Chronic Glucocorticoid Exposure-Induced Epididymal Adiposity Is Associated with Mitochondrial Dysfunction in White Adipose Tissue of Male C57BL/6J Mice

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Abstract

Prolonged and excessive glucocorticoids (GC) exposure resulted from Cushing’s syndrome or GC therapy develops central obesity. Moreover, mitochondria are crucial in adipose energy homeostasis. Thus, we tested the hypothesis that mitochondrial dysfunction may contribute to chronic GC exposure-induced epididymal adiposity in the present study. A total of thirty-six 5-week-old male C57BL/6J mice (~20 g) were administrated with 100 μg/ml corticosterone (CORT) or vehicle through drinking water for 4 weeks. Chronic CORT exposure mildly decreased body weight without altering food and water intake in mice. The epididymal fat accumulation was increased, but adipocyte size was decreased by CORT. CORT also increased plasma CORT, insulin, leptin, and fibroblast growth factor 21 concentrations as measured by RIA or ELISA. Interestingly, CORT increased plasma levels of triacylglycerols and nonesterified fatty acids, and up-regulated the expression of both lipolytic and lipogenic genes as determined by real-time RT-PCR. Furthermore, CORT impaired mitochondrial biogenesis and oxidative function in epididymal WAT. The reactive oxygen species production was increased and the activities of anti-oxidative enzymes were reduced by CORT treatment as well. Taken together, these findings reveal that chronic CORT administration-induced epididymal adiposity is, at least in part, associated with mitochondrial dysfunction in mouse epididymal white adipose tissue.

Introduction

Glucocorticoids (GC) are adrenal cortex-secreted steroid hormones stimulated upon hypothalamic-pituitary-adrenal (HPA) axis activation due to endocrine disorder and/or stress [1–3]. Patients with chronic exposure to excess GC, either resulted from Cushing’s syndrome or receiving GC therapy for the purposes of anti-inflammatory and/or immunosuppression, develop numerous metabolic changes including obesity, fatty liver, dyslipidemia, and glucose intolerance [4–6]. Obesity is part of metabolic syndrome characterized by central adiposity caused by distributing fat mass to the metabolically more active visceral fat [8,9]. In addition, mice selectively expressing the glucocorticoid-activating enzyme, 11 β-hydroxysteroid dehydrogenase type 1 (11βHSD1), in adipose tissue shows susceptibility of high-fat diet-induced obesity [23,24]. Thus, mitochondrial play prominent role in WAT lipid homeostasis.
The objective of the present study was to test the hypothesis that impaired mitochondrial functions may contribute to chronic GC exposure-induced epididymal adiposity.

Materials and Methods

Ethics statement
All animal-related procedures were approved by Sichuan Agricultural University Institutional Animal Care and Use Committee (M2012-07-08). All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

Animals, housing, and treatment
Thirty-six 5-week-old male C57BL/6J mice (~20 g) that are originally purchased from Jackson Laboratory (Bar Harbor, ME, USA) were obtained from Sichuan Academy of Medical Sciences (Chengdu, Sichuan, China). Mice were group-housed (3 mice/cage) in standard polycarbonate cages at 21 ± 2°C on timed 12-h dark, 12-h light cycles and had free access to standard rodent chow and tap water. After 1-week acclimation, animals were assigned into 2 treatments by body weight. According to previous report [25], 100 μg/ml of CORT (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in ethanol, and then diluted in tap water to a final ethanol concentration of 1% (v/v). Mice were provided with drinking water containing 1% ethanol by CORT or 1% ethanol solution alone (vehicle). During the experimental period, ad libitum diet remained available.

Animals were weighed weekly. After 4-week of the treatment, mice were anesthetized and sacrificed. Blood was collected and centrifuged at 3000 g for 15 min at 4°C. Plasma samples were stored at −20°C for future measurements. Epididymal, retroperitoneal and inguinal subcutaneous WAT depots were dissected and weighed as previously described [26]. The tissue samples were frozen and stored at −80°C for further analysis.

Histological analysis
The collected epididymal WAT was fixed in formalin solution, dehydrated, paraffin-embedded, and sectioned. Sections (10 μm thick) were stained with hematoxylin and eosin, then examined and photographed under Olympus Microscope Digital Camera DP21 (Olympus China, LTD., Beijing, China). Adipocyte sizes were measured for at least 200 individual cells per mice with Image J software (National Institutes of Health, Bethesda, MD, USA).

