CHRONIC UNPREDICTABLE MILD STRESS PROGRESSIVELY DISTURBS GLUCOSE METABOLISM AND APPETITE HORMONES IN RATS

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Abstract

Context. Chronic stress is characterized by increased release of catecholamines, glucocorticoids and other neurohumoral factors, predisposing individuals to obesity, insulin resistance and vascular disease, pathologies considered priority health problems. Study of alterations induced by stress on metabolism in association with food intake modulatory hormones (insulin, leptin and ghrelin) is mandatory.

Objective. This research studied temporal course during 60 days of chronic unpredictable mild stress (CUMS) on glucose and lipids metabolism, and on the neuroendocrine system that regulates appetite-satiety balance.

Materials and Methods. Wistar rats were exposed to CUMS for 20, 40 and 60 days. Corticosterone stayed high during 60 days of CUMS; after 40 days, body weight, cholesterol and triglycerides decreased and glucose intolerance was evident at day 60; insulin and ghrelin increased at 20 and 40 days, respectively; leptin decreased after day 20. Data suggest that 60 days of CUMS progressively disturb metabolism of carbohydrates and lipids as well as food intake regulatory hormones, affecting the metabolism, and can lead to the development of chronic degenerative diseases, such as cardiovascular disease, metabolic syndrome and type 2 diabetes.

Key words: stress, insulin, ghrelin, Leptin, chronic stress, metabolism alterations.

INTRODUCTION

Several genetic and physiological factors play a central role in the development of obesity, hypertension, insulin resistance and diabetes, disorders commonly grouped as metabolic syndrome. In particular, physiological stress, characterized by increased release of corticosteroids and other neurohumoral factors, is considered a fundamental risk factor of these disorders (1-3).

To understand the pathophysiological mechanisms underlying metabolic disorders, attention has been paid to the central regulatory elements of energy homeostasis, including food intake and energy expenditure. Although in regulating energy balance the relationship between processes of feeding and metabolism suggests involvement of the hypothalamic-pituitary-adrenal axis (HPA), this association has been little explored (4-6).

Thus for example, feelings of hunger and satiety result from orchestrated communication between peripheral signals (leptin, insulin and ghrelin) and sensory neurons in hypothalamus, among other regions (7). Ghrelin levels are increased in a negative energy balance, such as in fasting and anorexia nervosa, and are decreased in positive energy balance, as in obesity. Therefore, together with leptin and insulin, ghrelin belongs to a set of peripheral signs that inform to the brain about status of energy stores and contributes to long-term weight regulation.

In relation to HPA axis, it is well known that corticosteroids stimulate liver glycogenolysis and gluconeogenesis and inhibit insulin sensitivity and glucose uptake in skeletal muscle, leading to impaired glucose tolerance and insulin resistance (8). Corticosteroids also stimulate breakdown of stored triglycerides in adipose tissue, augmenting plasma free fatty acids (9). In addition, liver triglyceride synthesis and very low-density lipoproteins are increased. Insulin-resistance states are characterized by increased lipolysis in adipocytes, and raised free fatty acids and lipoproteins (9).

In this sense, rats submitted to immobilization during 15 days showed just a small gain of body weight, changes in carbohydrate metabolism and insulin resistance. However, an adaptive response at

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30 days of treatment was observed (10, 11). In other study, rats submitted to intense noise stress during 90 days showed elevation in corticosterone level and modifications in serum leptin (12). Thus, although the association between physiological stress and metabolic disorders is commonly accepted, when experimental animals are submitted to monotonous stressor, in many cases habituation is observed, which delays the metabolic abnormalities progress.

In contrast, the model of chronic unpredictable mild stress (CUMS) has been validated like a model with a well-characterized symptomatology and in which the habituation is absent still at 60 days of stress (13-16). In any way, the progression of the effects of chronic stress on metabolism for time periods longer than 40 days has not been explored yet in CUMS.

