Abstract
Several epidemiological studies described beneficial effects of chronic coffee intake on type 2 diabetes mellitus. Recently we demonstrated that chronic caffeine intake prevents the development of insulin resistance induced by hypercaloric diets and also reverses age-induced insulin resistance in rats. Herein, we tested the therapeutic dose of caffeine that restores insulin sensitivity in a prediabetic animal model. We also investigated the expression of proteins involved in insulin signaling pathways in adipose tissue. Rats submitted to a high sucrose (HSu) were submitted to different caffeine concentrations in drinking water, during 12 weeks. Insulin sensitivity and glucose tolerance were monitored during all the experimental period. Body weight, visceral and total fat, lipid profile and adipose tissue glut4, insulin receptor (IR) and protein kinase B (Akt) protein expression and activity were assessed. Chronic caffeine administration improved whole-body glucose homeostasis and insulin signaling pathways in adipose tissue in HSu animals in a concentration dependent manner. Our results suggest that this may be one of the mechanisms for caffeine-induced improvement of insulin sensitivity.

Keywords — Adipose tissue, caffeine, glucose tolerance, high-sucrose diet, insulin resistance

Cite this article as: Coelho JC, Melo BF, Rodrigues T, Matafome P, Sacramento JF, Guarino MP, Seiça R and Conde SV. Caffeine restores insulin sensitivity and glucose tolerance in high-sucrose diet rats: effects on adipose tissue. JCVd 2016: 440-449

I. INTRODUCTION
Metabolic diseases like type 2 diabetes mellitus (T2DM) and metabolic syndrome affect millions of individuals globally, contributing to significant morbidity and mortality worldwide.

Insulin resistance and pancreatic β-cell dysfunction are the core metabolic abnormalities that play a central role in the pathogenesis of T2DM[1], being insulin resistance defined as a state of reduced responsiveness, by insulin sensitive tissues (liver, muscle and adipose tissue), to normal circulating levels of insulin[2,3].

Although the mechanisms that lead to the appearance of insulin resistance remain unknown, several factors have been proposed to explain it, among which are, for example, obesity and lipotoxicity/hyperlipidemia[4]. In fact, the relationship between insulin resistance and overall obesity is well established since several studies have demonstrated that there is a strong independent relationship between both visceral adipose tissue and subcutaneous adipose tissue and insulin resistance, and that visceral fat accumulation decreases peripheral insulin sensitivity and enhances hepatic gluconeogenesis[5,6]. Moreover, it is known that dyslipidemia is strongly associated with metabolic diseases, like T2DM and is a major risk factor for coronary heart disease[7,8]. The typical dyslipidemia in T2DM is characterized by increased fasting and postprandial plasma triglycerides and free fatty acids, low levels of high density lipoprotein cholesterol (with dysfunction) and normal or slightly increased low density lipoprotein cholesterol with increased concentration of small dense LDL-cholesterol particles (SD LDL)[7,9].

In the past two decades, there has been an increase in the number of people diagnosed with T2DM all over the world[10]. This increase represents an alarming health problem, urging quick clarification of the pathophysiological mechanisms that underlie these diseases in an attempt to develop therapeutic interventions. The first therapeutic approach for T2DM is based on alterations in lifestyle[11,12] and during many years, one of the lifestyle modifications among subjects with metabolic diseases, was the reduction of coffee consumption, based on studies that showed that caffeine, when consumed acutely, impaired insulin sensitivity[13] and increased blood pressure[14]. However, in the last decade, several epidemiological studies have shown a beneficial relationship between long-term coffee consumption and T2DM, which represented a paradigm shift...
on the effects of coffee consumption on glucose homeostasis [16-19].

Besides the discussion on the protective versus deleterious effects of coffee in T2DM, there is also controversy concerning the nature of the compound involved in it. Some authors claim that the beneficial effects of coffee are mediated by caffeine[16, 19, 20] while others support that coffee components, apart from caffeine, are responsible for the protection against T2DM[21, 22].

Recent studies from our group demonstrated that chronic caffeine intake (1g/l during 15 days), on Wistar rats, prevents the development of insulin resistance induced by hypercaloric diets in prediabetes animal models, effect which appears to be mediated by a decrease in sympathetic nervous system activity [23]. Moreover, we have also shown that chronic caffeine intake reverses age-induced insulin resistance in rats [24].

