate of cortisol infusion} \quad (2)$$

$$R_a \text{ d3-cortisol} = \frac{\text{rate of d4-cortisol infusion}}{(\text{d4-cortisol:d3-cortisol})} \quad (3)$$

$$\text{Net } R_a \text{ cortisone} = \frac{\text{rate of d2-cortisone infusion}}{(\text{d2-cortisone:cortisone})} \quad (4)$$

Subcutaneous adipose (pmol/100 g tissue/min) and muscle tissue (pmol/100 mL tissue/min) production of cortisol, cortisone, and d3-cortisol were calculated using arterialized and venous samples (superficial epigastric vein for adipose and deep forearm vein for forearm skeletal muscle) and blood flow (L/min) at steady state (180–210 min) using the mean of four samples. Splanchnic glucocorticoid production (nanomoles per min) was calculated using the mean of three samples and correcting for hepatic blood flow, calculated from the concentrations of ICG in the hepatic vein and femoral artery as previously described (4). The following equations were used to calculate tissue glucocorticoid release:

$$\begin{aligned} \text{Cortisol release} &= (\text{BF} \times [\text{cortisol}_A]) \times \left( \frac{\text{d4-cortisol:cortisol}_A}{\text{d4-cortisol:cortisol}_V} \right) \\ &\quad - (\text{BF} \times [\text{cortisol}_A]) \end{aligned} \quad (5)$$

$$\begin{aligned} \text{d3-cortisol release} &= (\text{BF} \times [\text{d3-cortisol}_A]) \times \left( \frac{\text{d4-cortisol:d3-cortisol}_A}{\text{d4-cortisol:d3-cortisol}_V} \right) \\ &\quad - (\text{BF} \times [\text{d3-cortisol}_A]) \end{aligned} \quad (6)$$

$$\begin{aligned} \text{Net cortisone release} &= (\text{BF} \times [\text{cortisone}_A]) \times \left( \frac{\text{d2-cortisone:cortisone}_A}{\text{d2-cortisone:cortisone}_V} \right) \\ &\quad - (\text{BF} \times [\text{cortisone}_A]) \end{aligned} \quad (7)$$

where A is arterialized, V is venous, and BF is blood flow.

Data are presented as means ± SEM and were compared using paired Student *t* tests. Differences from zero were calculated using a single-sample *t* test. Significance was accepted at *P* < 0.05.

## RESULTS

**Validation of d2-cortisone tracer.** d2-Cortisone was a substrate for human 11βHSD1 *in vitro*. Incubation of HEK293/h11βHSD1 cells with d2-cortisone or cortisone resulted in production of d2-cortisol or cortisol, respectively (0.17 ± 0.05 vs. 0.41 ± 0.15 pmol/10<sup>5</sup> cells/min, *P* = 0.11). d2-Cortisone and cortisone had comparable effects in competition with [<sup>3</sup>H]<sub>2</sub>-cortisone: for formation of [<sup>3</sup>H]<sub>2</sub>-cortisol in the presence of d2-cortisone or cortisone, *V<sub>max</sub>* was 0.83 ± 0.34 and 0.73 ± 0.26 pmol/10<sup>5</sup> cells/min (*P* = 0.75) and apparent *K<sub>m</sub>* 1.60 ± 0.84 vs. 0.91 ± 0.39 µmol/L (*P* = 0.51), respectively. In human hepatic cytosol, [<sup>3</sup>H]<sub>2</sub>-cortisone was converted also to [<sup>3</sup>H]<sub>2</sub>-3α,5β-tetrahydrocortisone; the rates of this reaction, catalyzed by 5β-reductase/3αHSD, were similar for d2-cortisone and cortisone (318.8 ± 37.1 vs. 347.3 ± 37.9 pmol/mg/h, *P* = 0.68). [<sup>3</sup>H]<sub>2</sub> Products were not formed in control samples without cells, cytosol, or cofactor.

Three men aged 35 ± 7 years with BMI 22.8 ± 2.1 kg/m<sup>2</sup> participated in studies of *in vivo* pharmacokinetics of d2-cortisone. After bolus administration, elimination of d2-cortisone from blood could be fitted with first-order kinetics and the tracer could be detected for 1.5 h after injection (Fig. 1A). Mean plasma half-life was 57.5 min, volume of distribution 47.0 L, and area under the curve 494 nmol/L per min. Priming dose and steady-state infusion rates were estimated from these values.