Therefore, to study the alterations due to stress on metabolism and its association with food-intake modulatory hormones (insulin, leptin and ghrelin) is mandatory. The aim of this research was to establish the temporal course of the changes produced by 60 days of CUMS on the metabolism of carbohydrates, lipids and food-intake modulatory hormones in rats.

MATERIALS AND METHODS

Animals

Thirty adult male Wistar rats aged 3 months and weighing 250-300 g, were obtained from the experimental animal center at the UAM-Iztapalapa. They were housed with 5 rats per cage (50×30×20 cm) throughout the study, separated into control and experimental groups with 15 rats each, and kept under standard vivarium conditions. The colony room was maintained on a 12:12 light-dark cycle (lights on from 9:00 a.m. to 9:00 p.m.) and controlled temperature (24±1°C). Food and water were available *ad libitum* throughout the experiments. A Harlan diet for rodents with 22% protein, 5.5% fat and 3.9% fiber was utilized. The handling of the laboratory animals and experimental procedures were performed according to national and international rules (NIH guidelines for handling and care of animals), including the Official Mexican Rule (NOM-062-ZOO-999, revised 2001) and the National Institutes of Health (NIH Publication No. 8023, revised in 1996) for the health, safety and comfort of experimental animals.

Chronic unpredictable mild stress (CUMS)

The two groups of animals (control and experimental) were placed in separate rooms. The

control group (n=15) was separated into three subgroups of five rats each (subgroups 1-3), and the rats were only handled to register their weight and change the box. The control animals of these subgroups were sacrificed by decapitation at 20 (subgroup 1), 40 (subgroup 2) and 60 days (subgroup 3) after initiation of the study. The experimental group (n=15) was also separated into three subgroups of five rats each and were subjected to a CUMS protocol for 20, 40 and 60 days following the protocol described in Table 1.

This CUMS protocol is a slightly modified version of that developed by Willner (13) and Gamaro (14). It includes different stressors and variable exposure times, as follows: food deprivation, water deprivation, immobilization, cold (4°C), immersion in cold water (15°C), persistent light, isolation, the presence of a foreign object (10-cm diameter plastic balls placed), cage tilting (40°), overcrowding (15 animals per box), wet bedding and noise (40 dB).

Stressors were applied randomly, preferentially at the beginning of the light phase, between 9 and 11 a.m. During the periods of stress application, body weight and food intake were monitored every 5 days. To exclude the possible effect of acute stress, the rats were sacrificed by decapitation one day after completing the stress procedure. Serum was obtained by centrifuging the blood at 1500xg for 10 minutes and stored at -70°C to quantify corticosterone, insulin, ghrelin and leptin.

Oral glucose tolerance tests (OGTT)

The OGTT were performed in control and experimental groups at 20, 40 and 60 days. Tests were conducted after 12 h of fasting, and consisted of the intragastric administration of anhydrous dextrose (2g/kg), with glycemia measurements at T=0 and 30, 60, 90 and 120 min. The experimental design was performed to determine the OGTT at the same time in all groups submitted to chronic stress and the control group. Glycemia was measured with blood samples taken from the tail vein by the glucose dehydrogenase method (Accu Chek Performa, Roche). The area under the curve during the OGTT was computed according to the Tai model (17).

Food intake and body weight

The animals were separated into groups of 5 rats each and maintained in 50 × 30 × 20 cm boxes (5 rats/box). The quantity of food was weighed and given *ad libitum* for each box, weighing the remaining food each 24 h beginning at 9:00 A.M. Food intake was

Table 1. Chronic unpredictable mild stress applied for 20, 40 and 60 days to three month old male Wistar rats

