The antifungal agent ketoconazole protects against adiposity induced by a cafeteria diet

Short running title: Ketoconazole and obesity prevention

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ABSTRACT

The anti-glucocorticoid agent Ketoconazole is widely used in the human being as an antifungal agent, by inhibiting the synthesis of ergosterol, and in the treatment of Cushing´s Syndrome, by reducing the levels of cortisol. The aim of this work was to study in rats, the possible preventive effect of this drug against the adiposity induced by a high-fat diet (Cafeteria). Female Wistar rats were fed on standard pelleted diet or cafeteria diet during 42 days in the presence or absence of an oral treatment with ketoconazole (24 mg/kg of body weight). The cafeteria diet induced a higher energy intake and an increase of the body weight gain. In addition, the weight of the body fat and the analyzed adipose tissue depots were increased by the consumption of the high-fat diet. Interestingly, ketoconazole was able to protect against the increased total body fat and the enlargement of the adipose depots induced by the cafeteria feeding. Moreover, *ex vivo* isoproterenol-induced lipolysis was reduced in the adipocytes from the cafeteria fed animals and this decrease was reverted by the treatment with ketoconazole. Thus, the antiglucocorticoid agent ketoconazole was able to protect against the adiposity induced by a cafeteria diet, revealing an interaction between fat intake and glucocorticoids on adipose deposition.

Key words: High fat diet, glucocorticoids, lipolysis, adipocytes, obesity, rat

INTRODUCTION

Obesity has been recognized as a health problem of pandemic proportions [1]. Increased food intake and decreased energy expenditure associated to modern lifestyles have contributed to the spread of this disease. Thus, a number of studies have suggested that environmental factors, and notably dietary habits, are associated with the energy metabolism unbalance [2]. In this context, cafeteria-fed rats represent a useful model for human obesity studies because a high-fat intake appears to be a key factor in the development of human obesity [3-5].

Other identified cause of obesity and metabolic complications in humans is exposure to excessive levels of glucocorticoids (GC) in the circulating blood [6-8]. Thus, the involvement of these hormones is a necessary condition for the development of diet-induced obesity [9], while adrenalectomy (ADX) prevents the development of many forms of rodent obesity, including those induced by a high-fat intake [10, 11]. Also, hepatic glucocorticoid receptors are modulated during dietary restriction [12]. Furthermore, the blockade of the glucocorticoid receptor (GR) with antagonists inhibits dietary and genetic induced obesity [13, 14].

On the other hand, ketoconazole (KCZ) has been widely used as an antimycotic agent because of its effectiveness after oral administration against a wide range of fungal pathogens [15]. KCZ effects in fungi includes the inhibition of the synthesis of ergosterol by blocking 14-demethylation of lanosterol and the inhibition of the transformation of yeast to the mycelial form [16]. This drug is also considered as an anti-glucocorticoid agent by interfering with several cytochrome P-450 dependent enzymes involved in adrenal and gonadal steroid synthesis [17-19]. Due to its ability to reduce circulating GCs, KCZ has been successfully used as a palliative treatment of Cushing's syndrome [20, 21].

Taken together all of this, in this research we studied the possible protective effect of an oral treatment with KCZ in a rodent model of obesity, which was induced by a high-fat intake by specifically analyzing in such animals different indicators of adiposity and *ex vivo* lipolytic activity.

