


Differential regulation of glyceroneogenesis by glucocorticoids in epididymal and retroperitoneal white adipose tissue from rats

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Abstract

Purpose Investigate the glycerol-3-phosphate generation pathways in epididymal (EPI) and retroperitoneal (RETRO) adipose tissues from dexamethasone-treated rats.

Methods Rats were treated with dexamethasone for 7 days. Glycerol-3-phosphate generation pathways via glycolysis, glyceroneogenesis and direct phosphorylation of glycerol were evaluated, respectively, by 2-deoxyglucose uptake, phosphoenolpyruvate carboxykinase (PEPCK-C) activity and pyruvate incorporation into triacylglycerol (TAG)-glycerol, and glycerokinase activity and glycerol incorporation into TAG-glycerol.

Results Dexamethasone treatment markedly decreased the body weight, but increased the weight and lipid content of EPI and RETRO and plasma insulin, glucose, non-esterified fatty acid and TAG levels. EPI and RETRO from dexamethasone-treated rats showed increased rates of de novo fatty acid synthesis (80 and 100%) and basal lipolysis (20%). In EPI, dexamethasone decreased the 2-deoxyglucose uptake (50%), as well as glyceroneogenesis, evidenced by a decrease of PEPCK-C activity (39%) and TAG-glycerol synthesis from pyruvate (66%), but

increased the glycerokinase activity (50%) and TAG-glycerol synthesis from glycerol (72%) in this tissue. In spite of a similar reduction in 2-deoxyglucose uptake in RETRO, dexamethasone treatment increased glyceroneogenesis, evidenced by PEPCK activity (96%), and TAG-glycerol synthesis from pyruvate (110%), accompanied by a decrease in glycerokinase activity (50%) and TAG-glycerol synthesis from glycerol (50%). Dexamethasone effects on RETRO were accompanied by a decrease in p-Akt content and by lower insulin effects on the rates of glycerol release in the presence of isoproterenol and on the rates of glucose uptake in isolated adipocytes.

Conclusion Our data demonstrated differential regulation of glyceroneogenesis and direct phosphorylation of glycerol by glucocorticoids in EPI and RETRO from rats.

Keywords Adipose tissue · Glucocorticoids · Glyceroneogenesis · Glycerokinase

Introduction

The mobilization of fatty acids (FAs) incorporated into triacylglycerol (TAG) to attend the energy demands of peripheral tissues is a main function of white adipose tissue (WAT). The maintenance of adequate stores of TAG therefore seems essential for the normal functioning of WAT. The preservation of WAT TAG reserves requires a continuous supply of glycerol-3-phosphate (G3P, Fig. 1) to esterify preformed FAs, taken up from circulating lipoproteins or recycled after hydrolysis of endogenous TAG, or newly synthesized FAs. Because adipose tissue has relatively low levels of glycerokinase (GyK), the use of glycerol as a source of G3P for FA esterification and TAG formation is considered negligible. The generally

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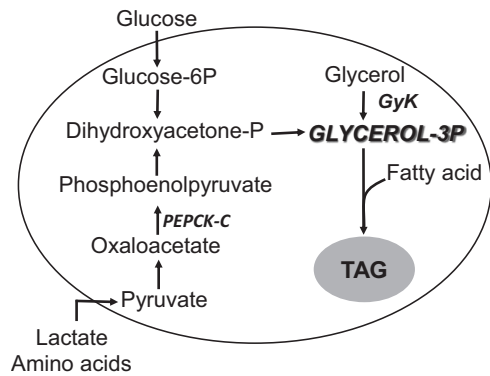


Fig. 1 Sources and pathways of glycerol-3-phosphate (G3P) generation

recognized source of G3P for this purpose has been glucose, via dihydroxyacetone in the glycolytic pathway and conversion to G3P by glycerophosphate dehydrogenase. However, G3P synthesis also occurs by glyceroneogenesis, which involves the carboxylation of pyruvate to oxaloacetate, decarboxylation of oxaloacetate to phosphoenolpyruvate, and the subsequent production of G3P [1]. The measurement of phosphoenolpyruvate carboxykinase (PEPCK-C) activity has been considered a marker of the rate of this metabolic pathway [1].

The three metabolic pathways involved in the G3P generation of adipose tissue seem to be adjusted to maintain the supply of this metabolite and TAG synthesis. We demonstrated several years ago that the adaptation of rats to a high-protein, carbohydrate-free diet increases WAT glyceroneogenic activity, evidenced by increased activity of PEPCK-C [2] and by increased rates of incorporation of non-glucose substrates into TAG-glycerol *in vivo* [3] and *in vitro*, by adipose tissue fragments [2] or isolated adipocytes [4]. Because the use of glucose *in vivo* and glycolytic flux are reduced in WAT from high-protein, carbohydrate-free diet-adapted rats [5], the increased glyceroneogenesis seemed to represent a compensatory mechanism for the reduced generation of G3P via glycolysis [3]. Similar results were found in WAT and liver from food-deprived and streptozotocin-induced diabetic rats, two other situations in which insulin levels are reduced [6, 7]. On the other hand, the cafeteria diet increases plasma insulin levels and, in adipose tissue, decreases glyceroneogenesis activity, evidenced by decreased activity of PEPCK-C and pyruvate incorporation into TAG-glycerol, and increases the *in vivo* glucose uptake and direct phosphorylation of glycerol, evidenced by increased GyK activity and glycerol incorporation into TAG-glycerol [8]. Denervation of adipose tissue from cafeteria diet-fed rats decreases the direct phosphorylation of glycerol, but not glyceroneogenesis or glucose uptake [8]. These findings suggest reciprocal changes in the generation of G3P from glucose via

glycolysis and from glyceroneogenesis, independently from G3P production by GyK [8], and that GyK is regulated by the sympathetic nervous system [6, 8]. Similar results were obtained in brown adipose tissue, a tissue highly innervated by the sympathetic nervous system [9, 10].