Day	Stressor (time)	Day	Stressor (time)
1	Food deprivation (12 h)	31	Immobilization (2 h)
2	Water deprivation (12 h)	32	Persistent light (24 h)
3	Persistent light (24 h)	33	Food deprivation (12 h)
4	Overcrowding (24 h)	34	Wet bet (12 h)
5	Overcrowding (24 h)	35	Isolation plus foreign object (5 h)
6	Without stressor	36	Immersion in cold water (15 min)
7	Immersion in cold water (15 min)	37	Water deprivation (24 h)
8	Immobilization (1.5 h)	38	Immobilization plus cold (1.5h)
9	Isolation (24 h)	39	Without stressor
10	Isolation (24 h)	40	Food deprivation (12 h)
11	Food deprivation (12 h)	41	Overcrowding (24 h)
12	Immobilization (2 h)	42	Persistent light (24 h)
13	Overcrowding (24 h)	43	Without stressor
14	Overcrowding (24 h)	44	Noise (3 h)
15	Persistent light (24 h)	45	Isolation (24 h)
16	Wet bet (12 h)	46	Water deprivation (24 h)
17	Immersion in cold water (15 min)	47	Immobilization plus cold (2 h)
18	Immobilization (1.5 h)	48	Foreign object plus noise (5 h)
19	Without stressor	49	Cage tilting 45° (5 h)
20	Food deprivation (12 h)	50	Food deprivation/overcrowding (24 h)
21	Persistent light (24 h)	51	Cold (3 h) plus persistent light (24 h)
22	Immobilization (2 h)	52	Water deprivation (24 h)
23	Water deprivation (12 h)	53	Persistent light (24 h)
24	Isolation (24 h)	54	Overcrowding (24 h) plus noise (5 h)
25	Isolation (24 h)	55	Immobilization (2 h)/overcrowding (24 h)
26	Cage tilting 45° (5 h)	56	Isolation (24 h)
27	Cold (3 h)	57	Foreign object (5 h)
28	Food deprivation (12 h)	58	Cold plus immobilization (2.5 h)
29	Immersion in cold water (15 min)	59	Noise (5 h) plus immersion cold water (15 min)
30	Overcrowding (24 h)	60	Without stressor

measured by subtracting the final food weight against the initial for box, divided between 5 animals. In Figure 3 food intake is given in g for rat/day. Body weight was recorded every 5 days between 9:00 and 10:00 a.m. since the first day until the last 60 days.

Cholesterol and triglycerides

Serum cholesterol and triglyceride levels were assessed in serum samples drawn from the rats subjected to 20, 40 and 60 days of CUMS using the Reflotron System (Roche).

Corticosterone, insulin, ghrelin and leptin

Quantification of serum insulin, leptin, ghrelin and corticosterone levels was performed by means of ELISA tests Merk Millipore (Darmstadt, Germany) for leptin, total ghrelin and insulin; ALPCO immunoassays (Sale N.H. USA) for corticosterone. All kits used were designed for rats. The ELISA plates were read by a prism spectrophotometer (Thermo Scientific Multiskan GO) at the wavelengths specified by each supplier.

Statistical analysis

Data were presented as the mean $\pm$ SEM. For the statistical analysis, because the control subgroups did not vary over the three time points ($p > 0.05$), they were grouped into a single group with $n=15$ for corticosterone, leptin, ghrelin, insulin, triglyceride and cholesterol levels, and OGTT. All of these findings were analyzed using one-way ANOVA followed by a Tukey- Kramer test. Body weight comparisons were assessed with Student's t-test for independent data. In both cases, NCSS 2007 (NCSS software, Kaysville, UT, USA) was used. Statistical significance was set at $p < 0.05$.

RESULTS

Effect of CUMS on corticosterone levels

CUMS increased more than 200% corticosterone levels at 20 days ($p < 0.05$), and this increase was maintained at 40 and 60 days [$F(3.26)=3.85$, $p < 0.05$] (Fig. 1).

