Leptin induces the expression of functional tissue factor in human neutrophils and peripheral blood mononuclear cells through JAK2-dependent mechanisms and TNFα involvement


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Abstract

Introduction: Leptin is an adipocyte-derived cytokine primarily involved in the regulation of body weight and energy balance. In vivo studies suggest that leptin promotes platelet aggregation and thrombosis. Neutrophils are involved in the crosstalk between inflammation and thrombosis in clinical disorders. Leptin is also involved in the regulation of inflammation.

Aim: We examined the in vitro effects of leptin on the expression of tissue factor (TF), the primary initiator of coagulation, in healthy neutrophils.

Materials and methods/results: The effects on TF expression were assayed functionally using a modified prothrombin time (mPT), as well as at mRNA and protein levels. The same experiments were performed in parallel with PBMC. Leptin induced functional TF and increased TF mRNA and protein expression in both cell types, as determined by mPT, real-time RT-PCR, western blot, flow cytometry, immunocytochemistry. Inhibition studies revealed that the effect of leptin on TF expression is
Introduction

Obesity is associated with elevated cardiovascular morbidity and mortality [1] including an increased incidence of arterial and venous thrombosis [2]. It has also been shown that the adipose tissue is capable of producing and secreting various hormones, growth factors and cytokines, many of which appear to be directly involved in the cardiovascular complications associated with increased body fat mass [3].

Leptin is primarily produced in white adipose tissue, and from there it is secreted into the bloodstream [4]. Plasma leptin concentrations are strongly correlated with total body fat mass, and elevated plasma leptin levels are frequently observed in obese individuals [5]. While the interaction of leptin with its receptor (ObR) on hypothalamic neurons is involved in the regulation of food intake and energy expenditure [6], ObR is also expressed on a variety of different cell types and tissues [7,8]. Recently, it was suggested that hyperleptinemia may contribute to the atherothrombotic risk associated with obesity [9,10]. Experimental studies revealed that leptin stimulates both arterial and venous thrombosis in mouse models of vascular injury [11,12], and that it promotes ADP-induced human platelet aggregation in vitro [11,13]. However, it is uncertain that the pronounced prothrombotic effects of leptin in vivo depend entirely on its relatively weak action as a platelet agonist and it is reasonable to assume that other types of cells and procoagulant factors may also be involved in this process.

Tissue factor (TF) is the primary in vivo initiator of the extrinsic coagulation cascade, leading to fibrin formation [14]. Recently, an alternatively spliced form of human tissue factor (asHTF) has been reported which contains most of the extracellular but lacks the transmembrane domain, and circulates in the peripheral blood [15]. This isoform also exhibits procoagulant activity and has been found to be incorporated into thrombi [15]. Increased adipose tissue expression of TF has also been reported [16,17] and weight loss was shown to reduce plasma TF levels in morbidly obese persons [18].

TF is expressed, among others, by inflammatory cells (activated endothelial and monocytes) involved in the pathogenesis of atherothrombosis. TF expression can be induced by various stimuli [19]. Recently, polymorphonuclear cells (PMN) under certain stimuli were also shown to produce functional TF [20–22]. Blood leukocytes express the leptin receptor [23–25] and stimulation of these cells with leptin was shown to upregulate the expression of inflammatory mediators such as TNFα and IL-6 [24] with subsequent activation of neutrophils, suggesting thus an indirect crosstalk between PMN and leptin [26]. Although Napoleone et al. [27] showed an induction of TF by leptin in monocytes, the effect of the adipokine in PMN is still unknown.

In the present study, we examined the effect of leptin on TF expression by PMN isolated from healthy individuals in an effort to elucidate the role of PMN as a source of circulating TF in hyperleptinemic disorders. At the same time, we also confirmed previous results in peripheral monocytes. The signal transduction downstream of the leptin receptor is mediated through the Janus kinase 2 (JAK2) pathway [28], so molecules involved in the pathway were inhibited in order to establish their involvement in TF expression. Since leptin induces the expression of TNFα and IL-6 in monocytes [24,29] and these cytokines induce TF [19], we also examined whether leptin’s action on TF expression is mediated through these two proteins in both cell types.