MATERIAL AND METHODS

Animals

Female Wistar rats, supplied by Harlan Iberica (Barcelona, Spain), weighing about 185 g, where housed at 21-23ºC with a 12 hours light cycle (8 a.m. to 8 p.m.) and distributed in different groups. A group of animals (Control and Control + KCZ groups, n=14) were fed on standard pelleted diet (Harlan Iberica, Barcelona, Spain) containing 16.6% of energy as protein, 73.1% of energy as carbohydrate and 10.3% of energy as lipid by dry weight. The remaining animals (Cafeteria and Cafeteria + KCZ groups, n=14) were fed on a high-fat diet in order to generate a diet induced obesity model. High-fat diet components were pate, bacon, chips, cookies, chocolate and chow with proportions 2:1:1:1:1:1, which was given to each rat daily as published elsewhere [22, 23]. The composition of the cafeteria diet was 9.3% of energy as protein, 31.5% of energy as carbohydrates and 59.2% of energy as lipids by dry weight (462 Kcals per 100 g in the cafeteria diet vs. 349 Kcals per 100 g in the pelleted diet). Animals had *ad libitum* access to water and food during the experimental trial. Body weight and food intake were recorded daily. In order to evaluate the effects of the administration of KCZ, which was generously supplied by VITA (Barcelona, Spain), both dietary groups were divided into two new subgroups, which received daily at 4:00 p.m. a dose (24 mg/kg per day) of KCZ (Control + KCZ and Cafeteria + KCZ groups, n=14) or vehicle, by oral gavage (Control and Cafeteria groups, n=14) as described elsewhere [19, 24]. At the end of the experimental period (42 days), rats were anaesthetized in the fasted state with Ketamine (50 mg/kg *ip*, Parke-Davis, Madrid, Spain) and Medetomidine (0.025 mg/kg *ip*, Pfizer S.A., Madrid, Spain) for the analysis of the body composition. After that, animals were sacrificed by decapitation and blood and tissue samples were immediately collected and

weighed. All the procedures were performed according to national and institutional guidelines of the Animal Care and Use Committee at the University of Navarra.

Body Composition

Body composition was assessed at the end of the experimental period, in fasted and anaesthetized animals, by using a non-invasive electromagnetic apparatus devised for rodents (EM-SCAN model SA-2, Springfield, IL, USA) as described and validated elsewhere [5].

Organ Weight

After the animals were sacrificed, periovarian, retroperitoneal and subcutaneous white adipose tissue (WAT), liver and ovaries were carefully excised, isolated and weighed.

Serum measurements

Serum free fatty acids were measured by the NEFA C ACS-ACOD method (Waco Chemicals USA, Inc., Richmond, VA, USA). Glycerol and triacylglicerides were determined with the RANDOX kit for *in vitro* glycerol and triacylglicerides diagnostic, respectively (Randox Laboratories LTD, Ardmore Road, UK). Glucose was measured with the GOD-PAP kit (ABX diagnostic, Montpellier, France). All of those measurements were adapted for a COBAS MIRA (Roche, Basel, Switzerland) equipment. Serum corticosterone was assayed by radioimmunooassay (RIA) as described by the supplier (Diagnostic Products Corp., Los Angeles, CA, USA).

Adipocyte isolation and lipolysis measurements

Adipocytes were isolated according to previously described methods [22, 25]. After collecting the adipose tissue, periovarian WAT samples were cut into small pieces and the fragments were digested at 37ºC with collagenase I (1.25 mg/ml) as the digestive enzyme (Worthington Biochemical Corp., Lakewood, NJ, USA) in Krebs-Ringer Bicarbonate Buffer containing albumin (3.5 g/100 ml) and glucose (6 mM) at 7.4 pH. The ratio of digestion solution for the adipose tissue mass was 2 ml per gram. After 30 min of incubation under continuous vigorous shaking (60 cycles/min), the fat cells were filtered through a nylon mesh and washed three times with KRBA to eliminate the stroma-vascular fraction and collagenase. The fat cells (5-15 mg of total cell lipids), were then incubated in polyethylene tubes (1 ml of incubation medium) with continuous gentle shaking (40 cycles/min) in a water bath at 37ºC in presence (10^{-10} to 10^{-6} M) or absence of isoproterenol (Sigma Chemical Co, Saint Louis, MO, USA). After 90 min, the incubation tubes were placed in an ice bath and 200 µl of the infranatant were removed for the enzymatic determination of the glycerol released into the incubation medium, which was taken as an index of lipolytic rate [22]. Total lipid content was evaluated gravimetrically after extraction as previously described [26]. Data were expressed as µmoles of glycerol released per 100 mg of total lipids after 90 min of incubation in KRBA.

Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM). Data and interactions were evaluated through a factorial two-way ANOVA (Diet, KCZ, and Diet x KCZ), which were followed by Tukey or U-Mann Whitney tests as appropriate. The SPSS 11.0 package for Windows was used for the statistical analysis. The level of probability was set at $P<0.05$ as statistically significant.

RESULTS

Effects on Body Weight and Food intake.