However, we [25] and other authors have shown that acute caffeine administration impairs insulin sensitivity both in healthy animals [26] and subjects [14, 26, 27] and in T2DM patients [28, 29]. Also it was shown that FFA increased in T2DM and obese patients after acute caffeine administration being also observed an increase in insulin and glucose levels after an oral glucose tolerance test [28]. Likewise, it was also described an increase of glucose and insulin concentrations after an oral glucose tolerance test preceded by an acute caffeine administration in T2DM patients [28]. These effects of acute caffeine administration contrast with the long-term caffeine consumption observed by our group, and also with other studies where beneficial actions have been reported. In elderly glucose intolerant subjects, higher caffeine consumption has been associated with a lower incidence of glucose intolerance and a beneficial effect on insulin sensitivity [30]. The same conclusions were taken in other cohort study in healthy younger and middle-age women [19] where the risk of development of T2DM was lower in the groups where the coffee consumption was higher.

Herein we tested the effects of different concentrations of long-term caffeine administration on insulin sensitivity in order to identify the therapeutic dose of caffeine that restores insulin action in a prediabetic animal model. Also, in order to understand if the beneficial effect of caffeine is due to alterations in adipose tissue, we investigated the expression of some important proteins involved in insulin pathway in adipose tissue.

II. METHODS

A. Animals and experiment procedures

The experiments were performed in Wistar rats of both genders aged 8-12 weeks old (250-450g), obtained from the animal house of Nova Medical School. The animals were maintained under temperature and humidity control (21 ± 1°C; 55 ± 10% humidity), with a 12h cycle of light and darkness.

Two groups of rats were used: a group submitted to a high-sucrose hypercaloric diet (Hsu) to obtain a lean animal model of insulin resistance and hypertension [23, 30, 31] and the respective control group. The Hsu model was achieved by administering 35% sucrose in drinking water during 28 days to the animals, additionally to the standard chow (7.4% fat, 75% carbohydrate (4% sugar) and 17% protein; SDS diets RM1; Probiológica, Sintra, Portugal). The control group fed standard chow and drank regular tap water.

After 28 days of diet, caffeine (0.5, 0.75 and 1g/l) was administered in drinking water to both Hsu and control animals, during 12 weeks. Insulin sensitivity and glucose tolerance were monitored prior to submit the animals to diet, 28 days after animals initiated the diets and each 2/3 weeks after the beginning of caffeine intake, being glucose tolerance monitored two days after performing insulin tolerance test (ITT). Caloric and liquid intake were monitored throughout the experimental procedure in all groups of animals. Body weight and animal behavior were monitored twice per week.

At the end of the experimental period, rats were anaesthetized with pentobarbital (60mg/kg, i.p.). Blood was collected by heart puncture and visceral, renal and genital fat were weighted and collected for Western Blot analysis of insulin signaling pathway proteins. All tissue samples were snap frozen and stored at -80ºC.

Principles of laboratory care were followed in accordance with the European Union Directive for Protection of Vertebrates Used for Experimental and Other Scientific Ends (2010/63/EU). Experimental protocols were approved by the Ethics Committee of the Faculty of Medical Sciences.

B. Measurement of insulin sensitivity

The insulin tolerance test (ITT) was used to measure insulin sensitivity. The ITT is one of the earliest methods developed to assess insulin sensitivity in vivo and provides an estimate of overall insulin sensitivity, correlating well with the ‘gold standard’ hyperinsulinaemic–euglycaemic clamp [32].

ITT was performed in conscious animals submitted to an overnight fasting of approximately twelve hours. The test consists in the measurement of basal glycaemia, followed by the administration of an insulin bolus (0.1 U/kg) in the tail vein and the measure of the decline in plasma glucose concentration over 15 min at 1 min intervals. The constant rate for glucose disappearance (KITT) was calculated using the formula 0.693/t1/2. Glucose half-time (t1/2) was calculated from the slope of the least-square analysis of plasma glucose concentrations during the linear decay phase [23, 30]. Blood samples were collected by tail tipping and glucose levels were measured with a glucometer (Abbott Diabetes Care, Portugal) and test strips (Abbott Diabetes Care, Portugal).