The hormonal control of these pathways is still not well established and the role of glucocorticoids in lipid metabolism of adipose tissue is an issue of great interest in basic and clinical research due to its contrasting metabolic effects depending on WAT locations in mammals. It is well known that glucocorticoids reduce peripheral glucose uptake by adipocytes [11], but pioneering studies have reported that this steroid hormone decreases the rate of the glyceroneogenesis pathway and PEPCK-C activity in epididymal adipose tissue (EPI) of rats [12–15]. These observations conflict with our previous findings that suggest reciprocal changes between glycolysis and glyceroneogenesis for G3P generation. Many studies have been performed to clarify the molecular mechanisms that underlie the differential regulation of PEPCK-C gene transcription by glucocorticoids in liver and adipose tissue [1, 16, 17]; however, few studies have been reported about the regulation of glyceroneogenesis by this hormone in different WAT pads. Thus, to obtain more information about the control of G3P supply for TAG synthesis in WAT by glucocorticoids, we investigated the production pathways of this metabolite in retroperitoneal (RETRO) and EPI adipose tissues from rats treated with dexamethasone (DEXA), a glucocorticoid widely used in medical clinics. Interestingly, although RETRO and EPI are considered visceral depots, there were many different responses to hormones, diets, food deprivation or sympathetic neural stimulation, when we compared these two tissues [18, 19]. It is already known that glucocorticoids induce TAG accumulation in visceral WAT and lipodystrophy in subcutaneous WAT, but the mechanisms involved in these responses are not yet clarified. In this work, our main objective was to evaluate the role of glucocorticoids in the three G3P generation pathways (glycolysis, direct phosphorylation of glycerol and glyceroneogenesis) that could explain the increase in lipid content and visceral WAT weight induced by this hormone. The effects of this glucocorticoid on *in vivo* rates of FA synthesis and on lipolysis in these two adipose tissue pads were also investigated.

Materials and methods

Animals and treatment

Male Wistar rats weighing 150–200 g and aged 6–7 weeks were housed (up to three per cage) in a room kept at 25 ± 2 °C on a 12:12 h light:dark cycle. The rats were fed with a commercial balanced diet [Nuvilab CR1, Nuvital, Brazil

(22% protein, 55% carbohydrate and 4.5% lipid)] and with water ad libitum. Rats were randomly divided into DEXA group (intraperitoneal (i.p.) injection of 1 mg/kg dexamethasone phosphate, Sigma® for 7 consecutive days) and control group (i.p. injection of saline in the same volume). On the 8th day, the animals were euthanized in order to collect blood and tissues. All the experiments were performed between 8:00 and 10:00 am. The care and treatment of the rats received prior institutional approval by the Ethical Committee of Ribeirão Preto Medical School of the University of São Paulo, Brazil (#120/2010).

In vivo rates of FA synthesis

Fed rats were injected intraperitoneally with $^3\text{H}_2\text{O}$ (3 mCi in 0.5 mL saline). After 1 h they were killed by decapitation, blood samples were collected for plasma water-specific radioactivity determination, and RETRO and EPI WAT were rapidly removed for measurement of label incorporation into TAG. The techniques used for lipid extraction, isolation of the TAG-FAs, radioactivity counting and plasma water-specific radioactivity determination have been previously described [20]. Rates of de novo FA synthesis were calculated following the assumptions of Windmueller and Spaeth [21].

Isolation of adipocytes

RETRO or EPI WAT samples from approximately eight rats were pooled and disaggregated with collagenase, according to the method of Rodbell [22], in a buffer containing 27 mmol/L HEPES, 137 mmol/L NaCl, 4.2 mmol/L NaHCO_3 , 0.4 mmol/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 mmol/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.4 mmol/L KH_2PO_4 , 5.4 mmol/L KCl and 1.3 mmol/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, pH 7.4, supplemented with 10 g/L FA-free albumin and 0.5 mmol/L glucose. After continuous shaking for 25–35 min at 37 °C, the adipocytes were filtered through a 300- μm nylon mesh and washed three times with the same medium.

In vitro rates of glucose uptake by WAT

Adipocytes (approximately 600,000 cells) were incubated for 3 min at 37 °C with constant shaking in 1 mL of Krebs–Henseleit buffer at pH 7.4, supplemented with 1% FA-free albumin, 27 mmol/L HEPES and 1 mmol/L of 2-deoxy-1- ^{14}C glucose (2-DG- ^{14}C ; 0.5 $\mu\text{Ci}/\text{vial}$). The procedure used was based on the method of Foley et al. [23] with some modifications. Briefly, 15 s before the end of the incubation, a 250 μL aliquot of medium was added to a microcentrifuge tube containing 100 μL of dinonyl phthalate (density of 0.98 g/L). The microcentrifuge tube was spun for 45 s, and the vial was cut with a blade in the

dinonyl phase, separating the medium from the cells. The cell pellet was added to a 5 mL vial with 4 mL of scintillation fluid, and the radioactivity of 2-DG- ^{14}C uptake by adipocytes was measured. To determine the blank, the reaction was performed on ice. The results are expressed as nmol of 2-DG- ^{14}C uptake by 10^6 cells per minute.

Lipolysis

Adipocytes (approximately 600,000 cells) were incubated for 1 h at 37 °C with constant shaking in 1 mL of Krebs–Henseleit buffer, pH 7.4, supplemented with 1% FA-free albumin, 27 mmol/L HEPES and 5 mM glucose in the presence or absence of 10 nM isoproterenol, a non-specific beta-receptor agonist. The effect of insulin (20 nM) on isoproterenol-stimulated lipolysis was also determined. Lipolysis was evaluated by glycerol release into the incubation medium, as previously described [24], because the rates of FA re-esterification by changes in glyceroneogenesis affect the FAs released by adipocytes [25].

Incorporation of 1- ^{14}C -pyruvate or U- ^{14}C -glycerol into TAG-glycerol

Aliquots of adipocyte suspension containing 10^6 cells were incubated for 1 h at 37 °C with constant shaking in 1 mL of glucose free Krebs–Henseleit buffer, pH 7.4, supplemented with 1% FA-free albumin and containing 1- ^{14}C -pyruvate or U- ^{14}C -glycerol (1 mmol/L, 1 mCi). The procedures used for lipid extraction, isolation and counting of ^{14}C -TAG-glycerol were as previously described [26].

