Large increases in adipose triacylglycerol flux in Cushingoid CRH-Tg mice are explained by futile cycling

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Harris C, Roohk DJ, Fitch M, Boudignon BM, Halloran BP, Hellerstein MK. Large increases in adipose triacylglycerol flux in Cushingoid CRH-Tg mice are explained by futile cycling. Am J Physiol Endocrinol Metab 304: E282–E293, 2013. First published December 4, 2012; doi:10.1152/ajpendo.00154.2012.—Glucocorticoids (GCs) are among the most effective therapies to treat inflammatory and autoimmune diseases, but their clinical utility is limited by a myriad of undesirable side effects. These effects include osteoporosis, muscle wasting, fat redistribution, and skin thinning. Here we use heavy water labeling and mass spectrometry to measure fluxes through metabolic pathways impacted by glucocorticoids. We combine these methods with measurements of body composition in corticotropin-releasing hormone (CRH)-transgenic (Tg) mice that have chronically elevated, endogenously produced corticosterone and a phenotype that closely mimics Cushing’s disease in humans. CRH-Tg+ mice had increased adipose mass, adipose glycerol synthesis, and greatly increased triglyceride/fatty acid cycling in subcutaneous and abdominal fat depots and increased de novo lipogenesis in the abdominal depot. In bone, CRH-Tg+ mice had decreased bone mass, absolute collagen synthesis rates, and collagen breakdown rate. In skin, CRH-Tg+ mice had decreased skin thickness and absolute collagen synthesis rates but no decrease in the collagen breakdown rate. In muscle, CRH-Tg+ mice had decreased muscle mass and absolute protein synthesis but no decrease in the protein breakdown rate. We conclude that chronic exposure to endogenous glucocorticoid excess in mice is associated with ongoing decreases in bone collagen, skin collagen, and muscle protein synthesis without compensatory reduction (coupling) of breakdown rates in skin and muscle. Both of these actions contribute to reduced protein pool sizes. We also conclude that increased cycling between triglycerides and free fatty acids occurs in both abdominal and subcutaneous fat depots in CRH-Tg+ mice. CRH-Tg mice have both increased lipolysis and increased triglyceride synthesis in adipose tissue.

glucocorticoids; corticosterone; triacylglycerol synthesis; lipolysis; stable isotopes; collagen synthesis; Cushing syndrome

GLUCOCORTICOIDS (GCs) are among the most effective therapies to treat inflammatory and autoimmune diseases, but their clinical utility is limited by a myriad of undesirable side effects. These effects include osteoporosis, skin thinning (1, 13) and increased bruising, muscle wasting, fat redistribution, hypertension, and insulin resistance (33). GCs have been shown to cause both decreased bone formation and increased bone resorption in humans and rodents (21, 26, 42). Reduction in bone collagen synthesis is a primary mechanism by which GCs decrease bone mass (7). GCs have been shown to induce muscle wasting through direct effects on muscle protein break-
understanding of the effects of GCs on target tissues will be integral to developing effective and safe SGRMs.

**METHODS**

*Animals.* CRH-Tg\(^+\) mice (kind gift of Mary Stenzel-Poore, Oregon Health and Science University) were maintained on the C57Bl/6 background. Because CRH-Tg\(^+\) females are not fertile, male CRH-Tg\(^+\) males were mated to WT C57BL/6 mice. CRH-Tg\(^+\) and WT littermates were housed in groups of no more than five under temperature-controlled conditions with a 12:12-h light-dark cycle. Mice were fed Picolab Rodent Diet 20 ad libitum. All studies received prior approval from the Animal Care and Use Committee at the University of California San Francisco.

**Body composition analysis.** Body composition analysis was performed using DEXA (Lunar Piximus). The head region was excluded from analysis using the region of interest feature of Piximus software.

**Micro-CT.** The distal femur was scanned in vivo and analyzed using a Scanco VivaCT 40 micro-CT as previously described (14).

**Ear skin thickness.** Ear skin thickness was measured using a Mitutoyo micrometer.

**Heavy water labeling protocol.** In the first cohort of mice, male 14-wk-old mice were labeled with heavy water (\(^2\)H\(_2\)O) for 1 wk to achieve a body water \(^2\)H enrichment of \(-5\%\) as described elsewhere (24). Mice were given a 3.5\% (volume/mass) intraperitoneal bolus of \(^2\)H\(_2\)O in saline and then given 8\% \(^2\)H\(_2\)O drinking water. This labeling period is ideal for analyzing TG kinetics in adipose tissue. A second separate cohort of animals only received the bolus label and were killed 5 h after the injection of label for the purpose of analyzing TG kinetics in the liver. \(^2\)H enrichment was measured in water distillate from plasma using gas chromatography-mass spectrometry (GC-MS) after conversion to tetrabromoethane, as described in detail elsewhere (9, 31, 37).

The basic principle of heavy water labeling can be summarized briefly as follows. The deuterium atoms become incorporated into covalent C-H bonds of metabolic precursors via a variety of metabolic reactions. When these precursors are used to synthesize molecules of interest such as TG in adipose or amino acids in proteins, the result is incorporation of heavy hydrogen label into these end products. By measuring the enrichment and labeling pattern of deuterium in these end products, with application of combinatorial analysis of mass isotope distributions (16), we can calculate their synthetic rates.

