To the Editor:

The enclosed manuscript *Leptin induces the generation of procoagulant, tissue factor bearing microparticles by human peripheral blood mononuclear cells* that we would like to submit to BBA general interest for possible publication represents original work. It has not been published before, except in abstract form, and is currently not under evaluation by other Journals.

The manuscript describes a novel mechanism that potentially links the adipokine, leptin, to cardiovascular and thrombotic disorders in obese individuals. The mechanism involves the generation of procoagulant, tissue factor-bearing vesicles, upon incubation of mononuclear cells with leptin via intracellular calcium mobilization and ERK1/2 activation. We therefore deem the manuscript, if considered scientifically sound, to be of general interest.

Leptin induces the generation of procoagulant, tissue factor bearing microparticles by human peripheral blood mononuclear cells

Silvia Petrini[a], Tommaso Neri[a], Stefania Lombardi[b], Cinzia Cordazzo[a*], Cristina Balìa[a], Valentina Scalise[a], Pierluigi Paggiaro[a], Roberto Pedrinelli[a], Alessandro Celi[a§]

Running title: leptin induces procoagulant microparticle shedding

a) Laboratorio di Biologia Cellulare Respiratoria; Dipartimento di Patologia Chirurgica, Medica, Molecolare e dell'Area Critica, Università di Pisa and Azienda Ospedaliero-Universitaria Pisana, Pisa, Italy

b) SSD Analisi ChimicoCliniche ed ImmunoAllergologia, USL 1, Massa e Carrara, Italy

* Present address: Sirius Biotech, Genova, Italy

None of the Authors has any conflicts of interest to disclose

§ To whom correspondence should be addrressed at Dipartimento di Patologia Chirurgica, Medica, Molecolare e dell'Area Critica, c/o Ospedale di Cisanello, Via Paradisa, 2 56124 - Pisa, ITALY

email: alessandro.celi@med.unipi.it

Abstract

Background: Obesity is linked to increased thrombotic risk. Circulating leptin concentration correlates with body mass index. Microparticles are small (.05-1 μm) vesicles shed by activated and apoptotic cells, involved in numerous pathophysiologically relevant phenomena including blood coagulation and thrombosis. We tested the hypothesis that leptin induces the shedding of procoagulant, tissue factor bearing microparticles by human peripheral blood mononuclear cells, and investigated the intracellular mechanisms leading to microparticle release upon incubation with leptin.

Methods: Peripheral blood mononuclear cells were isolated from healthy donors. Cells were incubated with leptin in the presence or in the absence of a phospholipase C inhibitor, U73122, a calmodulin inhibitor, W-7, and three inhibitors of mitogen activated protein kinases. Microparticle generation was assessed as phosphatidylserine concentration with a prothrombinase assay and by cytofluorimetric analysis. Tissue factor expression on microparticles was measured with a one-stage clotting assay. Intracellular calcium concentration was assessed by a fluorescent probe.

Results: Leptin increased intracellular calcium mobilization and stimulated the generation of tissue factor-bearing MP by peripheral blood mononuclear cells, as assessed by phosphatidylserine quantification, clotting tests and flow-cytometry. U73122, PD98059 (an extracellular signal-regulated kinase1/2 inhibitor), and W-7, significantly inhibited leptin-induced MP release. SB203580 (a p38 inhibitor), and SP600125 (a c-JunN-terminal kinase inhibitor) had no effect.

Conclusion: Leptin induced generation of procoagulant microparticles might

represent a link between obesity and atherothrombotic risk. Inhibition of leptin-induced microparticle generation might prove a viable strategy for the reduction of such risk in obese individuals.

Keywords: Leptin; Microparticles; Thrombosis; Tissue Factor; Obesity; Monocytes

List of non standard abbreviations: microparticles (MP); tissue factor (TF); peripheral blood mononuclear cells (PBMC); 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT); phosphatidylserine (PS); fluoresceinisothyocianate (FITC); phycoerythrin (PE); side scatter (SSc); forward scatter (FSc); phosphate buffered saline (PBS); mitogen activated protein kinases (MAPK); extracellular signal-regulated kinase (ERK); c-JunN-terminal kinase (JNK); phospholipase C (PLC); carboxyfluoresceinsuccinimidyl ester (CFSE).

1 Introduction

Obesity is a risk factor for vascular disorders, including hypertension, coronary artery diseases and thrombosis [1, 2]. The secretory products of adipose tissue contribute to the elevated risk of cardiovascular disease with mechanisms that are complex and partially unknown [3]. Leptin is a 16 kDa non-glycosylated polypeptide product of the *ob* gene and is mainly produced and secreted by fat cells in proportion to fat mass to signal the repletion of body energy stores to the hypothalamus [4]. Besides its role in regulating the body's energy balance, however, leptin is also involved in a variety of other biologically relevant phenomena, including the upregulation of the synthesis of interleukin-18 in monocytes [5], of Vascular Cell Adhesion Molecule-1 in mouse chondrocytes [6], of matrix metalloproteinases in cartilage [7] and of the initiator of the extrinsic pathway of blood coagulation, tissue factor (TF), in monocytes [8, 9] and neutrophils [10].

Microparticles (MP) are small membrane vesicles (0.05 - 1 μm) shed from cells following activation or during injury and apoptosis [11]. Long considered inert debris, MP are in fact involved in a number of physiologically relevant responses, including cell-cell communication [12], inflammation [13] and blood coagulation [14, 15]. The mechanisms leading to the generation of MP are only partially known; however, two distinct cellular processes have been characterized: chemical and physical cell activation, induced through an increase in intracellular calcium concentration by agonists or shear stress, respectively, and apoptosis, induced both by deprivation of growth factors or by apoptotic inducers [11, 16, 17]. MP express on their outer leaflet the negatively charged phospholipid, phosphatidylserine (PS), which represents an ideal

surface for the assembly of prothrombinase and tenase, the enzymatic complexes responsible for the activation of coagulation factors II and X, respectively [18]. Furthermore, the procoagulant potential of mononuclear cell-derived MP can be increased by the expression of TF [19]. In fact, the so called blood-borne TF [20] is probably, at least in part, TF bound to MP [14]. Several studies have shown that circulating MP are increased in most cardiovascular diseases and in patients with cardiovascular risk factors [21-23]. In obese and overweight subjects, an increased number of circulating MP has also been reported [24-26].

We investigated whether leptin induces the shedding of procoagulant, TF-bearing MP by human peripheral blood mononuclear cells (PBMC), thus lending further support to the hypothesis that these structures are, at least in part, responsible for the increased thrombotic risk in obese patients.

2 Materials and methods

2.1 Reagents and kits

Leptin, U73122, SP600125, SB203580, W-7, RPMI 1640 medium, penicillin, streptomycin, L-glutamine, fetal bovine serum, trypan blue, phosphate buffered saline (PBS), Ficoll-Hystopaque, dextran, (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide) (MTT), were obtained from Sigma (Milan, Italy). The absence of lypopolysaccharide in leptin powder was attested by Sigma (Milan, Italy). Thromboplastin standard was obtained from Beckman Coulter (Milano, Italy). Human anti-TF antibody was obtained from America Diagnostica (Instrumentation Laboratory, Milano, Italy). PD98059 was obtained from Cayman Chemical (Ann Arbor, MI, USA). The Zymuphen MP-Activity kit was

obtained from Hyphen BioMed (Neuville-sur-Oise, France). The Fluo-4 NW Calcium Assay kit was obtained from Molecular Probes (Invitrogen, Milan, Italy). Annexin V-fluoresceinisothyocianate (FITC), Annexin V-allophycocyanin (APC) and PE-labeled mouse anti human CD14 antibody was purchased from BD Bioscience (San Jose, CA, USA). Phycoerythrin (PE)-labeled propidium iodide was purchased from Immunological Science (Rome, Italy). Carboxyfluorescein diacetate succinimidyl ester (CFSE)-fluoresceinisothyocianate (FITC) was purchased from Miltenyi Biotec (Calderara di Reno, Italy). Megamix™, a blend of monodisperse fluorescent beads of three diameters (.5, .9 and 3.0 μm), was purchased from BioCytex (Marseille, France).

