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RESEARCH ARTICLE

Obesity and Chronic Stress Modulate Adenine Nucleotide Hydrolysis in Rat Blood Serum

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ABSTRACT

Obesity is a chronic disease which has become one of the most prominent public health concerns in the contemporary world. Obesity is associated with chronic stress, and both conditions have been related to other health complications, such as cardiovascular disease. NTPDases and alkaline phosphatases, among other enzymes, are known to hydrolyze adenosine 5'-triphosphate (ATP) and adenosine 5'-diphosphate (ADP) to AMP, while 5'-nucleotidase hydrolyzes adenosine 5'-monophosphate (AMP) to adenosine. In a previous study, we found that, after exposure to a hypercaloric diet combined with chronic stress for 6 weeks, rats exhibited several obesity-related, endocrine markers (hyperleptinemia, hypertriglyceridemia, and hypercholesterolemia). Therefore, given that a short period of exposure to a hypercaloric diet and chronic stress was already known to induce a model of obesity in rats, to in the present study, we evaluated adenine nucleotide hydrolysis, alkaline phosphatase levels, TNF-α serum levels, total serum proteins and obesity parameters in rats submitted to a model of obesity associated or not to restraint chronic stress for a period of 12 weeks. Obesity was assessed by weekly weight measurements, relative adipose tissue weight (mesenteric adipose tissue-MAT, subcutaneous adipose tissue-SAT and visceral adipose tissue-VAT) and lipid profiles. Obesity was associated with increased adipose tissue depots, dyslipidemia, but not showed effect on alkaline phosphatase, protein levels and TNF-α levels. Obesity and chronic stress led to decreased ADP and AMP hydrolysis, while only obesity was associated with decreased ATP hydrolysis. These effects were not observed in any of the other parameters evaluated. The effect of impaired homeostasis on nucleotide hydrolysis may consist of an adaptation to obesity and/or chronic stress which is likely to be caused by ectonucleotidases, since both alkaline phosphatase and total protein levels were normal in the animals studied. In conclusion, since adenine nucleotides promote a range of deleterious effects on platelets, endothelial tissue, and vascular smooth muscle (ATP and ADP), while adenosine has cardioprotective effect, we believe that altered nucleotides hydrolysis in the serum may be used as a biochemical marker for the cardiovascular risk induced by obesity and/or chronic stress.

KEYWORDS

Cardiovascular Disease, Cafeteria Diet, Chronic Stress, Nucleotides, NTPDases, Obesity

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INTRODUCTION

The combination of elevated daily stress levels and an increased consumption of highly caloric

palatable foods contributes significantly to the increasing prevalence of obesity in modern society[1,2]. The hypercaloric "cafeteria" diet is widely used to induce obesity in animal models, and has been shown by researchers to mimic food intake in modern humans[3-5]. Studies show that these diets lead to an increased accumulation of abdominal adipose tissue[4] as well as changes in lipid profile[4,6]. These are both risk factors for cardiovascular disease, which is the leading cause of morbidity and mortality in the world[7,8].

The chronic activation of the stress system has been associated with metabolic disorders and altered energy homeostasis[9]. The appetite-satiety centers in the hypothalamus have also been found to be influenced by exposure to stress[10] and persistently high concentrations of glucocorticoids (GCs) can increase "comfort food" intake and abdominal fat depots. Furthermore, stress is known to trigger hypercoagulable states, which are thought to be mediated by plasma catecholamine activity[11]. Blood coagulation is an essential determinant of cardiovascular risk[12].

Additionally, adenine (adenosine) nucleotides (ATP, ADP, AMP) and nucleosides play an important role in regulating the cardiovascular system[13,14]. The release of ATP and norepinephrine (NE) from the sympathetic nervous system into the blood stream causes vasoconstriction via P2X receptors on smooth muscle cells[15,16]. ATP also acts as a neurotransmitter in perivascular non-adrenergic non-cholinergic nerves, promoting vasodilation through direct effects on vascular smooth muscle, or indirect, P2Y receptor-mediated effects on the endothelium[16]. ATP and ADP are released into the bloodstream by vascular smooth muscle, endothelial, and circulating blood cells, as well as via outflow upon cell lysis, resulting in a range of effects on platelets, endothelial tissue, and vascular smooth muscle[17]. ADP is a potent platelet aggregator and vasoconstrictor[17,18] while adenosine inhibits platelet aggregation and induces vasodilatation[19].

