Greater replication and differentiation of preadipocytes in inherited corticosteroid-binding globulin deficiency

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1Department of Medicine, Redland Hospital, Cleveland, Queensland 4163; 2Department of Medicine, University of Queensland, and 3Department of Diabetes and Endocrinology, Princess Alexandra Hospital, Woolloongabba, Queensland 4102; and 4Greenslopes Private Hospital, Greenslopes, Queensland 4120, Australia

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Joyner, J. M., L. J. Hutley, A. W. Bachmann, D. J. Torpy, and J. B. Prins. Greater replication and differentiation of preadipocytes in inherited corticosteroid-binding globulin deficiency. Am J Physiol Endocrinol Metab 284: E1049–E1054, 2003. First published January 28, 2003; 10.1152/ajpendo.00262.2002.—Glucocorticoids are pivotal for adipose tissue development. Rodent studies suggest that corticosteroid-binding globulin (CBG) modulates glucocorticoid action in adipose tissue. In humans, both genetic CBG deficiency and suppressed CBG concentrations in hyperinsulinemic states are associated with obesity. We hypothesized that CBG deficiency in humans modulates the response of human preadipocytes to glucocorticoids, predisposing them to obesity. We compared normal preadipocytes with subcultured preadipocytes from an individual with the first ever described complete deficiency of CBG due to a homozygous null mutation. CBG-negative preadipocytes proliferated more rapidly and showed greater peroxisome proliferator-activated receptor-γ-mediated differentiation than normal preadipocytes. CBG was not expressed in normal human preadipocytes. Glucocorticoid receptor number and binding characteristics and 11β-hydroxysteroid dehydrogenase activity were similar for CBG-negative and normal preadipocytes. We propose that the increased proliferation and enhanced differentiation of CBG-negative preadipocytes may promote adipose tissue deposition and explain the obesity seen in individuals with genetic CBG deficiency. Furthermore, these observations may be relevant to obesity occurring with suppressed CBG concentrations associated with hyperinsulinemia.

Preadipocytes also express GRs, with regional and gender differences in GR complement (16).

Recent studies of lean and obese Zucker rats suggest that glucocorticoid action in adipose tissue may be modulated by corticosteroid-binding globulin (CBG). CBG is a 383-amino acid member of the serine protease inhibitor family of proteins. It is secreted by hepatocytes and binds over 90% of circulating cortisol under normal conditions. CBG constitutes a greater proportion of the total protein in rat white adipose tissue than in other tissues, including the liver (11). Furthermore, obese rats have less CBG in plasma and white adipose tissue than lean rats, and there is less CBG in visceral adipose tissue than in subcutaneous adipose tissue (12).

In humans, plasma CBG levels are inversely correlated with body mass and body mass index (20), and genetic CBG deficiency appears to be associated with obesity. An Italian-Australian family with a complete loss of function (null) mutation of the CBG gene, caused by a premature stop codon, has recently been characterized (34). Plasma CBG was undetectable by radioimmunoassay for three individuals homozygous for the null mutation, with ~50% normal CBG levels for null heterozygotes. Interestingly, individuals homozygous for the null mutation were relatively obese compared with other family members (34). A previous report of complete CBG deficiency described a boy, born to parents who were first cousins, who came to medical attention because he was obese. He was assessed as being CBG deficient on the basis of a lack of cortisol binding in serum and low total serum cortisol but with normal free cortisol levels (31). There are two CBG mutations associated with reduced cortisol-binding efficiency. With the CBG Lyon mutation, the homozygous individual was obese (9), but there was no comment on the weight of the individuals with the transcortin Leuven CBG variant (35). We hypothesized that CBG is an important modulator of cortisol action in preadipocytes and that CBG deficiency is associated with a change in glucocorticoid response in human preadipocytes that predisposes to obesity. We aimed to compare glucocorticoid-depen-

GLUCOCORTICOIDs ARE PIVOTAL FOR adipose tissue development. Endogenous or exogenous glucocorticoid excess is characterized by increased adipose tissue mass, especially centrally, with an increase in total fat cell number (19). Glucocorticoids act directly on adipose tissue. In vitro glucocorticoids increase both lipoprotein lipase activity in adipose tissue (27) and preadipocyte differentiation in a dose-dependent manner (13). Glucocorticoid receptors (GR) are present in human adipose tissue, with a greater GR density in visceral compared with subcutaneous adipose tissue (23, 29).

