Thrombosis Research 129 (2012) 74–79

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Caloric restriction improves coagulation and inflammation profile in obese mice

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ARTICLE INFO

Article history:
Received 22 March 2011
Received in revised form 26 May 2011
Accepted 30 May 2011

Keywords:
Obesity
Weight loss
Metabolism
Coagulation
Inflammation

ABSTRACT

To evaluate associations between adiposity and coagulation or inflammation profile, obese wild-type C57Bl/6 mice were subjected to drastic caloric restriction by switching from a high fat diet to restricted normal chow. After 6 weeks, total body weights as well as subcutaneous and gonadal adipose tissue mass were markedly reduced, associated with adipocyte hypotrophy (all $p<0.001$). Weight reduction was associated with markedly reduced plasma levels of plasminogen activator inhibitor-1, Factor VII and Factor VIII. Reduced oxidative stress and inflammation following weight reduction is supported by significantly lower expression in adipose tissues of pro-inflammatory interleukin-6, higher expression of anti-oxidant catalase, superoxide dismutase 1 and glutathione peroxidase 1, and lower plasma levels of C-reactive protein. Furthermore, reduced levels of leptin and enhanced levels of adiponectin were observed, whereas cholesterol and triglyceride levels were reduced. The content of structurally intact collagen fibers was significantly higher in subcutaeneous and gonadal adipose tissues after caloric restriction.

Thus, caloric restriction and drastic weight loss in obese mice is associated with improved plasma coagulation profile and with reduced oxidative stress and inflammation in the adipose tissues.

Introduction

High fat diet feeding results in development of excess adipose tissue and obesity, the most common metabolic disorder. The discovery of important signaling pathways originating from adipose tissue cells has advanced the concept that fat is a metabolically active organ rather than an inert tissue. Obesity is associated with reduced adipose tissue oxygenation, hypoxia, and increased chemotaxis of inflammatory cells [1]. Monocytes are recruited to adipose tissue where they secrete tumor necrosis factor-α and interleukin-6 [2] and, together with leptin and resistin produced by dysfunctional adipocytes [3], contribute to oxidative stress and to the pathogenesis of insulin resistance. Macrophages furthermore promote remodeling of adipose tissues by secreting matrix metalloproteinases (MMPs), preferentially at sites of adipocyte death [4]. In contrast, insulin-sensitizing adiponectin is downregulated during obesity, resulting in enhanced inactivation of the growth arrest specific protein 6 (GAS6)-mediated survival pathway and loss of protection against TNF-α and reactive oxygen species (ROS) [5]. In addition, ROS has pro-inflammatory and prothrombotic effects, as a result of decreased nitric oxide production and endothelial dysfunction [6].

Recent studies have evaluated the effect of diet-induced weight loss on serum adipokine levels and on oxidative stress in man and mice [7,8]. In these studies, however, analysis of adipose tissue composition and its relation with adipokine or inflammatory cytokine expression was not reported. In the present study we have subjected obese mice to rapid weight reduction by caloric restriction, and investigated the effects on coagulation, inflammation, oxidative stress, metabolism and adipose tissue composition.

Materials and methods

Animal model

Male wild-type C57Bl/6 mice were generated in the KU Leuven animal facility. Five weeks old male mice were kept in individual microisolation cages on a 12 h day/night cycle at 20–22 °C and fed with a high fat diet (HFD, Harlan Teklad TD88137, Zeist, The Netherlands; caloric value 20.1 kJ/g) during 50 weeks. At that time, the mice were either switched to a standard fat diet (SFD) (KM-04-k12, Muracon, Carfil, Oud-Turnhout, Belgium; caloric value of 10.9 kJ per g) restricted to 2.5 g/day ($n=8$), or continued on the HFD ad libitum for another 6 weeks ($n=7$). The HFD contains 42% kcal from saturated fat, 42.7% kcal from carbohydrate and 15.2% from proteins (for detailed composition: www.harlan.com). The SFD contains 13% fat, 44%

Abbreviations: aPTT, activated partial thromboplastin time; CLS, crown-like structures; CRP, C-reactive protein; HFD, high fat diet; HOMA-IR, homeostasis model assessment of insulin resistance; GON, gonadal; Gpx1, glutathione peroxidase 1; IL-6, interleukin-6; ISI, insulin sensitivity index; Nox4, nicotinamide adenine dinucleotide phosphate oxidase 4; PAI-1, plasminogen activator inhibitor-1; PT, prothrombin time; ROS, reactive oxygen species; SFD, standard fat diet; SC, subcutaneous; SOD1, superoxide dismutase 1; TNF-α, tumor necrosis factor-α; Xdh, xanthine dehydrogenase.