**Measurement of hydroxyproline enrichments in bone and skin by GC-MS.** \(\sim 4\)-\((\text{O}-\text{tert-butylmethylsilyl})\)-hydroxyproline pentafluorobenzyl ester was prepared from bone and skin by GC-MS as previously described (12, 31). Compounds were resolved on a DB-225 column. Mass spectrometry was performed in negative chemical ionization (NCI) mode with helium as the carrier gas, and mass-to-charge \((m/z\) ratios 424–426 corresponding to the \(M_0, M_1,\) and \(M_2\) mass isotopomers of derivatized hydroxyproline were analyzed by selected ion monitoring (SIM).

**Measurement of alanine enrichments from quadriceps mixed muscle proteins by GC-MS.** Pentafluorobenzyl-\(N,N\)-di(pentafluorobenzyll)-alanine was prepared from skeletal muscle and analyzed by GC-MS as previously described (5, 31). Compounds were resolved on a DB-225 column. Mass spectrometry was performed in NCI mode with helium as the carrier gas, and mass-to-charge \((m/z\) ratios 448–450 corresponding to the \(M_0, M_1,\) and \(M_2\) mass isotopomers were analyzed by SIM.

**Calculation of fractional synthesis of tissue collagen, muscle proteins, glycerol, and palmitate.** Fractional synthesis \(f\), representing the fraction of new collagen, protein, palmitate, or TG synthesized during the labeling period, was calculated for each analyte as the ratio of the measured excess \(M_1\) isotopomer \((EM_1)\) to the asymptotic value of \(EM_1\) \((EM_{1,max})\) as previously described (5, 25, 31). At steady state conditions with SIM of \(m/z\) ratios 270–272 (representing \(M_0-M_2\)) of palmitate-methyl ester.

**Fig. 1.** Body mass and body composition in wild-type (WT) and corticotropin-releasing hormone (CRH)-transgenic (Tg)\(^+\) mice. *A*; average group body mass. *B*; body composition by dual energy X-ray absorptiometry (DEXA), including lean mass, fat mass, and percent fat. \(n = 6\) for the WT group and \(n = 8\) for the CRH-Tg\(^+\) group. **C**: weights of individual adipose depots. BAT, brown adipose tissue. \(*P < 0.05\) compared with WT. \(****P < 0.001\) compared with WT by \(t\)-test.
for the pool size of a metabolic product, \( f \) represents the fraction of the pool replaced or the turnover (breakdown) rate. This calculation for \( f \) can be summarized using the following formula:

\[
f = \frac{EM_{1,\text{sample}}}{EM_{1,\text{max}}}
\]

where \( EM_{1,\text{sample}} \) is the fractional abundance of \( M_1 \) isotopomers in excess of that which occurs naturally. \( EM_{1,\text{sample}} \) is calculated for each analyte using the abundances of the \( M_0, M_1, \) and \( M_2 \) mass isotopomers measured in the sample and in an unenriched standard by GC-MS using the following formula:

\[
EM_{1,\text{sample}} = \left[ \frac{M_1}{(M_0 + M_1 + M_2)} \right]_{\text{sample}} - \left[ \frac{M_1}{(M_0 + M_1 + M_2)} \right]_{\text{standard}}
\]

\( EM_{1,\text{max}} \) was calculated for each analyte in each animal from the measured body \(^2\text{H}_2\text{O} \) enrichment, as described previously (5, 16), and represents the calculated \( EM_1 \) of the fully turned over analyte at the body \(^2\text{H}_2\text{O} \) enrichment measured in the animal.

**Calculation of TG half-life in adipose tissue.** As was shown previously (37), TG synthesis kinetics can be described using first-
order equations. Accordingly, we calculated TG half-life ($t_{1/2}$) using the following formula:

$$t_{1/2} (\text{wk}) = \frac{\ln(2)}{k},$$

where $k$ is the synthetic rate constant of TG, for which we used the fractional synthesis rate ($f$) of TG-glycerol (in units of fraction new/wk). A similar calculation was made for TG in liver.

**Calculation of absolute rates of bone collagen, skin collagen, muscle protein, and adipose TG synthesis.** Absolute synthesis rates of end products were calculated as the fractional synthesis multiplied by direct or indirect measurements of pool size. TG absolute synthesis rates in inguinal and epididymal fat depots were calculated, as previously described (31, 37, 39), from fractional TG synthesis ($f$) multiplied by adipose TG mass multiplied by a correction factor (0.9, which was measured and found not to be significantly different in CRH-Tg mice, data not shown) for the fractional mass of TG in adipose tissue:

Absolute synthesis ($g$/wk) = $f_{\text{TG}} \times$ adipose depot mass ($g$) \times 0.9.

Absolute rates of mixed muscle protein were calculated in a similar manner using a correction factor for estimated fraction of muscle protein (0.2, based on 20% protein in muscle cells, not significantly different in CRH-Tg mice, data not shown). Absolute rates of collagen synthesis were calculated from fractional collagen synthesis ($f$) multiplied by measured parameters of pool size, using arbitrary units relative to control animals.