2.2 PBMC isolation and culture

PBMC were isolated either from fresh buffy coats obtained from the local blood bank or from the peripheral blood of normal volunteers exactly as described [27]. The procedure was approved by the local ethics committee in accordance with the Declaration of Helsinki. Due to the strictly blind nature of the procedure, we were not informed whether some of the blood bank donors were taking medications or had some abnormal laboratory data; in contrast, the normal volunteers were recruited among the laboratory personnel and they had no relevant abnormalities that we are aware of. Briefly, a fresh buffy coat was mixed gently with an equal volume of 2,5% Dextran T500, and left for 40 minutes for erythrocyte sedimentation. Ten mL of leukocyte-rich supernatant was layered over 5 mL of Ficoll-Hystopaque and centrifuged for 30 minutes at 350 x *g* at 4°C. The PBMC-rich ring was washed twice in PBS. PBMC were then resuspended in RPMI/1% penicillin and streptomycin/1% L-glutamine and allowed to adhere for 30 minutes at 37°C on 96-well plates (.33x10^6 cells/well).

The cells were then washed two times with pre-warmed PBS, resuspended in RPMI/1% penicillin and streptomycin/1% L-glutamine/5% fetal bovine serum and allowed overnight at 37°C.

2.3 MP generation and purification

PBMC were washed twice with PBS at 37°C. Upon addition of leptin for the indicated times, the supernatants were recovered, centrifuged at 14,000 *x g* for 5 min at room temperature to remove dead cells and big cell fragments, and immediately used for further experiments. In some experiments, 12 mL of MP preparation were further purified by ultracentrifugation (100,000 x g for 2 hours, 4°C); the resulting pellet was resuspended in normal saline (250 µL) and used for the detection of TF activity (see below).

2.4 Measurement of MP

PS-positive MP in each sample were detected using the Zymuphen MP-activity kit according to the manufacturer's instructions and expressed as PS equivalents (nM PS).

2.5 Flow cytometry detection of MP

MP generated as described were analyze by flow cytometry. A mixture of 30 µL of supernatant, 3 µL of PE conjugated anti CD14 antibody, 3 µL of FITC labeled annexin V and 30 µL of annexin V 2x binding buffer was incubated for 15 minutes at room temperature in the dark. Four-hundred µL of PBS were added to each mixture immediately before analysis with a FACScanto^{tm}II flow cytometer (BD Biosciences, San Jose, CA, USA). Events acquisition was obtained at high flow rate and stopped after 210 seconds. The side scatter channel (SSc) and forward scatter channel (FSc) parameters were set at log scale. A PE conjugated mouse immunoglobulin G was used to confirm specificity of CD14 labeling. Monocyte-derived MP were discriminated first by size, as events conforming to a light scatter distribution within the .5-.9 µm bead range in a SSc vs. FSc window, as previously described [28], and further

identified as $CD14^+$ and annexin V^+ events in a PE vs. FITC window. In selected experiments, in addition to CD14-PE and annexin V-APC, CFSE was used to identify closed vesicles as $CD14^+$ and $CFSE^+$ events in PE vs. FITC windows.

2.6 Measurement of intracellular calcium concentration

The Molecular Probes Fluo-4 NW Calcium Assay kit was used to measure the changes in intracellular calcium concentration ($[Ca^{2+}]_i$) of PBMC. Pre-washed PBMC on 96-multiwell plate (.33 x 10^6 cells/well) were loaded with 100 µL of the dye loading solution containing Fluo-4 NW dye and probenecid, according to the manufacturer's instructions. The 96-well plate was incubated at 37 °C for 45-60 min in the dark and leptin (10 µg/mL) was added to the cells. The changes in Fluo-4 NW fluorescence were measured by the Wallac 1420 Victor 2 (PerkinElmer, Milan, Italy) at λ_{ex} 494 nm and λ_{em} 516 nm. Calcium mobilization was observed over time (up to 120 sec) and analyzed by the Wallac 1420 Software version 3 (PerkinElmer Life and Analytical Sciences, Wallac, Milan, Italy). The increase in $[Ca^{2+}]_i$ fluorescence was expressed as relative fluorescence units (RFU).

2.7 Assessment of MP-bound TF activity

TF activity was measured in MP generated in vitro from PBMC by a one-stage clotting time assay exactly as described [29]. Briefly, MP (100 µl) disrupted by 3 freeze-thaw cycles were mixed with an equal volume of MP-poor normal human plasma at 37°C; 25 mM $CaCl_2$ (100 mL) was added to the mixture and the time to clot formation in a 37°C water bath was recorded. The results were expressed in arbitrary units (AU) of procoagulant activity by comparison with a standard curve obtained using a human brain thromboplastin standard. This preparation was assigned a value of 1,000 AU for a clotting time of 30 s. An

anti-human TF antibody (30 μg/mL) was used to confirm identity of the procoagulant activity with TF (data not shown).

2.8 Assessment of apoptosis

Apoptosis was measured using a commercially available kit as previously described [30]. Briefly, 10 x 10^6 cells/well were applied to six-well plates and treated as described above, cisplatin (30 μM) treatment was used as positive control. After drug treatment, the PBMC were resuspended in 500 μl of binding buffer containing 5 μl of annexin V-FITC and 5 μl of propidium iodide-PE, and incubated in the dark at room temperature for 15 min. Cells were then analyzed in a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) to differentiate apoptotic cells (annexin V^+ and propidium $iodide^-$, lower right quadrant) from necrotic cells (annexin V^+/propidium $iodide^+$, upper right quadrant). Ten thousand events were recorded for each treatment group.

2.9 Data presentation and statistical analysis

Unless otherwise indicated, data are shown as mean ± SEM from n (as stated in the relevant figure legend) independent consecutive experiments; comparisons among groups were made by either ANOVA for repeated measures followed by Bonferroni's analysis or Student's paired two-tailed t-test, as appropriate, using Prism Software (GraphPad, San Diego, CA, USA). Values of $p < .05$ were considered statistically significant.

3 Results

3.1 Leptin induces MP generation by PBMC

To investigate whether leptin induces the release of MP, PBMC were stimulated with 3 different concentrations of leptin at different time points; the supernatants were collected, and MP were quantified as PS concentration. The initial concentrations of leptin were selected based on previously published work by different groups [7, 8, 31]. As shown in figure 1, leptin stimulates the generation of MP by PBMC in a dose dependent fashion. The effect was maximum at 4 hours and leveled off at later time points.