Hormone and biochemical measurements
Plasma CORT concentrations were measured using a commercial radioimmunoassay (RIA) kit from MP Biomedicals Inc. (Solon, OH, USA). Plasma insulin, leptin contents were determined with specific mouse enzyme-linked immunosorbent assay (ELISA) kits (Millipore, Billerica, MA, USA). For plasma fibroblast growth factor 21 (FGF21), ELISA kit (BioVendor, Candler, NC, USA) was applied to measure the concentrations. All measurements were performed according to respective manufacturer’s instructions.

The concentrations of triglyceride, NEFA and malondialdehyde (MDA) were measured using commercial assays (Jiancheng Institute of Bioengineering, Nanjing, China) as described previously [27]. The activities of superoxide dismutase (EC Number: 1.15.1.1), glutathione peroxidase (EC Number: 1.11.1.9) and catalase (EC Number: 1.11.1.6) were measured using specific commercial kits (Jiancheng Institute of Bioengineering, Nanjing, China) according to the provided instructions, respectively.

DNA and RNA extraction
Total RNA from frozen adipose tissues was extracted using TRI reagent (Takara, Dalian, China) and further purified with Qiagen RNeasy Mini kit (Qiagen, Valencia, CA, USA) [27]. Total DNA was isolated from epididymal WAT using a DNAiso Reagent (Takara). Concentration and integrity of the extracted RNA were determined with an Agilent 2100 Bioanalyzer (Santa Clara, CA, USA).

Quantitative real-time PCR
Quantitative Real-time PCR was conducted as previous report [28,29]. Briefly, the cDNA was synthesized from 1 μg total RNA using random primers and reverse transcriptase (Takara). Real-time quantitative PCRs were performed using Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems 7900HT real-time PCR system. The conditions for these PCRs were 40 cycles of 95°C for 15 sec and 60°C for 1 min. The real-time PCR of each sample were performed in duplicate. The data were analyzed using 18S rRNA as the internal control with the cycle threshold (2−ΔΔCT) method, as recommended by Applied Biosystems. Based on the Ct values, adipose expression of 18S rRNA was not affected by CORT treatment. The mtDNA analysis was performed as described [30], mtDNA was amplified using primers specific for the mitochondrial cytochrome b (Cyt B) gene and normalized to genomic DNA by amplification of the large ribosomal protein p0 (36B4). All primers used in this study are shown in Table 1.

Mitochondrial biogenesis methods
Citrarate synthase (EC Number: 2.3.3.1) activity in isolated mitochondria preparations of epididymal WAT was measured with spectrophotometer using citrate synthase kit (Sigma-Aldrich) following manufacturer’s instructions. NAD⁺ and NADH levels in isolated mitochondria preparations were determined using the NAD/NADH assay kit purchased from BioAssay Systems (Hayward, CA, USA). Mitochondria were isolated using the Qproteome Mitochondria Isolation Kit (Qiagen).

Statistical analysis
All statistical analyses were performed using SAS statistical packages (SAS Institute, Cary, NC, USA). Unpaired t test were applied to evaluate the comparisons between two groups. Data are presented as the mean ± SEM. Differences at P<0.05 are considered significant.

Results
Chronic CORT mildly decreases body weight
We treated adult mice with 100 μg/ml of CORT through drinking water for 4 weeks to determine the effect of chronic CORT exposure on body weight change in mice. Mice in CORT group lost weight after the first week of treatment, but this did not reach statistical significance (P>0.05, Fig. 1). In the subsequent weeks, CORT-treated animals were smaller than vehicle-treated controls, but there is no significance (P>0.05, Fig. 1). CORT exposure slightly decreased the daily food intake (5.30±0.33 g vs 4.46±0.28 g, P>0.05). No significant change in daily drinking water intake was observed after CORT treatment (6.95±0.43 ml vs 7.13±0.15 ml, P>0.05).
Chronic CORT exposure increases epididymal fat accumulation, but decreases adipocyte size

