MATERIAL AND METHODS

Subjects

For the present study, 59 patients with Metabolic Syndrome (MS), according to Adult Treatment Panel III definition,[1] were consecutively selected at the Hepatology Unit of Prof. Alejandro Posadas National Hospital (Buenos Aires, Argentina). All the MS patients presented hepatic steatosis which was diagnosed by abdominal ultrasonography performed by a single operator and in all cases confirmed by a liver biopsy showing macro vesicular steatosis in at least 5% of hepatocytes. In parallel, 36 subjects recruited among hospital employee volunteers were selected as controls; all of them underwent hepatic ultrasonography in order to discard steatosis.
The following exclusion criteria were considered for both groups: alcohol intake >20 g/day, diabetes, cardiovascular disease (CVD), neoplasia, hypothyroidism, recent history of acute illness, renal disorders, seropositive hepatitis B or C. None of the subjects received corticosteroids, immunosuppressive agents or drugs known to influence lipid metabolism such as statins or fibrates.

The weight, height and blood pressure of each participant were measured, and body mass index (BMI) was calculated to evaluate obesity degree. Waist circumference was taken midway between the lateral lower rib margin and the superior anterior iliac crest in a standing position, always by the same investigator.

According to BMI, all the subjects were subdivided in: -Normal Weight (NW, 18.5≤ BMI≤24.9 kg/m^2), n= 27; Overweight (OW, 25 kg/m^2≤ BMI≤29.9 kg/m^2), n= 20 and Obese (OB ≥30 kg/m^2), n=48.

Written informed consent was obtained from all the participants included in the study. The study had the approval of the Ethics Committees from the Faculty of Pharmacy and Biochemistry, University of Buenos Aires.

Samples

After a 12-h overnight fast, blood samples were drawn. Serum was kept at 4ºCwithin 48 h for the evaluation of glucose, lipids and lipoproteins or stored at -70 ºC for further determination of insulin, free fatty acids (FFA), adiponectin, high sensitivity C-reactive protein (hs-CRP) and VLDL, IDL and small and dense LDL isolation.
To measure LPL, HL and EL activities, heparin (60 IU/kg body weight) was administered intravenously. After 10 minutes, blood from the contralateral arm was collected in tubes on ice. Post-heparin plasma (PHP) was obtained by centrifugation at 1500 g at 4°C for 10 minutes and kept at −70°C.

Measurements

Total cholesterol, TG, and fasting glucose were measured using commercial enzymatic kits (Roche Diagnostics, Mannheim, Germany) in a Cobas C-501 autoanalyzer, coefficient of variation (CV) intra-assay<1.9%, CV inter-assay<2.4%, and averaging CV values of these parameters. HDL and LDL-cholesterol were determined by standardized selective precipitation methods, using phosphotungstic acid/$MgCl_2$ [2] and polyvinylsulfate [3] as precipitating reagents, respectively CV intra-assay<2.0%, and CV inter-assay<3.0%. Serum hs-CRP, apoA-I, and apoB-100 were determined by immunoturbidimetry (Roche Diagnostics, Mannheim, Germany), CV intra-assay<1.9%, and CV inter-assay<2.5% for the 3 parameters. FFA were determined by a spectrophotometric method (Randox, UK) CV intra-assay <2.6% and CV inter-assay <3.9% and insulin was measured with Immulite/Immulite 1000 Insulin (Siemens, USA), CV intra-assay<2.6%, and CV inter-assay<3.9%. To estimate IR, the homeostasis model assessment for insulin resistance (HOMA-IR) index was calculated as fasting

insulin (μU/mL)×fasting glucose (mmol/L)/22.5. TG/HDL-cholesterol index was also used as a surrogate marker of IR. Sera levels of adiponectin were determined by monoclonal antibody–based ELISA (R&D Systems, USA).

Lipoprotein measurements

VLDL and IDL isolation. VLDL [density (d) <1.006 g/ml] and IDL [d:1.006–1.019 g/ml] were isolated by sequential preparative ultracentrifugation,[4] in a Beckman XL-90 using a fixed-angle rotor type 90 Ti. Each run was performed at 105.000×g, for 18 h, at 14 °C. Purity of lipoprotein was tested by agarose gel electrophoresis. Isolated VLDL composition was characterized by the following parameters: cholesterol and TG, using the methods previously described, phospholipids by the Bartlett method[5] and proteins by the Lowry method.[6] Data was expressed as the percentage of each component. VLDL TG/protein (TG/PT) ratio was calculated as estimator of lipoprotein size. In order to assess IDL concentration, its cholesterol content was measured, as described before.

VLDL size by high performance liquid chromatography (HPLC). In brief, isolated VLDL were injected in a column TSK-Gel Lipopropack XL, 7.8 mm ID×30 cm(Tosoh, Japan) and runs were performed using as mobile phase: Tris acetate buffer 0.05 mol/l (pH 8) containing 0.3 mol/l sodium acetate,0.05% sodium azide and 0.005% Brij-35. Flow rate was 0.5 ml/min and the column eluate was monitored at 280 nm. [7] For the conversion of elution time in particle diameter, a standard curve was used, constructed with the logarithm of retention time and the logarithm of the diameter of standard diameter latex particles, 100 nm in diameter (Fluka, Sigma-Aldrich) and of 27 and 39 nm in diameter (MagsphereINC).

