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Effect of insulin-resistance on circulating and adipose tissue MMP-2 and MMP-9 activity in rats fed a sucrose-rich diet

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Abstract *Background and aim:* Adipose tissue produces different metalloproteinases (MMPs), involved in adipogenesis and angiogenesis. Different studies have shown that in obesity the behavior of different MMPs may be altered. However there are scarce data about the effect of insulin-resistance (IR) on MMP-2 and MMP-9 activity in adipose tissue. Our aim was to determine whether sucrose induced IR modifies MMP-2 and MMP-9 behavior in expanded visceral adipose tissue and the contribution of this tissue to circulating activity of these gelatinases.
Methods and results: Male Wistar rats were fed with standard diet (Control) or standard diet plus 30% sucrose in the drinking water throughout 12 weeks (SRD). In epididymal adipose tissue vascular density, size and adipocyte density, PPARγ expression and MMP-2 and -9 were measured. Adipose tissue from SRD presented higher adipocyte size (6.32 ± 8.71 vs $4.33 \pm 2.17 \times 10^3$ μm^2, $p = 0.001$) lower adipocyte density (164 (130–173) vs 190 (170–225) number/mm^2, $p = 0.046$) and lower vascular density (16.2 (12.8–23.5) vs 28.1 (22.3–46.5) blood vessels/mm^2, $p = 0.002$) than Control. MMP-2 and MMP-9 activity was decreased in SRD (1.93 ± 0.7 vs 3.92 ± 0.9 relative units, $p = 0.048$ and 1.80 ± 0.8 vs 5.13 ± 1.7 relative units, $p = 0.004$ respectively) in accordance with lower protein expression (0.35 ± 0.20 vs 2.71 ± 0.48 relative units, $p = 0.004$ and 1.12 ± 0.21 vs 1.52 ± 0.05 relative units, $p = 0.036$ respectively). There were no differences in PPARγ expression between groups.
Conclusion: Insulin resistance induced by SRD decreases MMP-2 and MMP-9 activity in adipose tissue which would not represent an important source for circulating MMP-2 and -9. In this state of IR, PPARγ would not be involved in the negative regulation of adipose tissue gelatinases.

Introduction

Matrix metalloproteinases (MMPs) constitute a family of zinc-dependent endopeptidases able to degrade extracellular matrix (ECM) components [1]. These enzymes are synthesized by multiple vascular cell types, including endothelial cells, vascular smooth muscles cells, circulatory monocyte, as well as the local tissue macrophages. MMPs play an important role during physiological tissue remodeling in embryonic development [2], in bone resorption [3] and in angiogenesis [4]. Moreover, during the last decade, MMPs have been extensively studied in the pathogenesis of the atherosclerosis process and cardiovascular disease (CVD) because of their major significance in vascular remodeling. Different MMPs have been identified in atherosclerotic plaques and in regions of foam cell accumulation and have been directly associated with plaque remodeling as well as plaque vulnerability [5–7].

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Gelatinases (MMP-2 and MMP-9) in general are highly expressed in fatty streaks and atherosclerotic plaques compared to normal regions of the vessel [8,9].

Abdominal obesity is one of the main risk factor of CVD. Expansion of fat cell size would require a pliant ECM, and recent studies have suggested that the absence of such pliant matrix could lead to adipose tissue inflammation, characteristic of insulin resistance (IR) states [10]. In this process, MMPs are involved in the control of proteolysis and adipogenesis [11]. In adipose tissue of an animal model of obesity, induced by high fat diet, it has been reported an up-regulation of mRNA levels of some MMPs (MMP-3, MMP-11, MMP-12, MMP-13, and MMP-14) and down-regulation of others (MMP-7, MMP-9, MMP-16, and MMP-24) as well as of their inhibitors [12]. These modulations differed according to the origin of the adipose tissue (gonadal vs subcutaneous), supporting the concept that the different localization of fat deposits presents different metabolic behavior [13]. In contrast, in a genetic obesity model, other authors described up-regulation of mRNA MMP-2 transcription without changes in mRNA MMP-9 transcription, in parallel with no changes in MMP-2 and MMP-9 activity [14].