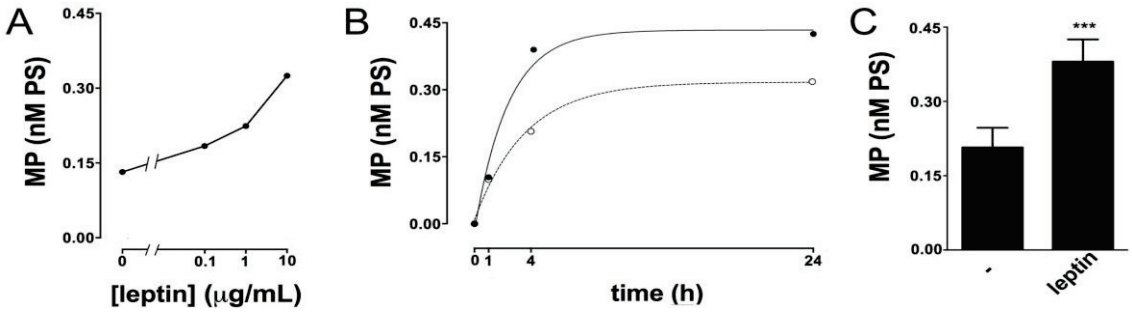

figure 1. MP generation, expressed as PS concentration, by PBMC incubated with leptin. (A) Dose-response curve for leptin (4 h). (B) time-response curve for leptin (10 μg/mL). Data are from one experiment representative of 2. Open circles, baseline; closed cicles: leptin-stimulated cells. (C) MP generation by PBMC treated with leptin (4 h; 10 μg/mL). ***p<.001 for leptin treated cells compared with baseline (Student's paired t-test); n=8

To confirm the results and to investigate the cellular origin of these MP, flow cytometry was used. MP generation was expressed as events conforming to light scatter distribution within the .5-.9 μm bead range in a SSc vs FSc window and further identified as annexin V positive events and CD14 positive events. Figure 2 shows that leptin increases the number of annexin V^+ MP (total MP) and of annexin $V^+/CD14^+$ MP (MP-derived MP). In selected experiments, we

also used CFSE to label MP in order to confirm that the events detected are closed vesicles with an intact membrane rather than cell debris [32]. $CFSE^+$, i.e. intact, closed MP containing cytoplasm, represented the majority of events detected by flow cytometry (87.5% and 91.6% of total $annexinV^+/CD14^+$ events recorded within the appropriate physical gate in the experiments depicted in panels E and F, respectively).

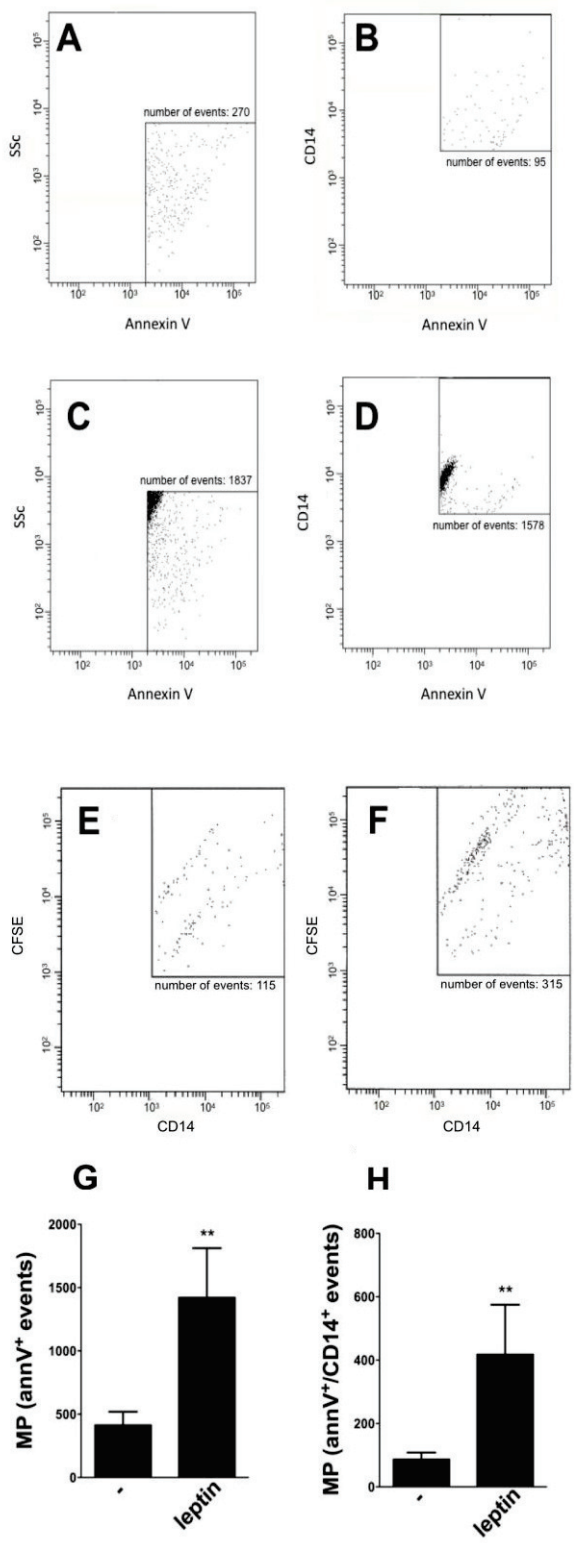

Figure 2. Flow cytometric analysis of leptin-induced MP generation by PBMC. Cells were incubated in the absence (A, B and E) or in the presence (C, D and F) of leptin (10 µg/mL; 4 h). MP were analyzed by flow cytometry as described to identify all SSc^+/annexin V^+ events (A and

C), $SSc^+/CD14^+$ events (B and D), and $CD14^+/CFSE^+$ events (E and F). Representative data from one experiment. (G) Statistical analysis of leptin-induced (10 µg/mL; 4 h) SSc^+/annexin V^+ MP generation. (H) Statistical analysis of leptin-induced (10 µg/mL; 4 h) $SSc^+/CD14^+$ MP generation. **p<.01 for leptin treated cells compared with baseline (Student's paired t-test); n=11.

3.2 Leptin induces the expression of MP-bound TF by PBMC

To evaluate whether leptin-induced MP express TF activity, a one-stage clotting test was used. As shown in figure 3, leptin induces an increase in MP-associated procoagulant activity. A monoclonal antibody to TF (30 µg/mL) inhibited most of the procoagulant activity (not shown), confirming the identity of this activity with TF.

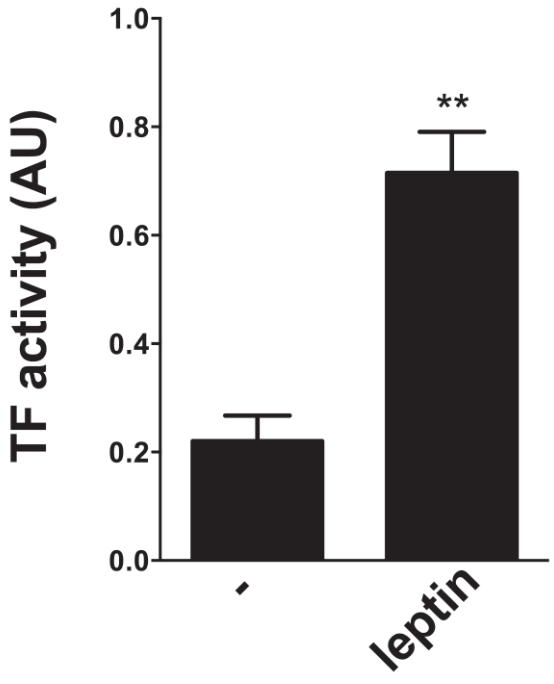

Figure 3. Leptin induces the generation of TF bearing MP by PBMC. Cells were incubated with leptin (10µg/mL; 4 h).The supernatants were then tested for TF activity with a one-stage clotting assay. **p<.001 for leptin treated cells compared with baseline (Student's paired t-test); n=3.