The body composition of CORT-treated mice was subsequently analyzed to determine the relative amounts of WAT. CORT exposure significantly increased epididymal WAT mass \((P\lt 0.05, \text{Fig. 2A})\), but did not alter retroperitoneal WAT \((0.154 \pm 0.012 \, \text{g vs} \, 0.149 \pm 0.007 \, \text{g}, \, P>0.05)\) and inguinal WAT \((0.287 \pm 0.048 \, \text{g vs} \, 0.267 \pm 0.043 \, \text{g}, \, P>0.05)\) weight compared with controls. Moreover, epididymal WAT made up a significantly larger proportion of their total body weight when compared to control mice \((P\lt 0.05, \text{Fig. 2B})\). Interestingly, in the histological analysis, CORT-treated mice had significantly smaller adipocytes in their epididymal WAT compared with vehicle-treated animals \((P\lt 0.05, \text{Fig. 2C and 2D})\), suggesting that the increased epididymal WAT mass is resulted from adipocyte hyperplasia.

Chronic CORT exposure changes plasma hormone levels and lipid profile

To determine the circulating levels of hormones included in the regulation of lipid metabolism, plasma concentrations of CORT, insulin, leptin, and FGF21 were measured. Meanwhile, plasma triacylglycerols and NEFA levels were tested to determine the blood lipid profile. As expected, chronic CORT exposure induced significant increase in plasma CORT concentration compared with the control group \((P<0.05, \text{Table 2})\). CORT also significantly increased plasma concentrations of insulin, leptin and FGF21 when compared to control mice \((P<0.05, \text{Table 2})\). CORT group showed significantly higher triacylglycerols and NEFA levels compared with the vehicle group \((P<0.05, \text{Table 2})\).

### Table 1. Primer sequences used in this study.

| Gene       | Sequence (5'-3')                        | Accession No. | Amplicon size (bp) |
|------------|------------------------------------------|---------------|-------------------|
| CytB       | F: TGAGGGGGCCCTTCCTCAGTGA               | NC_005089     | 118               |
|            | R: CTGTTTTGTGGAGGAAGAGG                 |               |                   |
| 36B4       | F: TGCCACACTCCCATCATCAA                 | NM_007475     | 240               |
|            | R: CAAAGACCGCAATCCTCATA                 |               |                   |
| PGC-1α     | F: AAAGCCGCTGGCTTACGAC                  | NM_008904     | 193               |
|            | R: GTGGAGGAGGGTGCTATG                   |               |                   |
| NRF-1      | F: CCAGTGGATGAGTACAGCG                  | NM_01164226   | 120               |
|            | R: GCACACATTCTCCTCAAGG                  |               |                   |
| CytC       | F: CCAATCTCCACGGCTCTGTT                 | NM_007808     | 100               |
|            | R: TACTCTCTCCCCAGTGATG                  |               |                   |
| IDH3α      | F: GAGGTGTGCTGTGTTGT                   | NM_026973     | 155               |
|            | R: TCCTCTCTCTTCCTGAAAGT                |               |                   |
| SIRT1      | F: AGTTCACCAGCTCTGCTGTG                 | NM_019812     | 198               |
|            | R: CTCCAGCAACAGCTCTCACA                |               |                   |
| PPARγ      | F: CCTCTGCCAAAGCATTTTGT                | NM_001172330  | 225               |
|            | R: GAACCTCTGCCACCTCTGAAA                |               |                   |
| CPT-1α     | F: CCAGGCTACAGTGAGGACATT               | NM_013495     | 100               |
|            | R: AAGGAATGCAAGCTCAACAT                |               |                   |
| HSL        | F: TGCTCTCTCTGAGGTGTA                  | NM_017619     | 183               |
|            | R: TCCTGCTGCTTTGTGAT                   |               |                   |
| ATGL       | F: TACCGGTGATGAGAAAGAGC                | NM_00116389   | 112               |
|            | R: CAGTCCACCTCTCAGACA                  |               |                   |
| FAS        | F: CCTGTGATAGAGGGATGAGA                | NM_007988     | 115               |
|            | R: ACTCCACAGGTGGAGAAAAGA               |               |                   |
| m18S rRNA  | F: ACCGGGTTCTTATTTTGTG                 | NR_003278     | 181               |
|            | R: TCGTCTCGAAACTCCGACT                 |               |                   |

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![Figure 1. Effect of CORT on body weight of mice.](image1.png)
Figure 2. Effect of CORT on epididymal WAT weight and adipocyte size of mice. (A) Weight of epididymal white adipose tissue (WAT) and (B) relative contribution of epididymal WAT to total body weight in mice treated with 100 μg/ml corticosterone (CORT) or vehicle for 4 weeks. (C) Paraffin sections of epididymal WAT of mice treated with CORT or vehicle were stained with hematoxylin and eosin. Scale bar: 100 μm. (D) The average adipocyte cell area of epididymal fat in mice treated with CORT or vehicle. Data are expressed as group means ± SEM (n = 6 by group). * P < 0.05 compared with vehicle.