Absolute skin collagen synthesis ($U$/wk) = $f_{\text{collagen}} \times$ relative skin thickness, where skin thickness is expressed as the fraction of control values, set as 1.0.

Absolute bone collagen synthesis ($U$/wk) = $f_{\text{collagen}} \times$ relative bone area, where bone area is expressed as the fraction of control values, set as 1.0. We used absolute measurements for adipose TG and muscle protein pool sizes based on organ weights and relative measurements for skin and bone.

**Calculation of absolute rates of DNL.** Absolute rates of DNL in inguinal and epididymal fat depots were calculated as previously described (3, 31). Briefly, absolute rates of DNL were calculated from fractional palmitate synthesis ($f$), adipose TG mass, the fraction of TG mass in adipose tissue (0.9) and the fraction of palmitate relative to other FAs in TG using the following formula:

Absolute DNL ($g$/wk) = $f_{\text{DNL}} \times$ adipose TG mass ($g$) \times fraction TG \times fraction TG-palmitate,

The fraction of TG-palmitate present in adipose TG was taken to be 20% (19, 28).

**Calculation of percent contribution of DNL to newly synthesized TG.** Percent contribution of DNL to newly synthesized TG in each adipose depot was calculated, as described previously (36, 38), as the fraction of newly synthesized palmitate divided by the fraction of newly synthesized TG and represented as a percentage:

%Contribution of DNL = $f_{\text{DNL}} / f_{\text{TG}} \times 100%.

**Measurement of glyceroenogenesis in adipose tissue and liver.** The relative contribution of glyceroenogenesis vs. glycolysis to TG-glycerol (%glyceroenogenesis) was calculated using mass isotopomer distribution analysis (MIDA) algorithms and $M_0$, $M_1$, and $M_2$ isotopomer data from derivatized TG-glycerol as measured by GC-MS as previously described (8, 16, 31, 37).

**Statistical analyses.** Statistical analyses were performed using GraphPad Prism. Unpaired t-tests were performed for each parameter to assess statistically significant differences between WT and CRH-Tg $^+$ groups. To assess statistically significant differences between more than two groups for a single parameter, specifically for percent glyceroenogenesis in inguinal fat, epididymal fat, and liver for both genotypes, one-way ANOVA was performed followed by Tukey’s posttests. $P < 0.05$ was considered to be significant for all tests.

**RESULTS**

**Body weights and body composition.** CRH-Tg $^+$ mice weighed the same as their WT littermates (Fig. 1A). However, CRH-Tg $^+$ mice had significantly more fat mass (4.8 ± 0.7 g for WT vs. 9.6 ± 1.9 g for CRH-Tg $^+$, $n = 6–8$, $P < 0.001$, a difference of 4.8 ± 2.0 grams; Fig. 1B) and a reciprocal decrease in lean tissue (23.7 ± 1.5 g for WT vs. 18.6 ± 2.1 g for CRH-Tg $^+$, $n = 6–8$, $P < 0.001$, a difference of 5.1 ± 2.6 grams; Fig. 1B) and therefore had approximately two times the percentage of body fat by weight (16.9 ± 1.8% for WT vs. 33.8 ± 2.6%, $n = 6–8$, $P < 0.001$; Fig. 1B) and ~23% lower lean tissue mass as measured by DEXA. We examined the weights of five white adipose tissue depots (inguinal, epididymal, perirenal, mesenteric, and dorsocervical) and the dorsocervical brown adipose depot. All depots were three- to sixfold larger in CRH-Tg mice (Fig. 1C). CRH-Tg mice ate an equal mass of chow diet compared with their WT littermates (data not shown). Thus, CRH-Tg $^+$ mice weighed the same as their WT littermates but had significantly less lean tissue and significantly more fat mass.