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doi:10.1016/j.thromres.2011.05.023

carbohydrate and 16.5% proteins, supplemented with minerals and vitamins (for detailed composition: www.carfil.be). Water was always available ad libitum. Food intake and body weight were measured daily, and body temperature was measured at weekly intervals using a rectal probe (TR-100, Fine Science Tools, Foster City, CA). Physical activity at night was monitored in cages equipped with a turning wheel linked to a computer to register full turns/12 h (19:00–07:00). Mice were scanned by non-invasive magnetic resonance imaging (MRI) and total body fat as well as volumes of inguinal subcutaneous (SC) and intra-abdominal (ABD) fat were determined as described [9].

At the end of the experiments, after 18 h fasting, mice were euthanasized by intraperitoneal injection of 60 mg/kg Nembutal (Abbott Laboratories, North Chicago, IL). Blood was collected via the retro-orbital sinus on trisodium citrate (final concentration 0.01 M) and plasma was stored at −80 °C. SC and gonadal (GON) fat pads were removed and weighed; portions were snap-frozen in liquid nitrogen for protein or RNA extraction, and paraffin sections (10 μm) were prepared for histology and immunohistochemistry. Other organs including kidneys, lungs, spleen, pancreas, liver, heart and brain were also removed and weighed.

All animal experiments were approved by the local ethical committee (KU Leuven P07071) and performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (1996).

Assays

The size and density of adipocytes or blood vessels in the adipose tissues were determined by staining with haematoxylin/eosin under standard conditions or with the Bandeiraea Simplicifolia lectin, followed by signal amplification with the Tyramide Signal Amplification Cyanine System (Perkin Elmer, Boston, MA), as described [10]. Blood vessel density was normalized to the adipocyte number. Analysis was performed using a Zeiss Axioplan 2 Imaging microscope with the AxioVision rel. 4.6 software (Carl Zeiss, Oberkochen, Germany).

Macrophage density of adipose tissues was quantified following staining with anti-Mac-3 antibody (Pharmingen, San Diego, CA) and normalized to adipocyte density. The occurrence of crown like structures (CLS) was also scored quantitatively [11]. Collagen content was determined by staining with Sirius red and quantified as percentage stained area per total tissue section area. Quality of collagen fibers was estimated by Sirius red polarization microscopy, allowing to quantify thick, tightly packed collagen fibers (orange-red) and thin loosely assembled fibers (yellow-green) [12].

Blood glucose concentrations were measured using Glucocard strips (Menarini Diagnostics, Firenze, Italy). Other metabolic parameters, including triglycerides, total cholesterol, HDL cholesterol and LDL cholesterol were determined using standard laboratory assays. Insulin (Mercodia, Uppsala, Sweden), C-reactive protein (CRP), leptin, and adiponectin (R&D Systems Europe, Lille, France) levels were measured using commercial ELISA's and plasminogen activator inhibitor-1 (PAI-1) antigen with a specific home-made ELISA [13]. The homeostasis model assessment was used to calculate the insulin resistance index [HOMA-IR as (fasting glucose level in mmol/l x fasting insulin level in mIU/l)/22.5] and the insulin sensitivity index [ISI as 1/(fasting glucose level in mg/dl x fasting insulin level in mIU/l) x 1000].

Platelet count, haemoglobin and haematocrit levels, were determined on a Cell-Dyn 3500 R (Abbott Diagnostics, Abbott Park, IL). Coagulation parameters including fibrinogen, Factor VII, Factor VIII, activated partial thromboplastin time (aPTT) and prothrombin time (PT) were determined on a Dade Behring BCSXP system (Siemens Healthcare Diagnostics, Deerfield, IL).