Extracellular adenine nucleotides are inactivated by enzymes of the nucleoside triphosphate diphosphohydrolase family (NTPDases), alkaline phosphatases (APs) and 5′-nucleotidase. The NTPDases hydrolyze ATP and ADP, while 5′-nucleotidase hydrolyzes AMP to adenosine[20]. This pathway has the dual function of reducing ATP levels and generating adenosine[21], while enzymes control the duration of receptor activation[22]. Our previous research has demonstrated the presence of NTPDases and 5'nucleotidase as well as ectonucleotidase activity in rat blood[23-26]. These factors also play an important role in pathological conditions such as cardiovascular diseases[27]. Alkaline phosphatases occur widely in nature, and are found in species ranging from bacteria to man. This enzyme catalyzes the hydrolysis of phosphoric acid monoesters as well as transphosphorylation reactions when in the presence of large concentrations of phosphate acceptors. Mammalian APs are membrane-bound, have higher a K_m values and require an optimal alkaline pH[28]. These enzymes are also responsible for the hydrolysis of adenine nucleotides[29].

In a previous study, we found that, after exposure to a hypercaloric diet combined with chronic stress for 6 weeks, rats exhibited several obesity-related endocrine markers (hyperleptinemia, hypertriglyceridemia, and hypercholesterolemia)[4]. Based on these important findings and the above considerations, this study aims to evaluate lipid profile and other parameters (adenine nucleotide hydrolysis, alkaline phosphatase levels, total proteins and TNF-α serum levels) in rats submitted to same model for a period of 12 weeks.

MATERIALS AND METHOD

Animals and Experimental Design

Thirty-two 60-day-old male Wistar rats (weight 200–250 g) were randomized by weight and housed in polypropylene cages (49 x 34 x 16cm). All animals were kept in a standard 12-hour light/dark cycle (lights on from 7.00 a.m. to 7.00 p.m.), in a temperature-controlled environment (22 ± 2°C), and were provided

with water and chow *ad libitum* (cafeteria diet and/or standard rat chow). All experiments and procedures were approved by the Institutional Animal Care and Use Committee (GPPG-HCPA protocol No. 11.0455) and were compliant with Brazilian guidelines on the use of experimental animals (Law No. 11,794. Vigorous attempts were made to minimize animal suffering and decrease external sources of pain and discomfort, and to use only the number of animals necessary to produce reliable scientific data. Rats were allowed 1 week to acclimate to their environment before the start of the experiment. The animals were divided into control and stress groups. Each group was further subdivided according to chronic stress exposure and diet: standard chow (control - C, control plus restraint stress - S) and cafeteria diet (cafeteria diet - CD, cafeteria diet plus restraint stress - CDS). The animals were weighed weekly and food intake was recorded daily. This experiment was carried out for 12 weeks. The animals were housed in groups of four animals per cage.

Stress Procedure and Experimental Diets

The animals were subjected to chronic restraint stress[30] using a plastic tube (25 x 7 cm) fixed with adhesive tape on the outside to avoid discomfort, but limiting the movements of the animal. One of the ends of the tube remained open to allow breathing. The animals were exposed daily to 1 hour of stress in the morning (between 9.00 and 12.00), 5 days a week for 12 weeks (no stress on weekends). All animals were returned to their home cages immediately after stress exposure. Control animals were kept in their home cages throughout the experimental period. The apparatus was ventilated and did not cause physical compression, avoiding hyperthermia and sweating. The standard rat diet (Nuvilab CR-1, NUVITAL®, Curitiba, PR, Brazil) was composed of 55.0% carbohydrates, 22.0% protein, 4.5% lipids, and 18.5% other constituents (fiber and vitamins); the diet contained a total of 2.93 kcal/g (information provided by the manufacturer). The palatable high-calorie diet (cafeteria diet) consisted of about 60.0% carbohydrates, 20.0% lipids, 15.0% protein, and 5.0% other constituents (sodium, calcium, vitamins, preservatives, minerals, etc.); the diet contained a total of 4.18 kcal/g (including soda - 0.42 kcal/mL). Calories were calculated based on information provided on the package label). The palatable diet was adapted based on a diet known as cafeteria diet or Western diet[3,4]. The cafeteria diet includes foods such as crackers, wafers, sausages, chips, condensed milk, and soda. Food was replaced daily for both diet groups. All animals had access to standard chow and water, including those who received the cafeteria diet.

Weighing and Tissue Collection and Blood Sampling

The animals were weighed on a weekly basis, and at the end of the experiment, adipose tissues (mesenteric, subcutaneous and visceral) were dissected and weighed on a semi-analytical scale. Data were expressed as grams of tissue per 100 g of body weight. The perigonadal and retroperitoneal fat pads were also removed and weighed. The animals were killed by decapitation, and blood and tissue samples were collected 24h after the last session of restraint stress, following 12-hour fast. Trunk blood was collected and centrifuged for 5 minutes at 5000 rpm at room temperature. This method was used to facilitate the collection of large volumes of blood serum for analysis. Most importantly, this model provides larger quantities of serum for biochemical analysis, including the assessment of hormone levels. Serum was frozen at -70 ° C until assayed.