**Bone parameters.** Using DEXA we found that CRH-Tg $^+$ mice had 29% less bone mineral content (0.450 ± 0.021 g for WT vs. 0.320 ± 0.032 g for CRH-Tg $^+$, $n = 6–8$, $P < 0.001$; Fig. 2A), 21% less bone area (8.67 ± 0.31 cm$^2$ for WT vs. 6.61 ± 0.47 cm$^2$ for CRH-Tg $^+$, $n = 6–8$, $P < 0.001$; Fig. 2B), but only 8% less BMD (0.512 ± 0.018 g/cm$^2$ for WT vs. 0.470 ± 0.017 g/cm$^2$ for CRH-Tg $^+$, $n = 6–8$, $P < 0.001$; Fig. 2C) than their WT littermates. We further analyzed the proximal femurs of CRH-Tg $^+$ and WT mice by micro-CT analysis (Fig. 2, E–F, and Table 1). This demonstrated that CRH-Tg $^+$ mice had 27%
less tissue volume [TV, equivalent to the bone volume (BV) plus the bone marrow volume] in the bone analyzed (0.46 ± 0.06 mm³ for WT vs. 0.34 ± 0.02 for CRH-Tg+, n = 6–8, P < 0.01; Table 1), 40% less BV (0.30 ± 0.03 mm³ for WT vs. 0.18 ± 0.01 mm³ for CRH-Tg+, n = 6–8, P < 10⁻³; Table 1), and 33% decreased cortical thickness of bone (0.247 ± 0.009 mm for WT vs. 0.164 ± 0.013 for CRH-Tg+, n = 6–8, P < 10⁻⁸; Table 1) compared with WT mice. This results in 20% less proportion of tissue that is bone (BV/TV), since there was no difference in the marrow volume (0.16 ± 0.03 mm³, n = 6–8) between WT and CRH-Tg+ mice. The density of the tissue was 19% lower in CRH-Tg+ mice (902 ± 44 mg/ml for WT and 736 ± 53 mg/ml for CRH-Tg+, n = 6–8, P < 10⁻⁴), but the BMD was similar in both genotypes (1,313 ± 22 mg/ml for WT and 1,302 ± 9 mg/ml for CRH-Tg+, n = 6–8). We then used our heavy water labeling approach combined with mass spectrometry to determine collagen synthesis rates in the bone of CRH-Tg+ mice. We found that CRH-Tg+ mice had fractional rates of bone collagen synthesis that were 36% lower (5.9 ± 2.0% for WT vs. 3.8 ± 1.4% for CRH-Tg+, n = 6–8, P < 0.05; Fig. 2D) compared with their WT littermates. Assuming 20% less total bone collagen in CRH-Tg+ mice (based on bone area), the absolute rate of bone collagen synthesis, calculated as f × relative bone area, was ~49% reduced in CRH-Tg+ mice (0.038 × 0.8 = 0.030 U/wk in CRH-Tg+ vs. 0.059 × 1.0 = 0.059 U/wk in WT, P < 0.05; Fig. 2G). Thus, CRH-Tg+ mice had less bone mass, synthesized bone collagen at a slower fractional rate, and turned over bone collagen more slowly compared with WT mice.

**Skin parameters.** We used ear skin thickness as a measure of total body skin thickness (20). The ear skin of CRH-Tg+ mice was 19% thinner than that of WT mice (188 ± 15 µm for WT vs. 153 ± 14 µm for CRH-Tg+, n = 10–11, P < 0.0001; Fig. 3A). Using our heavy water labeling approach combined with mass spectrometry, we found that CRH-Tg+ mice had similar fractional rates of skin collagen synthesis in a dorsal skin flap as their WT littermates (10.3 ± 2.1% for WT vs. 11.0 ± 1.8% for CRH-Tg+, n = 6–8; Fig. 3B). The estimated absolute rate of skin collagen synthesis was 15% lower in CRH-Tg+ mice (0.110 × 0.81 = 0.089 units in CRH-Tg+ mice vs. 0.103 × 1.0 = 0.103 units for WT, P < 0.05; Fig. 3C). We conclude that CRH-Tg+ mice had reduced absolute rates of skin collagen synthesis with no compensatory reduction in breakdown rate (similar fractional synthesis rates between genotypes). Thus, while CRH-Tg+ mice had thinner skin and synthesized skin collagen at a reduced absolute rate, there was no compensatory reduction in the skin collagen turnover (breakdown) rate constant compared with WT mice.

**Muscle protein synthesis.** CRH-Tg+ mice had significantly less lean tissue than WT mice (Fig. 1). In addition, the quadriceps muscles of CRH-Tg+ were 26% smaller than those of WT mice (155 ± 21.8 mg for WT vs. 114 ± 14.4 mg for CRH-Tg+, n = 6–8, P < 0.001; Fig. 4A). To understand the basis for this difference, we measured rates of protein synthesis of mixed proteins in quadriceps muscle of CRH-Tg+ mice and their WT littermates. We did not observe any significant difference in fractional muscle protein synthesis rates between the two groups (14.4 ± 0.1% for WT vs. 13.6 ± 2.6% for CRH-Tg+; Fig. 4B). The estimated absolute rate of muscle protein synthesis in quadriceps was 30% lower in CRH-Tg+ mice (0.136 × 114 mg × 0.2 = 3.10 mg/wk for CRH-Tg+ vs. 0.144 × 155 mg × 0.2 = 4.46 mg/wk for WT, n = 6–8, P < 0.05; Fig. 4C) with no compensatory reduction in breakdown rate. Thus, while CRH-Tg+ mice had less whole body lean tissue mass, smaller quadriceps muscles, and synthesized mixed muscle protein from quadriceps at a reduced absolute rate, there was no compensatory reduction in muscle protein turnover (breakdown) compared with WT littermates.

**Adipose and liver tissue lipid dynamics.** Inguinal fat pads were 3.2-fold larger in the CRH-Tg+ mice compared with WT mice (182 ± 105 mg for WT vs. 581 ± 301 mg for CRH-Tg+, n = 6–8, P < 0.05, Table 2). Fractional TG synthesis rates were similar in the inguinal depot of both genotypes (0.35 ± 0.09/wk for WT vs. 0.41 ± 0.06/wk for CRH-Tg+; Table 1). Accordingly, the half-life of TG in the inguinal depot was not significantly different between genotypes (2.1 ± 0.6 wk for

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**Figure 3.** Skin parameters in WT and CRH-Tg+ mice. A: ear skin thickness in µm, n = 10 for WT and n = 11 for CRH-Tg+. B: fraction of new collagen in skin. C: estimated absolute rate of skin collagen synthesis in arbitrary units/wk. *P < 0.05 and ***P < 0.001 compared with WT by t-test.
WT vs. 1.7 ± 0.2 wk for CRH-Tg⁺; Table 2). By combining fat pad masses with fractional synthesis, we calculated that total retained TG synthesis was 4.0-fold greater (49.8 ± 9.4 mg/wk for WT vs. 202 ± 83.6 mg/wk for CRH-Tg⁺, n = 6–8, P < 0.01) in the inguinal depots of CRH-Tg⁺ mice (Fig. 5A).