Effect of CUMS on carbohydrate management

In OGTT, no changes were observed in the basal glucose of either group. Basal glycemia in controls was 74.72 ± 3.0 mg/dL, and the hyperglycemic peak was reached at 60 min after dextrose administration

Figure 1. Effect of CUMS on corticosterone in rats (20, 40 and 60 days). Mean $\pm$ SEM, Control: n=15; CUMS: n=5. ANOVA, Tukey-Kramer. *Significant difference against control ($p < 0.05$).

Figure 2. A) Effect of CUMS on the Oral glucose tolerance test (OGTT). B) Area under the curve of the OGTT in rats subjected to CUMS (20, 40 and 60 days). Mean $\pm$ SEM, Control: n=15; CUMS: n=5. ANOVA, Tukey-Kramer. *Significant difference against control ($p < 0.05$).

(133.06 ± 7.9 mg/dL). This concentration decreased significantly ($p < 0.05$) at 90 (119 ± 5.7 mg/dL) and 120 min (97.63 ± 2.7 mg/dL) (Fig. 2A). The behaviour of the rats subjected to CUMS was similar to that of the control group after 20 days of stress; however, after 40 days, the hyperglycemic peak increased significantly ($p < 0.05$) at 60 min, showing a clear alteration of the glucose tolerance. This alteration was more pronounced after 60 days of stress. The area under the curve increased 21% at 40 days and 23% at 60 days of CUMS [$F(3.26) = 12.52$, $p < 0.05$] (Fig. 2B).

Effect of CUMS on body weight gain and food intake

The weight gain observed in the control group was similar to weight gain seen in the stress-treatment group up to day 40; however, at 50 days of CUMS, body weight decreased compared to controls ($p < 0.05$) (Fig. 3A). With respect to food intake, the chronic stress did not modify this parameter, as shown in Figure 3B.

Effect of CUMS on cholesterol and triglycerides

Chronic stress showed an effect on cholesterol levels at 40 and 60 days of exposure to CUMS [$F(3.26) = 8.47$, $p < 0.05$]. Cholesterol decreased 15% compared to control (Fig. 4A), while triglycerides decreased 34% after 60 days of stress [$F(3.26) = 4.17$, $p < 0.01$] (Fig. 4B).

Figure 3. Effect of CUMS on body weight gain (A) and food intake (B) after 60 days of CUMS. Mean $\pm$ SEM, (n=5). Student's t-test for independent data. *Significant difference against control ($p < 0.05$).

Effect of CUMS on insulin, leptin and ghrelin levels

CUMS significantly modified insulin, leptin and ghrelin levels ($p < 0.05$). Insulin increased significantly at 134% after 20 days of exposure to stress [$F(3,26)=13.17$, $p < 0.05$] (Fig. 5A). Leptin levels decreased 70% from 20 to 60 days of stress [$F(3,26)=23.8$, $p < 0.05$] (Fig. 5B), whereas ghrelin increased 149% after 40 days of CUMS [$F(3,26)=3.95$, $p < 0.05$] (Fig. 5C).

DISCUSSION

This research was planned to establish the progression of the changes produced by 60 days of CUMS on the metabolism of carbohydrates, lipids and food-intake modulatory hormones (ghrelin and leptin) in rats. The results showed gradual changes in corticosterone, body weight metabolism of carbohydrates and lipids, and food-intake regulatory hormone balance suggesting a gradual deregulation of HPA axis through negative feedback mechanisms that

chronically for 60 days induced a progressive change in metabolic response without habituation (Fig. 6). This is the first study in which it is approached the association among chronic stress (CUMS), metabolism and the food-intake regulatory hormone system that at long term generate degenerative disorders through of inflammatory mechanisms and redox misbalance, as it was already previously reported (16), without habituation until 60 days of stress. In addition, this is the first study in which the effects of CUMS are studied

Figure 4. Effect of CUMS on cholesterol (A) and triglycerides (B) of rats subjected to CUMS (20, 40 and 60 days). Mean $\pm$ SEM, Control: $n=15$; CUMS: $n=5$. ANOVA, Tukey-Kramer. *Significant difference against control ($p < 0.05$).