During primed infusion of d2-cortisone for 3 h, cortisone, cortisol, d2-cortisone, and d2-cortisol were readily measured in plasma (Fig. 1B and D). Steady-state d2-cortisone

concentrations were achieved after 15 min. The dilution of d2-cortisone by endogenous cortisone resulted in a calculated net  $R_a$  of cortisone of  $35.6 \pm 8.8$  nmol/min (Table 1). Licorice administration had effects consistent with inhibition of renal  $11\beta$ HSD2 dehydrogenase activity (30), lowering endogenous cortisone concentrations without affecting tracer d2-cortisone concentrations or clearance, and thereby increasing d2-cortisone enrichment and decreasing the calculated  $R_a$  of cortisone (Table 1 and Fig. 1B and C). Given the small number of participants ( $n = 3$ ), not all of these effects of licorice were statistically significant.

**Cortisol and cortisone recycling in key metabolic tissues in vivo.** For the adipose and skeletal muscle arteriovenous sampling, study participants ( $n = 6$  men) were aged 30–49 years (mean  $\pm$  SEM  $42 \pm 4$ ), with BMI  $21\text{--}28$  kg/m<sup>2</sup> ( $24.6 \pm 0.9$ ) and fat mass of  $10.5\text{--}30.5$  kg ( $16.5 \pm 2.9$ ), and took no regular medications. For the hepatic vein study, participants ( $n = 4$ ) were aged 43–70 years ( $60 \pm 6$ ), with BMI  $24\text{--}26$  ( $25.3 \pm 0.6$ ), being investigated for ischemic heart disease and taking statins ( $n = 4$ ) and  $\beta$ -adrenergic receptor blockers ( $n = 3$ ).

**Whole body.** Plasma d4-cortisol concentrations and enrichment of plasma with d4-cortisol and d3-cortisol were in steady state by 3 h, while plasma d2-cortisone concentrations and enrichment were in steady state by 1 h (Fig. 2). From tracer enrichments at steady state (Table 2), whole-body cortisol, d3-cortisol, and cortisone production were calculated (Table 3). Cortisol production was similar in the two studies and as previously reported (14). d3-cortisol production was approximately twice as high in the adipose and muscle study than in the splanchnic study, consistent with infusion of a higher enrichment of d4-cortisol tracer (40 vs. 20%) in the former to ensure detection in small volumes obtained from adipose veins. Cortisone production was somewhat higher when d4-cortisol was coinfused with d2-cortisone, consistent with the additional source of cortisol as substrate for  $11\beta$ -dehydrogenase activity from the cortisol infusion.

**Adipose.** Across adipose tissue, there were no arteriovenous differences in endogenous steroid or tracer concentrations (Table 2). Although there were trends for dilution of d4-cortisol with both cortisol and d3-cortisol, and once corrected for blood flow ( $5.7 \pm 1.3$  mL/min/100 g adipose) the mean cortisol and d3-cortisol release was consistent with a magnitude of reductase activity similar to that detected previously (4), these did not achieve statistical significance (Table 3). By multiplication of dual-energy X-ray absorptiometry-derived fat mass and local adipose tissue measurements, whole-body adipose tissue glucocorticoid

release was extrapolated to be  $\sim 10\%$  of whole-body values. However, there was a statistically significant fall in plasma d2-cortisone enrichment (Table 2) and detectable production of cortisone across subcutaneous adipose tissue (Table 3), consistent with adipose  $11\beta$ -dehydrogenase activity. The ratio of  $11\beta$ -reductase activity, as measured by  $R_a$  of cortisol, to  $11\beta$ -dehydrogenase activity was  $\sim 1:1.3$ .

**Skeletal muscle.** Endogenous and tracer steroid concentrations did not change across the forearm circulation/skeletal muscle except for a fall in d2-cortisone concentration (Table 2). However, there were significant differences in tracer enrichment, indicating dilution of d4-cortisol with both cortisol and d3-cortisol and dilution of d2-cortisone with cortisone (Table 2). Once adjustment was made for forearm blood flow ( $2.8 \pm 0.2$  mL/100 mL/min), there was significant release of cortisol, d3-cortisol, and cortisone, indicating both reductase and dehydrogenase activities (Table 3). The ratio of  $11\beta$ -reductase activity to  $11\beta$ -dehydrogenase activity was  $\sim 1:1$ .