Epididymal fat pads were 3.9-fold larger in CRH-Tg⁺ mice compared with WT mice (355 ± 116 mg for WT vs. 1,370 ± 691 mg for CRH-Tg⁺, n = 6–8, P < 0.01; Table 2). Fractional TG synthesis rates were 1.9-fold higher in the epididymal depot of CRH-Tg⁺ mice than in their WT littermates (0.20 ± 0.04/wk for WT vs. 0.38 ± 0.04/wk for CRH-Tg⁺, n = 6–8, P < 0.01; Table 2). Therefore, the half-life of TG in the epididymal depot was significantly shorter by 50% in CRH-Tg⁺ mice compared with WT mice (3.6 ± 0.7 wk for WT vs. 1.8 ± 0.2 wk for CRH-Tg⁺, n = 6–8, P < 0.01; Table 2). By combining fat pad masses with fractional synthesis, we calcu-
Fractional rates of palmitate DNL in the inguinal depots of CRH-Tg+ mice were 62% lower than WT mice (0.18 ± 0.07/wk for WT vs. 0.07 ± 0.02/wk for CRH-Tg+, n = 6–8, P < 0.001; Table 2). Adjusting for fat pad mass, absolute retained palmitate DNL was similar in the inguinal depots of CRH-Tg+ and WT mice (4.8 ± 0.7 mg/wk for WT vs. 6.4 ± 2.4 mg/wk for CRH-Tg+; Fig. 5C). Thus, CRH-Tg+ mice have larger inguinal fat pads, the total retained de novo palmitate synthesized in the inguinal depot was similar between the two genotypes, and therefore the relative contribution from FAs synthesized via the DNL pathway was lower. Percent contribution of DNL to total TG synthesized was significantly lower in the inguinal depots of CRH-Tg+ mice (48.6 ± 7.8% for WT vs. 16.4 ± 2.9% for CRH-Tg+, n = 6–8, P < 0.001; Table 2).

Measured fractional TG synthesis rates in the liver were significantly higher in CRH-Tg+ mice compared with their WT littermates (fraction new was 0.72 ± 0.11/wk in WT and 0.91 ± 0.07/wk in CRH-Tg+, n = 6–8, P < 0.01). Measured liver palmitate fractional DNL was not significantly different between the two groups (fraction new was 0.59 ± 0.06/wk in WT and 0.51 ± 0.10/wk in CRH-Tg+). However, given that liver TGs, including TG-glycerol and TG-palmitate, were almost fully turned over after 1 wk, making comparisons of fractional rates of liver TG synthesis unreliable for calculating half-lives. Because it has been shown that glyceroneogenesis plays a crucial role in TG/FA cycling (30), and that GCs decrease and increase glyceroneogenesis in the adipose tissue and liver, respectively (15), we used MIDA to identify the source of the TG-glycerol in adipose tissue and in liver and expressed this as percent glyceroneogenesis (8). Percent glyceroneogenesis indicates what percent of the TG-glycerol came from glyceroneogenic, rather than glycolytic, pathways. Percent glyceroneogenesis was not significantly different between the two genotypes in either fat depot or in liver (Fig. 6). Percent glyceroneogenesis was much higher in the liver than either fat pad of both genotypes (n = 6–8, P < 0.001) (Fig. 6). In summary, we observed that TG-glycerol in liver came mostly from glyceroneogenic pathways, whereas TG-glycerol in white adipose tissue came mostly from glycolytic pathways. Furthermore, chronic exposure to corticosterone did not significantly change the source of TG-glycerol in either tissue.

Because the 1-wk labeling study is well suited for adipose TG dynamics, but not liver kinetics, we performed an addi-
tional heavy water labeling experiment with a short labeling period to examine hepatic TG dynamics. Hepatic lipid content was analyzed in a separate cohort of WT and CRH-Tg mice 5 h after administration of a $D_2O$ injection bolus. The total deposition of TG in WT livers (expressed as mg/wk, for comparison with adipose TG deposition rates) was $1,042 \pm 235$ mg/wk in WT and $1,058 \pm 269$ mg/wk in CRH-Tg mice (Table 3). There was a trend toward increased hepatic DNL of palmitate in CRH-Tg mice (CRH-Tg 33.6 \pm 21.8 vs. WT 13.4 \pm 6.1 mg, $P = 0.052$; Table 3). The TG content of WT mice was 43 mg/liver and in CRH-Tg mice was 108 mg/liver (Table 3). The TG half-life in WT liver was 4.7 h, and it was significantly higher in CRH-Tg mice (10.0 h, $P < 0.05$; Table 3). Hepatic palmitate content was increased in CRH-Tg mice (10.5 mg/liver in WT vs. 32.5 mg/liver in CRH-Tg, $P < 0.05$; Table 3).