3.3 Leptin-induced MP generation is mediated by activation of extracellular signal regulated kinase (ERK) 1/2 but not by activation of c-Jun N-terminal kinase (JNK) and p38

To investigate the implication of various proteins that compose the leptin intracellular pathway, we focused on mitogen activated protein kinases (MAPK). To this end, we used 3 different inhibitors of MAPK, an extracellular signal-regulated kinase (ERK)1/2 inhibitor (PD98059), a c-JunN-terminal kinase (JNK) inhibitor (SP600125), and a p38 inhibitor (SB203580). Cells were pre-treated with PD98159 (1 μM), SP600125 (.65 μM) and SB203580 (1μM) for 30 minutes. All inhibitors were found non toxic by MTT assay [30]; furthermore, none caused MP release (data not shown).

As shown in figure 4, only PD98159 inhibited MP generation by PBMC.

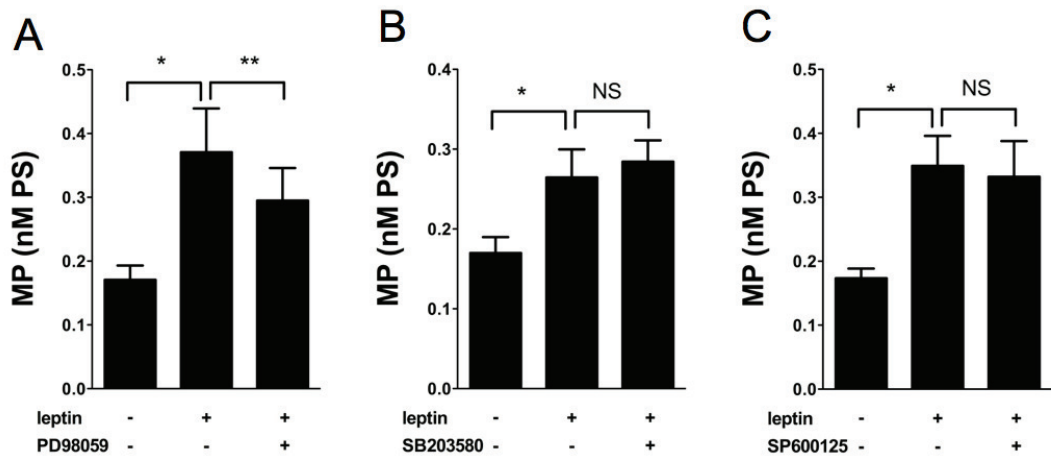

Figure 4. MP generation, expressed as PS concentration, by PBMC incubated in the absence and in the presence of leptin (4 h; 10 μg/mL) and (A) PD98059 (1 μM; 30 minutes preincubation); (B) SB203580 (1 μM 30 minutes preincubation); (C) SP600125 (.65 μM; 30 minutes preincubation). *p<.05 for leptin treated cells compared with baseline; ** p<.05 for leptin+PD98059 treated cells compared with leptin treated cells; NS: not significant (ANOVA analysis with Bonferroni post test); n=7.

3.4 Leptin induces the mobilization of intracellular calcium in PBMC

Leptin has been shown to induce an increase in intracellular calcium concentration [33, 34]. Because calcium mobilization is involved in MP generation [11, 27, 28, 35], we investigated whether leptin increases $[Ca^{2+}]_i$ in our experimental conditions. Figure 5 shows that leptin (10 µg/mL) induces a sustained increase in $[Ca^{2+}]_i$.

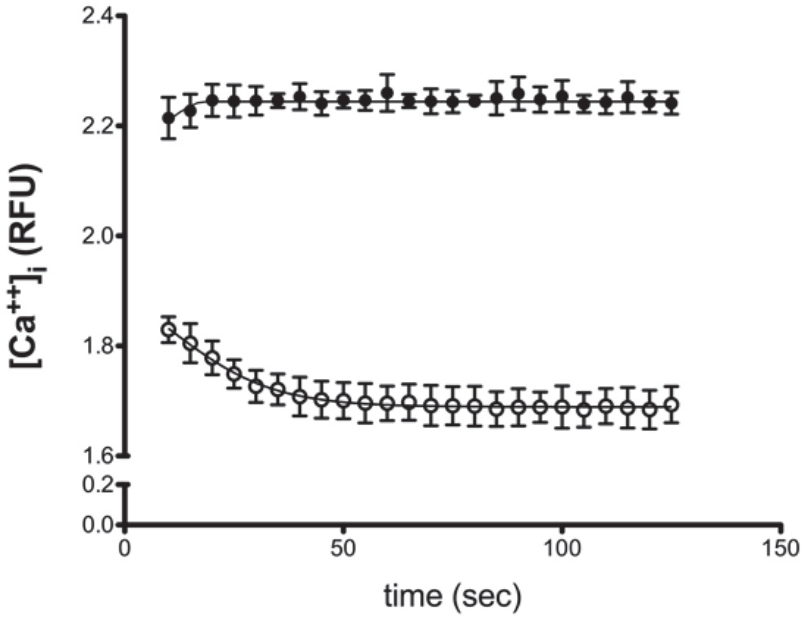

Figure 5. Comparison of intracellular calcium concentration in PBMC in baseline conditions (open circles) and after leptin (10 µg/mL) treatment (solid circles), as assessed by Fluo4-NW incorporation (RFU: relative fluorescence units); n= 3.

3.5 Leptin does not induce apoptosis is PBMC

Besides calcium mediated cell activation, apoptosis is a well known mechanism of MP generation [11]. We investigated if in our experimental condition leptin induced MP generation through this mechanism. Figure 6 shows that leptin did not induce apoptosis.