Measurement of enzyme activity

Using the method described by Chang and Lane [27], PEPCK-C activity was assayed in 100,000 g supernatant after homogenization of WAT (0.5 g/mL) in 20 mmol/L triethanolamine buffer at pH 7.5, containing 0.2 mol/L sucrose, 5 mmol/L 2-mercaptoethanol and 1 mmol/L EDTA. The incorporation of ^{14}C -bicarbonate (2 $\mu\text{Ci}/\text{tube}$) into an acid-stable product was determined in an assay mixture with an identical composition to that used previously [26]. The protein content of the homogenates was determined by the bicinchoninic acid method [28].

According to the recommendation of Newsholme et al. [29], GyK activity was measured in 2000 g supernatant obtained after tissue homogenization in ice-cold 1% KCl in 1 mmol/L EDTA. The composition of the assay mixture, which contained U- ^{14}C -glycerol (1 $\mu\text{Ci}/\text{tube}$), and the isolation of labeled glycerol phosphate have been previously described [9]. The protein content of the homogenate used in the GyK assay was determined by the method of Lowry et al. [30].

Quantitative real-time RT-PCR (qRT-PCR)

Glucocorticoid receptor, 11 β -hydroxysteroid dehydrogenase type 1 (11 β HSD1) and PEPCK-C mRNA levels were determined by qRT-PCR in WAT from all groups. WAT was immediately frozen in liquid nitrogen and RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA). Reverse transcription into cDNA was performed using 4 μ g of RNA, 20 pmol oligo(dT) primer (Invitrogen) and Advantage ImProm-II reverse transcriptase (Promega, Madison, WI, USA). Real-time RT-PCR was carried out on an ABI7000 sequence detection system (Applied Biosystems, Foster City, CA, USA), using a SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit with ROX (Invitrogen) and rat primers for: 11 β HSD1 (forward: 5'-CTC CAG GGC GCA TTC CT-3' and reverse: 5'-TCA TAG ACA CAG AAA CAG CTT TGA AA-3'), glucocorticoid receptor (forward: 5'-CGC GTA ATG ACA TCC TGA AGC-3' and reverse: 5'-GGG TAG AGC CCT GGA ATG-3'), PEPCK-C (forward: 5'-ATC GAA GGC ATC ATT TTT GG-3' and reverse: 5'-CAT GGC AGC TCC TAC AAA CA-3') and GAPDH (forward: 5'-TGC ACC ACC AAC TGC TTA GC-3' and reverse: 5'-GGC ATG GAC TGT GGT CAT GAG-3'). The mRNA was quantified by measuring the threshold cycle normalized with GAPDH and expressed relative to the control group. The level of target transcript expression was calculated using the standard curve method [31].

Western blotting analysis

EPI and RETRO WAT were homogenized in 10 mmol/L Tris-HCl buffer (pH 7.4) at 4 °C containing 250 mmol/L sucrose, 1 mmol/L EDTA, 10 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 10 mmol/L sodium orthovanadate, 5 μ g/mL of aprotinin, 1 mg/mL of leupeptin and 1 mmol/L phenylmethyl-sulfonyl fluoride. The homogenate was centrifuged at 3000 g at 4 °C for 15 min and protein content was determined by the method of Lowry et al. [30]. An equal volume of sample buffer (20% glycerol, 125 mmol/L Tris-HCl, 4% SDS, 100 mmol/L dithiothreitol, 0.2% bromophenol blue, pH 6.8) was added to the supernatant and the mixture was boiled. Twenty micrograms of total proteins was separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with primary IgG antibodies against PEPCK-C (sc-32879, Santa Cruz Biotechnology), GyK (ab-126599, Abcam), GLUT4 (#2213, Cell Signaling), Akt total (#9272, Cell Signaling), pAkt^{Ser473} (#9271, Cell Signaling) and α -tubulin (sc-32293, Santa Cruz Biotechnology). Primary antibodies were detected by a peroxidase-conjugated secondary antibody and visualized by ECL reagents in Molecular Imager ChemiDoc XRS (Bio-Rad). Band intensities were quantified

using Image Lab software (version 5.2.1, Bio-Rad). Subsequently, the same membrane was incubated with a commercial stripping solution (Restore Plus Western Blot Stripping Buffer, Thermo Scientific) and incubated with tubulin antibody.

Insulin tolerance tests (ITT) and HOMA index

Rats fasted overnight (12 h without food) were used for the ITT; the animals were fed and then fasted for 3 h. Human normal insulin (0.75 IU/kg body weight) was injected intraperitoneally and blood was collected from the tail vein at different time points, as indicated. Plasma glucose was measured using a glucose meter (Accu-Chek Performa, Roche). Values were normalized considering the start value equal to 100%. The areas under the curves for ITT were determined with GraphPad Prism software. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using glucose and insulin determinations in 24 h-fasted rats using the following formula: fasting glucose (mmol/L) \times fasting insulin (mIU/mL)/22.5.

Other methods of chemical analysis

Serum glucose concentration was determined enzymatically using a commercial kit from Labtest (Lagoa Santa, Brazil). The concentration of serum insulin was measured with a radioimmunoassay using a commercial kit (Coat-a-10 Count Insulin) from Diagnostic Products Corporation, Los Angeles, CA, USA. Adipose tissue lipids were extracted by the procedure of Folch [32] and determined gravimetrically.

Statistical methods

The data are expressed as means \pm SEM. Except for glucose uptake and lipolysis, which were analyzed using two-way ANOVA followed by Student–Newman–Keuls, the differences between groups were analyzed using Student's *t*-test. The criterion for significance was $P < 0.05$.

Results

DEXA treatment decreased the mRNA expression of glucocorticoid receptor by approximately 80% in both adipose tissues (Fig. 2a). This effect was accompanied by increases of approximately 2-fold and 4.5-fold in the mRNA expression of 11 β HSD1 in EPI and RETRO, respectively (Fig. 2b). These findings show the responsiveness of WAT to glucocorticoid treatment.