**DISCUSSION**

Although CRH-Tg$^+$ mice weigh the same as their WT littermates (Fig. 1A), DEXA revealed that CRH-Tg$^+$ mice have less lean tissue, more adipose tissue, and less bone mass, bone area, and BMD than their WT littersates (Fig. 1B), whereas micro-CT analysis revealed that CRH-Tg$^+$ mice had decreased bone cortical thickness at the proximal femur (Fig. 2D and Table 1). Our DEXA and micro-CT analyses differed with respect to BMD, with DEXA showing a decrease and micro-CT showing no difference in CRH-Tg$^+$ mice (Fig. 2C and Table 1, respectively). Because DEXA covers the entire skeleton and our micro-CT analysis was only done in proximal femur, it is possible that greater losses occur elsewhere in the skeleton to explain these observed differences. However, more likely this can be explained by a size artifact from DEXA, as has been described (10, 29), since CRH-Tg$^+$ bones are smaller than WT bones (Table 1). In accordance with this, the decrease in BMD was less than the decrease in bone area and bone mineral content. Unlike humans with Cushing’s disease, CRH-Tg$^+$ mice accumulate rather than lose fat in subcutaneous depots, so instead of redistributing fat from subcutaneous depots to other areas of the body as humans do, CRH-Tg$^+$ mice accumulate fat in both subcutaneous and abdominal depots (Table 2) although the various depots differ in their response to GCs (Fig. 1C). For the depots examined using stable isotopes, there was a small preferential increase in epididymal depot size compared with the inguinal depot. Although the phenotype of the CRH-Tg mouse is striking with respect to body composition, their overall body weights are not different from WT mice. More specifically, the replacement of 5 grams of lean mass with 5 grams of fat (as observed in CRH-Tg mice compared with WT mice) is equivalent to an added $\sim$3 grams of fat energy in WT mice, assuming caloric values of 9 and 4 kcal/g of fat (adipose) and lean mass (protein), respectively. As a result, the actual disruption in energy balance is mild in CRH-Tg mice. In accordance with this, we did not observe significant differences in the absolute mass of chow consumption. However, if one normalizes food consumption to lean body mass, CRH-Tg mice do eat more chow, given their decreased lean body mass (Fig. 1B). We observed that CRH-Tg$^+$ mice also have thinner skin than their WT littermates (Fig. 3A), just as patients with Cushing’s syndrome typically have thin skin.

It is worth briefly reviewing the significance and interpretation of metabolic labeling results under near-steady-state conditions present in this study (adult animals, body composition, and weight-stable over a 7-day labeling period). Absolute rates of synthesis of an end product, such as tissue collagen, muscle protein, and adipose TG, are measured from the fractional replacement rate per unit time multiplied by the pool size (17).
The fractional replacement rate constant ($k$) under steady-state conditions, in turn, can be shown to be determined by the breakdown rate ($41$). Many metabolic pathways exhibit coupling of synthesis and breakdown, such that increases or decreases in one result in parallel changes in the other ($11$). The effects of GCs on these processes are of potential interest for the pathophysiology of reduced protein and increased TG pool sizes in Cushing's syndrome.

CRH-Tg$^+$ mice had lower fractional synthesis rates, or replacement rates, of bone collagen and less bone mass compared with WT mice (Fig. 2 and Table 1) and therefore less absolute collagen synthesis. In addition, because fractional bone collagen synthesis was lower in CRH-Tg$^+$ mice, we conclude that this reduction in bone collagen synthesis was accompanied by a compensatory reduction in bone collagen degradation. CRH-Tg$^+$ mice had similar fractional synthesis rates of skin collagen synthesis (Fig. 3B) but had thinner skin compared with WT mice (Fig. 3A). Thus, we conclude that CRH-Tg$^+$ mice had reduced absolute rates of skin collagen synthesis with no compensatory reduction in breakdown rate (similar fractional synthesis rates between genotypes). CRH-Tg$^+$ mice had similar fractional muscle protein synthesis rates (Fig. 4B), less lean tissue mass (Fig. 1B), and smaller quadriceps muscles (Fig. 4A) compared with WT mice. Therefore, CRH-Tg$^+$ mice have absolute muscle synthesis in quadriceps with no compensatory reduction in breakdown rate.

Our estimates of absolute synthesis are indirect, in that we did not measure the actual pool size of collagen or muscle protein biochemically, but rather measured them by physical or radiographic methods. The tissue measurements are robust, however, and unlikely to be qualitatively incorrect.

It is possible that, in younger CRH-Tg mice, there would be a decrease in the fractional synthesis of skin collagen and muscle protein that would be responsible for the smaller pool size as adults, whereas, during the chronic phase of GC exposure, there was a normalization of the fractional synthesis rates. Indeed, we have previously observed decreases in fractional replacement rates of skin collagen and muscle protein in mice acutely exposed to GCs ($31$). Thus a lower absolute rate of synthesis is likely responsible for establishing a decreased pool size, and decrease in absolute rates of collagen synthesis in bone and skin and of protein in muscle contribute, at least in part, to maintaining GC-dependent decreases in bone mass and skin thickness and muscle mass, respectively, in a chronic setting. Interestingly, these results also point out that a failure to match or couple reduced breakdown rates to reduced synthesis rates contributes to the smaller pool size of skin collagen and muscle protein. In contrast, bone collagen did exhibit some compensatory reduction in fractional breakdown rate ($k$), or coupling, although not enough to maintain normal bone mass. Loss of the normal coupling mechanism in skin and muscle is an interesting action of GCs that requires further exploration.

