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Chronic low-dose glucocorticoid treatment increases subcutaneous abdominal fat, but not visceral fat, of male Wistar rats

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ABSTRACT

Aim: Most studies developed to investigate the effects of glucocorticoids chronic treatment on white adipose tissue uses high doses of these hormones. This study analyzes some effects of a chronic, continuous and steady infusion of low-dose hydrocortisone and the relationship with lipid accumulation in white adipose depots in rats.
Main methods: Nineteen male Wistar rats were divided into control (CON) and cortisol (CORT) groups. Along six weeks CORT group received continuous infusion of 0.6 mg/kg/day of hydrocortisone, while CON group received saline. After euthanasia, subcutaneous and visceral (retroperitoneal and mesenteric) fat pads were excised, weighted and analyzed for: lipogenic enzymes activity; molecular changes of 11-hydroxysteroid dehydrogenase type 1 (11βHSD1) enzyme; enzymes involved in lipid uptake, incorporation, and metabolism and in fatty acids esterification. Besides, morphometric cell analysis was performed.
Key findings: CORT group showed increased triglycerides, changes in lipoprotein profile and 26,8% increment in central subcutaneous (SC) mass, while visceral fat pads masses remained unchanged. Adipocytes from SC, only, presented increased fatty acid synthase, ATP-citrate lyase and glucose-6-phosphate dehydrogenase activity, in addition to reduced AMP-activated protein kinase and 11βHSD1 enzymes content.
Significance: Chronic low-dose hydrocortisone treatment consequences seem to be different from those commonly seen in long term hypercortisolism. While high doses promote lipid accumulation in visceral depots, a low dose showed an increase in central SC depot only. This appears to involve an increment in lipid storage and in *de novo* lipogenesis enzymes activity.

1. Introduction

Glucocorticoids (GC) are steroid hormones synthesized in the cortex of the adrenal glands and its basal secretion is rhythmic and occurs in a circadian fashion under the influence of a neuroendocrine control system - the hypothalamic-pituitary-adrenal axis (HPA). GC can also be locally activated in various tissues through 11β-hydroxysteroid dehydrogenase-1 (11βHSD1) enzyme action, which converts circulating inactive versions of these hormones in their active forms (cortisol in humans and corticosterone in rats) [1]. The increased content or activity of this enzyme increases intracellular GC concentration, amplifying their actions.

At the physiological level, GCs are considered catabolic hormones for their action in obtaining substrates (glucose, glycerol, free fatty acids and amino acids) for glucose maintenance and energy mobilization in stressful conditions, in order to supply the increasing energy demand in these situations [2]. Although GCs perform several vital functions to the organism [3] their primary function is to provide glucose production for maintenance of its blood levels.

White adipose tissue (WAT) is also a target organ of GC action, where they exert pleiotropic effects [2,4–6], among which lipid mobilization is included. Although the effect of these hormones in inducing lipolysis is well documented [7–9], the result of its action is complex involving both fat degradation or storage and appears to depend on physiological context. GCs influence on fat accumulation was clearly demonstrated in previous studies. In humans, cortisol excess at a systemic level results in two to five-fold increase in central fat, particularly visceral fat, in patients with Cushing's syndrome [10–12]. Rosmond et al. [13] found that individuals under chronic stress (with increased circulating cortisol) are more likely to increase visceral fat. According

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to the authors, one possible explanation is the fact that cortisol and insulin synergistically increase lipoprotein lipase (LPL) activity.

However, most studies developed with GC chronic use, intending to investigate its effects on WAT, uses high doses of these hormones. As described by Lee et al. [14] in an extensive review, works seeking to investigate the effects of chronic body exposure to lower concentrations are lacking. In this context, the aim of this study was to analyze some effects of a chronic, continuous and steady infusion of a low-dose hydrocortisone and the relationship with lipid accumulation in rats. For this purpose, male adult Wistar rats were subjected to infusion of 0.6 mg/kg/day of hydrocortisone over six weeks. The dose was established based on previous definition of high ([15] [~120 mg/kg/day], [16] [~33 mg/kg/day]) and low ([17] [0.75 mg/kg/day]) glucocorticoid dose and considering the pump capacity. We investigated possible changes in the activity of lipogenic enzymes; molecular changes of enzymes involved in lipid metabolism, besides the expression of 11βHSD1 enzyme.

Different from the most studies found in the literature, our results showed an increase in central subcutaneous fat depot, while visceral depots remained unchanged.

2. Materials and methods

2.1. Animals

The experiments were carried out on 19 adults, male Wistar rats, weighing 300–350 g, housed individually under standard environmental conditions (lights on from 7 PM to 7 AM [inverted cycle], temperature 21 ± 1 °C, water and food *ad libitum* - Nuvital® balanced standard chow pellets, Nuvital SA, Colombo, Brazil). All experimental procedures were reviewed and approved by the Ethical Committee for Animal Research (CEEA) (n° 010/10) of the Institute of Biomedical Sciences of University of São Paulo. After 2 weeks of acclimatization they were randomly divided into 2 groups: control (CON, $n = 11$) and cortisol (CORT, $n = 8$). Then, the animals received intraperitoneal anesthesia with ketamine and xylazine (Anasedan®, Seropédica, RJ, Brazil - 0,15 mL/100 g of body mass), prior to a surgical subcutaneous implant (dorsal interescapular region) of an osmotic minipump (Alzet®, Cupertino, CA, United States – model 2006 with 200 µL capacity) containing a 0,9% saline solution for CON group, while CORT group received hydrocortisone (hydrocortisone 21-hemisuccinate sodium salt – Sigma Aldrich Co®, São Paulo, SP, Brazil) diluted in distilled water at the dose of 0,6 mg/kg/day (or 0,18 mg/day). The treatment lasted for 6 weeks during which food and water intakes and body mass were measured weekly.