Biochemical Serum Assays

Lipid Profile

Triglycerides (TG), total cholesterol (TC) and high-density lipoprotein (HDL) levels were measured using a commercial Bioliquid kit (Laborclin, PR, Brazil) and read in a spectrophotometer. Low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) levels were calculated using the Friedewald formula (VLDL= TAG/5 and LDL Total cholesterol – (HDL-VLDL)[31]. Values are expressed as mg/mL.

Nucleotides Hydrolysis Assay

ATP and ADP hydrolysis were evaluated using the method described by Oses et al. (2004). The reaction mixture contained 0.5 to 1.0 mg serum protein in 112.5 mM Tris-HCl at pH 8.0, and was preincubated for 10 min to equilibrate the mixture. The reaction was started by adding ATP or ADP to a final concentration 3.0 mM; the incubation was performed at 37.0 °C in a final volume of 200.0 µL for 40 min. The reaction was stopped by the addition of 200.0 µL of 10% trichloroacetic acid (TCA). All samples were centrifuged at 5000 x g for 5min to eliminate precipitated protein. The supernatant was used for colorimetric assay. Inorganic phosphate (Pi) release was measured by the Malachite green method[32]. AMP hydrolysis was carried out using the same method employed for ATP and ADP hydrolysis. The reaction mixture, containing 3.0 mM AMP as substrate in 100.0 mM Tris-HCl at a pH of 7.5, was incubated with 0.5 to 1.0 mg serum protein at 37.0 °C in a final volume of 200.0 µL. All subsequent procedures involved in AMP hydrolysis were the same as those described for ATP and ADP hydrolysis. Protein concentration was measured using the Bradford method with bovine serum albumin as a standard[33]. Enzyme activity was expressed as nmol of inorganic phosphate (Pi) released per /min/ mg protein (nmolPi/min/mg protein).

Alkaline Phosphatase Enzyme Assay

Alkaline phosphatase was measured using a Labtest® kit (Alkaline phosphatase ref. 40 - ANVISA - 10009010038, Minas Gerais, Brazil). Enzyme activity was assayed according to the release of thymolphthalein monophosphate, which is colored blue in an alkaline medium. The color produced is directly proportional to enzyme activity, and is measured at 590 nm. The blue color is clearly visible in the end product of the reaction. Prior to the assay, the substrate was stored at 15-25 º C in a buffer solution containing 22mmol/L thymolphthalein monophosphate, 300 mmol / L pH 10.1 color reagent, 94 mmol / L with sodium carbonate, 250 mmol / L sodium hydroxide and 45 U/L standard. The container was then tightly sealed to prevent evaporation. All reagents were stored at 15-25 º C until needed. Samples were read in a spectrophotometer with a standard 590 nm or orange filter (580-590 nm), adjusting the zero with the blank control tube. Under these conditions, the color is stable for 120 minutes.

Total Protein Assay

Total protein content was measured using a Labtest® kit (Total Proteins, Ref. 99, ANVISA - 10009010080, MG, Brazil). In this method, the copper (Cu^{+}) in a biuret reagent reacts with the peptide bonds of the serum proteins. The resulting purple color is measured at a maximum absorbance wavelength of 545 nm, which is proportional to the protein concentration in the sample. The kit contains biuret, which must be stored between 15-30ºC, and 600mmol/L sodium hydroxide, 12mmol/L copper sulfate, as well as preservatives and antioxidants. The product is corrosive and must be handled with care, never making contact with the mouth. The reaction involves 4.0g/dL standard, containing 4g/dL bovine albumin 4g/dL and 15.4mmol/L azide sodium. The standard must be stored at 15-30ºC and sealed to avoid evaporation. Since azide sodium is a toxic chemical, and reacts with metals to form explosive compounds, it must be used with care and discarded with large amounts of water. Samples were read in a spectrophotometer with a standard 545 nm or green filter (430-550 nm), adjusting the zero with the blank control tube. Under these conditions, the color is stable for 60 minutes.