Previous studies have demonstrated that rats fed sucrose-rich diet (SRD) exhibited impaired insulin activity and expanded adipose tissue [15,16]. However, scarce studies have been developed to evaluate the effect of sucrose induced IR on MMPs behavior. Until now, to our knowledge, the effect of SRD has only been evaluated on MMPs cardiac expression [17], resulting in increased MMP-2 and MMP-9 levels [18].

Our aim was to determine whether sucrose induced IR modifies MMP-2 and MMP-9 behavior in expanded visceral adipose tissue and the contribution of this tissue to circulating activity of these gelatinases.

Methods

Animals

Male Wistar rats ($n = 18$) obtained from the animal laboratory of the Department of Biochemistry, Faculty of Dentistry, University of Buenos Aires (Argentina) were maintained under controlled temperature (22 ± 1 °C), humidity (50–60%), and air flow conditions, with a fixed 12-h light/dark cycle (light on7:00 AM to 7:00 PM). Until the beginning of the experiment, all animals were fed a standard rat laboratory chow and had free access to food and water to standardize their nutritional status. This diet provided approximately 2.9 kcal/g chow. When the rats' weight was 175–190 g, they were randomly divided into two groups: Control group ($n = 9$) and Sucrose rich diet (SRD) group ($n = 9$). Both continued to receive the preweighed standard diet, but the SRD group also received 30% sucrose in the drinking water throughout 12 weeks. In this period, the rats evolve to an early insulin resistance state [15,16], which was confirmed with insulin tolerance test (ITT) as previously described [19]. Body weight and caloric intake were monitored weekly throughout the experimental period. Possible dehydration was checked by plasma sodium measurement. All the procedures were carried out according to the National Institute of Health Guide for the Care and Use of Laboratory Animals [20] and the protocol was approved by the Local Committee of the Faculty of Pharmacy and Biochemistry, University of Buenos Aires.

Samples

After 12 weeks of treatment, food and water were removed at the end of the dark period (7:00 AM). After 4 h of fasting, animals were anesthetized with an intraperitoneal injection of pentobarbital (60 mg/kg body weight). Blood samples were obtained and rapidly centrifuged at 1.500× *g* during 15 min; serum was kept at 4 °C within 48 h for the evaluation of glucose, lipids and lipoproteins or stored at −70 °C for further determination of insulin and free fatty acids (FFA). Plasma was obtained and stored at −70 °C for further MMP-2 and MMP-9 activity determination.

Epididymal, perirenal and intestinal fat tissue was removed and weighed in order to evaluate visceral adiposity. Epididymal adipose tissue (EAT) was fractioned and stored in liquid nitrogen for zymographic and Western blotting analysis and one sample was fixed in 4% formalin buffer, pH 7.0 and conserved at 4 °C for histological evaluation.

Assessment of body fat by X-ray absorptiometry

Body composition was assessed by dual energy X-ray absorptiometry (DXA) using a total body scanner Lunar DPX (DPX Alpha 8034, Lunar Radiation Corp., Madison, Wisc., USA), with software specifically designed for small animals. All DXA determinations were done by one investigator to avoid inter-assay error. Scans were analyzed by ultrahigh-resolution analysis software, and values for percent total fat DXA, which was the current interest, were recorded. The coefficient of variation was 2.2%.

Biochemical determinations

On the day of sacrifice serum glucose, total cholesterol and triglycerides (TG) levels were measured using commercial enzymatic kits (Roche Diagnostics GmbH, Mannheim, Germany) in a Cobas C-501 autoanalyser; the intra-assay CV was <1.9% and the inter-assay CV was <2.4% for all parameters. HDL-cholesterol was determined by standardized selective precipitation method using phosphotungstic acid/$MgCl_2$ as a precipitating reagent [21]. Given the naturally low plasma concentration of LDL-cholesterol in rats, no HDL-cholesterol was calculated as the difference between total-cholesterol and HDL-cholesterol as approximation of atherogenic lipoprotein levels.

FFAs were determined by a spectrophotometric method (Randox, UK), and insulin was measured with a sandwich ELISA kit using a monoclonal antibody against rat insulin and an enzyme-linked polyclonal antibody (Rat/Mouse

ELISA kit, Linco Research, USA). In order to estimate IR, the HOMA-IR (homeostasis model assessment for insulin resistance) was calculated [22].