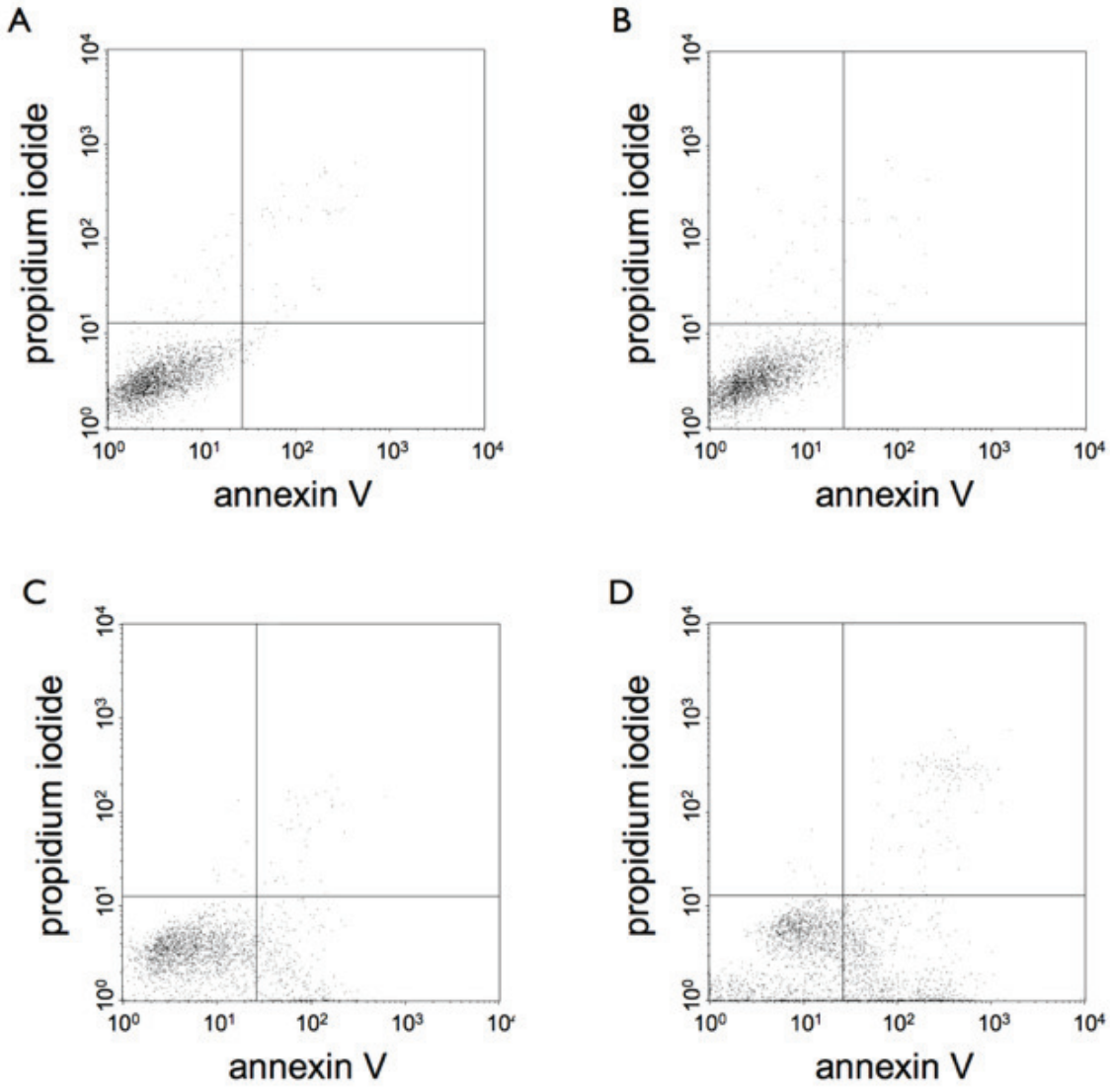

Figure 6. Flow cytometry analysis of apoptosis of mononuclear cells. (A) PBMC cultured for 4 hours in the absence of stimuli. (B) PBMC cultured for 4 hours in the presence of leptin (10 μg/mL); (C) PBMC cultured for 20 hours in the absence of stimuli. (D) PBMC cultured for 20 hours in the presence of cisplatin (12 μM) (positive control). Data from one experiment representative of two.

3.6 Leptin-induced MP generation is mediated through phospholipase C (PLC) and calmodulin

Numerous biological actions of leptin are mediated through calcium mobilization [33, 34]**;** furthermore, our data confirm that leptin causes an increase in $[Ca^{2+}]_i$

in PBMC in our experimental conditions. We therefore set up to investigate the role of some of the molecular pathways involved in calcium signaling in MP generation. We used a PLC inhibitor, U73122, to investigate the role of calcium ions stored in the endoplasmic reticulum. Cells were pre-treated with U73122 (1 μM) for 30 minutes. To look into how calcium ions may act after their release we used a calmodulin inhibitor, W-7 (.650 μM for 30 minutes). Both inhibitors were found non toxic by MTT assay [30]; furthermore, neither caused MP release (data not shown).

Cell treatment with both U73122 (fig. 7) and W-7 (fig. 8) inhibited MP production induced by leptin

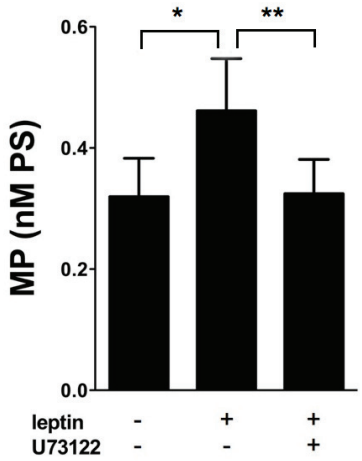

Figure 7. MP generation, expressed as PS concentration, by PBMC incubated in the absence and in the presence of leptin (4 h; 10 μg/mL) and U73122 (1 μM; 30 minute pretreatment). *p<.05 for leptin treated cells compared with baseline; ** p<.05 for leptin+U73122 treated cells compared with leptin treated cells (ANOVA analysis with Bonferroni post test); n=7.

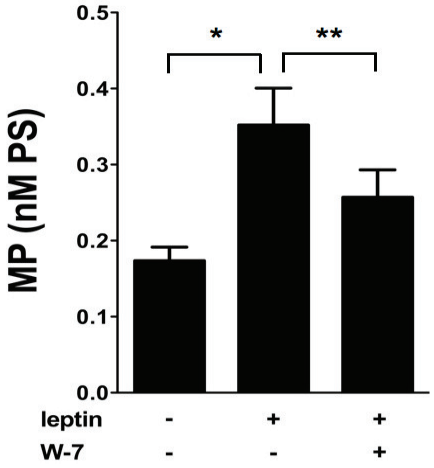

Figure 8. MP generation, expressed as PS concentration, by PBMC incubated in the absence and in the presence of leptin (4 h; 10 µg/mL) and W-7 (.65 µM; 30 min pretreatment). *p<.01 for leptin treated cells compared with baseline; ** p<.05 for leptin+W-7 treated cells compared with leptin treated cells (ANOVA analysis with Bonferroni post test); n=8.

Inhibition of leptin-induced MP generation by PD98059, U73122 and W-7 was confirmed by flow cytometry (fig. 9).

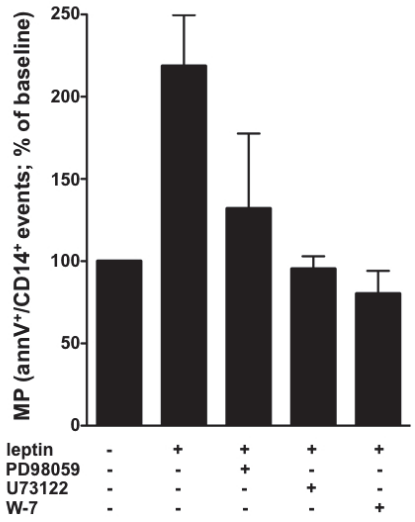

Figure 9. Flow cytometry analysis of the inhibitory effects of PD98059, U73122, and W-7 in leptin-induced MP generation by human mononuclear cells; n=3. MP were analyzed as described in figure 2. Experimental conditions were identical to those described in figure 4, 7 and 8.