C. Measurement of glucose tolerance

Glucose tolerance was evaluated by an oral glucose tolerance test (OGTT). The animals were submitted to an overnight fast of approximately twelve hours. Glucose (2g/kg) was administered by gavage and plasma glucose was measured at 0, 15, 30, 60, 120 and 180 minutes. Blood samples were collected by tail tipping and glucose levels were measured with a glucometer (Freestyle Precision, Abbott Diabetes Care, Portugal) and test strips (Freestyle Precision™, Abbott Diabetes Care, Portugal). Glucose excursion curves (plasma glucose vs time) were plotted and the area under the curve (AUC) was compared.
D. Plasma lipid profile

Plasma was collected after heart puncture to EDTA precoated tubes. The lipid profile was assessed using a RANDOX kit (RANDOX, Irlaodox, Porto, Portugal) to determine total cholesterol and triglycerides by Trinder-based colorimetric end-point assays, and HDL and LDL by a direct-HDL and direct-LDL clearance method, respectively.

E. Western Blot analysis of Glucose Transporter type 4 (glut4), Insulin Receptor (IR) and Protein Kinase B (Akt) in adipose tissue

Visceral adipose tissue samples (100mg) were homogenized in Zurich (10mM Tris-HCl, 1mM EDTA, 150mM NaCl, 1% Triton X-100, 1% sodium cholate, 1% SDS) with a cocktail of protease inhibitors (trypsin, pepstatin, leupeptin, aprotinin, sodium orthovanadate, PMSF). Samples were centrifuged (Eppendorf, Madrid, Spain) at 13000g for 20 minutes and the supernatant was collected and frozen at -80°C until further use.

The evaluation of the expression of the total glut4, insulin receptor, and insulin receptor phosphorylated at Tyr1163, Akt and Akt phosphorylated at Ser473 were performed according to Rodrigues et al. (2013)33. Briefly, after blocking for 1 h at room temperature with 5 % non-fat milk in Tris-buffered saline, pH 7.4 containing 0.1 % Tween 20 (TBBS) (BioRad, Spain), the membranes were incubated overnight at 4°C with the primary antibodies against glut4 (1:200, Sta Cruz Biotechnology, USA), insulin receptor (1:200, Sta Cruz Biotechnology, USA), insulin receptor phosphorylated (phospho-Tyr1361, 1:500; Abcam, UK), Akt (1:1000, Cell Signalling, USA) and Akt phosphorylated (phospho-Ser473, 1:1000, Cell Signalling, USA). The membranes were washed with Tris-buffered saline with Tween (TBST) (0.1%) and incubated with donkey anti-goat (1:2000, Sta Cruz Biotechnology, USA) or goat anti-mouse (1:2000, Sta Cruz Biotechnology, USA) or goat anti-rabbit (1:5000, Rockland, USA) in TBS for 2h at room temperature and developed with enhanced chemiluminescence reagents according to the manufacturer’s instructions (ClarityTM Western ECL substrate, BioRad, United States). Intensity of the signals was detected in a Chemidoc Molecular Imager (Chemidoc; BioRad, Madrid, Spain) and quantified using the Quantity-One software (BioRad). The membranes were re-probed and tested for Calnexin immunoreactivity (bands in the 83 kDa region) to compare and normalize the expression of proteins with the amount of protein loaded.

F. Statistical analysis

Data were analyzed with Graph Pad Prism Software, version 5 (GraphPad Software Inc., San Diego, CA, EUA) and represented as mean ± SEM. The significance between means difference was estimate through One and Two-Way Analysis of Variance (ANOVA) with Bonferroni, and Dunnett’s and Bonferroni multicomparsion test, respectively. Differences were significant when p<0.05.

III. RESULTS

A. Effect of caffeine administration on caloric intake, weight gain and fat mass, in animals submitted to a high-sucrose diet

The effects of chronic caffeine administration on caloric intake and on weight and fat (total and visceral) gain, both in control and Hsu groups are depicted in table I. As expected, HSu diet significantly increased the caloric intake by 55.6% (control caloric intake = 196.4±7.6 Kcal/kg/day and HSu caloric intake = 305.68±14.58 Kcal/kg/day). Caloric intake was not modified by chronic caffeine intake at any of the doses tested either in control or in Hsu groups.