Histological evaluation

The histological examination by light microscopy was performed in a blinded manner. Fixed EAT samples were dehydrated in ethanol, embedded in paraffin wax, and cut with a microtome Reichert (Austria). The resulting 5 micro-sections were stained with hematoxylin and eosin reagent and periodic acid-schiff (PAS) stain for the determination of size and density of adipocytes and vascular density. In both cases the quantification was performed in at high power field (HPF), 20 fields at ×400 for each animal using a computerized image analyzer (Image Pro Plus, Media Cybernetics Corp). Blood vessel density was normalized to the adipocyte number.

Immunohistochemistry

To localize MMP-2 and MMP-9 and evaluate their protein concentrations, immunohistochemistry was performed. All EAT sections were processed simultaneously under identical conditions. Streptavidin–biotin–peroxidase technique was applied. Slides were incubated in a humidified chamber overnight with either a mouse monoclonal anti-MMP-2 antibody (Novocastra, Leica microsystems) or a mouse monoclonal anti-MMP-9 antibody (Vector Laboratories). The reaction was revealed with diaminobenzidine and contrasted with Harris's hematoxylin. Negative controls were performed by omitting the primary antibody. Immunoreactivity intensity was quantified using the Image Pro Plus software (Media Cybernetics Corp).

Gelatinolytic zymography

MMP-2 and MMP-9 activity from adipose tissue and plasma was measured by zymography [23]. For the adipose tissue, briefly, 150 mg was homogenized in 50 mM Tris buffer, pH 7.4, containing 5 mM $CaCl_2$, 1 μM $ZnCl_2$ and 1% Triton X-100. Twenty micrograms of protein was applied to non-reduced sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using 7.5% gels copolymerized with gelatin 0.1% (G-8150, Sigma). For the measurement of circulating MMPs activities, plasma was diluted 1/10 in saline and 10 μl was applied in the gels. Gels were run 3 h in 25 mM Tris, 192 mM Glycine, 0.1% SDS at 4 °C, pH 8.3, in a Mini Protean-3 (Bio-Rad Laboratories). After running, gels were rinsed with 2.5% Triton X-100 for 30 min and then incubated 18 h in 0.15 M NaCl, 10 mM $CaCl_2$, TrisHCl pH 7.4 at 37 °C. After staining with Coomassie blue R-250 (B-0149, Sigma) and destained with acetic acid–methanol–water (1:3:6), enzyme activity was detected as colorless bands against the blue-stained background. MMP-2 (67 kDa, active form) and MMP-9 (84 kDa, active form) were identified by molecular weight. Conditioned media from the promyelocyte U-937 cell lines was used as activity standard. Coefficients of variation were 4.8% (intra-assay) and 8.6% (inter-assay). Band intensities were quantified using Scion-Image J software (Scion Corporation), and relative activity was expressed as a ratio to the internal standard.

Western blots

Epididymal adipose tissue (400 mg) was homogenized and sonicated in 20 mM Tris buffer, pH 7.4, containing 150 mM NaCl, 1% Triton X-100 and 2% protease inhibitor cocktail (Sigma Aldrich, USA). Tissue homogenates were centrifuged and protein concentrations were determined by Lowry's method in the supernatant. Equal amounts of protein samples (50 mg per lane) were separated in 12% SDS-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membrane. Blots were blocked with 5% skimmed milk for 1 h and incubated overnight at 4 °C with a polyclonal rabbit IgG antibody either against PPARγ (Santa Cruz Biotechnology, Inc., Germany) or against β-actin (Sigma Aldrich, USA). After washing with Tris buffer saline and Tween 0.1%, the blots were incubated with HRP conjugated secondary antibody (BioRad, USA) for 1 h at room temperature. The specific signals were visualized using the ECL Western Blotting Analysis System (Thermo Scientific, Pierce, USA) enhanced chemiluminescence system. PPARγ band at the expected size of 67 kDa was identified by the use of pre-stained molecular weight standards (Thermo Scientific, Pierce, USA), which was absent in the negative control experiments performed in the absence of primary antibody. The relative intensity of protein signal was quantified by densitometric analysis using Fluorchem program (Alpha Innotech Corp). Results are expressed as a PPARγ protein/actin protein ratio.