Serum TNF-α Assay

TNF-α levels were determined by enzyme-linked immunosorbent assay (ELISA) using the ChemiKine kit (Millipore), according to manufacturer recommendations. The serum was centrifuged at 14,000 X g for 30 min at 4 °C. The resulting supernatant was used for the TNF-α assay. Samples were incubated on 96-well flat bottom plates coated with anti-BDNF polyclonal antibody at 2–8 °C overnight. After this first incubation, biotinylated mouse anti-BDNF monoclonal antibody was added to each

well and incubated for 2–3 h. The plates were further incubated for 1h following the addition of streptavidin-horseradish peroxidase conjugate. TMB/E Substrate was added to each well for 15 min until the 500 ng/mL standard reached a deep blue color. The reaction was stopped by the addition of stop solution (HCl) to each well, which led the standard to change to a yellow color. The color reaction was immediately measured by spectrophotometry in a plate reader at 450 nm. A standard TNF-α curve ranging from 0 to 500ng/mL was prepared on each plate.

Statistical Analysis

Results were expressed as mean ± standard error of the mean (S.E.M.). The weight of animals at baseline was compared between groups using a one-way ANOVA. Changes in measurements over time were assessed through a two-way repeated measures ANOVA (effect of time, cafeteria diet, stress, time x stress, time x cafeteria diet and time x stress x cafeteria diet interactions), followed by Bonferroni posthoc tests when necessary. Interactions were analyzed at the end of the treatment through a two-way ANOVA (effect of obesity, stress, obesity x stress) followed by Bonferroni's tests for multiple comparisons when necessary. Results were expressed as the percentage of TNF-α serum levels. Statistical analyses were performed using the IBM SPSS software, version 19.0, and differences between groups were considered significant at $P < 0.05$.

RESULTS

Weekly weight (g)

The animals were randomized by weight, and basal weight did not differ significantly between groups (Data not showed, one-way ANOVA, $P>0.05$). The weight of animals in the cafeteria diet group exhibited a noticeable increase starting in the third week of the study. A two-way repeated measures ANOVA showed effects of time ($F_{11,30}= 128.167$, $P<0.001$) and of the cafeteria diet ($F_{(1,14)}= 4.199$, $P=0.04$), but no effects of stress ($F_{(1,14)}= 2.692$, $P>0.05$) on the weight of rats. There were no interactions between stress and the cafeteria diet ($F_{(2,22)}= 0.040$, $P>0.05$). There were interactions between time x cafeteria diet ($F_{(12,14)}= 4.275$, $P<0.001$), but not between time x stress ($F_{(12,14)}= 1.714$, $P>0.05$), and between time x stress x cafeteria diet ($F_{(14,22)}= 1.448$, $P>0.05$). These results indicated the presence of obesity in the animals in the cafeteria diet group. See figure 1 below.

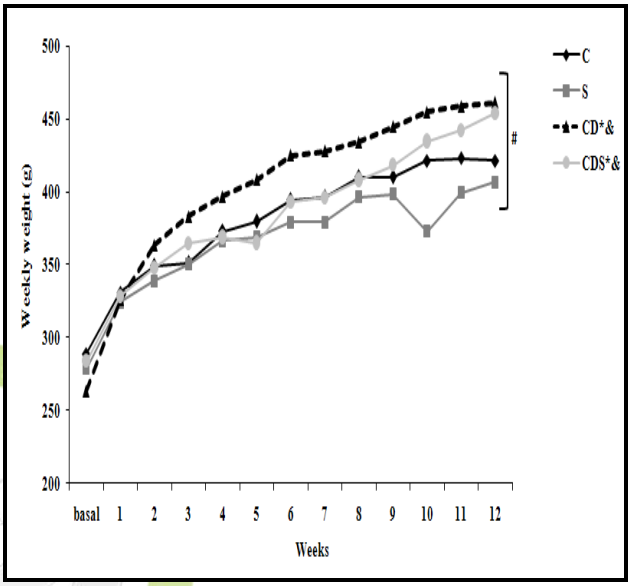

Figure 1: Weekly body weight. Data expressed as mean ± S.E.M. (two-way repeated measures ANOVA followed by Bonferroni's tests, n= 8 animals/group). ($^{\#}$) Significant effect of time. (*) Significant effect of the cafeteria diet. ($^{\&}$) Interactions between time x stress, time x cafeteria diet and time x stress x cafeteria diet

Relative Adipose Tissue Weight (g/100g)

Obesity was defined as the presence of increased adipose tissue in specific regions of the body (two-way ANOVA followed by Bonferroni's tests). MAT values were influenced by the cafeteria diet on MAT (obesity) ($F_{(1,14)}= 25.612$, $P<0.001$); but not by stress ($F_{(1,14)}= 0.084$, $P>0.05$), and there were no interactions between these independent variables ($F_{(2,22)}= 0.979$, $P>0.05$). SAT weight was influenced by the cafeteria diet (obesity) ($F_{(1,14)}= 37.170$, $P<0.001$), but not by stress ($F_{(1,14)}= 0.016$, $P>0.05$), and there were no significant interaction between these independent variables ($F_{(2,22)}= 7.157$, $P>0.05$).