After 7 days of DEXA administration, body weight was approximately 30% lower than controls (Table 1). However, the weight of EPI and RETRO adipose tissues (g/100 g

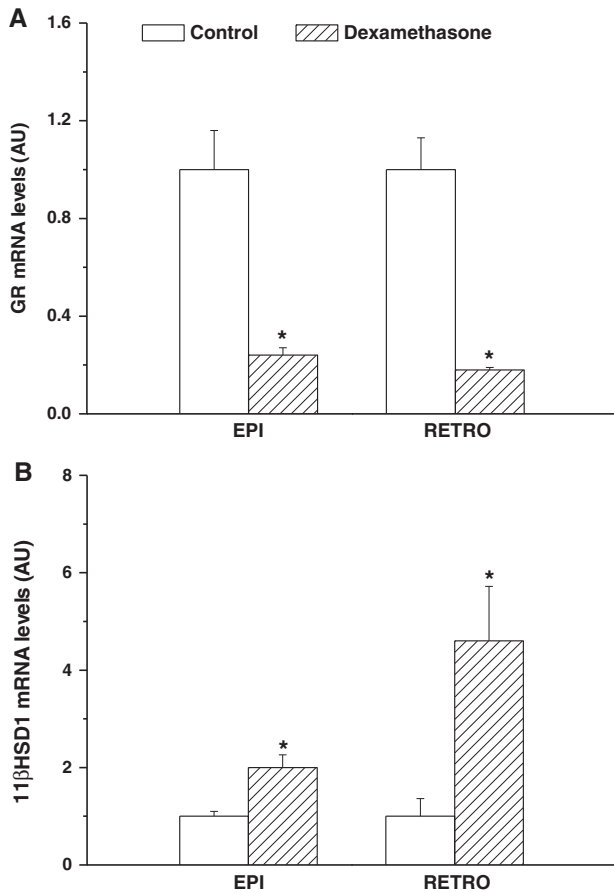


Fig. 2 Effect of dexamethasone treatment (1 mg/kg) on mRNA expression of glucocorticoid receptor (a) and 11beta-hydroxysteroid dehydrogenase type 1 (11βHSD1) (b) in epididymal (EPI) and retroperitoneal (RETRO) adipose tissue from Wistar rats. Bars are means ± SEM; n = 10. *P < 0.05 vs. control

Table 1 Body weight, weight and lipid content of epididymal (EPI) and retroperitoneal (RETRO) white adipose tissue (WAT) and serum concentrations of insulin, glucose, non-esterified fatty acids and triacylglycerol and HOMA-IR index from rats treated with dexamethasone and controls

	Control	Dexamethasone
Body weight (g)	220.0 ± 1.6	150.0 ± 3.5*
EPI WAT (g/100 g)	0.57 ± 0.04	1.13 ± 0.14*
RETRO WAT (g/100 g)	0.43 ± 0.05	0.82 ± 0.09*
EPI lipid content (g/total tissue)	1.10 ± 0.03	1.49 ± 0.05*
RETRO lipid content (g/total tissue)	0.83 ± 0.03	1.08 ± 0.04*
Insulin (μU/mL)	35.5 ± 3.7	203.1 ± 28.1*
Glucose (mg/dL)	108 ± 4	266 ± 26*
HOMA-IR index	10.65 ± 1.20	106.63 ± 8.90*
Non-esterified fatty acids, (μmol/mL)	0.34 ± 0.02	0.46 ± 0.06*
Triacylglycerol (mg/dL)	54.72 ± 5.33	87.40 ± 7.83*

Data are presented as means ± SEM of ten rats. *P < 0.05 vs. control

body weight) was approximately 100% greater in DEXA-treated rats compared to control animals (Table 1). Lipid content was approximately 33% higher (g/total tissue) in both WAT pads from DEXA-treated rats (Table 1). DEXA treatment increased the concentrations of serum insulin (approximately 600%), glucose (approximately 150%), non-esterified FAs (approximately 36%) and TAG (approximately 60%) (Table 1).

DEXA treatment increased the rates of in vivo FA synthesis in EPI (approximately 80%) and RETRO (approximately 100%) (Fig. 3a). The basal lipolysis in both WAT depots was 20% higher in DEXA-treated animals compared to controls (Fig. 3b). Isoproterenol increased glycerol release into the incubation medium, but by a lower degree of magnitude in WAT of DEXA-treated rats (Fig. 3b). The presence of insulin, a very well-known antilipolytic hormone, inhibited the beta-adrenergic agonist effect in EPI from both groups and in RETRO from control rats; however, this insulin effect was not observed in RETRO adipose tissue from DEXA-treated rats (Fig. 3b).