Statistical analysis

Data are presented as mean ± SD or median (range) according to normal or skewed distribution, respectively. Differences between groups were tested using the unpaired Student's t test or the Mann–Whitney U-test according to the data distribution. Pearson or Spearman analyses, for parametric or non-parametric variables, were used to determine correlations between parameters. The SPSS 19.0 software package (Chicago, IL) was used for statistical analysis. A $p < 0.05$ was considered significant.

Results

Nutritional induced insulin resistance

At the end of the experimental period, no differences in body weight and energy intake were observed between groups (Table 1). Although there was no difference in body weight, SRD group showed an increase of total fat evaluated by DXA ($p = 0.01$) as well as an increase of visceral adipose tissue ($p < 0.001$), estimated by adipose tissue

Table 1 Effect of 12 weeks of sucrose rich diet on body weight, visceral adipose tissue mass and serum parameters.

	Control ($n = 9$)	SRD ($n = 9$)	p
Body weight (g)	251 ± 46	283 ± 66	ns
Energy intake (Kcal/100 g/d)	23.6 ± 5.4	24.1 ± 6.9	ns
Plasma sodium (mmol/l)	145.3 ± 2.3	142.0 ± 3.4	ns
Total fat (%)	18.9 ± 5.4	27.4 ± 4.6	0.01
Visceral adipose tissue (g)	16.7 ± 5.8	29.9 ± 11.4	0.001
Epididymal adipose tissue (g)	6.3 ± 1.9	10.4 ± 3.6	0.001
Perirenal adipose tissue (g)	5.9 ± 2.5	10.4 ± 4.7	0.001
Intestinal adipose tissue (g)	4.5 ± 1.7	8.6 ± 4.3	0.001
TG (mmol/L)	0.67 ± 0.27	1.44 ± 0.62	0.001
Total-chol (mmol/L)	1.33 ± 0.23	1.25 ± 0.18	ns
HDL-chol (mmol/L)	0.83 ± 0.21	0.67 ± 0.21	0.038
No HDL-chol (mmol/L)	0.52 ± 0.23	0.55 ± 0.21	ns
Glucose (mmol/L)	6.49 ± 0.99	6.55 ± 1.55	ns
FFA (mmol/L)	0.51 ± 0.16	0.69 ± 0.26	0.032
Insulin (pmol/L)	153 (52–184)	476 (143–1120)	0.03
HOMA-IR	9.8 (2.5–14.2)	27.7 (7.6–70.2)	0.047

Data is expressed as mean ± SD or median (range) for skewed distributed data.
TG, triglyceride; FFA, free fatty acids; HOMA-IR, homeostasis model assessment insulin-resistance index.

removed from the epididymus, intestine and kidneys (Table 1).

Regarding to serum metabolites, SRD group presented higher TG ($p < 0.001$) and lower HDL-cholesterol ($p = 0.038$) compared to the Control group, without differences in total and non-HDL cholesterol (Table 1). In turn, insulin ($p = 0.03$), FFA ($p = 0.032$) and HOMA-IR ($p = 0.047$) were significantly increased in SRD group, without differences in glucose concentration (Table 1). Plasma sodium levels were similar in both groups and did not reveal signs of dehydration (Table 1).

Histological characteristics of visceral adipose tissue

Histological characteristics of EAT from Control and SRD group are illustrated in Fig. 1A and B, respectively. As expected, the average adipocyte size in SRD group was significantly higher than Control ($p < 0.001$). Positive associations with FFA ($r = 0.64$, $p = 0.048$) and HOMA-IR ($r = 0.68$, $p = 0.031$) were observed. SRD group presented lower adipocyte density than Control ($p = 0.046$) (Table 2).

Regarding vascular density, it was significantly lower in EAT from SRD compared to Control ($p = 0.002$), and inversely correlated with adipocyte size ($r = -0.75$, $p = 0.013$) and HOMA-IR ($r = -0.68$, $p = 0.032$). This difference did not remain significant after normalize to the adipocyte number (Table 2).