Table 2. Effect of CORT on plasma hormone levels and lipid profile of mice.

|                  | Vehicle   | CORT      |
|------------------|-----------|-----------|
| Corticosterone (ng/ml) | 23.0 ± 2.5 | 195.1 ± 16.6* |
| Insulin (ng/ml)    | 1.52 ± 0.21 | 7.49 ± 0.15* |
| Leptin (ng/ml)     | 1.61 ± 0.06 | 7.37 ± 0.90* |
| FGF21 (pg/ml)      | 165.0 ± 8.7 | 355.1 ± 14.0* |
| Triacylglycerols (mM) | 0.58 ± 0.16 | 1.20 ± 0.16* |
| NEFA (μM)          | 449.1 ± 70.7 | 686.4 ± 77.7* |

Plasma concentrations of corticosterone, insulin, leptin, fibroblast growth factor 21 (FGF21), triacylglycerols and nonesterified fatty acids (NEFA) in mice treated with 100 μg/ml CORT or vehicle for 4 weeks.

Data are expressed as group means ± SEM (n = 6 by group).

* P < 0.05 compared with vehicle.

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Chronic CORT exposure alters the expression of lipolysis and lipogenesis-related genes in epididymal WAT

We next determined the effects of chronic CORT exposure on the expression of genes related to lipolysis and lipogenesis. The expression of hormone-sensitive lipase (HSL) in epididymal WAT was significantly increased by CORT treatment \( (P<0.05, \text{Fig. 3A}) \). CORT also induced a 40% decrease in citrate synthase activity \( (P<0.05, \text{Fig. 4B}) \). Furthermore, CORT exposure increased the activities of superoxide dismutase, glutathione peroxidase and catalase in epididymal WAT of CORT-treated mice compared with vehicle \( (P<0.05, \text{Fig. 5B, 5C and 5D}) \).

**Discussion**

The metabolic syndrome presented in the patients suffering from either chronic endogenous hypercortisolism or exogenous GCs treatment contributes to the greater risk of cardiovascular diseases, diabetes and obesity, and reduces the life quality \([5,31]\). In this study, our intriguing findings showed that chronic administration of CORT, the principal GC in mice, increases epididymal WAT amount without body weight gain, decreases epididymal adipocyte sizes, increases the expression of both lipogenic and lipolytic genes, impairs mitochondrial biogenesis, functions and antioxidative process when compared with control mice. Considering the crucial role of mitochondria in energy homeostasis, therefore, we have supposed that the epididymal adiposity appears during chronic GC exposure might be associated with GC-induced mitochondrial dysfunction.

Recent study has developed a potential model of the metabolic syndrome resulted from chronic CORT treatment \([25]\). This mouse model was applied in the present study to investigate the metabolic outcomes of mice under chronic CORT exposure. The CORT-treated mice received 713 \( \mu \text{g/d} \) of CORT through drinking water according to the calculation from water intake. This oral dose is comparable with other studies conducted with subcutaneous GC pellets in male mice and female rats \([32,33]\). As expected, plasma concentrations of CORT, insulin, leptin and FGF21 were elevated in mice treated with CORT. Although single plasma CORT levels give little information, previous studies using similar approach have demonstrated the hormone availability and endocrine physiology \([25,34]\). Therefore, mice exposed to CORT through drinking water could be a suitable model for human hypercortisolism.