**Splanchnic tissues.** Across the splanchnic circulation, there was a fall in d4-cortisol, cortisone, d3-cortisone, and d2-cortisone concentrations. Enrichment of cortisol with d4-cortisol and of cortisone with d2-cortisone decreased, indicating endogenous glucocorticoid production. After adjustment for hepatic blood flow ( $0.64 \pm 0.11$  L/min), there was significant splanchnic release of cortisol, d3-cortisol, and cortisone (Table 3). The ratio of  $11\beta$ -reductase to  $11\beta$ -dehydrogenase activity was  $\sim 3:1$ .

## DISCUSSION

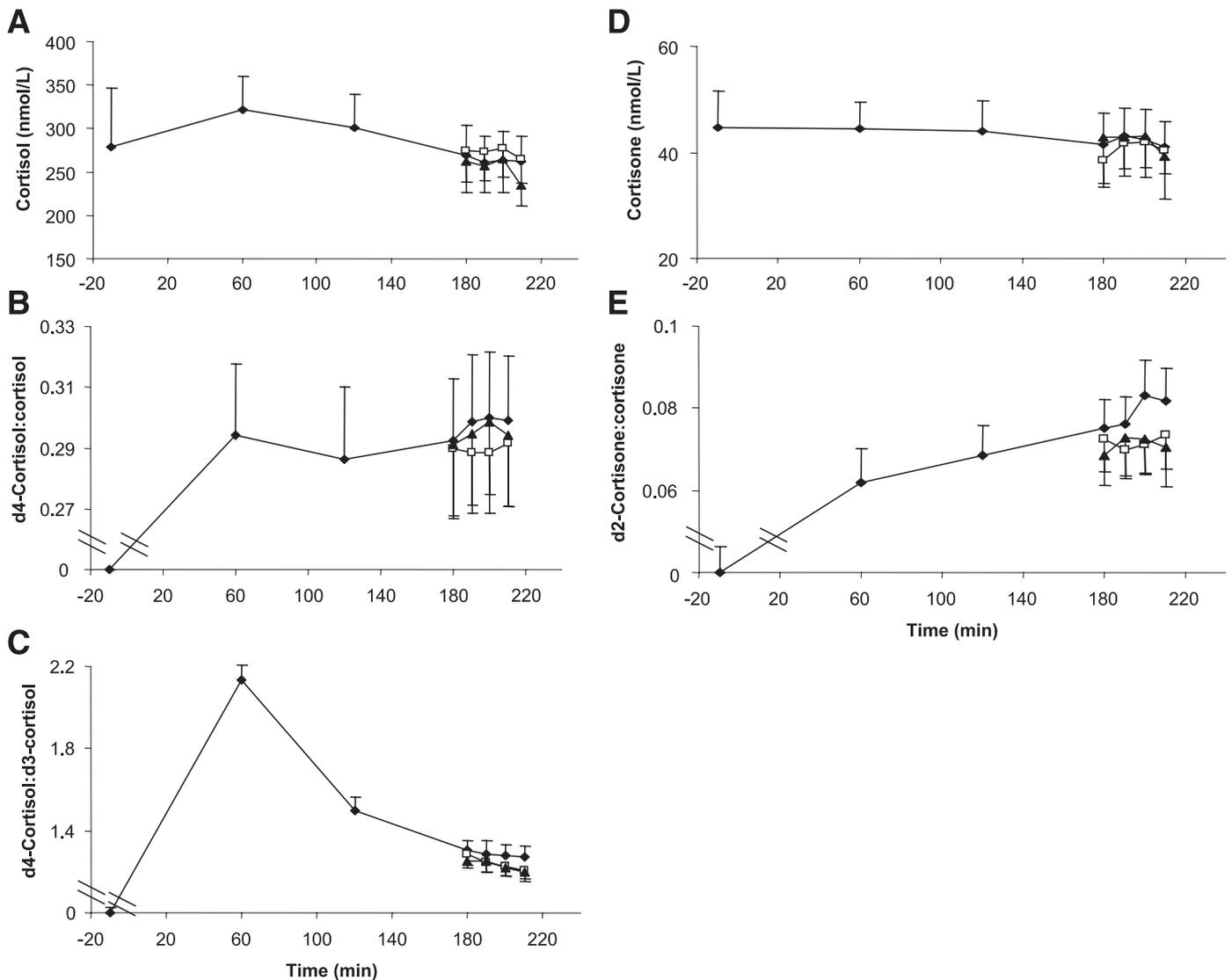
We developed a novel tracer, d2-cortisone, to quantify cortisone generation for the first time in vivo in humans. This can be attributed to  $11\beta$ -dehydrogenase activities of  $11\beta$ HSD2 and/or  $11\beta$ HSD1, since these are the only enzymes known to catalyze cortisone generation. Substantial  $11\beta$ -dehydrogenase activity is present not only in whole body (presumably reflecting kidney  $11\beta$ HSD2 activity [31]) but also in the splanchnic circulation (potentially reflecting  $11\beta$ HSD2 activity in the gut) and in sites where  $11\beta$ HSD2 is not expressed significantly, including subcutaneous adipose tissue and forearm skeletal muscle. Although cortisol regeneration by  $11\beta$ -reductase predominates in the splanchnic circulation, there is balanced recycling between cortisol and cortisone in skeletal muscle and predominant inactivation of cortisol by  $11\beta$ -dehydrogenase in adipose tissue. These data challenge the concept that  $11\beta$ HSD1 is exclusively a physiological amplifier of glucocorticoid action, suggesting instead that active recycling between cortisol and cortisone provides the opportunity for an amplified dynamic response to alterations in  $11\beta$ HSD1. Moreover, they suggest, as has been demonstrated empirically in vitro (32), that  $11\beta$ HSD1 inhibitors may be required to inhibit reductase activity selectively over dehydrogenase activity in order to lower intracellular cortisol concentrations and be efficacious; failure to achieve this might explain lack of efficacy in phase 2 clinical trials (10).