Figure 5. Effect of CUMS on insulin (A), leptin (B) and ghrelin (C) in rats subjected to CUMS (20, 40 and 60 days). Mean $\pm$ SEM, Control: $n=15$; CUMS: $n=5$. ANOVA, Tukey-Kramer. *Significant difference against control ($p < 0.05$).

Figure 6. Behaviour of physiological metabolic parameters in A) the control group and B) animals subjected to 20, 40 and 60 days of physiological stress. BW=body weight; TG=triglycerides; Chol=cholesterol; AUC= area under the curve; Lep=leptin; Ghrl=ghrelin; Ins=insulin; COR=corticosterone.

for periods of time longer than 40 days, implicating the food-intake regulatory hormone system.

Stress response depends on the nature and time of exposure to the stressor. The “stress spectrum hypothesis” establishes that acute psychological stress induces an immediate physiological response (18). In contrast, increased corticosterone has been observed after chronic exposure to stress, which is considered a beneficial stimulus in stressed rats that promotes adaptation and survival, and is a characteristic of the “eustress spectrum”. The progression of chronic stress over 20 days probably makes that animals respond in the middle of this spectrum (i.e., “resistance”), which supports survival for longer periods under increasingly demanding conditions. When stress continues beyond of 40 days, the stress response probably will progress to one extreme of “stress spectrum” –i.e., “distress”– where the precipitation or exacerbation of metabolic impairment is evident; and when stress is more prolonged, it generate degenerative disorders, endangering the life of the individual.

In this work, exposure time was extended to determine whether stress causes progressive alterations

or habituation of the experimental animals. High corticosterone levels are considered markers of stress. Previous experiments showed that different types of acute stress in rats significantly increased plasma corticosterone levels (11, 18, 19). In this regard, previous studies showed that chronic exposure using the same stressor under conditions of low-intensity or of psychological nature, may induce an adaptation (20, 21), resulting in reduced corticosterone levels (22). However, in CUMS applied for 60 days, high corticosterone levels were observed due to different stressors that were used through the assay to prevent adaptation (16).

CUMS reduced body weight after 40 days without modifying food consumption. In contrast, chronic stress due to electrical shocks or immersion in cold water and immobilization increased food-intake in rats, without significant changes in body weight (11, 23). These data seem to suggest that the effects on the body weight depend on the intensity of the stressor. In fact, different stressors can have different metabolic consequences, which may be influenced by the type, intensity, and duration of the stressor. Therefore, unpredictable stressors may lead to weight loss, whereas predictable ones may lead to weight gain. In addition, although corticosterone levels were elevated after 20 days of stress, no changes in body weight were observed at 20 days, this probably due to resistance systems that participate to compensate the stress effects. Nevertheless, after 40 days of stress, these compensating mechanisms are suppressed, resulting in a loss of body weight, probably due to the generation of a resistance to anabolic action of corticosterone.

Furthermore, psychological stressors may be associated with weight gain, whereas physical or immune stressors may lead to weight loss (10,24,25). In the present work, although in CUMS submitted stressed organism the neuroendocrine energy balance (by increasing ghrelin and decreasing leptin) tends to be re-established, a suitable integration of responses at the hypothalamic level does not occur, resulting in a null gain of body weight.

Reduced plasma triglycerides may result in enhanced activity of lipoprotein lipase, an enzyme regulated by cortisol that may influence on low body weight (26, 27). Reductions in body weight and adipose tissue could be due to some degree of decreased food intake; however, in the present study, food intake did not change. Although oscillatory variations in food intake were observed in control and experimental group, these were homogeneous in both groups until 40 days, oscillating between 30 ± 10 g per animal, which fell within

normal range of food intake for this species (28). After 40 days the stress group showed decreased weight body despite the fact that food intake did not modify.