4 Discussion

The aim of this work was to test the hypothesis that leptin induces the generation of procoagulant MP. Indeed, we show that leptin causes the release of procoagulant MP by PBMC in a dose- and time-dependent fashion; flow cytometric analysis identified a portion of these MP as of monocytic origin. Although linear membrane fragments can be generated upon cell manipulation and stimulation, we confirmed that the vast majority of PS generated in our experimental conditions was associated to microparticles. Because MP of monocytic origin may express TF [19], which adds to their procoagulant potential, we also investigated the presence of TF activity on leptin-stimulated PBMC-derived MP, and confirmed the presence of functionally active TF on the surface of leptin-induced MP. Freeze-thaw cycles have been proposed as a suitable way to externalize PS and decrypt TF on intact cells prior to functional analysis with clotting assays [36]. This step is probably irrelevant with MP and in fact Lee and Mackman have shown that, contrary to what anticipated, freeze/thaw has no effect on MP-bound TF [37]. However, we have chosen to perform the standard freeze/thaw cycles for consistency with our previous work [27-29, 35, 38]. PBMC, like other cell types, generate MP upon stimulation with various stimuli, including bacterial lipopolysaccharide [39], the calcium ionophore A23187 [40], angiotensin II [35], peroxisome proliferator activated receptor-γ agonists [29], cigarette smoke extract [28, 41]. The role of leptin in TF regulation has been previously extensively analyzed [8, 10]. Therefore, our experimental design was not devised to investigate the mechanisms of leptin-induced TF synthesis. In contrast, because different stimuli induce generation of TF-bearing MP with different mechanisms, we investigated the specific

mechanism(s) whereby leptin induces MP generation in our experimental conditions. Cell activation and apoptosis can cause MP generation [11]. Proteins of the MAPK family are involved in both cell activation and apoptosis [42] and act in the intracellular leptin pathway [43]. We therefore investigated the role of the members of the MAPK family, ERK1/2, JNK and p38, on leptin-induced MP release by PBMC. We found that only an ERK1/2 inhibitor, PD98059, decreases MP generation. This observation led us to conclude that leptin-induced MP generation is mediated by the activation of ERK1/2. This conclusion is consistent with the previous observation that ERK1/2 is phosphorylated by leptin stimulation in human PBMC [44].

The relatively short time-frame of MP generation upon stimulation with leptin, as well as the observation that this phenomenon is not affected by inhibition of JNK and p38, members of the MAPK family mostly involved in apoptotic phenomena [45], suggests cell activation, rather than apoptosis, as the relevant mechanism. A critical step in the process that leads to cell activation, and eventually to MP release, is an increase in $[Ca^{++}]_i$ [11, 29]. The observation that leptin induces calcium mobilization led us to conclude that the release of MP by PBMC takes place through cell activation. The observation that the number of apoptotic cells, as assessed by annexin-V/propidium iodide binding, is not affected by leptin treatment in our experimental conditions is in agreement with this conclusion. Based on the known role of PLC in the intracellular signaling pathways activated by leptin [43, 46], we postulated a role for this protein on leptin-induced MP generation. The observation that pretreatment with the PLC inhibitor, U73122, significantly downregulates MP generation is consistent with the hypothesis that MP shedding requires calcium mobilization from intracellular

storage pools that takes place via phosphatidylinositol (3,4,5) trisphosphate [43].

Calmodulin, a small protein of 148 aminoacids, functions as a sensor that couples Ca^{2+} signals to biochemical and cellular changes and represents the most abundant Ca^{2+} sensor in non muscle cells. Calmodulin participates in the regulation of the Ras signaling pathways, leading to MAPK activation [47]. Our observation that calmodulin inhibition with W-7 reduces MP generation confirms a role for this protein as a Ca^{2+} sensor in leptin-induced MP generation by PBMC.

In conclusion, our data demonstrate that leptin induces the generation of procoagulant, TF-bearing MP by PBMC through a mechanism involving calcium mobilization by PLC and the calmodulin-mediated activation of the MAPK-ERK1/2 pathway.

Procoagulant MP likely contribute to the increased atherothrombotic risk in obese patients. Pharmacological modulation of MP generation, for example through the inhibition of ERK1/2 and/or of calcium mobilization, or calmodulin mediated signaling, might prove helpful in preventing such events. Although great care must be taken when inferring in vivo relevance from data generated in vitro, the recent observation that during weight loss leptin plasma levels decrease in parallel with procoagulant platelet- , leukocyte-, and endothelial cell-derived MP [48, 49] lends support to the physiologic relevance of our data.

5 References

[1] W.G. Haynes, D.A. Morgan, S.A. Walsh, W.I. Sivitz, A.L. Mark, Cardiovascular consequences of obesity: role of leptin, Clin Exp Pharmacol Physiol. 25 (1998) 65-69.

[2] F. Samad, W. Ruf, Inflammation, obesity, and thrombosis, Blood. 122 (2013) 3415-3422.

[3] D.C. Lau, B. Dhillon, H. Yan, P.E. Szmitko, S. Verma, Adipokines: molecular links between obesity and atheroslcerosis, Am J Physiol Heart Circ Physiol. 288 (2005) H2031-41.

[4] H. Munzberg, C.D. Morrison, Structure, production and signaling of leptin, Metabolism. 64(1) (2015) 13-23.

[5] P. Jitprasertwong, K.M. Jaedicke, C.J. Nile, P.M. Preshaw, J.J. Taylor, Leptin enhances the secretion of interleukin (IL)-18, but not IL-1beta, from human monocytes via activation of caspase-1, Cytokine. 65 (2014) 222-230.

[6] X. Kang, Q.Y. Xie, J.S. Zhou, B. Zhang, D.F. Liao, H.H. Wu, W. Zheng, X.M. Pan, Y. Quan, C/EBP-alpha, involvement of a novel transcription factor in leptin-induced VCAM-1 production in mouse chondrocytes, FEBS Lett. 588 (2014) 1122-1127.

[7] W. Hui, G.J. Litherland, M.S. Elias, G.I. Kitson, T.E. Cawston, A.D. Rowan, D.A. Young, Leptin produced by joint white adipose tissue induces

cartilage degradation via upregulation and activation of matrix metalloproteinases, Ann Rheum Dis. 71 (2012) 455-462.

[8] E. Napoleone, A. Dl Santo, C. Amore, G. Baccante, C. di Febbo, E. Porreca, G. de Gaetano, M.B. Donati, R. Lorenzet, Leptin induces tissue factor expression in human peripheral blood mononuclear cells: a possible link between obesity and cardiovascular risk?, J Thromb Haemost. 5 (2007) 1462-1468.

[9] R. Lorenzet, E. Napoleone, A. Cutrone, M.B. Donati, Thrombosis and obesity: cellular bases, Thromb Res. 129 (2012) 285-289.

[10] S. Rafail, K. Ritis, K. Schaefer, I. Kourtzelis, M. Speletas, M. Doumas, S. Giaglis, K. Kambas, S. Konstantinides, G. Kartalis, Leptin induces the expression of functional tissue factor in human neutrophils and peripheral blood mononuclear cells through JAK2-dependent mechanisms and TNFalpha involvement, Thromb Res. 122 (2008) 366-375.

[11] M.J. VanWijk, E. VanBavel, A. Sturk, R. Nieuwland, Microparticles in cardiovascular diseases, Cardiovasc Res. 59 (2003) 277-287.

[12] J.H. Distler, L.C. Huber, S. Gay, O. Distler, D.S. Pisetsky, Microparticles as mediators of cellular cross-talk in inflammatory disease, Autoimmunity. 39 (2006) 683-690.

[13] J.H.W. Distler, O. Distler, Inflammation: Microparticles and their roles in inflammatory arthritides, Nature Reviews Rheumatology. 6 (2010) 385-386.

[14] A. Celi, R. Lorenzet, B.C. Furie, B. Furie, Microparticles and a P-selectin-mediated pathway of blood coagulation, Dis Markers. 20 (2004) 347-352.

[15] A.S. Leroyer, F. Anfosso, R. Lacroix, F. Sabatier, S. Simoncini, S.M. Njock, N. Jourde, P. Brunet, L. Camoin-Jau, J. Sampol, F. Dignat-George, Endothelial-derived microparticles: biological conveyors at the crossroad of inflammation, thrombosis and angiogenesis, Thromb Haemost. 104 (2010) 456-463.