Regarding weight and fat gain, Hsu diet did not significantly change weight gain throughout the experiment, as compared to the control group. Also, chronic caffeine intake did not modify weight gain in both control and Hsu groups. Hsu diet increased total and visceral fat by 34.4 and 32.0%, respectively, (total fat control = 59.8±1.60 g/kg and visceral fat control = 13.70±0.31 g/kg), being this increment in visceral and total fat reversed by caffeine administration at the highest concentrations (7.5 and 1g/l), for total fat, and at all concentrations for visceral fat.

B. Caffeine restores insulin sensitivity in animals submitted to...
The effect of different doses of caffeine on insulin sensitivity is represented in Fig. 1, both in control and in HSu animals.

In the control group, chronic caffeine intake did not modify insulin sensitivity, throughout the experimental period (Fig. 1a). After 4 weeks of HSu diet, insulin sensitivity decreased from 4.95±0.49% glucose/min to 2.36±0.21% glucose/min and remained diminished during the course of the experiment (Fig. 1b). In the sucrose-fed animals, continuous caffeine administration progressively increased insulin sensitivity leading to a full reestablishment of insulin action to control values at 9 and 5 weeks of 0.5 and 0.75g/l caffeine administration, respectively (Fig. 1b). Furthermore, it was observed that 1g/l of caffeine increased insulin sensitivity earlier than lower concentrations, with insulin action brought back to control values after 3 weeks of administration (K_{ITT} = 4.44±0.36% glucose/min).

C. Caffeine improves glucose tolerance in animals submitted to high-sucrose diet

Fig. 2 represents the effect of chronic caffeine intake on glucose tolerance both in control and HSu groups. In the control group, chronic caffeine administration did not alter glucose tolerance (Fig. 2a). HSu diet progressively impaired glucose tolerance, becoming significantly different at the 16th week of diet, in comparison with animals fed with the standard diet (control AUC = 23726; HSu AUC = 25902) (Fig. 2b). Also, Fig. 2b shows that caffeine administration ameliorated glucose tolerance, overtime, in the animals fed with HSu diet, being this effect dependent on the dose of caffeine administered (HSu AUC = 25902; HSu plus 0.5g/l of caffeine AUC = 23074; HSu plus 0.75g/l of caffeine AUC = 22352; HSu plus 1g/l of caffeine AUC = 21486).

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**Figure 1** - Effect of chronic caffeine intake on insulin sensitivity, in control animals (A) and in animals submitted to a high-sucrose diet (B), determined by an Insulin Tolerance Test (ITT) and expressed as the constant for glucose disappearance (K_{ITT}). Lines represent means ± s.e.m. One-Way ANOVA with Dunnett’s Multiple Comparison Test ***p<0.001 (comparing HSu values without caffeine after 4 weeks of diet with HSu values without caffeine before diet); ##p<0.05 (comparing HSu plus 1g/l of caffeine values, 9 weeks of diet, with HSu plus 1g/l of caffeine, 4 weeks of diet); ##p<0.01 (comparing HSu plus 0.5g/l of caffeine values, 4 weeks of diet, with HSu plus 0.5g/l of caffeine values, 4 weeks of diet); HSu plus 0.75g/l of caffeine values, 9, 13 and 16 weeks of diet, with HSu plus 0.75g/l of caffeine, 4 weeks of diet; HSu plus 1g/l of caffeine values, 11 weeks of diet, with HSu plus 1g/l of caffeine, 4 weeks of diet); ###p<0.001 (comparing HSu plus 0.5g/l of caffeine values, 11 weeks of diet, with HSu plus 0.5g/l of caffeine, 4 weeks of diet; HSu plus 0.75g/l of caffeine values, 11 weeks of diet, with HSu plus 0.75g/l of caffeine, 4 weeks of diet; HSu plus 1g/l of caffeine values, 11 weeks of diet, with HSu plus 1g/l of caffeine, 4 weeks of diet).