VAT weight was influenced by the cafeteria diet (obesity) ($F_{(1,14)}$= 24.425, P<0.001), but not by stress ($F_{(1,14)}$= 0.007, P>0.05), and there were no significant interactions between these variables ($F_{(2,22)}$= 1.158, P>0.05). See figure below.

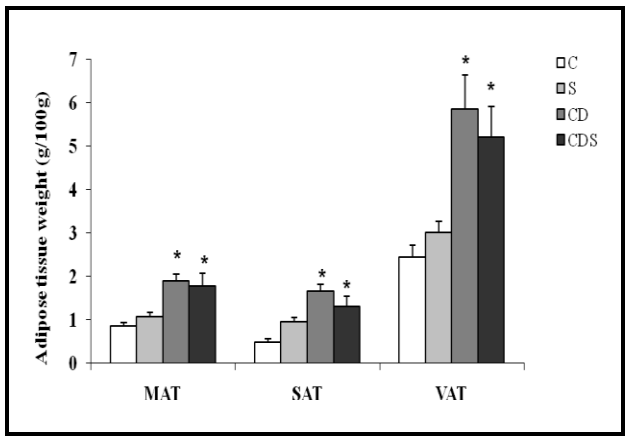

Figure 2: Relative weight of MAT, SAT and VAT in Wistar rats. Weights of visceral adipose tissue, as well as perigonadal and retroperitoneal fat pads. Data expressed as mean ± S.E.M. (two-way ANOVA followed by Bonferroni's tests, n = 8 animals/group). (*) Significant effect of the cafeteria diet

Lipid Profile (mg/mL)

Obesity following treatment was associated with increased triglycerides and total cholesterol (and thus LDL and VLDL levels) and decreased serum HDL levels (two-way ANOVA). Triglyceride levels were influenced by the cafeteria diet (obesity) ($F_{(1,14)}$= 12.865, P=001), but not by stress ($F_{(1,14)}$= 0.062, P>0.05), and there were no significant interactions between these variables ($F_{(2,22)}$= 0.000, P>0.05). Total cholesterol levels were influenced by the cafeteria diet (obesity) ($F_{(1,14)}$= 4.407, P=0.04), and chronic stress had a nearly significant influence on these values ($F_{(1,14)}$= 3.916, P=0.056). However, there were no interactions between these independent variables ($F_{(2,22)}$= 0.094, P>0.05). HDL concentrations were influenced by the cafeteria diet (obesity) ($F_{(1,14)}$= 4.861, P=001), but not by stress ($F_{(1,14)}$= 1.434, P>0.05), and there were no significant interactions between these variables ($F_{(2,22)}$= 0.623, P>0.05). Calculated LDL cholesterol was influenced by the cafeteria diet (obesity) ($F_{(1,14)}$= 11.682, P=001), but not by stress ($F_{(1,14)}$= 0.007, P>0.05), and there were no significant interactions between these variables ($F_{(2,22)}$= 0.220, P>0.05). The cafeteria diet (obesity) ($F_{(1,14)}$= 12.865, P=0.01), but not stress ($F_{(1,14)}$= 0.062, P>0.05), also had an impact on calculated VLDL-cholesterol. There were no significant interactions between these independent variables ($F_{(2,22)}$= 0.000, P>0.05). See table below.

Table 1: Lipid profile (mg/dL)

	C	***S***	***CD***	***CDS***
Triglycerides	169.00±20.52	159.87.00±20.32	302.00±37.65*	293.50±60.04*
Cholesterol	53.80±1.47	57.62±3.31(1)	57.90±2.51*	63.12±1.39*(1)
HDL	19.30±0.33	21.12±0.91	18.00±1.37*	18.37±0.77*
LDL	14.98±2.32	13.17±2.21	16.97±4.11*	17.42±2.33*
VLDL	33.96±4.10	31.97±3.31	60.40±6.36*	58.70±12.00*

(*) Significant effect of the cafeteria diet. ($^{\#}$) Significant effect of chronic stress.[1] Tendency to have an effect on levels of total cholesterol (P=0.056). Data expressed as mean ± S.E.M. (two-way ANOVA n = 8 animals/group).

Nucleotide Hydrolysis (nmolPi/min/mg protein)

ATP Hydrolysis

A two-way ANOVA revealed an association between decreased ATP hydrolysis and obesity in the groups exposed to the cafeteria diet ($F_{1,8}$= 5.038, P=0.03), but no association between these variables and chronic stress ($F_{(1,8)}$= 1.846, P>0.05); we also found no interactions between obesity and chronic stress ($F_{(2,13)}$= 0.993, P>0.05). See figure 3 below.