Localization of MMP-2 and MMP-9 in EAT

The protein localization and expression of MMP-2 and MMP-9 were evaluated by immunohistochemistry and quantified by densitometry analysis. In EAT from Control and SRD group, MMP-2 and -9 were localized in the region of the capillaries, perivascular connective stroma and in the basement membrane surround adipocytes (Fig. 2). In turn, immunohistochemical analysis showed that MMP-2 and MMP-9 protein expression was significantly decreased in SRD group ($p = 0.004$ y $p = 0.036$, respectively) (Fig. 2C and F).

MMP-2 and MMP-9 activity

In accordance with the lower MMPs expression, in SRD group, EAT presented lower MMP-2 ($p = 0.048$) and MMP-9 activity ($p = 0.004$) than Control (Fig. 3). In turn, both enzyme activities were negatively associated with FFA (MMP-2: $r = -0.72$, $p = 0.029$; MMP-9: $r = -0.88$, $p = 0.002$) and adipocyte size (MMP-2: $r = -0.79$, $p = 0.036$; MMP-9: $r = -0.70$, $p = 0.036$), meanwhile MMP-9 positively correlated with vascular density

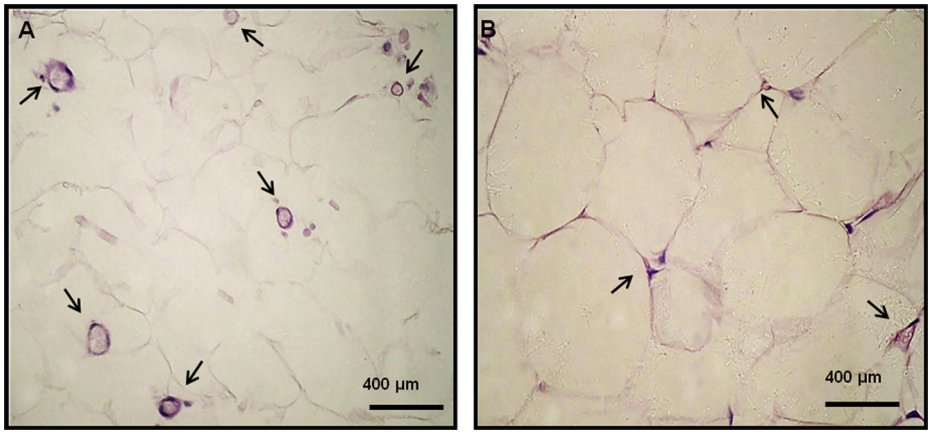

Figure 1 Histological characteristics of visceral adipose tissue of animals fed with control diet (A) and animals fed with sucrose rich diet (B). Arrows indicate blood vessels.

Table 2 Effect of 12 weeks of sucrose rich diet on adipocyte size and adipocyte and vascular density in adipose tissue.

	Control ($n = 9$)	SRD ($n = 9$)	p
Adipocyte size ($\times 10^3$ μm^2)	4.33 ± 2.17	6.32 ± 8.71	0.001
Adipocyte density (number/mm^2)	190 (170–225)	164 (130–173)	0.046
Vascular density (blood vessels/mm^2)	28.1 (22.3–46.5)	16.2 (12.8–23.5)	0.002
Normalized vascular density	0.13 ± 0.02	0.13 ± 0.04	ns

Data is expressed as mean ± SD or median (range) for skewed distributed data.

($r = 0.71$, $p = 0.014$). In plasma, no differences in MMPs activities were observed between SRD and Control group (MMP-2: 0.99 (0.81–1.00) vs 0.82 (0.79–0.96); MMP-9: 0.97 (0.94–1.00) vs 1.04 (1.03–1.05) relative units; p = ns).

On the other hand, there were no associations between plasma MMPs activity vs tissue MMPs activity (MMP-2, $r = 0.28$ and MMP-9, $r = 0.08$; p = ns), or between plasma MMPs activity and EAT mass (MMP-2, $r = 0.54$ and MMP-9, $r = -0.65$; p = ns).

PPARγ expression

PPARγ expression at 12 weeks of diet did not show differences between groups (Control vs SRD: 0.15 ± 0.05 vs 0.11 ± 0.07 relative units; p = ns). No association of the receptor levels with MMP-2 ($r = 0.45$; p = ns) or MMP-9 ($r = 0.30$; p = ns) activity was observed.