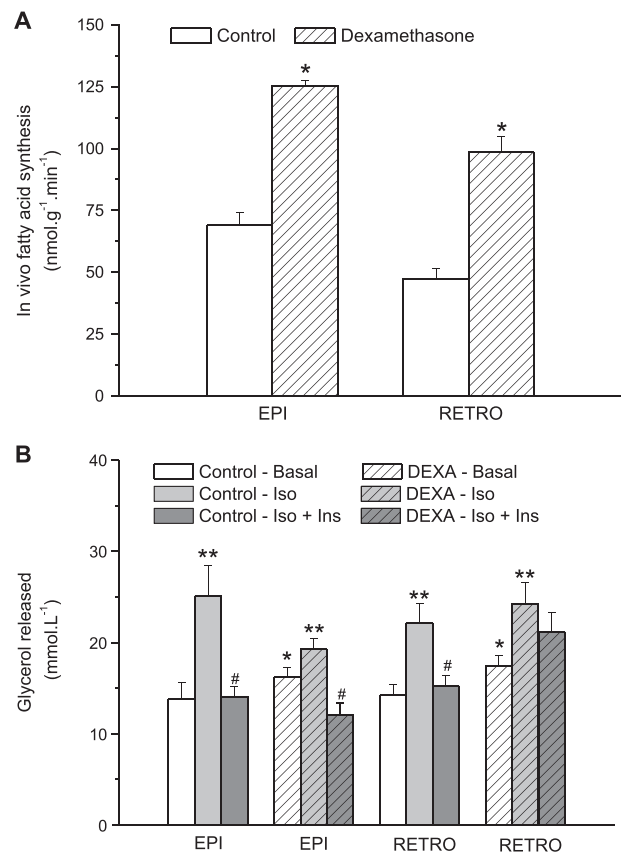


Fig. 3 Effect of dexamethasone treatment (1 mg/kg) on in vivo rates of fatty acid synthesis (a) and in vitro lipolysis (b) in the absence (basal) or presence of isoproterenol (10 nM) and insulin (20 nM) in EPI, and RETRO adipose tissue from Wistar rats. Bars are means ± SEM; n = 10 rats (a) or six tubes of pooled adipocytes from approximately eight rats (b). *P < 0.05 vs. control, **P < 0.05 vs. basal, #P < 0.05 vs. isoproterenol

ITT and HOMA-IR index demonstrated a marked reduction in the glucose homeostasis induced by DEXA treatment for 7 days. After insulin injection, the normalized serum glucose concentration was higher after 10, 15, 20, 25 and 30 min in DEXA-treated rats than in controls, resulting in an increase (20%) in the area under the curve (Fig. 4a). The 10-fold increase in HOMA index (Table 1) confirmed the effect of DEXA treatment on body insulin resistance compared to controls.

Lower insulin sensitivity after DEXA administration was also observed in WAT. In basal conditions the rates of in vitro glucose uptake by EPI and RETRO were approximately 50% lower in DEXA-treated rats than in controls (Fig. 5a). The addition of insulin to the incubation medium increased the glucose uptake by adipocytes. In EPI and RETRO from control rats, as well as in EPI from DEXA-treated rats, the saturation of glucose uptake occurred at insulin concentrations greater than 20 nM (Fig. 5a); however, in RETRO from DEXA-treated rats, the addition of 10 nM insulin induced a maximum increase in glucose uptake (Fig. 5a).

Lower glucose uptake in adipose tissue from DEXA-treated rats was accompanied by a decrease in GLUT4 content in both tissues (Fig. 5b). Akt-p content was reduced (approximately 40%) only in RETRO by treatment with DEXA (Fig. 5c).

In spite of the similar reduction in glucose uptake in EPI and RETRO, the effect of DEXA treatment on G3P generation by glyceroneogenesis and by direct phosphorylation of glycerol was opposing between these two adipose tissue depots. EPI from DEXA-treated rats showed 39 and 66% lower rates of ¹⁴C-pyruvate incorporation into TAG-glycerol and PEPCK-C activity, respectively, without changes in PEPCK-C content and expression (Fig. 6). This

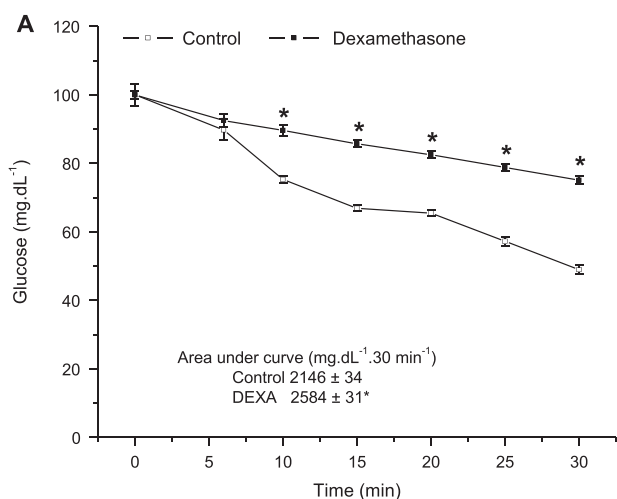


Fig. 4 Effect of dexamethasone treatment (1 mg/kg) on insulin tolerance test of Wistar rats. Bars are means ± SEM; n = 10. *P < 0.05 vs. control