ADP Hydrolysis

A two-way ANOVA showed an association between obesity and decreased ADP hydrolysis in the groups exposed to the cafeteria diet ($F_{1,8}$= 14.210, P=0.002). The same effects were

observed for chronic stress ($F_{(1,8)}$= 23.562, P<0.001). Additionally, a significant interaction was identified between chronic stress and obesity ($F_{(2,13)}$= 42.909, P<0.01) See figure 3 below.

AMP Hydrolysis

A two-way ANOVA showed an association between obesity and decreased AMP hydrolysis in the cafeteria diet groups ($F_{1,8)}$= 23.410, P<0.001). The same effects were observed for chronic stress ($F_{(1,8)}$= 8.946, P=0.009). A significant interaction between chronic stress and obesity was also found with regard to AMP hydrolysis ($F_{(2,13)}$= 14.426, P=0.002). See figure 3 below.

Alkaline Phosphatase (U/L), Total Protein Concentration (g/dL)

The results from a two-way ANOVA demonstrated that alkaline phosphatase concentrations were not affected by the cafeteria diet ($F_{(1,10)}$= 1.157, P>0.05) or chronic stress ($F_{(1,10)}$= 0.79, P>0.05). There was also no interaction between these independent variables ($F_{(2,16)}$= 0.84, P>0.05). A two-way ANOVA also demonstrated that total protein concentration was not affected by the cafeteria diet ($F_{(1,10)}$= 1.157, P>0.05) or by chronic stress ($F_{(1,10)}$= 0.79, P>0.05). There was also no interaction between these independent variables ($F_{(2,16)}$= 0.84, P>0.05). See figures 4 and 5 below.

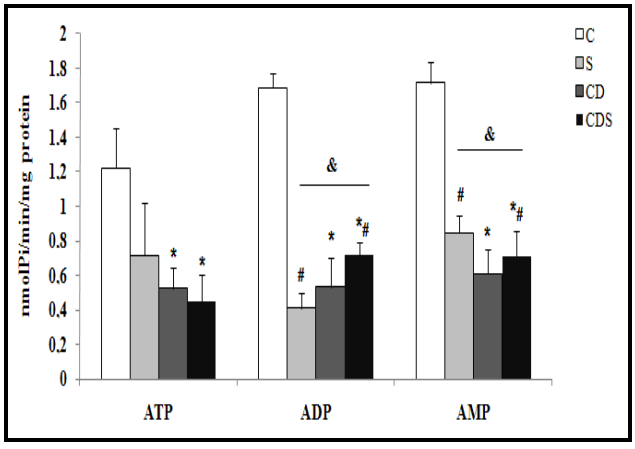

Figure 3: Nucleotide hydrolysis in the blood serum, data expressed as mean ± S.E.M. (Two-way ANOVA, n= 5 animals/group). ($^{\&}$) Interactions between cafeteria diet x stress. (*) Significant effect of the cafeteria diet. ($^{\#}$) Significant effect of chronic stress.

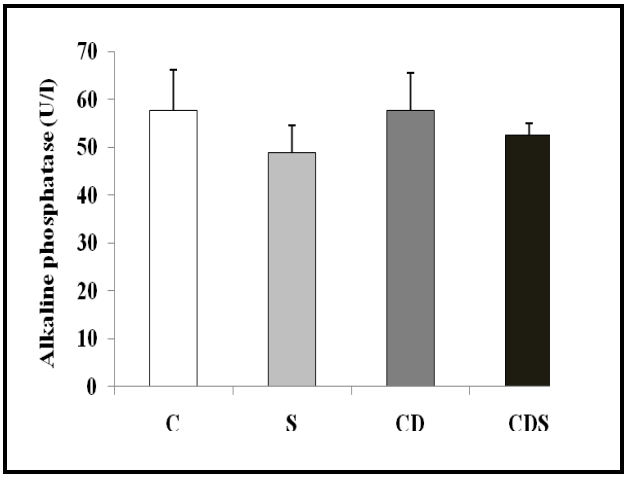

Figure 4: Alkaline phosphatase activity, expressed as mean ± S.E.M. (two-way ANOVA, n= 6 animals/group)