Discussion

In the present study we have evaluated MMP-2 and MMP-9 activity in visceral adipose tissue in an animal model of early IR induced by sucrose rich diet. We found that gelatinases activity was decreased in this tissue; however this was not associated with changes in MMPs plasma activity. During the last years, circulating levels of MMPs have emerged as potential biomarkers of CVD [24,25], however, in obesity, the expanded adipose tissue could be an important source of circulating MMPs. Our question was whether the expanded visceral adipose tissue in IR induced by SRD, could significantly contribute to circulating activity of gelatinases. Our data show that in this model of IR, the decrease of MMP-2 and -9 activity observed in visceral adipose tissue is not subsequently reflected in plasma activity changes.

MMPs are involved in the adipogenesis and angiogenesis of adipose tissue [26]. Previous studies in cell culture showed different expression profile during adipogenesis, with high MMP-2 and -9 expressions in very early stages of differentiation [12]. Maquoi et al. reported that mRNA levels for MMP-2 increased throughout the process of preadipocyte differentiation, with a maximal expression in mature adipocytes (between day 12 and 19 of differentiation). In reference to mRNA levels for MMP-9, the authors described a decrease in the first days of differentiation and a subsequent increase in the mature adipocyte [12]. However, in gonadal adipose tissue of obese mice fed under high fat diet for 15 weeks, a decrease in the mRNA levels of MMP-9 and no change in the mRNA levels of MMP-2 were observed [12]. Other authors described up-regulation of mRNA MMP-2 transcription without changes in mRNA MMP-9 transcription, in parallel with no

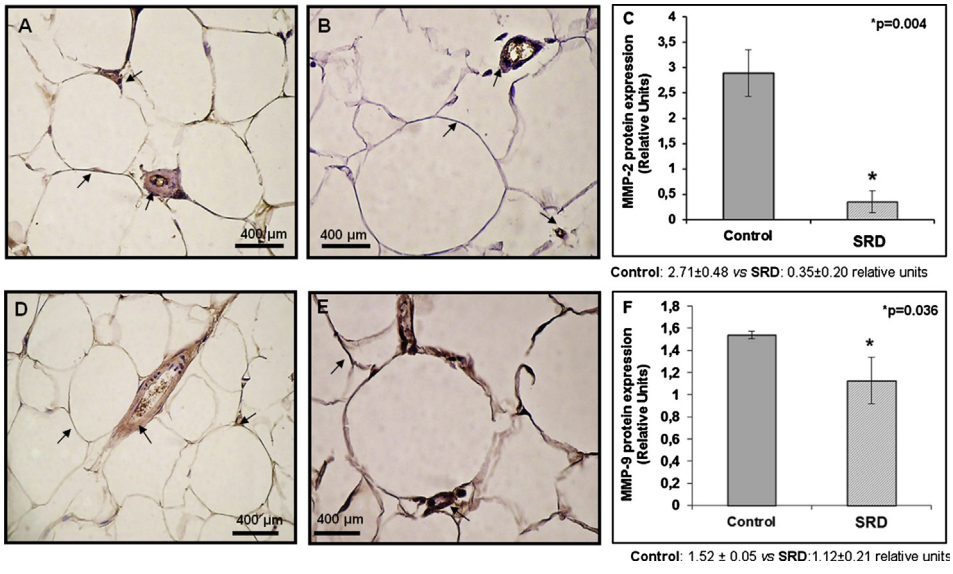

Figure 2 Representative immunohistochemistry for MMP-2 and MMP-9 by immunoperoxidase technique. MMP-2 (A: Control and B: SRD) and MMP-9 (D: Control and E: SRD) were localized in the region of the capillaries, perivascular connective stroma and in the basement membrane surround adipocytes. Immunohistochemical analysis showed that MMP-2 (C) and MMP-9 (F) protein expression was significantly decreased in SRD group. Data are shown as mean ± SD.