Small and dense LDL isolation. The small and dense LDL subfraction was measured by a precipitation commercial kit (sdLDL-C, Denka Seiken, Japan) using heparin sodium salt and $MgCl_2$. After precipitation LDL-cholesterol was measured by a direct and selective homogenous assay method (LDL-EX; Denka Seiken, Japan).[8] Results were calculated as sdLDL-chol mg/dl serum and expressed as percentage.

Lipase Activities

Lipoprotein Lipase and Hepatic Lipase as TG hydrolase. LPL and HL as TG hydrolase were determined in PHP by measuring the oleic acid produced by the enzyme-catalyzed hydrolysis of an emulsion containing [^{3}H]-triolein (Amersham TRA191; Amersham, Buckinghamshire, UK) according to Nilsson-Ehle method.[9] The assay mixture for LPL activity contained labelled and unlabelled Triolein (Sigma T-7140) (1.3 mmol/ml of glyceryltrioleate with a specific activity of 10 x10^{6}cpm/mmol), mixed with 0.11 mmol/ml of L-lysophosphatidylcholine (Sigma L-4129), 4% bovine serum albumin (Sigma A-6003), 10% v/v of human serum as source of apoC-II in 0.2 M buffer Tris–HCl pH 8.0 with NaCl 0.3 M. This mixture was incubated with PHP in saline solution 1:10 to determine the total lipolytic activity, and simultaneously with PHP in saline solution 1:5 and NaCl 1M (as inhibitor of LPL) for 30 minutes at 37°C. After incubation, the reaction was stopped and the released fatty acids were isolated by extraction with a carbonate-borate buffer, pH 10.5. The [^{3}H] oleic acid was quantified by counting with a Liquid Scintillation Analyzer (Packard TRI-CARB 2100; Packard Instruments, Meridian, CT). LPL activity of each individual was calculated from the difference between total activity and the activity measured in presence of NaCl 1M.
For HL, the assay mixture was prepared as previously described using 0.2 M buffer Tris–HCl pH 9.0 with NaCl 0.15 M and without human serum. In this case, the mixture was incubated with PHP in saline solution 1:10 and NaCl 1M (as inhibitor of the other lipases) for 30 min at 30ºC. After the incubation, the extraction and measurement of

free fatty acids was perform as described above. Results were expressed as μmolFFA released per milliliter of PHP per hour. CV intra-assay was 4% and CV inter-assay 9%. Because of the complexity of this assay, the CV is considered to be quite satisfactory.

Endothelial Lipase and Hepatic Lipase as Phospholipase. EL and HL as phospholipase were determined as previously described.[10] Briefly, total SN1-specific phospolipase activity was determined using (1-decanoylthio-1-deoxy-2-decanoyl-sn-glycero-3-phosphoryl) ethylene glycol (ThioPEG) as the substrate.[11] An emulsion of 4.09mmol/L ThioPEG (Avanti) in 100mmol/L HEPES, pH 8.3, and 7mmol/L Triton X-100 was prepared by sonication with a Bransom Sonifier. A 1:1 mol mixture of ThioPEG to 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) was prepared by adding the DTNB 271mmol/L (Sigma) solution to the ThioPEG emulsion, resulting in a chromogenic substrate solution. Total SN1 phospholipase activity was measured by adding 20 μL of a one-tenth dilution of PHP and 80 μL of chromogenic substrate to the wells of a 96-well plate and following the absorbance at 412 nm for 30 minutes in a Molecular Devices Spectra Max 250 microplate reader. To determine the HL phospholipase activity, the PHP dilutions were pre-incubated in ice for 15 minutes with 1mol/L NaCl to inhibit the EL activity. Then, the chromogenic substrate solution was added, and the plate was read at 412nm as described previously. Finally, EL activity was calculated as the difference between total phospholipase activity and hepatic phospholipase activity. The intra-assay CV for HL and EL were 3.1%, and 15.7%, respectively. The inter-assay CV for HL and EL were 5.3% and 28.8%, respectively. The EL CV is higher because it is the difference between total lipase and HL. All enzyme activities were calculated using the molar adsorption coefficient value for the nitro-5-thiobenzoate anion, the depth of solution, and the volume of the enzyme source. Results were expressed in μmol of FFA per milliliter of PHP per hour.

Statistical Analysis

Data are presented as mean±SD or median (range) according to normal or skewed distribution, respectively. Differences between Control and MS group were tested using the unpaired Student t test, $\chi 2$ test, or the Mann–Whitney U test, according to the data distribution. One-way analysis of variance was used to test differences among patients with different obesity degree. Previously, each variable was examined for normal distribution, and abnormally distributed variables were log transformed. Further evaluation was performed using Scheffé multiple comparison test. Pearson or Spearman analysis, for parametric or non-parametric variables, was used to determine correlations between parameters. To verify the difference of lipases activity between groups, we performed an analysis of covariance, controlling for necessary confounders such as age and gender. Stepwise and multiple linear regression analyses were used to indentify independent correlates of enzymes activities. Previously, each variable was examined for normal distribution, and abnormally distributed variables were log-transformed. The SPSS 19.0 software package (Chicago, IL) was used for statistical analysis.

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