Therefore, it is likely that other glucocorticoid metabolic effects are participating too. For example, cortisol decreases body mass, suggesting that the declination in food intake should be accompanied by a change in metabolism, which fosters weight loss. Indeed, chronic glucocorticoid release increases body temperature and overall energy expenditures (24) and alters sleep patterns, all of which may promote decreases in body mass, even under conditions of normal food consumption.

Although CUMS did not cause impaired fasting glycemia, increased hyperglycemic peaks and areas under the curve during OGTT suggest impaired glucose tolerance after of 40 days, which might progress to insulin resistance. This alteration may be related to the high metabolic wear to which the animals were subjected to chronic stress, which increased liver gluconeogenesis by excessive release of glucocorticoid, inhibiting insulin sensitivity (29). Insulin action is the principal determinant of glycemia; however, a decrease in insulin action is accompanied by hypersecretion of insulin (30), to abolish insulin resistance and to maintain normoglycemia.

In the present research, increased insulin levels at day 20 were observed, whereas impaired glucose tolerance was observed after 40 days, which might lead to long-term insulin resistance (9). Glucocorticoids may thus be common mechanistic mediators in the pathophysiological consequences of T2D (25, 30, 31). However, progression effect of chronically elevated glucocorticoid levels upon insulin regulation and the generation of insulin resistance as well as on the behavioural changes in rodents remains unclear. In this study, reduced plasma triglycerides and cholesterol were also observed at 60 days of CUMS. Other models of acute and chronic stress have been shown that can affect the lipid metabolism, mainly decreasing triglycerides (11, 25, 32, 33). Although data on cholesterol levels due to chronic stress in humans are ambiguous (34), these results clearly show the importance of the type of stressor in the alterations of lipid metabolism.

Ghrelin levels increased at 40 days; the same was observed due to stress by caloric restriction (35). The increment in ghrelin may reflect an attempt by the body to regulate the HPA axis, weight loss and impaired glucose tolerance under conditions of metabolic resistance that begin to become chronic and whose effects may even threaten survival, as has been

observed in ghrelin knock-out mice (35-38).

There is a negative correlation between ghrelin and leptin, both of which are considered metabolism-modulating hormones. Leptin decreased after 20 days, while ghrelin increased after 40 days of stress. Accumulating evidence has shown that leptin, like other peptides involved in the central regulation of food intake, controls the function of the HPA axis. Several studies have shown that leptin reduces the HPA axis' response to stress at different levels of the HPA axis (39, 40). In this study, decreased leptin secretion was observed during stress that may facilitate the responsiveness of HPA axis, which seems to be important for survival.

These results suggest gradual deregulation of HPA axis through negative feedback mechanisms that chronically induce a progressive change in metabolic response (41). On the other hand, recent studies showed that at 60 days of CUMS are evident damages to the pancreas and liver by oxidative stress (16). These organs may be indirectly associated with the progression of metabolic changes in the system of neuroendocrine regulation of appetite-satiety.

Furthermore, CUMS represents an animal model that in addition to avoiding habituation activates damage mechanisms that involve a systemic chronic inflammation of low-grade, allowing long-term studies about stress physiopathological aspects and its association with other risk factors (hyper caloric diets, diabetogenic agents, obesity, etc.) of metabolic disease. In consequence, the evaluation of the susceptibility of this model of CUMS for developing insulin resistance, diabetes and metabolic syndrome is mandatory.

In conclusion, CUMS progressively disturbs the metabolism of carbohydrates, lipids, and hormones that regulate food-intake in rats till 60 days. These metabolic alterations could be associated with chronic degeneration, in which risk factors such as diet, sedentary lifestyles, and genetic makeup, in conjunction with chronic stress, predispose individuals to develop diseases. This study shed light on the effects of chronic stress on the progression of long-term metabolic disorders.

Conflict of interest

The authors declare no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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