[16] T. Benameur, R. Andriantsitohaina, M.C. Martinez, Therapeutic potential of plasma membrane-derived microparticles, Pharmacol Rep. 61 (2009) 49-57.

[17] O. Morel, L. Jesel, J.M. Freyssinet, F. Toti, Cellular mechanisms underlying the formation of circulating microparticles, Arterioscler Thromb Vasc Biol. 31 (2011) 15-26.

[18] O. Morel, F. Toti, B. Hugel, B. Bakouboula, L. Camoin-Jau, F. Dignat-George, J.M. Freyssinet, Procoagulant microparticles: disrupting the vascular homeostasis equation?, Arterioscler Thromb Vasc Biol. 26 (2006) 2594-2604.

[19] S. Falati, Q. Liu, P. Gross, G. Merrill-Skoloff, J. Chou, E. Vandendries, A. Celi, K. Croce, B.C. Furie, B. Furie, Accumulation of Tissue Factor into Developing Thrombi In Vivo Is Dependent upon Microparticle P-Selectin Glycoprotein Ligand 1 and Platelet P-Selectin, J. Exp. Med. 197 (2003) 1585-1598.

[20] P.L.A. Giesen, U. Rauch, B. Bohrmann, D. Kling, M. Roque, J.T. Fallon, J.J. Badimon, J. Himber, M.A. Riederer, Y. Nemerson, Blood-borne tissue factor: Another view of thrombosis, Proc Natl Acad Sci. 96 (1999) 2311-2315.

[21] C.M. Boulanger, N. Amabile, A. Tedgui, Circulating microparticles: a potential prognostic marker for atherosclerotic vascular disease, Hypertension. 48 (2006) 180-186.

[22] A.P.r. Owens, N. Mackman, Role of tissue factor in atherothrombosis, Curr Atheroscler Rep. 14 (2012) 394-401.

[23] N. Amabile, S. Cheng, J.M. Renard, M.G. Larson, A. Ghorbani, E. McCabe, G. Griffin, C. Guerin, J.E. Ho, S.Y. Shaw, K.S. Cohen, R.S. Vasan, A. Tedgui, C.M. Boulanger, T.J. Wang, Association of circulating endothelial microparticles with cardiometabolic risk factors in the Framingham Heart Study, Eur Heart J. (2014)

[24] T. Murakami, H. Horigome, K. Tanaka, Y. Nakata, K. Ohkawara, Y. Katayama, A. Matsui, Impact of weight reduction on production of platelet-derived microparticles and fibrinolytic parameters in obesity, Thromb Res. 119 (2007) 45-53.

[25] A. Stepanian, L. Bourguignat, S. Hennou, M. Coupaye, D. Hajage, L. Salomon, M.C. Alessi, S. Msika, D. de Prost, Microparticle increase in severe obesity: not related to metabolic syndrome and unchanged after massive weight loss, Obesity (Silver Spring). 21 (2013) 2236-2243.

[26] E. Campello, E. Zabeo, C.M. Radu, L. Spiezia, S. Gavasso, M. Fadin, B. Woodhams, R. Vettor, P. Simioni, Hypercoagulability in overweight and obese subjects who are asymptomatic for thrombotic events, Thromb Haemost. 113 (2014)

[27] T. Neri, C. Armani, A. Pegoli, C. Cordazzo, Y. Carmazzi, S. Brunelleschi, C. Bardelli, M.C. Breschi, P. Paggiaro, A. Celi, Role of NF-kappaB and PPAR-gamma in lung inflammation induced by monocyte-derived microparticles, Eur Respir J. 37 (2011) 1494-1502.

[28] C. Cordazzo, S. Petrini, T. Neri, S. Lombardi, Y. Carmazzi, R. Pedrinelli, P. Paggiaro, A. Celi, Rapid shedding of proinflammatory microparticles by human mononuclear cells exposed to cigarette smoke is dependent on Ca mobilization, Inflamm Res. (2014)

[29] T. Neri, C. Cordazzo, Y. Carmazzi, S. Petrini, C. Balia, F. Stefanelli, A. Amoruso, S. Brunelleschi, M.C. Breschi, R. Pedrinelli, P. Paggiaro, A. Celi, Effects of peroxisome proliferator activated receptors-gamma agonists on the generation of microparticles by monocytes/macrophages, Cardiovasc Res. 11 (2012) 243-247.

[30] Y. Carmazzi, M. Iorio, C. Armani, S. Cianchetti, F. Raggi, T. Neri, C. Cordazzo, S. Petrini, R. Vanacore, F. Bogazzi, P. Paggiaro, A. Celi, The mechanisms of nadroparin-mediated inhibition of proliferation of two human lung cancer cell lines, Cell Prolif. 45 (2012) 545-556.

[31] M.S. Mutabaruka, M. Aoulad Aissa, A. Delalandre, M. Lavigne, D. Lajeunesse, Local leptin production in osteoarthritis subchondral

osteoblasts may be responsible for their abnormal phenotypic expression, Arthritis Res Ther. 12 (2010) R20.

[32] G. Grisendi, E. Finetti, D. Manganaro, N. Cordova, G. Montagnani, C. Spano, M. Prapa, V. Guarneri, S. Otsuru, E.M. Horwitz, G. Mari, M. Dominici, Detection of microparticles from human red blood cells by multiparametric flow cytometry, Blood Transfus. 13 (2015) 274-280.

[33] J.T. Padra, I. Seres, G. Foris, G.J. Paragh, G. Konya, G. Paragh, Leptin triggers Ca(2+) imbalance in monocytes of overweight subjects, Neuropeptides. 46 (2012) 203-209.

[34] V. Rajapurohitam, F. Izaddoustdar, E. Martinez-Abundis, M. Karmazyn, Leptin-induced cardiomyocyte hypertrophy reveals both calcium-dependent and calcium-independent/RhoA-dependent calcineurin activation and NFAT nuclear translocation, Cell Signal. 24 (2012) 2283-2290.

[35] C. Cordazzo, T. Neri, S. Petrini, S. Lombardi, C. Balia, S. Cianchetti, Y. Carmazzi, P. Paggiaro, R. Pedrinelli, A. Celi, Angiotensin II induces the generation of procoagulant microparticles by human mononuclear cells via an angiotensin type 2 receptor-mediated pathway, Thromb Res. 131 (2013) e168-74.

[36] A. Celi, G. Pellegrini, R. Lorenzet, A.D. Blasi, N. Ready, B.C. Furie, B. Furie, P-Selectin induces the expression of tissue factor on monocytes, Proc Natl Acad Sci. 91 (1994) 8767-8771.

[37] R.D. Lee, D.A. Barcel, J.C. Williams, J.G. Wang, J.C. Boles, D.A. Manly, N.S. Key, N. Mackman, Pre-analytical and analytical variables affecting the measurement of plasma-derived microparticle tissue factor activity, Thromb Res. 129 (2012) 80-85.

[38] F. Novelli, T. Neri, L. Tavanti, C. Armani, C. Noce, F. Falaschi, M.L. Bartoli, F. Martino, A. Palla, A. Celi, P. Paggiaro, Procoagulant, tissue factor-bearing microparticles in bronchoalveolar lavage of interstitial lung disease patients: an observational study, PLoS One. 9 (2014) e95013.