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dent activities of preadipocytes from an individual who is homozygous for the CBG null mutation (34) with those of normal preadipocytes. We compared rates of replication and differentiation capacity of these cells. Upon finding differences in these activities, we studied whether human preadipocytes expressed CBG, and we compared GR characteristics and 11β-hydroxysteroid dehydrogenase (11β-HSD) activity as possible mediators of the differences found.

METHODS

Subjects and sample preparation. Abdominal subcutaneous fat of $-1\, \text{cm}^3$ was obtained (with informed consent) by biopsy through a 1-cm periumbilical incision from a 57-yr-old male [body mass index (BMI) 39.4, waist 125 cm] homozygous for the CBG null mutation. As controls, abdominal subcutaneous adipose tissue samples of $-1\, \text{cm}^3$ were collected (with informed consent) during elective surgery from Caucasian male subjects (see Table 1 for age, BMI, and waist data). It was important to compare samples from the same gender and site, because there are regional differences in rates of differentiation (1) and 11β-HSD activity (6) and regional and gender differences in GR (16). All subjects provided informed written consent. The Greenslopes Private Hospital Ethics Committee and the Princess Alexandra Hospital Research Ethics Committee provided ethical approval for the work. The normal subjects were not specified according to race for the work. The normal subjects were not specified.

Samples were transported to the laboratory in Ringer solution and were processed within 15 min by collagenase digestion, as previously described (30). The stromovascular fraction was plated into culture solution and were processed within 15 min by collagenase work and the work of others suggest that severe mutations of the CBG gene are quite rare (26).

Differentiation. Confluent monolayers of subcultured preadipocytes in 25-cm² flasks were washed, and differentiation medium (DMEM-Ham’s F-12 with 100 nM rosiglitazone, 0.25 mM IBMX for the first 2 days, 100 IU of penicillin, 100 µg/ml streptomycin, 2 mM l-glutamine, 100 nM cortisol, 0.2 nM triiodothyronine, 500 nM insulin, 17 µM pantothenate, 33 µM biotin, 10 µg/ml transferrin, and 15 mM HEPES) or a differentiation medium without rosiglitazone was applied. Rosiglitazone is a peroxisome proliferator-activated receptor-γ (PPARγ) activator that has been previously demonstrated to enhance the differentiation of normal human preadipocytes that have been grown in SCM (1). Differentiation was assessed after 14 days by measuring glycerol-3-phosphate dehydrogenase (G3PD) activity, as previously described (15). Briefly, the SDS-soluble protein sample was assayed using the Bio-Rad 3550 microplate reader. The MTS proliferation assay was validated by confirming that the formazan absorbance vs. direct cell counts were linear over the range studied.

Table 1. Characteristics of normal preadipocyte sample donors

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<th>Sample</th>
<th>Age, yr</th>
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<td>61</td>
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BMI, body mass index.
CBG gene expression. Total RNA was extracted from confluent preadipocytes and stored at −70°C before being reverse-transcribed using the random hexamer priming option of a commercially available kit (SUPERSCRIPT, Life Technologies, Gaithersburg, MD). The integrity of the reverse-transcription step was checked by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RT-PCR. A negative control, with no reverse transcriptase added, was included for each RNA sample. Previously published CBG (24) and GAPDH (8) primer sequences and a second set of CBG primers were designed (sense 5′-GACAAAGGAAGATGACAC-3′, antisense 5′-GCACAGCTTTATGCGACC-3′) were obtained commercially. The PCR was performed in a Corbett Research PC-90 microplate thermal sequencer with an annealing temperature of 55°C and 2 mM MgCl₂, these parameters having been optimized for the primers by use of HepG2 cDNA as the positive control. The PCR products were visualized under ultraviolet illumination after electrophoresis on an ethidium bromide-labeled 2% DNA agarose gel.

GR assessment. Dexamethasone binding as a measure of GR number was assessed using a whole cell-binding assay, as previously described (16). Dexamethasone has minimal affinity for CBG (26). Briefly, 25-cm² flasks of confluent cells were washed with PBS and preincubated in DMEM-Ham’s for 30 min at 37°C. This was followed by a 60-min incubation with one of six serial dilutions of 0.78–25 nM tritiated dexamethasone ([³H]Dex; 70–110 Ci/mm, Amersham Australia) or 0.78–25 nM [³H]Dex with a 250 times excess of unlabeled dexamethasone (Sigma-Aldrich) in DMEM-Ham’s. After the cells were washed with ice-cold PBS, they were lysed, and an aliquot was taken from each flask to count bound [³H]Dex and for protein determination (3). A Scatchard plot provided the binding characteristics for each sample.