**Figure 2** - Effect of chronic caffeine intake on glucose tolerance, in control animals (A) and in animals submitted to a high-sucrose diet (B), determined by an Oral Glucose Tolerance Test (OGTT). Each line represents the plasmatic glucose excursion curve in the absence or in the presence of caffeine measured during 180 minutes. Lines represent means ± s.e.m. One-Way ANOVA with Dunnett’s Multiple Comparison Test *p <0.05; **p <0.01; ***p <0.001, comparing HSu values after 16 weeks of diet with control values and comparing HSu plus 1g/l of caffeine values with HSu without caffeine.
Table II - Effect of chronic caffeine intake on lipid profile, in animals submitted to a high-sucrose diet. Data is presented as means of 5-6 values ± s.e.m. One-way ANOVA with Dunnet’s multicomparison tests *p<0.05 (comparing HSu values with control values), **p<0.01, comparing HSu plus 1g/l values with HSu without caffeine values.

<table>
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<th>[Caffeine]g/l</th>
<th>Plasma Cholesterol (mg/dl)</th>
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D. Effect of caffeine on lipid profile

The effect of caffeine administration on lipid profile is represented in Table II. HSu diet significantly increased triglycerides by 81% (control triglycerides levels = 42.7±5.2 mg/dl and HSu triglycerides levels = 77.58±5.89 mg/dl). Caffeine administration had no significant effect on lipid profile both in control and HSu groups; however, triglycerides values showed a decreasing trend in the HSu group after caffeine treatment (see Table II). In the HSu group, plasma cholesterol significantly decreased in rats treated with 0.75 g/l caffeine (HSu cholesterol levels = 76.45±4.3 mg/dl).

E. Effect of chronic caffeine intake on the expression of glut4, IR and IR phosp Tyr1163. Akt and Akt phosp Ser473 in the visceral adipose tissue

In Fig. 3 is represented the effect of caffeine intake in the expression of Glut4 transporters in the adipose tissue in controls and in the HSu animals. Neither control nor HSu diet promoted alterations in glut4 expression in adipose tissue (control expression = 100; HSu expression = 78.09±8.54). Also, the administration of 0.5 and 0.75g/l of caffeine did not alter glut4 expression in both groups. However, 1g/l caffeine significantly increased glut4 levels by 133%, in control group, and by 100.5% in HSu group (control plus 1g/l caffeine = 233.78±28.09; HSu plus 1g/l caffeine = 156.62±31.47) (Fig. 3).

Fig. 4 shows the expression of IR (Fig. 4a) and its phosphorylation (Fig. 4b). HSu diet did not cause any change in IR expression or in its phosphorylation (control IR expression = 100; HSu IR expression = 96.45±19.81; control IR phosphorylation = 100; HSu IR phosphorylation = 96.72±4.24). Chronic caffeine administration at the concentrations of 0.5 and 0.75 g/l had no significant effect in IR expression and phosphorylation, either in control or in HSu groups. However, the highest caffeine concentration (1g/l) increased IR expression in adipose tissue by 59.2% and 37.8% in control and HSu, respectively (control plus 1g/l caffeine = 159.20±13.08; HSu plus 1g/l = 132.87±6.72) (Fig. 4a) and also increased IR phosphorylation by 55.7% and 68.7% in both control and HSu group, respectively (control plus 1g/l caffeine = 155.69±17.40; HSu plus 1g/l caffeine = 163.21±11.99) (Fig. 4b).

Fig. 5 represents the effect caffeine administration in Akt expression (Fig. 5a) and its phosphorylation (Fig. 5b) in control and HSu groups. Both control and HSu diets did not alter Akt expression (control expression = 100; HSu expression = 105.70±7.41), however, in control group, the concentration of 1g/l of caffeine significantly increased Akt expression by 46, 9% (control plus 1g/l caffeine = 146.92±9.73). Moreover, in the HSu group, chronic caffeine administration showed a trend to
progressively increase Akt expression (Fig. 5a). Regarding Akt phosphorylation, HSu diet significantly decreased it by 21.8% (control Akt phosphorylation = 100; HSu Akt phosphorylation = 78.16±2.75).

Chronic caffeine administration progressively increased Akt phosphorylation both in control and HSu groups (Fig. 5b). In the HSu group, caffeine administration not only restored Akt phosphorylation, but also increased the Akt phosphorylation levels in relation to control group not submitted to caffeine treatment (HSu plus 0.5g/l caffeine = 106.48±8.80; HSu plus 0.75g/l caffeine = 113±17.18; HSu plus 1g/l caffeine = 155.40±24.58) (Fig. 5b).