Both groups of animals fed on the high-fat diet (Cafeteria and Cafeteria + KCZ groups) during the 6 weeks gained more than those animals (Control and Control + KCZ groups) fed on the standard-fat diet (Table 1). On the other hand, KCZ treatment was unable to prevent this increase in body weight induced by the cafeteria diet (Cafeteria + KCZ vs. Cafeteria group). Although food intake (expressed in g and g/day) was significantly decreased in rats fed on cafeteria diet as compared to controls (-10.6 ± 2.7%, $P<0.01$), the energy consumed (expressed in Kcal and Kcal/day) by these animals was significantly higher (17.4 ± 3.6%, $P<0.01$). Moreover, cafeteria groups consumed 6.8 times more dietary fat and the energy efficiency (g of body weight gained per 100 Kcal consumed) was 19.9 ± 5.7% greater for the high-fat fed animals than for controls ($P<0.05$).

Effects on Body Composition.

The assessment of the body composition by using the EM-SCAN showed (Table 2 and Figure 1) that the proportion of total fat from overweight rats (Cafeteria group) was 50% greater ($P<0.001$) than in lean animals (Control and Control + KCZ groups). The treatment with KCZ (Cafeteria + KCZ group) was able to prevent the increase of total fat the induced by the cafeteria diet intake ($P<0.001$) without affecting this measurement in the Control + KCZ group. On the other hand, the lean mass was not apparently affected neither by the diet nor by the treatment when was expressed in grams (Figure 1), although a statistically marginal tendency ($P<0.10$, interaction diet x KCZ) was observed for an increased lean mass in the Cafeteria + KCZ group (194.7 ± 4.9 g) in comparison to the others groups (Control: 182.7 ± 2.5, Control + KCZ: 177.2 ± 6.7, and Cafeteria: 181.5 ± 5.2 g).

Effects on tissue weights

When rats were fed on the high-fat diet (Cafeteria group), there was a significant enlargement of the fat pads in comparison to Control and Control + KCZ groups (Table 2). Thus, periovarian, retroperitoneal and subcutaneous adipose tissues were significantly increased ($P<0.001$) by 98.4 ± 12.4, 129.2 ± 12.7, and 70.0 ± 6.3%, respectively by the high-fat dietary intake. However, the KCZ administration to high-fat fed animals (Cafeteria + KCZ group) was able again to prevent this higher fat deposition induced by the diet ($P<0.01$) without affecting this variable in these animals receiving KCZ and fed on the standard-fat diet (Control + KCZ group), which was confirmed by the statistical interaction found ($P<0.05$). The analysis of other tissues, showed a slight decrease (-11.6 ± 1.3 %, $P<0.05$) of the liver proportions by the cafeteria diet intake and an increase of liver and ovarian weights (17.8 ± 5.3 and 45.2 ± 2.3 respectively) by KCZ dosage ($P<0.001$). Moreover, although the differences did not reach statistical significance, it appears that the KCZ prevented the observed diet-reduced liver weight.

Effects on serum measurements

Serum fasting lipid measurements (Free fatty acids, triglycerides, glycerol, and cholesterol) and glucose levels were not significantly affected by the high-fat diet and KCZ treatment, according to the ANOVA 2x2 design (Table 3). The corticosterone levels (ng/ml) were high in all experimental groups as compared with reference values, presumably because the stressful sacrifice procedure, although non-statistical differences were observed among them. Thus, the circulating corticosterone concentrations when expressed as a % of the control group (100%), in order to circumvent the stress influence, were 90% and 95% for the Cafeteria and cafeteria + KCZ groups, respectively in relation to the reference group (Control).

Effects on lipolysis measurements.

The possible interaction of KCZ and the experimental diet on the lipolysis was analyzed in isolated adipocytes from the periovarian WAT, which were incubated in the absence and presence of different concentrations of isoproterenol, a β-adrenergic agonist, to determine the glycerol release as an indicator of the lipolytic rate. Basal data (without isoproterenol) were not significantly different in the *ex vivo* lipid mobilization in adipocytes from the four groups of the experimental design (Control: 0.21 ± 0.02, Control + KCZ: 0.24 ± 0.05, Cafeteria: 0.11 ± 0.02 and Cafeteria + KCZ: 0.21 ± 0.08 μmols of glycerol per mg of lipids). In addition, the increasing concentrations of isoproterenol (10^{-10} to 10^{-6} M) produced a significant enhancement of the glycerol release in the four groups (Figure 2). This stimulation was significantly impaired at two concentrations of isoproterenol (10^{-7} and 10^{-6} M, $P<0.05$) by the diet (Cafeteria group) respect to the rest of the groups. Interestingly, KCZ protected this reduction of the lipolysis induced by the high-fat diet without affecting *per se* the rise of the isoproterenol–induced glycerol release.