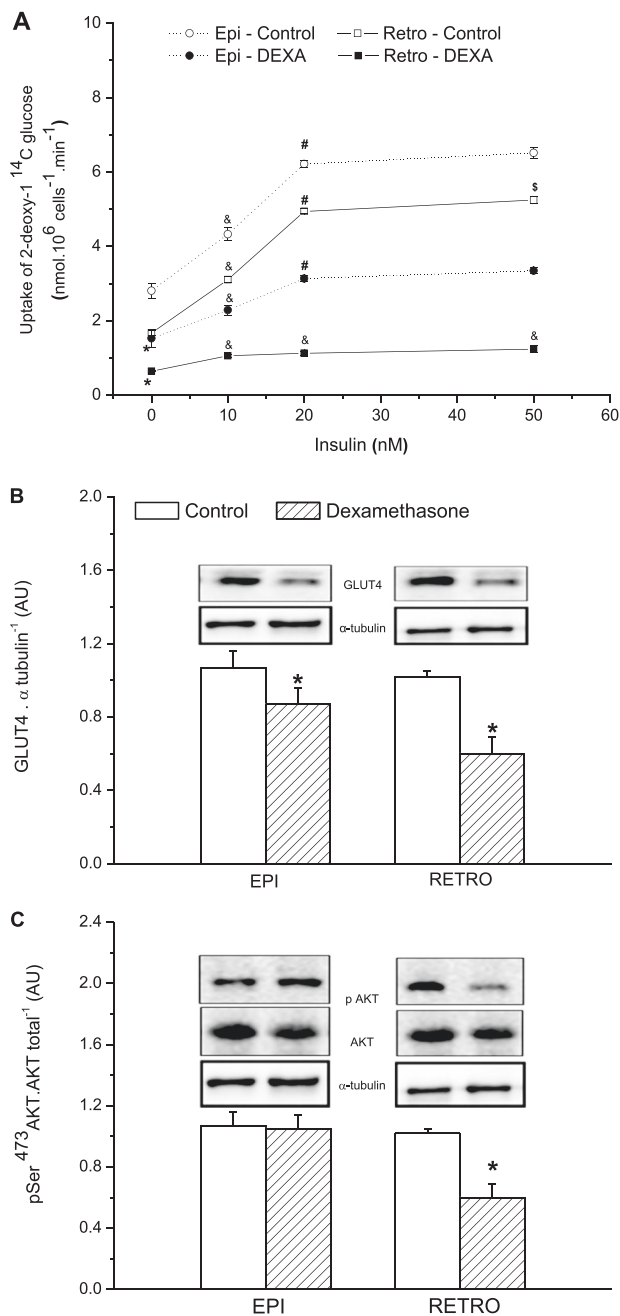


Fig. 5 Effect of dexamethasone treatment (1 mg/kg) on the rates of glucose uptake in the absence or presence of insulin by isolated adipocytes (a) and GLUT4 (b) and p-Akt^{Ser473} (c) content in EPI and RETRO adipose tissue from Wistar rats. Each point represents the mean ± SEM of six tubes of pooled adipocytes from approximately eight rats (a) or five rats (b, c). *P < 0.05 vs. control, &P < 0.05 vs. basal, #P < 0.05 vs. 10 nM, \$P < 0.05 vs. 20 nM

effect was accompanied by faster rates of ¹⁴C-glycerol incorporation into TAG-glycerol (approximately 50%), GyK activity (72%) and GyK content (40%, Fig. 7). On the other hand, RETRO from DEXA-treated rats showed faster rates of ¹⁴C-pyruvate incorporation into TAG-glycerol (110%), PEPCK-C activity (96%) and expression

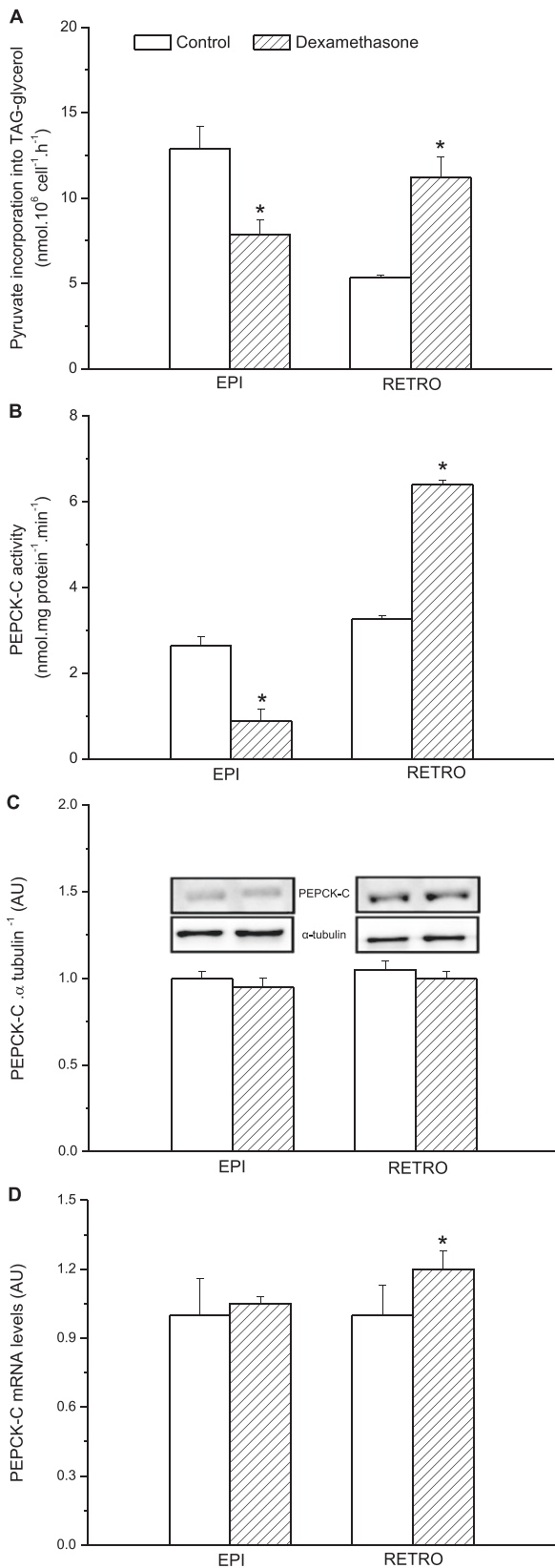


Fig. 6 Effect of dexamethasone treatment (1 mg/kg) on rates of ¹⁴C-pyruvate incorporation into TAG-glycerol (a), PEPCK-C activity (b), content (c) and expression (d) in EPI and RETRO adipose tissue from Wistar rats. Bars are means ± SEM; n = 5–10. *P < 0.05 vs. control