IV. DISCUSSION

In the present study, we demonstrated that chronic caffeine intake restores whole-body insulin sensitivity and glucose tolerance in prediabetic rats in a dose dependent manner. In addition, we have shown that the latency time needed to restore insulin sensitivity and glucose tolerance is inversely correlated with the caffeine dose administered. We also showed that chronic caffeine intake reduced the fat mass in HSu animals and it also increased the expression and phosphorylation of some important proteins involved in insulin pathway in adipose tissue, namely glut4 transporters, IR and Akt.

The concentrations of caffeine tested were chosen based on the literature, and the higher dose used (1g/l) corresponds to a consumption of three to four cups of coffee per day, 0.75 g/l to 2/3 coffees per day and 0.5 g/l to 1/2 coffees per day[37]. It is known that at concentrations in the millimolar range, caffeine promotes the release of intracellular calcium and that it has commonly been used as a Ryanodine receptors (RyR) calcium channels agonist[34], being this RyR channels already associated with insulin secretion regulation and glucose homeostasis[35,36]. However, it should be noted that, at these doses, the only mechanism significantly affected by caffeine is the antagonism of adenosine receptors[37].

In the present work we observed, as expected[23, 30], that HSu diet decreased insulin sensitivity after 4 weeks of diet (28 days), an effect that was sustained throughout the 16 weeks of the experimental procedure in the HSu group. In our experimental setting, chronic caffeine administration did not alter insulin sensitivity in control animals, although it completely restored insulin sensitivity in a concentration dependent-manner in HSu animals, being the latency time necessary to restore this parameter inversely correlated with the caffeine dose. Our results are in agreement with recent studies where it was shown that chronic caffeine intake restores and ameliorates metabolic syndrome in diet-induced obese Wistar rats[38] and improves insulin sensitivity in Wistar-Kyoto rats[39].
In our experimental setting, glucose tolerance gradually decreased with HSu diet consumption, showing a trend to diminish at 4 weeks of diet and being significantly decreased at 16 weeks of diet, which is concurring with previous studies\(^{[40, 41]}\). Chronic caffeine intake in control animals did not alter glucose tolerance, which is in agreement with findings by other authors\(^{[42, 43]}\), where they have shown that chronic caffeine intake in different concentrations tested (37.5, 56.2, 75.0 or 93.0 mg/kg per day) did not promote alterations on glucose tolerance\(^{[42]}\). Similar results were presented by Morakinyo et al.\(^{[43]}\) study, where it was shown that chronic coffee intake (during 12 weeks) did not alter glucose tolerance in control animals. Herein we have also showed in HSu animals, that chronic caffeine intake (1g/l), completely restored glucose tolerance. These results are compatible with the study of Morakinyo et al.\(^{[44]}\) in HSu rats where they show that coffee administration prevent the development of glucose intolerance\(^{[45]}\) and with the description of the beneficial effects of coffee on glucose intolerance obtained in the several epidemiological studies in Humans\(^{[18, 44, 45]}\).

Herein we also showed that chronic caffeine consumption produced no alterations on daily calorific intake. The effects of caffeine/coffee administration on appetite and food/caloric intake are not consensual, with some authors reporting that acute caffeine administration reduces food intake in rats\(^{[46]}\) while others state that chronic administration of caffeine does not affect standard chow intake in rodents, although it diminishes food intake in relation to palatable food\(^{[47]}\). The basis of these controversial results may rely on the differences between acute and chronic caffeine administration, since the majority of the effects of caffeine on food intake were observed as a result of acute caffeine intake.