Materials and methods

Study population

Leukocytes from seven healthy donors (BMI=25) were used in the study. The peripheral blood collection protocol was approved by the Institutional Review Board, and all other procedures were in compliance with institutional guidelines. Informed consent was obtained from all subjects.

Isolation of mononuclear and polymorphonuclear cells from peripheral blood

Blood was collected in EDTA-treated tubes (Vacuette, Grenier bio-one, Austria), and PMN and peripheral blood mononuclear cells (PBMC) were immediately separated by 1077/1119 Histopaque double-gradient density centrifugation (Histopaque; Sigma-Aldrich) and washed thoroughly in PBS. Moreover, PMN were assessed for purity (~98%) using cytopsin films, for viability

mediated, at least in part, by JAK2 and PI3K. Our findings, after neutralising TNFα in supernatants of leptin-treated cells, also suggest the involvement of TNFα in the leptin-induced TF expression in leukocytes.

Conclusions: This study indicates a novel link between inflammation, obesity and thrombosis by showing that leptin is able to trigger the extrinsic coagulation cascade. This work suggests a possible mechanism of the thrombotic effects of hyperleptinemic-associated clinical disorders.

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(97%) by trypan blue exclusion, and for platelet contamination using the May–Grünwald–Giemsa staining (−2 platelets/100 PMN cells).

Stimulation and inhibition studies

Washed cells (0.8–1 × 10^6 in 250 μL PBS) were incubated with agonists or antagonists (see below) for 120 min at 37 °C. In particular, cells were stimulated with recombinant leptin (R&D Systems), in dilutions ranging from 17.5 nM to 1800 nM. Based on the findings of preliminary studies, a leptin concentration of 300 nM was used for all subsequent experiments. Serum (50 μL in 200 μL PBS) from patients with antiphospholipid syndrome (APS) was used as positive control [20].

In order to analyse the signal transduction pathways involved in the effects of leptin on TF production, cells were pretreated with the JAK2 pathway inhibitor AG490 (100 μM), the phosphoinosytide-3 OH kinase (PI3K) inhibitor LY294002 (50 μM), or the MEK inhibitor PD98059 (25 μM), respectively (all from Calbiochem, La Jolla, CA, USA), 30 min prior to the addition of PBS, leptin or APS serum. Preliminary experiments revealed that all inhibitors worked in a dose-dependent manner (with leptin stimulation for AG490 and LY294002 and with APS stimulation for PD98059), reaching peak activity at the doses which were subsequently used throughout the present study. For TNFα blocking assays, 0.2 μg/μL recombinant anti-human antibody (HUMIRA, Abbott) was used and 0.01 μg/μL IL-6 monoclonal antibody mouse-anti-human (MAB 206, R&D) was used for IL-6 inhibition studies (the dose was assessed according to the manufacturer’s instructions). Of note, endotoxin contamination of all materials not tested by their respective manufacturers was excluded using the Limulus amebocyte assay (Sigma-Aldrich).

At the end of the incubation period, supernatants of cells were collected by centrifugation at 800 × g for 10 min, and immediately analysed using the modified prothrombin time assay (see below). The cell pellets were used for FACS analysis or were stored at −80 °C and later processed for TF protein and mRNA expression analysis.

Modified prothrombin time assay (mPT)

The coagulation properties (TF/FVIIa binding activity) of the cell supernatants were determined using an mPT assay as described previously [20]. In order to artificially increase the PT time, thromboplastin, ISI 1.9 (Instrumentation Laboratory, Milan, Italy) was diluted in PBS. In particular, 120 μL PBS and 80 μL thromboplastin were added to 100 μL PPP, thus increasing the PT to approximately 30–32 s. The coagulation properties of samples were measured by replacing PBS with equal volumes of cell supernatants.