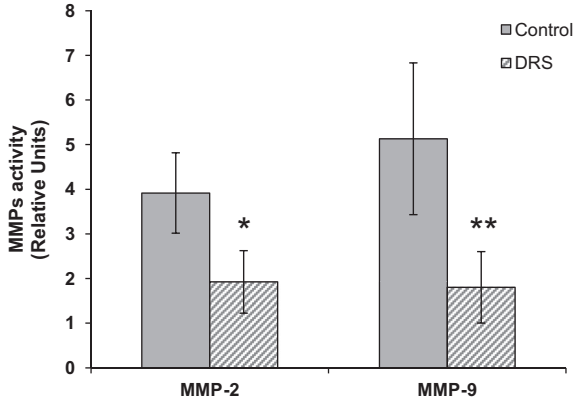

Figure 3 MMPs activity. MMP-2 Control: 3.92 ± 0.9 vs SRD: 1.93 ± 0.7 relative units, $^{*}p = 0.048$. MMP-9 Control: 5.13 ± 1.7 vs SRD: 1.80 ± 0.8 relative units, $^{**}p = 0.004$.

changes in MMP-2 and MMP-9 activity, in genetic obese mice [14].

In our model, after 12 weeks of diet, we observed a decrease in MMP-2 and MMP-9 levels in EAT from SRD group in accordance with the lower gelatinolytic activity. In both groups MMP-2 and -9 were located mainly in the region of the capillaries, perivascular connective stroma and in the basement basal membrane surrounding adipocyte, as previously described [12,14].

As MMPs are involved in the expansion of adipose tissue, we evaluated the association between MMP-2 and -9 and the size of adipocytes and vascular density in this tissue. As expected, the adipocytes of SRD group were found to be larger than those of the control group. Both enzymes activities were negatively associated with adipocyte size, furthermore, other studies showed that the mRNA levels of MMP-9 were negatively correlated with gonadal fat weight, meanwhile no association was found with MMP-2 levels [12]. Other MMPs have been described in adipose tissue remodeling, like MMP-3, -11, -12 and -13 in mice maintained on high fat diet for 15 weeks [12]. It could be supposed that at early IR stage induced by SRD other MMPs different from MMP-2 and MMP-9 may help adipose tissue growth.

When analyzing the vascular density, there was a significant reduction in the number of blood vessels in the EAT from SRD group which was lost after normalize to the adipocytes number. The vascular density was inversely associated with the size of adipocytes and directly with MMP-9 activity. It should be kept in mind that vascular density may be affected by the number and/or size of adipocytes as well as by the angiogenic activity [27].

Given that the PPARγ receptor is an important regulator of MMPs, its expression was evaluated in EAT. In vitro studies have reported an increase in PPARγ expression during adipocyte differentiation and its subsequent decrease in mature adipocytes [12]. In this study we found no significant differences in PPARγ levels between Control and SRD group at 12 weeks of diet. Our data suggest that other mechanisms would be involved in the negative regulation of MMPs at this stage of IR. In turn, we observed a significant negative association between FFA and MMPs activities in EAT. In accordance, Boden et al. [28] reported that FFA decreases MMP-2 activity in rat EAT, through an inhibitory effect on insulin stimulation of MMPs, this effect is opposed to the observed in rat aorta, where FFA augmented insulin stimulation of MMP-2 activity.

Concerning MMP-2 and -9 plasma activities, the lack of differences between groups and the absence of association between adipose tissue and plasma activity of the gelatinases, suggest that this tissue is not a major contributor of these circulating enzymes.

Assuming that our results are applicable to human, we could suggest that the increase in MMP-2 plasma activity previously observed in IR patients [29] would not proceed from adipose tissue. Accordingly with our results, Gummesson et al. [30] studied the gene expression of MMP-9 in adipose tissue in men with and without metabolic syndrome and found a lack of association between adipose tissue mRNA and plasma levels of MMP-9.

It is important to take into account that although we have not measured MMPs plasma concentration, the evaluation of MMPs activity overcomes the protein mass study. Furthermore, in this state of IR, PPARγ would not be involved in the negative regulation of adipose tissue gelatinases. Ongoing research should include the study of TIMPs levels in EAT, which could contribute to the decrease of MMP-2 and -9 activity; in addition more time of exposure to SRD could reveal changes in these MMPs activity. Further studies are necessary to elucidate the effect of IR on adipose tissue MMPs behavior.

Conflict of interest

The authors have no conflict of interest.

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