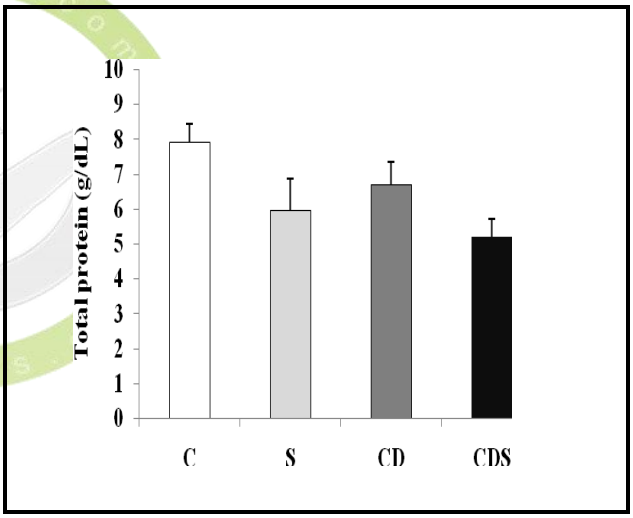

Figure 5: Total protein levels in the blood serum, expressed as mean ± S.E.M. (two-way ANOVA, n= 6 animals/group)

Serum TNF-α Levels (ng/mL)

The results from a two-way ANOVA demonstrated that alkaline phosphatase concentrations were not TNF-α concentrations were affected by the cafeteria diet ($F_{(1,13)}$= 1.140, P>0.05) or by chronic stress ($F_{(1,13)}$= 3.716, P>0.05). There was also no interaction between these independent variables ($F_{(2,21)}$= 1,154, P>0.05). See figure 6 below.

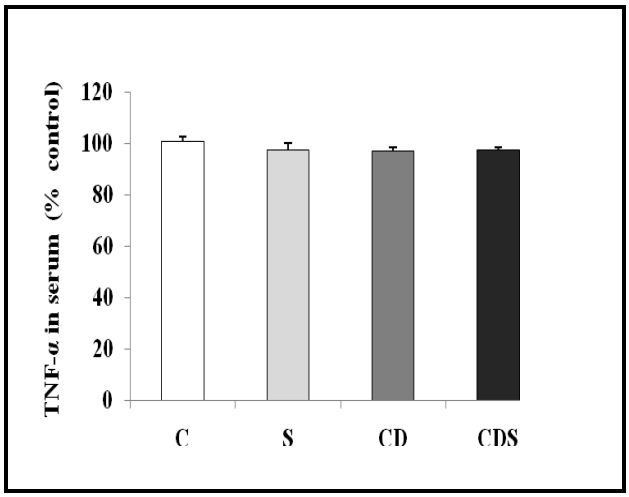

Figure 6: TNF-α concentrations in the blood serum (percentage of control), data expressed as mean ± S.E.M. (Two-way ANOVA, n= 7-8 animals/group)

DISCUSSION

The present study showed that, while obesity negatively modulates the hydrolysis of adenine nucleotides and chronic stress only reduces ADP and AMP hydrolysis, the interaction between obesity and chronic stress also appears to have an impact in the hydrolysis rates of these nucleotides. However, we found no evidence of an effect of obesity and/or chronic stress on total protein, alkaline phosphatase or TNF-α levels.

Circulating adenine nucleotides are known to play a role as signaling molecules in a variety of physiological and pathological processes[34]. The decreased ADPase activity in rats exposed to 12 weeks of restraint stress corroborated our previous results in rats exposed to the same stress model for 6 weeks[23]. However, the longer exposure period in the present study also had a negative impact on the hydrolysis of adenine nucleotides (ATP, ADP and AMP). We believe that these effects may be involved in the pathophysiology of obesity and may mediate the metabolic alterations induced by obesity and/or chronic stress. The reduced ATPase activity induced by obesity can result in increased ATP blood levels. During the inflammatory process, ATP is released into the extracellular environment and contributes to inflammation by the activation of purinergic P2X receptors[35]. It is also known that ATP, in conjunction with other neurotransmitters, may be released into the bloodstream from sympathetic nerves, causing vasoconstriction via binding to purinergic P2X receptors on smooth muscle cells[36,37]. A recent study has shown that diet-induced obesity showed sympathetic hyperactivity mediated vasoconstriction of small mesenteric arteries of the rat over a range of stimulation frequencies, and this effect in obesity involves enhanced purinergic neurotransmission, in addition to increased adrenergic function. The purinergic upregulation can be caused by postsynaptic changes in P2X receptor density or distribution in obese individuals. It should also be noted that neurally released ATP may only be able to reach extrasynaptic receptors after changes are made to the mechanisms controlling the breakdown of the substance[38].