Previously available methods for measuring  $11\beta$ -dehydrogenase activity and/or cortisone production are inadequate. Most investigators relied upon measuring urinary free cortisol-to-cortisone ratios as an index of renal  $11\beta$ HSD2 activity (33,34) or on urinary cortisol-to-cortisone metabolite ratios, which reflect predominantly intrahepatic steroid levels. However, these ratios only reflect net balance between the activities of multiple enzymes and do not quantify rates of turnover between cortisol and cortisone. Others have administered labeled substrates for

TABLE 1  
Concentrations of cortisone and d2-cortisone tracer and calculated kinetic parameters before and after licorice administration

|                                   | Without licorice | With licorice    | <i>P</i> |
|-----------------------------------|------------------|------------------|----------|
| $C_{ss}$ cortisone (nmol/L)       | $64.9 \pm 2.6$   | $24.8 \pm 4.7$   | 0.02     |
| $C_{ss}$ d2-cortisone (nmol/L)    | $9.3 \pm 2.1$    | $12.6 \pm 1.7$   | 0.25     |
| Enrichment d2-cortisone (%)       | $16.9 \pm 2.9$   | $43.5 \pm 8.0$   | 0.08     |
| Net $R_a$ of cortisone (nmol/min) | $35.6 \pm 8.8$   | $15.1 \pm 4.5$   | 0.17     |
| Clearance d2-cortisone (L/min)    | $1.04 \pm 0.8$   | $0.9 \pm 0.2$    | 0.83     |
| $C_{ss}$ cortisol (nmol/L)        | $238.0 \pm 33.7$ | $177.9 \pm 40.5$ | 0.18     |
| $C_{ss}$ d2-cortisol (nmol/L)     | $6.2 \pm 1.7$    | $18.5 \pm 5.8$   | 0.19     |

Data are means  $\pm$  SEM for  $n = 3$ .  $C_{ss}$ , concentration at steady state. d, deuterium.



**FIG. 2.** Unlabeled and tracer glucocorticoids in plasma from arterialized samples and veins draining skeletal muscle and subcutaneous adipose tissue. Data are means  $\pm$  SEM for  $n = 6$  volunteers. Statistical comparisons were conducted on average values in each subject (Table 2). **A:** Cortisol concentrations. **B:** d4-Cortisol-to-cortisol ratio. **C:** d4-Cortisol-to-d3-cortisol ratio. **D:** Cortisone concentrations. **E:** d2-Cortisone-to-cortisone ratios. ◆, arterialized vein; ▲, superficial epigastric vein; □, deep forearm vein. d, deuterium.