2.2. Ex vivo experiments

At the end of the 6th week, after 12 h of fasting, CON and CORT animals were decapitated under sodium thiopental anesthesia (Tiopentax®, São Paulo, SP, Brazil) (3%, 5 mg/100 g bw, ip) at 7 AM (early environmental dark period, ZT = 0). This time was defined based on the circadian rhythm of corticosterone release in the animals, when the serum concentrations are supposed to be highest in controls. Trunk blood was collected for glucose, insulin, corticosterone and lipids determinations. Median laparotomy was performed to excise the following adipose tissues: subcutaneous (SC) (inguinal), retroperitoneal (RP) and mesenteric (ME). Adrenal glands were also excised, cleaned from the surrounding fat and weighted. Subcutaneous, RP and ME fat pads were weighed, fragments were collected for adipocyte isolation and samples were stored at − 80 °C.

2.3. Adipocytes isolation and morphometric analysis

Adipocytes from each fat pad were isolated by collagenase tissue digestion as described by Rodbell [18]. Cell suspension aliquots were photographed and evaluated under an optical microscope (100 × magnification) coupled to the digital microscope camera 1.3 MP (Moticam 1000®; Motic, Richmond, British Columbia, Canada). Mean adipocyte diameters were determined by measuring 100 cells using MOTIC-IMAGES Plus 2.0® software.

2.4. Indirect calculation of cellularity

The number of cells in each fat pad was indirectly calculated dividing the total fat pad mass by the mean adipocytes mass. Adipocyte mass was calculated using the following formula: $\mathbf{d} = \mathbf{m}/\mathbf{v}$, where **d** is the mean density of an adipocyte (0,91 g/mL), **m** is adipocyte mass (in pg [10^{-9} mg]) and **v** is the mean adipocyte volume (in pL [10^{-9} mL]).

2.5. Hormones, glucose and lipids determinations

Serum glucose, triglycerides (TAG), total cholesterol and its fractions was determined as previously described [19]. Plasma corticosterone was determined by ELISA method using specific commercial kit for rats (IBL International, reference RE52061) following the manufacturer instructions (the kit percentage of cross reactivity to hydrocortisone is 0,3%). Serum insulin was also measured by ELISA method, using commercial kit (EMD Milipore Corporation, Cat. #EZRMI-13K). Insulin resistance was calculated using the homoeostasis model of assessment insulin resistance ($HOMA_{ir}$) index, as defined by the equation $HOMA_{ir}$ = (fasting glucose [mmol/L] × fasting insulin [µU/L])/22.5.

2.6. Oral glucose tolerance test

One week before euthanasia the animals underwent an oral glucose tolerance test (oGTT) at 7 AM (early dark environmental phase in inverted cycle) after 12 h of fasting. Tail blood was collected at time 0, then a glucose load was offered by gavage (75 mg/100 g of body mass) and new blood samples were collected at 5, 10, 20, 30, 60 and 90 min afterwards. Glucose concentration was determined using a glucometer (One Touch Ultra®, Johnson & Johnson®, São Paulo, SP, Brazil).

2.7. Maximal enzyme activity of the de novo fatty acid synthesis in adipose tissue samples

The activities of malic enzyme, glucose-6-phosphate dehydrogenase (G6PDH), fatty acid synthase (FAS) and ATP citrate lyase (ACL) that belong to the *de novo* lipogenesis (DNL) pathway were analyzed following the methods described in Chimin et al. [19]. Enzyme activities were expressed as millimoles per minute per total fat pad mass (mmol/min/total fat pad mass).

2.8. Western blotting

Tissue samples were homogenized in buffer at 4 °C (Triton-X 100 1%, 100 mM Tris (pH 7,45), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 2 mM PMSF and aprotinin 0.01 mg/mL) in Polytron (PT 3100, Kinematica AG, Littau-Lucerne, Switzerland). Tissues extracts were centrifuged at 12,000 rpm at 4 °C for 20 min to remove insoluble material. Sample's protein content (from the supernatant portion) was quantitated using Bradford reagent (Bio-Rad) and treated with Laemmli buffer containing 100 mM DTT. Aliquots (50 µg) of total protein were subjected to polyacrylamide gel electrophoresis (SDS-PAGE 10%, Invitrogen) and transferred to a nitrocellulose membrane. The antibodies used for obtaining the blottings are detailed in Table 1.

2.9. Statistical analysis

Results were expressed as mean ± SEM, using the StatGraphics Centurion XVI® program. Mann-Whitney test was used for comparisons