In order to verify that the procoagulant activity was due to TF alone, supernatants were incubated with 0.01 μg/μL (1/100) of neutralising anti-TF monoclonal antibody (No. 4509, American Diagnostica, Greenwich, CT, USA) for 30 min, followed by measurement of the PT as described above. The use of anti-TF monoclonal antibody, in dilutions 1/5, 1/50, did not neutralize the exogenous thromboplastin. Non-specific controls for anti-TF, anti-TNFα and anti-IL-6 were treated with the same subclass and concentration of mouse anti-human antibodies. None of the reagents, agonists and antagonists alone showed procoagulant activity or reacted with the neutralising antibody.

RNA extraction, RT and real-time RT-PCR

RNA isolation from PMN and PBMC was performed using a TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. cDNA was synthesised from 1 μg of RNA using Superscript III reverse transcriptase (Invitrogen). The detection of the leptin receptor was performed semi-quantitatively using 2 μL of cDNA and adding primers as previously described [30] under the following conditions: 94 °C 2 min, 94 °C 1 min, 62 °C 1 min, 72 °C 1 min, for 36 cycles. In the same reaction the addition of 18s primers in ratio 1/12 (primers/competimers) as well as, the optimal number of cycles were optimised according to the manufacturer’s instructions (Quantum RNA Classic II 18S, Ambion, Austin, TX). Results were analysed with 1D Image Analysis Software (Eastman Kodak Company, V3.0).

In order to quantify the relative expression levels of TF and asHTF mRNA, real-time PCR was performed with RNA isolated from PMN and PBMC of 7 healthy individuals before and after exposure to 300 nM leptin. In each sample, specific primers and probes for the detection of full length TF, alternatively spliced TF (asHTF), or GAPDH mRNA sequences were used in the conditions as previously described [19, 20].

In order to quantify the relative target gene expression (i.e., TF, asHTF), the 2^−ΔΔCt method [31] was used. In brief, the target gene amount of stimulated cells was normalised relative to the respective amount in unstimulated cells (2^−ΔΔCt).

Immunocytochemistry

Immunocytochemical staining for TF was performed in cytospin films using the APAAP (Alkaline Phosphatase–Anti-Alkaline Phosphatase) method (K0670, DakoCytomation, Carpenteria, CA, USA) as previously described [32]. A monoclonal IgG1 mouse anti-human TF antibody (No 4509, American Diagnostica, Greenwich, CT, USA) was used. The secondary antibody from the kit alone was used as a negative control, while a monoclonal IgG1 mouse anti-human CD19 antibody (M0740, DakoCytomation, Carpenteria, CA, USA) was used as unrelated control for neutrophils. Images were captured by the Eclipse E200 microscope (Nikon, Japan) equipped with a DS-L1 camera (Nikon) and imported into ACT-1 software (ACT-1 version 2.1, Nikon).

Flow cytometry

Indirect intracellular FITC labelling was carried out since this protocol provided more efficient staining than using FITC-conjugated TF monoclonal antibodies. Briefly, staining was performed for 4 h at 4 °C in the dark using 1.8 μg unconjugated anti-TF antibody (same as in immunocytochemistry) per 2.5 × 10^5 cells, followed by a 30 min incubation with 10 μL secondary polyclonal goat anti-mouse FITC-conjugated antibody (555988, BD Pharmingen). Incubation with the secondary antibody was performed for 30 min at RT, followed by addition of 0.2 μg/μL unconjugated rabbit anti-goat antibody (P0375, DakoCytomation) and incubation for 30 min at RT. After washing the cell pellet, stained cells were resuspended in PBS and analysed by flow cytometry (BD FACSCalibur, BD Biosciences). Representative data showing the expression of both isoforms of the leptin receptor in PBMC (lanes 1, 2) and PMN (lanes 3, 4) with 18s as internal standard. Lanes 5 and 6 are blank and no RT control respectively. Ten microliter of PCR products were analysed in 1.5% agarose gel.