11 $\beta$ -dehydrogenase, including 11 $\alpha$ -[ $^3$ H]-cortisol (35), d4-cortisol (2), 11 $\alpha$ -[ $^2$ H]-cortisol (36), and d2-cortisol (37), but the removal of these compounds is not exclusively dependent on 11 $\beta$ -dehydrogenase and  $R_a$  of 11 $\beta$ -dehydrogenase product (labeled cortisone or liberated [ $^3$ H]) cannot be quantified accurately in steady state in the absence of simultaneous measurement of clearance of the product. Here, we used the gold standard approach, in which endogenous cortisone production is inferred from dilution of a “tracer” labeled cortisone, infused in steady state. We selected d2-cortisone because it is available, is distinguishable in assays from metabolites of d4-cortisol, and as a stable isotope can be safely administered and measured with high specificity. Cell-based assays confirmed similar metabolism of cortisone and d2-cortisone.

After in vivo administration, d2-cortisone had a longer half-life than has been reported for cortisone (58 vs. 28 min) (38). However, the earlier report from the 1950s relied on inferior assay technology. Cortisone production rate was also higher than the rate measured previously

with a d4-cortisol tracer (by extrapolation from generation of d3-cortisone) under conditions of non-steady-state kinetics (36 vs. 24 nmol/min, adjusted for substrate concentration and volume of distribution [2]). This likely reflects the advantages of making steady-state measurements based on dilution of tracer by tracee when both tracer and tracee are at physiological concentrations. A limitation of the d2-cortisone tracer is that it only measures net cortisone production and cannot account for recycling of cortisone-cortisone interconversion. Moreover, there was a hint in our data, albeit not statistically significant, that the addition of deuterium to cortisone makes it a worse substrate than cortisone for 11 $\beta$ -reductase activity. For these reasons, all 11 $\beta$ -dehydrogenase activities reported here may be somewhat underestimated. However, the cortisone production rate was similar in steady state to the cortisone production rate; approximately halved by licorice administration, consistent with the effect of licorice on 11 $\alpha$ -[ $^3$ H]-cortisol half-life (30); and increased by infusion of additional cortisol when d4-cortisol tracer was administered.

TABLE 2

Mean steady-state plasma steroid concentrations and ratios during deuterated cortisol and cortisone infusions

|  | Adipose and skeletal muscle study |                           |                      | Splanchnic study |              |
|--|-----------------------------------|---------------------------|----------------------|------------------|--------------|
|  | Arterialized vein                 | Subcutaneous adipose vein | Skeletal muscle vein | Femoral artery   | Hepatic vein |
| Cortisol (nmol/L)  | 263.8 ± 30.6                      | 254.1 ± 30.3              | 271.9 ± 31.2         | 368.9 ± 26.2     | 336.4 ± 49.6 |
| d3-Cortisol (nmol/L)   | 60.0 ± 6.0                        | 59.2 ± 5.9                | 63.0 ± 6.4           | 32.0 ± 3.9       | 37.6 ± 7.3   |
| d4-Cortisol (nmol/L)   | 76.3 ± 6.9                        | 73.3 ± 8.4                | 77.3 ± 7.9           | 50.6 ± 7.4       | 36.9 ± 9.0†† |
| d4-Cortisol-to-d3-cortisol ratio   | 1.3 ± 0.1                         | 1.2 ± 0.0                 | 1.2 ± 0.0†           | 1.47 ± 0.1       | 0.89 ± 0.1†  |
| d4-Cortisol enrichment [d4-cortisol/(cortisol + d4-cortisol) × 100], %     | 22.8 ± 1.3                        | 22.6 ± 1.4                | 22.4 ± 1.2†          | 11.9 ± 0.8       | 9.6 ± 0.7††† |
| Cortisone (nmol/L)   | 42.0 ± 5.3                        | 42.0 ± 7.8                | 40.6 ± 4.6           | 56.4 ± 8.3       | 9.2 ± 0.9††  |
| d2-Cortisone (nmol/L)  | 4.1 ± 0.4                         | 4.0 ± 0.7                 | 3.6 ± 0.6††          | 4.7 ± 0.3        | 0.4 ± 0.1††† |
| d2-Cortisone enrichment [d2-cortisone/(cortisone + d2-cortisone) × 100], % | 7.2 ± 0.6                         | 6.5 ± 0.7†                | 6.6 ± 0.6†           | 8.4 ± 1.6        | 4.0 ± 0.7    |

Data are means ± SEM for the average from each subject in serial samples obtained over 30 min after at least 3 h of tracer infusion in  $n = 6$  men for adipose and skeletal muscle study and  $n = 4$  men for splanchnic study. † $P < 0.05$ , †† $P < 0.01$ , ††† $P < 0.001$  comparing arterialized vs. venous measurements using Student paired  $t$  test. d, deuterium.