DISCUSSION

The current study has been designed to assess the effects of the anti-glucocorticoid KCZ administration on adiposity and lipolytic activity in rats with a diet-induced overweightness. High-fat diets are widely used, being an accepted strategy to induce fat accumulation in animals [3, 22]. As expected, the intake of this "cafeteria" diet during 6 weeks (Cafeteria group vs. Control group) produced an increase of body weight gain, total body fat percent and adipose tissue deposition. Thus, cafeteria diet intake induced a rise in total adiposity, which could be considered as a situation of overweightness rather than "obesity", since the final differences in body weight were small (10-15 g). This enhanced adiposity is related to the higher energy intake, fat consumption and energy efficiency observed in high-fat fed animals and is in good accordance with data from previous reports [3, 22, 23]. In addition, isoproterenol dependent lipolysis was *ex vivo* measured from adipocytes of the periovarian fat depot and the analysis revealed that this activity was reduced by a high-fat diet intake. This finding has been attributed to a loss of β_1 and β_3 adrenoceptor numbers in adipocytes from different regional areas induced by the cafeteria diet or to alterations of their coupling to the adenylate cyclase system resulting in a reduced β-adrenergic responsiveness [27, 28].

Regarding the antifungal agent KCZ, the oral treatment with this drug to control rats (Control + KCZ group vs. Control group) during 6 weeks did not produce *per se* any change on body weight [29, 30] as well as on other measurements usually affected by the cafeteria diet. Although KCZ is a potent inhibitor of gonadal and adrenal steroidogenesis, it was not previously reported any effect of KCZ on fat metabolism or food intake before. Concerning the other analyzed tissues, it was published a dose-dependent increase of the liver and ovaries weights after an *in vivo* treatment with KCZ to rats [17, 30, 31]. The effect on the liver is probably due to the influence of this drug on the hepatic microsomal

activity [31] and on the hepatocellular uptake of bile acids, producing hepatic injury [24]. Moreover, ovaries hypertrophy could be produced by a change of the progesterone/LH ratio as induced by the treatment of KCZ [17]. These results confirm the correct administration of this drug during 42 days, and no effects on the other analyzed measurements were found. Despite that no data of brown adipose tissue weight and leptin has been obtained in the current experiment, previous reports from our laboratory confirm that both measurements are increased by a high-fat intake [5]. Since this tissue contains glucocorticoid receptors and corticosterone is known to inhibit non-shivering thermogenesis [32], administration of an anti-glucocorticoid agent may be also involved in a brown adipose tissue enlargement.

The study of the combined effect of the diet and KCZ revealed that the drug administration protects to a large extent against the development of adiposity associated with high-fat feeding. Thus, KCZ was able to avoid the increase of fat-related indicators (% of total body fat, and adipose tissue weights) and the reduction of the lipolytic activity without affecting food and energy intake. It is assumed that KCZ treatment impairs steroid synthesis and decrease corticosterone values in the rat [18, 19]. The reasons because no statistical differences in the corticosterone levels were found among the experimental groups should be attributed to the fact that all of them were extremely high as a consequence of the sacrifice procedure (decapitation), which may mask the anti-glucocorticoid actions of the treatment on this determination. Other explanation could be ascribed to the fact of the long-term period of the treatment (42 days) as compared to other reports, which may trigger compensatory mechanism.

Indeed, previous studies concerning the *in* vivo effects of KCZ on corticosterone levels revealed contradictory results depending on the dose, the conditions of measurement (stress/normal) and the sex of the animals [19, 33, 34]. Classically, GCs are permissive

agents on the increase of adipose tissue depots induced by a high fat-induced intake. Thus, adrenalectomy impairs the increase of the fat depot induced by cafeteria diet in fatty Zucker rats [11], the use of other GR antagonists (DHEA and mifepristone) has a protective effect against accumulation of fat depots induced by cafeteria diet [13, 35], and high GC sensitivity in adipose tissue develops visceral obesity that is exaggerated by a high-fat diet [7]. Furthermore, glucocorticoid increases associated to stress appear to facilitate body weight gain in rats fed on a high-energy diet [36]. In this context, it has been reported that cafeteria diet increases the hypothalamic-pituitary-adrenocortical axis, enhancing GCs effects [37] and the increased levels of GCs could lead to a redistribution of stored energy towards an enlargement of the abdominal fat depots in the presence of insulin [38]. Thus, in the context of the cafeteria diet, where it has been reported a moderate hyperinsulinaemia [39], KCZ treatment could avoid the permissive action of the GCs in the increased adiposity and the redistribution of the fat within the intraabdominal region.