In CRH-Tg$^+$ mice, we observed increased fat mass by DEXA and increased weights of dissected fat depots. Whereas humans with GC excess redistribute fat from peripheral to central depots, all depots of CRH-Tg$^+$ mice are increased in size. Despite this difference, CRH-Tg$^+$ mice still display increased FA/TG futile cycling. Therefore, in humans, there is an imbalance between TG synthesis and lipolysis across depots, and, in mice, this imbalance is overcome by the net increase in synthesis. We measured rates of TG synthesis and DNL in subcutaneous and abdominal adipose depots of CRH-Tg$^+$ mice. Although fractional rates of TG synthesis were only slightly higher in CRH-Tg$^+$ mice relative to WT mice in the inguinal depot, they were two times as high in the epididymal depot, and incorporating the larger fat pad masses of CRH-Tg$^+$ mice, the total amount of retained TG synthesized, meaning newly synthesized TG that was not broken down, over the 7-day labeling period was four- and sevenfold higher in CRH-Tg$^+$ mice in inguinal and epididymal depots, respectively.

We have previously shown that, in obese, leptin-deficient, adult ob/ob mice, absolute TG synthesis rates in adipose tissue are higher because of increased fat mass, but fractional TG replacement rates are the same as WT controls ($38$), unlike the CRH-Tg$^+$ mice, which have increased fractional TG replacement rates. Because fat mass was stable for both genotypes during the labeling period, we conclude that both absolute synthesis and lipolysis are several times higher in CRH-Tg$^+$ mice, that is, CRH-Tg$^+$ mice have increased TG turnover. This process can also be described as TG/FA futile cycling and is cell autonomous, since it occurs ex vivo ($6$) and ($43$) in vitro. The finding of increased TG/FA cycling in CRH-Tg$^+$ is corroborated by our recent findings showing increased expression of genes involved in both TG synthesis and lipolysis in the adipose tissue of CRH-Tg$^+$ mice ($44$). Specifically, lipin 1 (Lpin1) expression was increased approximately threefold, whereas glycerol 3-phosphate acyltransferase (Gpat) 3 and Gpat4 were increased by approximately fivefold and sixfold, respectively ($44$). Simultaneously, there was an approximately fivefold and threefold increase in expression of the lipases hormone-sensitive lipase (Lipe) and monoacylglycerol lipase (Mgl1) ($44$). We have therefore demonstrated that the physiological sequelae of increasing enzymes of TG synthesis (Lpin1,
The DNL results support this interpretation. Despite the fact that the total amount of TG synthesized was much greater in both depots in the CRH-Tg+ mice, the fractional contribution from DNL was lower in the subcutaneous depots and similar in the epididymal depots of CRH-Tg+ mice compared with WT mice. The amount of total retained palmitate synthesized via the DNL pathway was similar in the inguinal depot and fourfold higher in the epididymal depot of CRH-Tg+ mice relative to WT mice, demonstrating that the DNL pathway and absolute rates of DNL in adipose tissue are not inhibited by GC treatment. Rather, the lower relative contribution from DNL despite higher absolute DNL flux supports the conclusion that input from FA that had been derived from lipolysis in other depots accounts for the majority of TG turnover. This form of “futile cycling” accounts for most of the increased TG synthesis as measured by $^2$H label incorporation.

We were able to quantify glyceroneogenesis as a source for TG-glycerol in the liver, as well as in the inguinal and epididymal fat depots. It has previously been reported that GCs increase glyceroneogenesis in the liver and decrease glyceroneogenesis in the adipose tissue via differential regulation of PEP-CK (15). We report here that, although the major source of TG-glycerol is indeed glyceroneogenesis in the liver and glycolysis in the adipose tissue, no significant differences in percent contribution from glyceroneogenesis were observed between CRH-Tg+ mice and WT mice in either tissue. Analysis of a second cohort of animals revealed that CRH transgenic mice exhibit markedly increased hepatic DNL (34 vs. 13 mg palmitate synthesis/wk). Quantitatively, however, the added ~20 mg palmitate synthesized/wk represents only a modest addition to the increased whole body fat stores (~10 g whole body fat stores in transgenic mice vs. ~5 g in WT) to the ~3.5 g TG deposited in adipose tissue/wk in transgenic mice or the ~1 g TG deposited/wk in WT (Table 3).

The role of lipid synthesis in hepatic steatosis is more complex. The transgenic animals exhibited modest hepatic steatosis so that the addition of 20 mg palmitate DNL/wk per liver might make an impact. Measurement of hepatic TG kinetics suggests another mechanism underlying increased hepatic TG stores, rather than overproduction of TG by the liver. TG synthesis rates were in fact identical in WT and transgenic mouse livers (1,042 vs. 1,058 mg/wk, respectively). The kinetic explanation for elevated hepatic fat stores in transgenic mice was a reduced clearance or turnover rate of hepatic TG stores ($k = 0.069$ vs. $0.147/h, P < 0.05$). These results suggest disordered lipid export from the liver rather than overproduction of TG.