The HSu rat is a lean animal model of insulin resistance\(^{[23, 31, 43]}\) therefore, as expected the daily weight gain in these animals was not significantly affected by the diet, despite increased caloric intake observed. Likewise, chronic caffeine intake did not affect daily weight gain in Hsu rats, which is consistent with results showing that long-term coffee consumption does not cause significant weight changes\(^{[23, 46]}\). Our results contrast with several epidemiological studies that attributed the beneficial effects of coffee intake on glucose homeostasis to weight loss, due to an increased thermogenesis, lipolysis and fat oxidation, induced by the drug\(^{[16, 19, 20, 39]}\). However, this studies evaluated coffee intake, and being coffee a mixed of many compounds, direct effect of caffeine was not evaluated. Also, these studies were performed in humans and not in Wistar rats, and that can contribute to the distinct results. In our experimental setting, caffeine did not alter caloric intake nor weight gain suggesting that the mechanism that contributes to the beneficial effects of chronic caffeine intake in glucose homeostasis does not involve a total body weight reduction or a decrease in the caloric intake. However, after 16 weeks on HSu diet, the animals presented an increased total and visceral fat that is reversed by caffeine intake at the highest concentrations (0.75 and 1g/l), for total fat, and at all concentrations for visceral fat. Regarding the lipid profile, our work confirmed that HSu diet significantly increased triglycerides\(^{[50]}\) and that serum triglycerides showed a trend to decrease in the HSu group after caffeine treatment, which is also in agreement with previous studies\(^{[51]}\), that show that chronic coffee intake (during 10 weeks), in
Sprague-Dawley rats, decreased the liver triglycerides, previously augmented by high-fat diet.

HSu diet did not promote alterations either in glut4 expression or in IR and Akt expression in adipose tissue, however it significantly decreased Akt phosphorylation. Several studies reported alterations in glut4 expression in adipose tissue in rats submitted either to high-fat diet[52] or to subcutaneous administration of monosodium glutamate[53]. In Ikemoto et al.[48] study, heterozygous transgenic mice harboring a 14-kb glut4 minigene and nontransgenic mice were submitted to a high-carbohydrate diet and it was observed a decrease in glut4 levels in adipose tissue. In Leguisamo et al.[49] study, neonate male SHR were submitted to subcutaneous administration of monosodium glutamate diluted in saline solution for 9 days, and it was also observed a decrease in glut4 expression in adipose tissue. Our results, as mentioned above, showed no alterations in glut4, insulin receptor and Akt expression. However, our data were obtained from animals submitted to a high-sucrose diet and, to the best of our knowledge, the effects of a high-sucrose diet on adipose tissue expression of insulin signaling proteins are not described in the literature.

The novelty of our work lies on showing that chronic caffeine stimulates insulin signaling on adipose tissue. In fact, regarding glut4 levels, 1g/l caffeine significantly increased its levels both in control and HSu groups. Chronic caffeine administration at the highest concentration (1g/l) increased IR expression in control and HSu and also increased IR phosphorylation in both control and HSu group.

Concerning Akt, in the control group, the concentration of 1g/l of caffeine significantly increased Akt expression. Moreover, in the HSu group, chronic caffeine administration showed a trend to progressively increase Akt expression. Regarding Akt phosphorylation, chronic caffeine administration progressively increased Akt phosphorylation both in control and HSu groups. In the HSu group, caffeine administration not only restored Akt phosphorylation, but also increased the Akt phosphorylation levels in relation to control group not submitted to caffeine treatment. This increase in Akt phosphorylation levels was also observed, in liver and skeletal muscle, in the Kobayashi et al.[54] study where it was shown that coffee ingestion (during 3 weeks) enhanced Akt phosphorylation. All together these results show, for the first time, that one of the mechanism that can contribute for the beneficial effects of chronic caffeine intake on glucose homeostasis might be the increase in insulin signaling in the visceral adipose tissue.

It should be noted that further studies are necessary in order to fully understand the effects of chronic caffeine intake on adipose tissue metabolism. Furthermore, is necessary to study other proteins involved in insulin signaling cascade, since we only studied glut4, Akt and IR, and investigate their function. Moreover, herein we only studied the effects of chronic caffeine intake on adipose tissue. Therefore, other insulin sensitive tissues like skeletal muscle and liver should be assessed to better understand the alterations that are occurring with the intake of caffeine.

V. CONCLUSIONS

We conclude that long-term caffeine administration restores insulin sensitivity and glucose tolerance in an animal model of prediabetes in a concentration-dependent manner and that the latency time needed to restore these parameters is inversely correlated with caffeine concentration. Also, we have shown that the caffeine concentration that achieved the better metabolic profile was the higher concentration tested (1g/l).

We also concluded that chronic caffeine administration avoids visceral and total fat accumulation in a concentration dependent manner in HSu animals, without altering animal’s body weight. Although caffeine did not directly affect IR expression in the adipose tissue, it stimulates the insulin signaling cascade. Therefore, we can suggest that this may be one of the mechanisms for caffeine-induced improvement of insulin sensitivity in HSu rats.

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