Regarding the reverted decrease by KCZ treatment of glycerol release in adipocytes from rats with cafeteria diet, the role of GCs in the regulation of lipid metabolism in adipose tissue is a matter of debate. While it has been published in humans that these hormones have no effect [40], stimulate [41, 42] or even inhibit lipolysis [43, 44], in rat adipose tissue, seem to increase the ability of lipolytic agents to activate triglyceride breakdown [45, 46]. However, regional variations in adipose tissue about GCs receptors expression [47], GCs effect/response [48, 49], and different fat accumulation GCs-mediated effects [50] have been described. Moreover, it has been reported in adipose tissue of rats treated with cafeteria diet regional variations (visceral *vs.* subcutaneous) regarding the lipolytic activity catecholamine-induced, and also on β1-α2-β3 adrenoceptors expression [28, 51-53], although the periovarian adipose tissue is considered a

representative area for carrying this kind of studies [5]. In this sense, GCs reduce in white adipose tissue the β3 adrenoceptors protein expression and β3-adrenoceptor-mediated adenylate cyclase activity in a 3T3-f442A mouse cell line [46, 54].

Taken together these studies, it is possible that, although stimulating overall lipolysis at the whole body level, GCs may specifically inhibit lipolysis at local level [55, 56] and produce a redistribution of white adipose tissue into the abdominal region [38]. The reduction of the lipolytic activity in periovarian fat by the high-fat diet could be mediated by the GCs, which induce a decrease of β_1-β_3 adrenoceptors protein expression and impairing the cAMP production and elimination rate [46]. Thus, KCZ-treatment could protect the reduction of the lipolysis induced by the cafeteria diet intake by lowering GC circulating levels and preventing the decrease of adrenergic receptors. Furthermore, since gonadal hormones are known to exert strong effects on energy balance [57-60], and that KCZ administration also decreases the level of these hormones [17, 30, 61], it can not be discarded a possible mediating effect of gonadal steroids to explain our results [62]. Finally the regulation by GCs of the antilipolytic effect of insulin and the lipolytic effects of both catecholamines and glucagon could be impaired by KCZ and should be not excluded to explain our experimental outcome [63-65].

In summary, the current findings suggest that KCZ could protect against the high-fat diet induced adiposity by impairing the permissive role of GC in this process [9]. On the other hand, although is possible to consider KCZ as an ADX-like chemical agent, others authors reported a possible role of KCZ directly antagonizing GR [66]. Clearly, further studies are needed to clarify the mechanisms by which KCZ participate in the protection of cafeteria-induced diet, focusing on the mechanisms affecting GC sensitivity, although this experiment have clearly demonstrated an interaction between fat intake and glucocorticoids in adiposity induced by a high fat diet.

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LEGENDS FOR FIGURES

Figure 1. Stacked bars showing the fasting weight of total body fat and lean mass of the experimental groups (Control, Control + KCZ, Cafeteria, and Cafeteria + KCZ). Data are given as the mean ± SEM for lean and fat mass. ¥, $P<0.001$ vs. Control group of fat mass; §, $P<0.001$ vs. Control + KCZ group of fat mass; #, $P<0.001$ vs. Cafeteria + KCZ group of fat mass ($P<0.10$ for the interaction diet x KCZ).

Figure 2. Isoproterenol-induced glycerol release in adipocytes from the experimental groups (Control, Control + KCZ, Cafeteria, and Cafeteria + KCZ). Adipocytes were isolated from periovarian adipose tissue as described under the *Material and Methods* section. Data are given as the mean ± SEM. ¥, $P<0.01$ vs. Control group; §, $P<0.01$ vs. Control + KCZ group; ‡, $P<0.05$ vs. Cafeteria + KCZ group; #, $P<0.01$ vs. Cafeteria + KCZ group.