In summary, livers of CRH transgenic mice synthesize more de novo FA and have higher TG levels, but the latter is due to impaired clearance (export) of hepatic TG, not TG overproduction by the liver.

Where does the added body fat in transgenic mice come from? The net TG deposition rate in measured adipose depots was significantly higher in transgenic mice compared with WT. Both measured depots of CRH-Tg+ mice had a fractional new synthesis of ~40% new TG, indicating ~3.5 grams of newly synthesized TG per week when extrapolated over their 10 grams of adipose fat. In WT mice, the fractional synthesis varied by depot. Because it was not possible to assay every depot, we must rely on data from the measured depots to estimate whole body adipose TG synthesis. Assuming superficial depots such as the inguinal depot account for one-third of adipose and visceral depots account for two-thirds yields an estimated whole animal adipose TG synthesis rate of 1.2 grams for WT mice. Furthermore, whereas adipose and hepatic TG synthesis rates were similar in WT mice (~1 g/wk for each tissue), they were three to four times higher in adipose compared with liver in transgenic mice (~3.5 g/wk in adipose vs. ~1 g/wk in liver). Therefore, we conclude that adipose tissue synthesis is the major contributor to the excess TG deposition in adipose tissue in CRH-Tg mice.

Palmitate DNL was a small fraction of synthesized TG in adipose tissue. The fractional synthesis of palmitate in the two depots was similar in CRH-Tg mice, and extrapolating to total body fat yields 135 mg new palmitate/wk. In WT mice, the fractional synthesis of palmitate varied by depot. Using the previously mentioned assumptions yields a level of adipose new palmitate of 100 mg/wk. The question, what tissue did the stored adipose FA derived from DNL come from, adipose itself or liver, was addressed indirectly by our data. Hepatic palmitate DNL in WT was 13% of the measured adipose DNL value (~13 mg/wk in liver compared with 100 mg/wk deposited in adipose tissue); and hepatic DNL was ~25% of deposited adipose DNL in transgenic mice (34 vs. 135 mg/wk). In summary, CRH transgenic mice exhibited markedly higher hepatic DNL than WT controls. The liver contributed 13–25% of accumulated DNL in adipose tissue, with a higher proportion observed in CRH transgenic mice.

In summary, we performed an evaluation of key metabolic pathways affected by chronic endogenous GC exposure in CRH-Tg+ mice, a mouse model that closely mimics the etiology and symptoms of Cushing’s syndrome in humans. We believe our novel study using stable isotope labeling of mice is the most complete study examining the complex multiorgan metabolic effects of GCs in vivo. In mice exposed to chronically high levels of endogenous GCs, bone mass, skin thickness, muscle mass, and absolute rates of bone and skin collagen synthesis and muscle protein synthesis were dramatically decreased compared with WT controls. In addition, there was a compensatory reduction in fractional breakdown (replacement) rate of bone collagen but not skin collagen or muscle protein. We conclude that reduced synthesis occurs for bone collagen, skin collagen, and muscle protein and that the expected physiological coupling of breakdown and synthesis is lost for skin collagen and muscle protein during chronic overproduction of endogenous GCs. Both reduced synthesis and failure to reduce breakdown contribute to the maintenance of reduced protein pool sizes in this model of chronic GC excess. In CRH-Tg+ mice we also observed more TG synthesis in subcutaneous and abdominal fat depots, more DNL in the abdominal depot only, and no difference in glyceroneogenesis in either depot compared with WT controls. Furthermore, consistent with our previous work showing increased expression of genes involved in both TG synthesis and lipolysis (44), we demonstrated that increased TG/FA futile cycling occurs in both abdominal and subcutaneous fat depots in CRH-Tg+ mice, and, in these mice, net TG storage is favored over lipolysis. Further examination of TG hepatic kinetics revealed that the liver was a minor player in the observed increased adipose TG/FA cycling observed in CRH-Tg mice. Our studies shed light on the molecular mechanisms of GC-mediated changes in metabolism, which may be helpful in designing safer GCs for the treatment of inflammatory disease. In addition, given the implication of GC action in the pathophysiol-

Gpat3, Gpat4) and lipolysis (Lipe, Mgl) is futile cycling of FA in and out of the TG pool.
ogy of metabolic syndrome (4, 40), these findings may give insight into potential therapies for this disease of epidemic proportions.

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DISCLOSURES

The authors have no conflicts of interest to report.

AUTHOR CONTRIBUTIONS

Author contributions: C.H. and D.J.R. conception and design of research; C.H., D.J.R., M.F., B.M.B., and B.P.H. performed experiments; C.H., D.J.R., M.F., B.M.B., and B.P.H. analyzed data; C.H., D.J.R., and M.K.H. interpreted results of experiments; C.H., D.J.R., and M.F. prepared figures; C.H. and D.J.R. drafted manuscript; C.H. edited and revised manuscript; C.H., M.F., and M.K.H. approved final version of manuscript.

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