Altogether, these data validate the use of d2-cortisone as a tracer.

The splanchnic circulation produces more cortisol than cortisone in individuals being investigated for coronary artery disease. Indeed, splanchnic release accounts for the great majority of whole-body d3-cortisol generation by 11 $\beta$ -reductase, as previously described (4,18,39), but accounts for less than one-half of whole-body cortisone generation. However, given the importance of 11 $\beta$ HSD2 in the kidney (35), which was previously thought to be the exclusive source of cortisone (31), it is notable that splanchnic cortisone production is substantial. Its origin is uncertain. 11 $\beta$ HSD1 is expressed in visceral fat and the liver (40,41) and may function as a dehydrogenase. However, although 11 $\beta$ HSD2 expression in adipose and liver is low (15,42,43), it is highly expressed in the epithelia of the gastrointestinal tract (44), and this is likely to be the principal mechanism accounting for cortisone release previously reported in the portal vein (3,4). Given this contribution of visceral 11 $\beta$ -dehydrogenase activity, it appears that liver 11 $\beta$ HSD1 is indeed a predominant reductase, as anticipated. Unlike skeletal muscle and adipose tissue, other enzymes are expressed in human liver that remove cortisol and cortisone, including A-ring reductases. It is possible that differential removal of cortisol or cortisone may influence the substrate concentrations and, hence, the balance between 11 $\beta$ -dehydrogenase and 11 $\beta$ -reductase, respectively.

In adipose tissue, results were not as anticipated, with substantial 11 $\beta$ -dehydrogenase activity. This confirms previous *ex vivo* (13) and *in vivo* findings with microdialysis (14) but using a technique that does not risk dissociation of

11 $\beta$ HSD1 from the colocalized H6PDH and, hence, loss of NADPH cofactor supply. Remarkably, the dehydrogenase activity exceeded 11 $\beta$ -reductase activity in adipose tissue in steady state. The magnitude of 11 $\beta$ -reductase activity as judged by d3-cortisol release might be underestimated owing to the recently discovered slow turnover of the intra-adipose glucocorticoid pool in humans (45) so that accumulation of d3-cortisone substrate to steady state in adipose may take longer than the duration of infusion. However, this is not a confounder of the measurement of cortisol released from adipose 11 $\beta$ -reductase, which was also slower than the release of cortisone. The source of dehydrogenase activity in adipose is likely to be 11 $\beta$ HSD1, as adipose 11 $\beta$ HSD2 transcript levels are very much lower than 11 $\beta$ HSD1 and may not be sufficient to produce measurable active protein (13,42,43,46). Adipose tissue 11 $\beta$ HSD1 has been a focus of research since the description of its upregulation in human obesity (7) and the potent effects of its manipulation in mice (47), but it has been assumed to be an exclusive 11 $\beta$ -reductase. However, an artificial increase in adipose dehydrogenase activity by transgenic overexpression of 11 $\beta$ HSD2 in adipocytes is protective against the adverse metabolic effects of high-fat feeding in mice (16). The current data suggest that there could be switching between reductase and dehydrogenase activities of 11 $\beta$ HSD1 in adipose tissue and that it can no longer be assumed that intra-adipose glucocorticoid concentrations are linearly related to 11 $\beta$ HSD1 protein levels.