[39] N. Satta, F. Toti, O. Feugeas, A. Bohbot, J. Dachary-Prigent, V. Eschwege, H. Hedman, J.M. Freyssinet, Monocyte vesiculation is a possible mechanism for dissemination of membrane-associated procoagulant activities and adhesion molecules after stimulation by lipopolysaccharide, J Immunol. 153 (1994) 3245-3255.

[40] C. Cerri, D. Chimenti, I. Conti, T. Neri, P. Paggiaro, A. Celi, Monocyte/macrophage-derived microparticles up-regulate inflammatory mediator synthesis by human airway epithelial cells, J Immunol. 177 (2006) 1975-1980.

[41] M. Li, D. Yu, K.J. Williams, M.L. Liu, Tobacco smoke induces the generation of procoagulant microvesicles from human monocytes/macrophages, Arterioscler Thromb Vasc Biol. 30 (2010) 1818-1824.

[42] R. Seger, E.G. Krebs, The MAPK signaling cascade, FASEB J. 9 (1995) 726-735.

[43] G. Fruhbeck, Intracellular signalling pathways activated by leptin, Biochem J. 393 (2006) 7-20.

[44] C. Martin-Romero, V. Sanchez-Margalet, Human leptin activates PI3K and MAPK pathways in human peripheral blood mononuclear cells: possible role of Sam68, Cell Immunol. 212 (2001) 83-91.

[45] S.H. Yang, A.D. Sharrocks, A.J. Whitmarsh, MAP kinase signalling cascades and transcriptional regulation, Gene. 513 (2013) 1-13.

[46] C. Dellas, K. Schafer, I.K. Rohm, M. Lankeit, M. Leifheit, D.J. Loskutoff, G. Hasenfuss, S.V. Konstantinides, Leptin signalling and leptin-mediated activation of human platelets: importance of JAK2 and the phospholipases Cgamma2 and A2, Thromb Haemost. 98 (2007) 1063-1071.

[47] N. Agell, O. Bachs, N. Rocamora, P. Villalonga, Modulation of the Ras/Raf/MEK/ERK pathway by Ca(2+), and calmodulin, Cell Signal. 14 (2002) 649-654.

[48] O. Morel, F. Luca, L. Grunebaum, L. Jesel, N. Meyer, D. Desprez, S. Robert, F. Dignat-George, F. Toti, C. Simon, B. Goichot, Short-term very low-calorie diet in obese females improves the haemostatic balance through the reduction of leptin levels, PAI-1 concentrations and a diminished release of platelet and leukocyte-derived microparticles, Int J Obes (Lond). 35 (2011) 1479-1486.

[49] E. Campello, E. Zabeo, C.M. Radu, L. Spiezia, M. Foletto, L. Prevedello, S. Gavasso, C. Bulato, R. Vettor, P. Simioni, Dynamics of circulating microparticles in obesity after weight loss, Intern Emerg Med. (2016)

Legend to the figures

Figure 1. MP generation, expressed as PS concentration, by PBMC incubated with leptin. (A) Dose-response curve for leptin (4 h). (B) time-response curve for leptin (10 µg/mL). Data are from one experiment representative of 2. (C) MP generation by PBMC treated with leptin (4 h; 10 µg/mL). ***p<0.001 for leptin treated cells compared with baseline (Student's paired t-test); n=8

Figure 2. Flow cytometric analysis of leptin-induced MP generation by PBMC**.** Cells were incubated in the absence (A, B and E) or in the presence (C, D and F) of leptin (10 µg/mL; 4 h). MP were analyzed by flow cytometry as described to identify all SSc^+/annexin V^+ events (A and C), SSc^+/$CD14^+$ events (B and D), and $CD14^+$/$CFSE^+$ events (E and F). Representative data from one experiment. (G) Statistical analysis of leptin-induced (10 µg/mL; 4 h) SSc^+/annexin V^+ MP generation. (H) Statistical analysis of leptin-induced (10 µg/mL; 4 h) SSc^+/$CD14^+$ MP generation. **p<.01 for leptin treated cells compared with baseline (Student's paired t-test); n=11.

Figure 3. Leptin induces the generation of TF bearing MP by PBMC. Cells were incubated with leptin (10µg/mL; 4 h).The supernatants were then tested for TF activity with a one-stage clotting assay. ***p<.001 for leptin treated cells treated cells compared with baseline (Student's paired t-test); n=3.

Figure 4. MP generation, expressed as PS concentration, by PBMC incubated in the absence and in the presence of leptin (4 h; 10 µg/mL) and (A) PD98059

(1 μM; 30 minutes preincubation); (B) SB203580 (1 μM 30 minutes preincubation); (C) SP600125 (.65 μM; 30 minutes preincubation). *p<.05 for leptin treated cells compared with baseline; ** p<.05 for leptin+PD98059 treated cells compared with leptin treated cells; NS: not significant (ANOVA analysis with Bonferroni post test); n=7.

Figure 5. Comparison of intracellular calcium concentration in PBMC in baseline conditions (open circles) and after leptin (10 μ /mL) treatment (solid circles), as assessed by Fluo4-NW incorporation (RFU: relative fluorescence units); n= 3.

Figure 6. Flow cytometry analysis of apoptosis of mononuclear cells. (A) PBMC cultured for 4 hours in the absence of stimuli. (B) PBMC cultured for 4 hours in the presence of leptin (10 μg/mL); (C) PBMC cultured for 20 hours in the absence of stimuli. (D) PBMC cultured for 20 hours in the presence of cisplatin (12 μM) (positive control). Data from one experiment representative of two.

Figure 7. MP generation, expressed as PS concentration, by PBMC incubated in the absence and in the presence of leptin (4 h; 10 μg/mL) and U73122 (1 μM; 30 minute pretreatment). *p<.05 for leptin treated cells compared with baseline; ** p<.05 for leptin+U73122 treated cells compared with leptin treated cells (ANOVA analysis with Bonferroni post test); n=7.

Figure 8. MP generation, expressed as PS concentration, by PBMC incubated in the absence and in the presence of leptin (4 h; 10 μg/mL) and W-7 (0.65 μM; 30 min pretreatment). *p<.01 for leptin treated cells compared with baseline; ** p<.05 for leptin+W-7 treated cells compared with leptin treated cells (ANOVA

analysis with Bonferroni post test); n=8.

Figure 9. Flow cytometry analysis of the inhibitory effects of PD98059, U73122, and W-7 in leptin-induced MP generation by human mononuclear cells; n=3. MP were analyzed as described in figure 2. Experimental conditions were identical to those described in figure 4, 7 and 8.

***Conflict of Interest form**

Click here to download Conflict of Interest form: COI Disclosure Balia.pdf

***Conflict of Interest form**

Click here to download Conflict of Interest form: COI Disclosure Celi.pdf

***Conflict of Interest form**

Click here to download Conflict of Interest form: COI Disclosure Cordazzo.pdf

***Conflict of Interest form**

Click here to download Conflict of Interest form: COI Disclosure Lombardi.pdf

***Conflict of Interest form**

Click here to download Conflict of Interest form: COI Disclosure Neri.pdf

***Conflict of Interest form**

Click here to download Conflict of Interest form: COI Disclosure Paggiaro.pdf

***Conflict of Interest form**

Click here to download Conflict of Interest form: COI Disclosure Pedrinelli.pdf

***Conflict of Interest form**

Click here to download Conflict of Interest form: COI Disclosure Petrini.pdf

***Conflict of Interest form**

Click here to download Conflict of Interest form: COI Disclosure Scalise.pdf