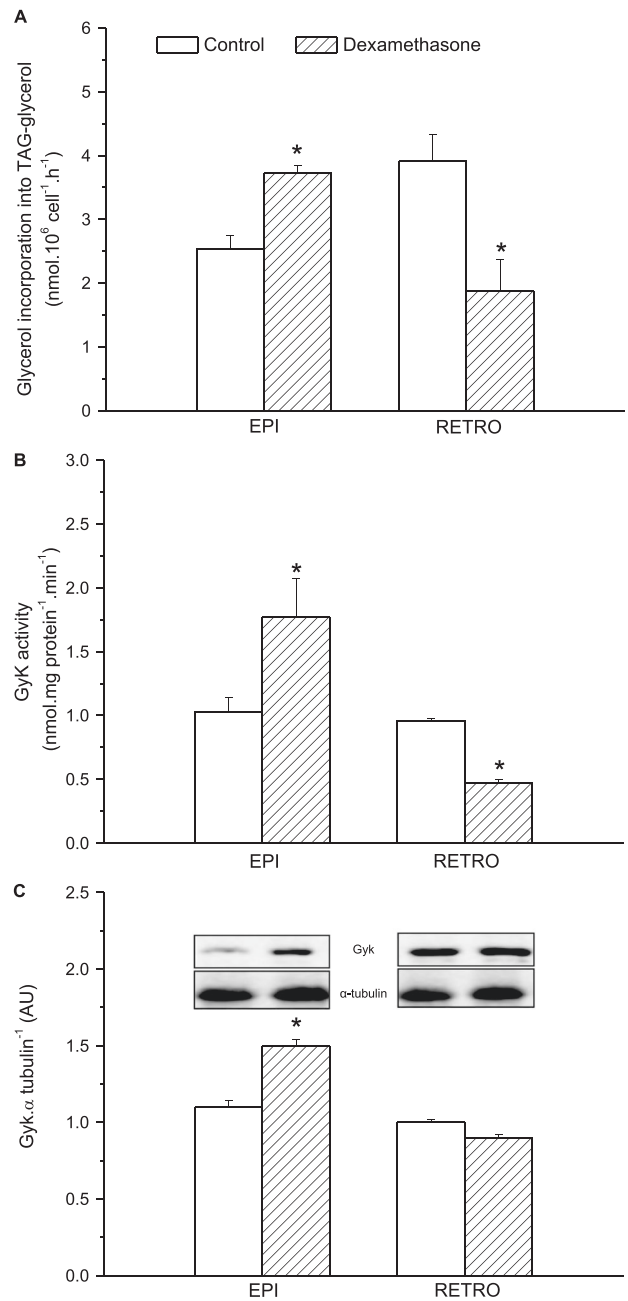


Fig. 7 Effect of dexamethasone treatment (1 mg/kg) on rates of ¹⁴C-glycerol incorporation into TAG-glycerol (a), GyK activity (b) and content (c) in EPI and RETRO adipose tissue from Wistar rats. Bars are means ± SEM; n = 5–10. *P < 0.05 vs. control

(approximately 20%) without changes in PEPCK-C content (Fig. 6). However, RETRO adipose tissue from DEXA-treated rats showed lower rates of ¹⁴C-glycerol incorporation into TAG-glycerol (approximately 50%) and GyK activity (approximately 50%), without changes in GyK content (Fig. 7). Similar results were also found in EPI and RETRO adipose tissues from adrenalectomized rats treated with DEXA for 7 days (data not shown).

Discussion

Our results on the effects of DEXA treatment for 7 days in male adult rats can be summarized as follows (Fig. 8): (1) Insulin sensitivity in the whole body and isolated adipocytes is decreased, as clearly indicated by ITT and glucose uptake by EPI and RETRO adipocytes; (2) Glyceroneogenic flux is decreased in EPI but increased in RETRO, as evidenced by PEPCK-C activity and rates of TAG synthesis from ^{14}C -pyruvate; and (3) The production of G3P by direct phosphorylation of glycerol is increased in EPI and decreased in RETRO, evidenced by GyK activity and rates of TAG synthesis from ^{14}C -glycerol.

Probably, DEXA-treated rats show reduced corticosterone values due to DEXA inhibition of the HPA axis, and an $11\beta\text{HSD1}$ mRNA increase may be a way to compensate them [33, 34]. It is also well established that the chronic use of glucocorticoids causes insulin resistance [35]. The administration of DEXA (1 mg/kg) in rats for 5 days induces peripheral insulin resistance, and increases glucose and insulin plasma levels [36, 37], similarly to our findings (Fig. 4). The higher plasma levels of insulin could be due to a response of islet pancreatic β cells to elevated values of circulating glucocorticoids and glucose [38]. Glucocorticoids increase hepatic glucose production, decrease peripheral glucose uptake by muscle and adipose tissue, and

increase muscle protein breakdown and TAG hydrolysis, providing additional substrates for energy generation [39]. Thus, DEXA effects in vivo on adipose tissues could be due to indirect mechanisms, in contrast to what was observed previously when WAT explants or cultured adipocytes were directly exposed to this hormone [15, 16, 40]. Previous studies have demonstrated that DEXA decreases glucose uptake [40] and GLUT4 expression [41] in rat adipose tissue. Our data show that DEXA treatment decreased the GLUT4 content (Fig. 5b) and basal glucose uptake (Fig. 5a) in RETRO and EPI; however, only in RETRO, DEXA treatment decreased the content of phosphorylated Akt (Fig. 5c), accompanied by lower insulin effects on the rates of glucose uptake (Fig. 5a) and on antilipolytic activity in isolated adipocytes (Fig. 3b). These data suggest a higher insulin resistance induced by DEXA in RETRO.

Our previous studies have suggested the existence of a reciprocal change in G3P generation from glycolysis and from glyceroneogenesis in WAT of fasted, diabetic, high-protein and carbohydrate-free diet-fed or cafeteria diet-fed rats [6, 8]. In this present work, DEXA effects on RETRO glyceroneogenesis (Fig. 6) and glucose uptake (Fig. 5a) were consistent with these previous findings, but these two metabolic pathways were both decreased in EPI from DEXA-treated rats (Fig. 8). Thus, the rat glucocorticoid treatment seems to be the first experimental model to show the loss of reciprocity between glycolysis and glyceroneogenesis in adipose tissue, as was seen in EPI. Actually, it is known that glucocorticoids reduce PEPCK-C gene expression in adipose tissue, while they are inducers in liver and kidney [1, 42]; however, there is no report comparing this effect of DEXA among different adipose tissue pads in rodents. It is known that glucocorticoids decrease PEPCK-C activity in EPI adipose tissue and culture adipocytes [12–15, 42]. In 3T3L1 adipocytes, glucocorticoids inhibit activation of the PEPCK-C gene promoter by C/EBP α or C/EBP β [16]. We believe that the absence of a DEXA effect on PEPCK mRNA and content in both WAT pads could be due to the much longer treatment time (7 days) than that used previously by others (hours), inducing some sort of adaptation of these biochemical processes. Interestingly, in this work, DEXA treatment increased glyceroneogenesis and PEPCK-C activity (Fig. 6) in RETRO, accompanied by a decrease in Akt phosphorylation (Fig. 5c), which could suggest insulin resistance in this tissue, as already commented. In the liver, insulin inhibits the expression of PEPCK-C and glucose-6-phosphatase, the rate-limiting enzymes of gluconeogenesis, via Akt-FoxO1 phosphorylation [43–45]. On the other hand, the inhibitory effect of DEXA on glyceroneogenesis from EPI occurs without changes in Akt phosphorylation (Fig. 5c). The involvement of Akt-FoxO1 in the control of PEPCK-C, a key enzyme of glyceroneogenesis, in WAT has not been reported before;