11β-HSD activity. Confluent preadipocytes in 25-cm² flasks were washed with PBS and DMEM-Ham’s F-12, with 500 nM tritiated cortisol or cortisone applied in triplicate for a 6-h incubation. The cortisone and cortisol in the media were measured by an improved method of HPLC, as previously reported (22). The cells were washed with PBS and lysed by sonication, and the protein was measured (3) to allow the calculation of cortisol/cortisone interconversion in femtomoles per milligram protein per hour. There were no measurable amounts of cortisol or cortisone in the PBS washes or the cell lysates.

Statistical analysis. Comparisons of replication and G3PD activity used the Student’s t-test (two-tailed). The statistical analyses were performed with the data analysis function of Microsoft Excel, version 5. Statistical significance was defined as P < 0.05.

RESULTS

Proliferation. The CBG-negative cells grew to confluence more quickly than normal preadipocytes, requiring subculturing more frequently. For this reason, the proliferation comparisons were done both with samples that had been grown in vitro for the same period of time and with separate samples that were the same passage number. The CBG-negative preadipocytes proliferated more quickly than any of the normal preadipocyte samples (P < 0.0001 for all; Fig. 1). Cortisol and the antiglucocorticoid RU-486 had no influence on proliferation rates for CBG-negative or normal preadipocytes in these short-term incubation studies (data not shown).

Despite proliferating more quickly, the CBG-negative preadipocytes were morphologically indistinguishable from normal preadipocytes, and premature senescence was not apparent. Their ongoing proliferation was noted for ≤5 mo in vitro compared with 4 mo for normal preadipocytes.

Differentiation. CBG-negative preadipocytes differentiated more readily than normal preadipocytes in differentiation medium containing rosiglitazone. CBG-negative preadipocytes had visibly greater lipid accumulation and had a 10-fold greater G3PD activity than similarly treated normal preadipocyte samples (1,333 vs. 52, 68, 118, 142, 198, and 254 mU/mg protein, or P = 0.005, 0.006, 0.006, <0.001, 0.001, 0.012, respectively; Fig. 2). In the absence of rosiglitazone, CBG-negative and normal preadipocytes showed similar low levels of G3PD activity (47 vs. 14, 36, and 68 mU/mg protein).

CBG gene expression. There was no evidence of CBG mRNA in normal human preadipocytes. With use of two different sets of CBG primers with expected PCR products of 247 and 296 base pairs, the PCR produced strong bands in the HepG2-positive control lanes, but there were no bands in any preadipocyte lane (Fig. 3).

GR. With use of dexamethasone whole cell-binding assays, the GR characteristics were the same for the CBG-negative and normal preadipocytes. For three assays, including both strains of CBG-negative preadipocytes, the dissociation constants (Kd) were 6.8, 9.5, and 12.1 nM vs. 5.1–12.6 nM for normal preadipocytes (Fig. 4), and the maximal binding capacities (Bmax)
were 263, 488, and 603 fmol/mg vs. 327–599 fmol/mg for normal preadipocytes (Fig. 5).

11β-HSD activity. Type 1 11β-HSD activity was normal, with cortisone-to-cortisol conversion for the two CBG-negative preadipocyte strains being 14 and 22 fmol·mg protein$^{-1}$·h$^{-1}$ vs. normal preadipocytes 10, 10, 17, and 18 fmol·mg$^{-1}$·h$^{-1}$ (Fig. 6). There was minimal cortisol-to-cortisone conversion (type 2 11β-HSD activity) in CBG-negative or normal cells.

DISCUSSION

Preadipocytes from an individual with complete CBG deficiency had increased proliferation and enhanced PPARγ-mediated differentiation compared with normal preadipocytes. These characteristics may promote adipose tissue deposition by increasing the number of preadipocytes and their conversion to mature adipocytes. The observations may be relevant to the role of CBG in obesity reported in genetic CBG deficiency (34) and the potential role of suppressed CBG levels in hyperinsulinemic humans (10).

The mechanism of hyperproliferation and accentuated thiazolidinedione-stimulated differentiation in preadipocytes from genetically CBG-deficient individuals is unclear, as CBG gene expression was not detected in cultured human preadipocytes. Furthermore, measures of GR binding and 11β-HSD activity were normal.