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antibody alone or FITC-labelled IgG1 antibody (345815, BD Pharmingen) were used as isotype controls. Fixation, permeabilisation and analysis were performed as previously described [20]. Flow cytometry was performed in a FACS Scan with CellQuest software (BD Biosciences). Cells were identified by their forward and side scatter characteristics and Median Fluorescence Intensity (MFI) ratio was applied as previously described [33].

Western blot analysis
Approximately 3×10^6 cells were re-suspended in lysis buffer, containing 1% Triton-X in 150 mM NaCl, 20 mM HEPES (pH 7.5) with protease inhibitors (Complete Protease Inhibitor Tablets, Roche). After freezing and thawing for a minimum of three times, lysates were centrifuged and supernatants were quantified using the BCA Protein Assay

Figure 2  Effect of leptin on the procoagulant activity of supernatants from PMN and PBMC. Panel A: TF-dependent procoagulant activity after stimulation of PMN and PBMC with increasing concentrations of recombinant human leptin. *: p<0.001. Panel B: The mean mPT of 7 healthy PMN and PBMC stimulated with leptin is significantly reduced (25.70±1.07 s, bar 3 and 27.20±2.60 s, bar 7 respectively) compared to the control unstimulated cells (32.00±0.90 s bar 1 and 32.50±0.80 s, bar 5) (*: p<0.005). Inhibition of supernatants with anti-TF specific antibody returns the mPT values back to the baseline (32.30±0.50 s, bar 5, 31.30±0.70 s, bar 8) Cells treated with serum from patients with APS were used as a positive control (20.60±0.50 s in PMN, bar 2 and 19.70±1.40 s in PBMC, bar 6).

Figure 3  \(2^{-\Delta\Delta CT}\) relative quantification of TF isoforms. Schematic presentation of difference expression (\(2^{-\Delta\Delta CT}\)) of TF and asTF mRNA levels in leptin-stimulated and unstimulated (control) PMN and PBMC.
Figure 4  Effect of leptin on TF protein expression. Panel A. Representative data of flow cytometry analysis of PMN (upper panel) and PBMC (lower panel), either unstimulated (treated only with PBS) or treated with leptin (300 nM) or a combination of leptin (300 nM) and anti-TNF (0.20 μg/μl). The populations were determined by their forward and side scatter characteristics. Panel B. Representative immunocytochemistry of leptin-stimulated PMN (7 total experiments) with monoclonal antibody against TF antigen in 1: unstimulated cells and 2: in leptin-stimulated cells. (Natural magnification, ×1000) Panel C. Representative western blot analysis of PMN and PBMC isolated from healthy individuals and incubated with APS (positive control), 300 nM recombinant leptin, or PBS (negative/unstimulated). The membrane was re-blotted for GAPDH in order to verify equal loading.
Western blot analysis was performed as previously described [20].

Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical analysis was carried out using Student’s t-test for paired means, using GraphPad Prism software (Version 4.03, GraphPad Software Inc.). For the relative quantification of mRNA expression the $2^{-\Delta\Delta C_{t}}$ equation, was selected for the comparison of data within each group. In all analyses, differences were considered statistically significant if the $p$ value was less than 0.05.

Results

Effect of leptin on the procoagulant activity of PMN and PBMC supernatants

PBMC are known to express both the long (OB-Rb) and short (OB-Ra) isoforms of the leptin receptor [26]. In PMN, the short isoform was shown to be expressed, while controversy exists as to the expression of the long isoform [26,34]. In order to confirm the presence of the leptin receptor, a semi-quantitative RT-PCR was performed in 7 samples of both cell populations, and both isoforms (OB-Ra and OB-Rb) were detected (representative data in Fig. 1).