Skeletal muscle has only been recognized relatively recently as a potentially important site for 11 $\beta$ HSD1 activity. Previous *in vivo* studies measuring arteriovenous gradients

TABLE 3

Calculated steady-state kinetic parameters for cortisol and cortisone generation in adipose tissue, skeletal muscle, and splanchnic tissues

|                     | Adipose and skeletal muscle study |                                       |                                   | Splanchnic study      |                       |
|---------------------|-----------------------------------|---------------------------------------|-----------------------------------|-----------------------|-----------------------|
|                     | Whole body (nmol/min)             | Subcutaneous adipose (pmol/100 g/min) | Skeletal muscle (pmol/100 mL/min) | Whole body (nmol/min) | Splanchnic (nmol/min) |
| $R_a$ cortisol      | 62.6 ± 8.4†                       | 29.3 ± 21.1                           | 19.7 ± 4.1†                       | 63.5 ± 2.3††          | 64.0 ± 11.4†          |
| $R_a$ d3-cortisol   | 24.9 ± 1.0††                      | 11.4 ± 7.9                            | 5.9 ± 1.8†                        | 11.0 ± 0.8††          | 12.9 ± 2.1†           |
| Net $R_a$ cortisone | 65.1 ± 6.9††                      | 38.7 ± 13.0†                          | 15.2 ± 5.8†                       | 65.2 ± 4.6††          | 19.5 ± 2.8†           |

Data are means ± SEM for  $n = 6$  men in the adipose and skeletal muscle study and  $n = 4$  in the splanchnic study. † $P < 0.05$ , †† $P < 0.001$  vs. zero using single-sample  $t$  tests. d, deuterium.

in total cortisol and cortisone concentrations failed to demonstrate forearm 11 $\beta$ HSD1-reductase activity (20,48,49), despite skeletal muscle 11 $\beta$ HSD1 expression (17). However, this may be explained by balanced recycling between cortisol and cortisone, as revealed here with stable isotope tracers. Using d4-cortisol, 11 $\beta$ -reductase activity has been detected in the leg of obese subjects (18,21). We show that there is both 11 $\beta$ -reductase and 11 $\beta$ -dehydrogenase activity in human forearm, albeit of lower magnitude than in adipose tissue. Since skeletal muscle blood flow accounts for most of forearm blood flow when the hand is excluded from the circulation, the 11 $\beta$ -reductase activity can most likely be attributed to 11 $\beta$ HSD1 in the rhabdomyocytes (17). The 11 $\beta$ -dehydrogenase activity may be due to reversibility of 11 $\beta$ HSD1 in rhabdomyocytes, as in adipose tissue, but might also be due to 11 $\beta$ HSD2 expressed within skeletal muscle. 11 $\beta$ HSD2 has been reported in skeletal muscle vascular and interstitial cells and in rhabdomyocytes (22), although other investigators have not replicated these findings in needle biopsy muscle samples (23,50).

In conclusion, these data obtained with novel tools provide key new insights into the physiology of glucocorticoid metabolism in key metabolic tissues in humans. The number of participants is relatively small and so, although sufficient for paired comparisons of reductase and dehydrogenase activities within individuals, is insufficient to estimate biological variability, for example in relation to obesity or variations in regulators of 11 $\beta$ HSD1 expression. In addition, the hepatic vein samples were obtained in different participants from the skeletal muscle and adipose samples, so comparison between tissues must be cautious. However, we speculate that since recycling between active cortisol and inert cortisone is an energy-consuming process, it is likely to have a physiological advantage. More work is required to understand whether glucocorticoid recycling occurs within each cell by 11 $\beta$ HSD functioning as both reductase and dehydrogenase (autocrine recycling) or whether recycling occurs in neighboring cells (paracrine recycling); this requires *in vivo* studies beyond the reach of current methodologies. As in other "futile recycling" in metabolic pathways, recycling between cortisol and cortisone within tissues may confer a more dynamic response to variation either in 11 $\beta$ HSDs themselves or in substrate availability. Moreover, in the basal state it appears that liver metabolism is set in favor of cortisol regeneration, while adipose metabolism may be set in favor of cortisol inactivation. This insight calls for a reappraisal of our understanding of the consequences of tissue-specific disruption of 11 $\beta$ HSD1 in obesity and the basis for targeting 11 $\beta$ HSD1 as a treatment for metabolic syndrome.

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K.A.H performed clinical and laboratory studies, data analysis, and wrote the manuscript. K.N.M. and J.I. performed clinical studies and data analysis. N.L.C. assisted with study design and performed clinical studies. R.H.S. and R.M.R. assisted with study design and data analysis and interpretation. D.E.N. assisted with study design and performed clinical studies. R.A. conceived the studies, assisted with study design and data analysis, and edited the manuscript. F.K. assisted with study design and performed clinical studies. B.R.W. conceived the studies, assisted with study design and data analysis, and edited the manuscript. B.R.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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