ADP is a potent platelet-recruiting factor and induces platelet aggregation through its interaction with two P2 platelet receptors, one of which is a P2Y1 receptor linked to phospholipase C pathways. ADP is an important secondary agonist which amplifies most platelet responses and contributes to the stabilization of the thrombus[39]. The hydrolysis of ADP by enzymes present in the serum removes ADP from circulation and produces adenosine, both of which contribute to the inhibition of platelet aggregation[19]. ADP P2Y12 receptor agonists, such as clopidogrel and prasugrel, share this same mechanism of action, and are prescribed as protection against vascular events[40,41]. The decreased AMP hydrolysis observed in this study also suggests a reduction in adenosine levels. This nucleoside plays an important cardioprotective role in the heart, and is involved in the prevention of atherosclerosis, the maintenance of hemostasis and thromboregulation[42].

The decreased adenine nucleotide hydrolysis in the presence of stress (ADP and AMP) and obesity (ATP, ADP and AMP) is not likely to be influenced by alkaline phosphatase levels. The fact that neither total protein concentration nor alkaline phosphatase levels were altered in the present study suggest the maintenance of the

expression of ectonucleotidases in the plasma membrane. These enzymes hydrolyze ATP and ADP to AMP (NTPDases) and hydrolyze AMP to adenosine (5′-nucleotidase)[20]. Some of our recent studies have shown that exposure to chronic stress by movement restriction for 12-weeks may increase serum corticosterone concentration, and lead to hypothalamic pituitary adrenal (HPA) axis hyperresponsiveness, as observed by higher peak corticosterone values[43]. Another one of our studies has also found that, when the same model is employed for only six weeks, no evidence of HPA axis adaptation is observed in terms of changes in corticosterone levels[4]. However, it has been suggested that homeostatic impairment is only observed following longer periods of chronic stress, demonstrating that the length of exposure is a crucial factor to be considered in chronic stress studies.

The present study also demonstrated that a hypercaloric diet and daily stress exposure, either alone or in interaction with one another, lead to increased caloric intake over time. These results corroborate previous findings on the relationship between chronic restraint stress and preference for high-calorie foods[44,45] and sweet foods[46] . Additionally, several studies have shown that the cafeteria diet is associated with increased food consumption[5,6]. The consistent increases in weekly weight gain observed in the cafeteria diet groups reinforce the ability of this diet to induce obesity. Despite the strong interaction between the cafeteria diet and chronic stress and its impact on calorie intake, only the cafeteria diet on its own led to significant increases in weekly body weight over time. The increase in mesenteric, subcutaneous and visceral adipose tissue weight (MAT, SAT and VAT) observed in animals exposed to the cafeteria diet confirmed the presence of obesity in these animals. The free fatty acids released by intra-abdominal tissues into the hepatic-portal system are responsible for the association between body fat distribution, metabolic complications, and increased cardiovascular risk[47,48]. Additionally, although both intra-abdominal (MAT and VAT in this study) and subcutaneous adipose tissues appear to contribute to increased rates of metabolic disease and, consequently, to a higher cardiovascular risk, the metabolic activity of SAT may have a more significant impact on the risk of these conditions, given the higher mass of SAT as compared to VAT[49,50]. It is important to note that in this study, samples of SAT were only collected from the abdominal, perigonadal, and retroperitoneal regions, as well as from mesenteric fat pads in the intra-abdominal region.

The levels of many inflammatory mediators have been found to be altered in obesity[51]. Normally, in response to excess lipid stores, visceral adipocytes secrete increasing amounts of inflammatory cytokines such as interleukin-6 and TNF-α[52]. Surprisingly, in this study, despite the significant increase in adipose tissue observed in obese animals, their TNF-α serum levels were found without changes.

Additionally, as expected, obesity had a significant effect on lipid profiles, leading to increased triglycerides, cholesterol, LDL and VLDL levels and decreased HDL levels. A strong tendency for high cholesterol levels (P=0.056) was found in animals exposed to stress. Similar findings have also been reported by studies in both humans and animals exposed to chronic stress[49,53]. It is important to highlight that dyslipidemia is a well-established risk factor for atherosclerosis and cardiovascular disease[54,55].

CONCLUSION

This study showed an increase in the adipose tissue depots of animals fed with the cafeteria diet, which were also more likely to become obese. These changes occurred simultaneously with alterations in lipid profile. When combined, these factors are known to lead to increased proinflammatory, prothrombotic and cardiovascular risks. Since stress and obesity are both involved in atherosclerosis, and given the role of ADP as a signaling molecule which activates platelet aggregation, the association between stress, obesity and ADP hydrolysis rates observed in the present study was an

especially interesting finding. The decreased ADP hydrolysis rates observed in obese and/or chronically stressed animals suggests that this process may play a role in the etiology of atherosclerosis induced by obesity and/or chronic stress exposure. If this is the case, ADP hydrolysis rates may consist of a possible biomarker of the cardiovascular risk induced by these factors.

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