Quantitative real time-PCR (RT-PCR)

For gene expression analysis, SC and GON fat pads were homogenized using lysing matrix tubes containing ceramic and silica beads in a Hybaid Ribolyser ((both from Hybaid Ltd., Thermo, Waltham, MA). Total DNA-free RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) and the concentration was determined spectrophotometrically (ND-2000, Nanodrop Technologies, Wilmington, DE). cDNA was obtained by incubating 50 ng total RNA with Taqman Reverse Transcription Kit reagents and 5 μM random hexamers (Applied Biosystems, Lennik, Belgium) at 48 °C for 60 min. mRNA expression was determined by quantitative RT-PCR in the ABI-prism Fast 7500 sequence detector using Taqman gene expression assays (Applied Biosystems) of adiponectin (Mm00456425_m1), resistin (Mm00445641_m1), PAI-1 (Mm00435860_m1), IL-6 (Mm00446190_m1), TNF-α (Mm00443258_m1), catalase (Mm00437992_m1), superoxide dismutase 1 (SOD1; Mm01700393_m1), glutathione peroxidase 1 (Gpx1; Mm00656767_g1), xanthine dehydrogenase (Xdh; Mm00442110_m1), nicotinamide adenine dinucleotide phosphate oxidase 4 (NADPH oxidase 4 or Nox4; Mm00479246_m1); for leptin primers (Fw: '5-CAA AAC CCT CAT CAA GAC CAT TG-3'; Rv: '5-AGT CCA AGC CAG TGA CCC TCT-3' and FAM/TAMRA-labeled probe ('5-TTC ACA CAC GCA GTC GGT ATC CGC-3') (Eurogentec, Seraing, Belgium) were used. As a housekeeping gene, the expression of β-actin mRNA was measured using a Taqman gene expression assay (Mm01205647_g1). PCR amplifications were performed in duplicate wells, using the following conditions: 2 min at 50 °C and 10 min at 94 °C followed by 40 two-temperature cycles (15 sec at 95 °C and 1 min at 60 °C). Data were obtained as cycle threshold (Ct) values and expressed as copy number of target mRNA relative to 10^5 copies of β-actin.

Statistical analysis

Data are shown as means ± SEM for the number of animals studied. Statistical significance between groups is evaluated by non-parametric Mann-Whitney *U*-test. Correlations are examined by the nonparametric Spearman's rank correlation coefficient. Comparison of progress curves was performed by two-way repeated-measures ANOVA. Values of $p<0.05$ are considered statistically significant.

Results

Effect of caloric restriction on adiposity

After 50 weeks of HFD, total body weight of C57Bl/6 mice was 59 ± 1.9 g (n = 15). Mice were transferred to individual cages and were continued on HFD (n = 7) or subjected to caloric restriction (2.5 g/day of SFD) for 6 weeks (n = 8); both groups were weight-matched at the start. Food intake in the HFD group was 4.08 ± 0.08 g/day/mouse, whereas in the SFD group all the food provided was eaten. Mice continued on HFD essentially kept their weight, whereas mice on the SFD showed drastic weight loss (Fig. 1).

Physical activity at night was lower for the mice on HFD (1215 ± 217 versus 2688 ± 639 turns/12 h for SFD; p = 0.014). Body temperature was not significantly different for both groups (37.1 ± 0.11 °C or 36.4 ± 0.44 °C for HFD or SFD). Caloric restriction of obese mice during 6 weeks resulted in significantly reduced SC and GON fat mass, as well as in weight of liver, spleen, kidneys and heart (Table 1). MRI scanning confirmed significantly reduced volumes of total body fat, SC and ABD fat depots after 6 weeks on SFD as compared to HFD (Fig. 1, inset).

Effect of caloric restriction on coagulation profile

Caloric restriction resulted in marked downregulation of the expression of PAI-1 mRNA in both SC (35-fold) and GON (12-fold) adipose tissues (Table 2). PAI-1 expression correlated positively with SC or GON fat mass (Table 3). PAI-1 protein levels in extracts of SC and GON adipose tissues (Table 4), as well as in plasma (Table 5), were significantly reduced by caloric restriction.