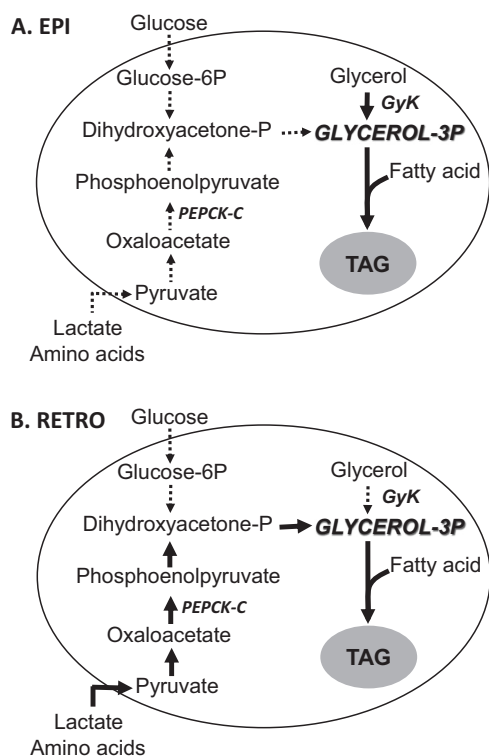


Fig. 8 Effect of glucocorticoids on G3P generation pathways in EPI (a) and RETRO (b) adipose tissue. GyK, glycerokinase; PEPCK-C, phosphoenolpyruvate carboxykinase

thus, further experiments using Akt and/or FoxO1 overexpression in adipocytes would be important to clarify this point.

Our data show that DEXA treatment induces opposing effects in the direct phosphorylation of glycerol and GyK activity, increasing these processes in EPI and decreasing them in RETRO. Our previous studies [6, 8, 9, 46] have suggested that GyK activity is regulated by neural mechanisms. The cafeteria diet increases GyK activity and glycerol incorporation into TAG-glycerol in WAT accompanied by an increase in sympathetic activity. This effect is blocked by denervation of WAT from cafeteria diet-fed rats [8]. Studies have demonstrated that sympathetic activity to WAT has a pad-specific pattern, for example increasing to RETRO, but not to EPI, after glucoprivation [18]. There are no studies investigating the effect of glucocorticoids on sympathetic activity to WAT. It is possible that an indirect mechanism regulates the glucocorticoid effect on WAT direct phosphorylation of glycerol. On the other hand, food-deprived rats have a decrease in GyK activity despite an increase in sympathetic activity to RETRO, but denervation of this tissue induces an additional decrease in the GyK activity [6]. These data suggest that other mechanisms contribute to the regulation of direct phosphorylation of glycerol in WAT. Recent studies have demonstrated in vivo that glucocorticoid and mineralocorticoid receptor antagonism markedly reduces high-fat diet-driven weight gain and fat mass expansion accompanied by a marked increase in PGC-1 α protein expression in adipose tissue from mice [47]. Thus, a direct effect of glucocorticoids regulating glycerol phosphorylation in WAT is also possible, because GyK expression is activated by PGC-1 α [48]. Glucocorticoids also regulate the expression of PGC-1 α in other cell types, inhibiting it in brown pre-adipocytes [49] and stimulating it in fetal cardiomyocytes [50].

Glucocorticoids increase the lipid content in both EPI and RETRO visceral adipose tissue, although by different mechanisms. The present data show that glucocorticoids differentially regulate glyceroneogenesis and direct phosphorylation of glycerol in RETRO and EPI adipose tissue from rats. This mechanism of glucocorticoids on G3P generation has presented a novel regulation of TAG synthesis in a depot-dependent manner.

In EPI WAT isolated from DEXA-treated rats there was a decrease in glyceroneogenesis and PEPCK-C activity, with a compensatory increase of ¹⁴C-glycerol incorporation into TAG-glycerol and GyK activity and content (Fig. 8a). In contrast, in RETRO WAT there was decreased ¹⁴C-glycerol incorporation and GyK activity, with a compensation of increased ¹⁴C-pyruvate incorporation into TAG-glycerol and PEPCK-C activity (Fig. 8b). These depot-dependent differences cannot be explained by the number of receptors, or by the 11 β HSD1 enzyme, but the

higher insulin resistance in RETRO could explain, at least in part, these results. Probably, the inhibitory insulin effect on PEPCK activity was not effective in these resistant cells, thus the effect of glucocorticoid prevails and glyceroneogenesis is increased in this tissue. In EPI, the direct phosphorylation of ¹⁴C-glycerol by GyK for incorporation into TAG is the predominant pathway, probably by an effect of glucocorticoids on GyK gene expression and content only in this tissue. This is the first time that a fundamental role of GyK in the regulation of G3P formation to maintain rates of TAG synthesis in one specific adipose tissue pad has been found. The explanation for this finding is still unknown. New experiments need to be performed to clarify the molecular mechanism of glucocorticoids regulating the G3P formation pathways in each depot, as well as to clarify the higher insulin resistance in RETRO than in EPI.

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Author contributions G.N.F., D.F., L.C.C.N., V.E.C. and I.C.K. conceived and designed the study, interpreted the results and drafted/edited the manuscript. G.N.F., R.R.V., S.L.B., S.P.G., N.M.Z. and M. A.R.G. collected data, conducted analyses and revised the manuscript. All authors approved the content of this manuscript. All authors contributed to the development, analysis and drafting of this article.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval The care and treatment of the rats received prior institutional approval by the Ethical Committee of Ribeirão Preto Medical School of the University of São Paulo, Brazil (#120/2010).

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