CBG may be narrowly expressed in human tissues, with definite tissue expression demonstrated in hepatocytes and in placental syncytiotrophoblasts (24) where CBG may modulate glucocorticoid and progesterone tissue interactions (2). CBG has been reported in rodent adipose tissue by corticosterone binding but not gene expression (11, 12), and this may represent circulating CBG from plasma taken into cells. There is speculation in the literature regarding this for other cell types with putative CBG membrane receptors involved (32). The fact that corticosterone binding in rodent white adipose tissue mirrors plasma CBG levels (12) supports the idea that CBG may be sequestered in adipose tissue and/or taken up by preadipocytes/adipocytes. The facts that there is less CBG in the adipose tissue of obese rats than in that of lean rats, and that there are regional differences in CBG in adipose tissue, subcutaneous greater than visceral, strongly suggest that CBG is an important modulator of glucocorticoid activity in adipose tissue (12).

However, this does not explain why preadipocytes from a CBG-negative individual should respond differently than normal preadipocytes under identical in vitro conditions if preadipocytes do not express CBG. CBG influences the kinetic parameters of cortisol transport and clearance. In studies comparing subjects with normal or high CBG concentrations (using estrogen-containing oral contraceptives), increased CBG levels were associated with a reduced rate of cortisol clearance and an increased mass of circulating cortisol in a smaller volume of distribution (4). It may simply be that long-term growth in a CBG-deficient environment, in vivo, causes changes in preadipocyte growth and differentiation that are sustained in vitro for at least four passages. This is indirectly supported by the fact that there were no short-term differences in glucocorticoid responsiveness and no change in replication

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*E1052* PREADIPOCYTE GROWTH AND DIFFERENTIATION IN CBG DEFICIENCY

AJP-Endocrinol Metab • VOL 284 • MAY 2003 • www.ajpendo.org
rate with additional glucocorticoid or with the antグルココルチコイド RU-486.

Alternatively, the lack of CBG during fetal development may modulate glucocorticoid action, causing permanent changes in gene expression in preadipocytes. CBG is present in a number of fetal tissues, with temporal and spatial changes in CBG localization suggesting that CBG influences steroid hormone activity in fetal tissues (33). Glucocorticoids are involved in gene programming, with one example being that of glucocorticoid exposure in late gestation causing permanent changes to rat hepatic phosphoenolpyruvate carboxykinase gene expression (25).

We looked for mechanisms that may be mediating the different responsiveness of the preadipocytes from a CBG-deficient individual compared with control preadipocytes. We studied GR number, because the GR is a significant regulator of glucocorticoid action in many tissues and because glucocorticoids downregulate their own receptor. We also compared their 11β-HSD activity, as local interconversion of cortisol and cortisone is known to have significant effects on glucocorticoid responsiveness at a tissue level, and again glucocorticoids regulate (increase) type 1 11β-HSD activity (6). There were no differences in either GR number or characteristics or in 11β-HSD activity between CBG-negative individuals and control subjects. The enhanced differentiation occurred only in the presence of rosiglitazone, suggesting that the effect requires the presence of PPARγ-mediated mechanisms. This is not surprising, as glucocorticoids induce CAAT-enhancing binding protein (C/EBP)-δ, which in turn induces PPARγ and C/EPBα. PPARγ and C/EPBα act synergistically to promote adipocyte differentiation (21).

It is important to consider whether the differences in the CBG-negative preadipocyte’s replication and differentiation could be due to effects other than CBG’s effects on cortisol action. CBG also binds 17α-hydroxyprogesterone, but as no progesterone binding has been shown in human adipose tissue (5, 28), it is unlikely that the differences we have demonstrated are secondary to direct changes in progesterone responsiveness.

Genetic abnormalities resulting in a reduced amount of CBG protein or cortisol-binding activity are rare (26), but hyperinsulinemia is associated with reduced CBG concentrations (10). In in vitro studies of HepG2 cells, insulin reduced CBG secretion in a dose-dependent manner, suggesting a causal relationship (7). If, as our study suggests, lower CBG concentrations influence adipose tissue development, this may be another aspect of the pathophysiology of the metabolic syndrome. This may be important in the treatment of type 2 diabetes mellitus when insulin-sensitizing agents are compared with agents that increase insulin concentrations. Furthermore, efforts to increase CBG concentrations toward normal may attenuate some aspects of the metabolic syndrome.

In summary, we have shown that genetic CBG deficiency is associated with enhanced proliferation and differentiation of human preadipocytes. We were unable to elicit mechanisms by which CBG deficiency influences preadipocyte metabolism. The effect of CBG deficiency on preadipocyte function may be due to altered circulating cortisol kinetics or an effect on adipocyte development/programming early in life. We propose that the increased proliferation and enhanced differentiation of CBG-negative preadipocytes may promote adipose tissue deposition and explain the obesity seen in individuals with genetic CBG deficiency. Furthermore, these observations may be relevant to obesity occurring with suppressed CBG concentrations associated with hyperinsulinemia.

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