Surprisingly, we did not find rapid increases in body weight caused by chronic CORT treatment as previously published work \([25]\). However, as shown by numerous reports \([8,15,25,35-37]\), increased intra-abdominal rather than subcutaneous WAT together with elevated plasma triacylglycerols and NEFA levels in CORT-treated mice were also observed in this study, pointing to a sarcopenic phenotype. Active GC contributes to the sarcopenia, since excess GC induce hypoandrogenism and insulin resistance, both of which tend to decrease protein retention and

**Figure 3. Effect of CORT on expressions of lipolytic and lipogenic genes in epididymal WAT of mice.** (A) Relative expression of lipolysis-related genes, and (B) lipogenesis-related genes in epididymal white adipose tissue (WAT) of mice treated with 100 \( \mu \text{g/ml} \) CORT or vehicle for 4 weeks. Data are expressed as group means \( \pm \) SEM \( (n=6 \text{ by group}) \). All the mRNA expression data are normalized to the house keeping gene \( (18S \text{ rRNA}) \). * \( P<0.05 \) compared with vehicle.

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Figure 4. Effect of CORT on mitochondrial biogenesis and function in epididymal WAT of mice. (A) Relative abundance of mitochondrial DNA (mtDNA), (B) relative mRNA levels of mitochondrial genes responsible for mitochondrial biogenesis and function, (C) activity of citrate synthase (CS), (D) NAD⁺ levels, and (E) NAD⁺/NADH ratio in epididymal white adipose tissue (WAT) of mice treated with 100 μg/ml CORT or vehicle for 4 weeks. Data are expressed as group means ± SEM (n = 6 by group). All the mRNA expression data are normalized to the housekeeping gene (18S rRNA), mtDNA is the relative amount to genomic DNA * P<0.05 compared with vehicle.

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Figure 5. Effect of CORT on ROS defense system in epididymal WAT of mice. (A) Concentration of malondialdehyde (MDA), and activities of (B) superoxide dismutase, (C) glutathione peroxidase, and (D) catalase in epididymal white adipose tissue (WAT) of mice treated with 100 μg/ml CORT or vehicle for 4 weeks. Data are expressed as group means ± SEM (n = 6 by group). * P<0.05 compared with vehicle.

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induce loss of minerals from bone, muscle and other tissues [39,39]. It’s suggested that CORT-induced sarcopenia leads to the relative increase in WAT at the expense of the rest of the body in the present study.

Another surprising finding in our study is that chronic CORT exposure reduced epididymal adipocyte size despite increased epididymal WAT weight. For exploring the mechanism underlying this phenomenon, the expression of genes related to lipolysis and lipogenesis were subsequently measured. In agreement with several recent studies [13,40], we showed the up-regulation of these gene expression by chronic CORT exposure. A number of studies have also demonstrated either adipolytic or adipogenic effect of GC in vivo and in vitro, depending on GC type, concentration, and duration [10,41–44]. However, GC are most likely acting as a lipolytic or lipogenic factor through separate pathways. Lipolysis and lipogenesis are putatively concomitant in epididymal WAT under GC administration, and like our data, GC prefer to stimulate epididymal adipocyte hyperplasia rather than hypertrophy to induce central obesity.

Chronic CORT induces epididymal fat enlargement, and mitochondrial defects cause adiposity [45], but the relevant point is the linkage between these manifestations. Previous studies reported the present of glucocorticoid receptor (GR) and glucocorticoid response elements (GREs) in mitochondria [46,47], suggesting glucocorticoids are able to directly act in mitochondria, and then regulate the energy metabolism, since mitochondria play key actors in global energy modulation [45]. Our results suggest that CORT impairs mitochondrial biogenesis and oxidative function in epididymal fat. In line with our data, a most recent report using rat pheochromocytoma PC12 cells has shown excessive glucocorticoid increases protein carbonylation, inhibits activities of mitochondrial complex I and superoxide dismutase [48]. Similarly, decreased activities of anti-oxidative enzymes and increased ROS levels were also observed in our CORT-treated epididymal WAT of mice, suggesting the failure of ROS defense system, which, on the other hand, is also an evidence of mitochondrial dysfunction [49]. Adipose tissue is peculiar in its physiological hypercortisolemia. Diabetes 57: 2028–2036.

In conclusion, the present results indicate that chronic CORT administration induces epididymal adiposity and adipocyte hyperplasia. This is, at least in part, associate with mitochondrial dysfunction in epididymal WAT. Our work offers a potential explanation for the underlying mechanism of metabolic complications of excess glucocorticoid.

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Author Contributions

Conceived and designed the experiments: JY DC. Performed the experiments: JY BY JH PZ. Analyzed the data: JY BY JH PZ. Contributed reagents/materials/analysis tools: XM GH. Wrote the paper: JY DC.

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