In order to examine the effects of leptin on the production of tissue factor (TF), PMN and PBMC from healthy donors were stimulated with recombinant human leptin (17.5–1800 nM) for 120 min at 37 °C, and the TF-dependent procoagulant activity was measured in the cell supernatants using the modified prothrombin time (mPT) assay. Incubation with PBS or serum from patients with APS was used as negative or positive control, respectively, in these analyses. The mean control mPT at baseline without stimulation was 40.00±0.90 s. Addition of leptin-induced a dose-dependent procoagulant activity resulting in a decrease in the mPT time that reached statistical significance after stimulation with 150 nM leptin ($p<0.001$; Fig. 2A). The effects of leptin reached a plateau at a concentration of 600 nM. Based on these findings, the concentration (300 nM) in the middle of the linear phase of the dose curve (Fig. 2A) was selected for all further experiments. Similar doses have been used previously in in vitro experiments [27,35]. Supernatant from PMN and PBMC treated with 300 nM leptin for 120 min reduced the mPT to 25.70±1.07 and 27.20±2.60 s respectively (Fig. 2B, bars 3 and 7 respectively; $p<0.0001$ compared to supernatant from unstimulated – control-cells). Supernatants from cells stimulated with APS serum as a positive control [20] demonstrated a significant increased procoagulant activity, as expected (Fig. 2B, bars 2 and 6, $p<0.0001$ compared to unstimulated cells). Importantly, addition of a monoclonal anti-TF antibody (0.01 µg/µL) resulted in the return of the mPT to baseline values (Fig. 2B, bars 4, 8) indicating that the procoagulant activity of leptin was entirely TF-dependent. At the same time, in concordance with previous results [20] it was shown that the trace amounts (~2%) of PBMC and platelets in the neutrophil population had no effect on the results (data not shown).

Effect of leptin on the expression of tissue factor at the mRNA and protein level

Prompted by these findings, we examined whether leptin induces TF mRNA expression in human leukocytes. The presence of TF mRNA expression was analysed by real-time RT-PCR in unstimulated PMN and PBMC isolated from 7 healthy individuals, and compared

Figure 5  Potential mechanisms involved in the effect of leptin on TF-dependent procoagulant activity. PMN (Panel A) and PBMC (Panel B) were isolated from 7 healthy individuals (bars 1–6 respectively). The effects of leptin on the mPT were studied in the presence of PBS (negative control – bar 1), recombinant leptin (positive control – 300 nM; bar 2) and leptin in the presence of inhibitors. In both panels (PMN and PBMC) the JAK2 inhibitor AG490 (100 µM; bar 3), the PI3 kinase inhibitor Ly294002 (50 µM; bar 4) abolished the effects of leptin ($p<0.001$ compared to leptin-stimulated cells), while the MEK inhibitor PD98059 (25 µM; bar 5) had no effect ($p=ns$ compared with leptin-stimulated cells). Co-incubation with leptin and anti-TNFα (0.20 µg/µL, bar 6) resulted to an inhibition of TF procoagulant activity ($p<0.001$ compared to leptin-stimulated cells).

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to those obtained in cells that were treated with 300 nM leptin. In contrast to unstimulated controls, cells that had been exposed to leptin showed increased mRNA expression levels of full length as well as of alternatively spliced TF. In particular, according to the 2−ΔΔCT analysis [31], stimulation of PMN with leptin led to a 4.58 ± 1.17-fold increase of full length TF (Fig. 3, bar 2 vs. 1; p<0.05) and a 2.89 ± 0.15-fold increase of asHTF (Fig. 3, bar 6 vs. 5; p<0.05) mRNA expression. Similarly, stimulation of PBMC with leptin resulted in a 3.10 ± 1.18-fold increase of full length TF (Fig. 3, bar 4 vs. 3; p<0.05) and a 2.00 ± 0.15-fold increase of asHTF (Fig. 3, bar 8 vs. 7; p<0.05) mRNA expression.

Flow cytometry analysis of both PMN and PBMC from 6 healthy donors confirmed the induction of TF protein expression after stimulation with leptin (Fig. 4A). The median fluorescent intensity (MFI) analysis revealed a significant increase of 3.94 ± 0.49-fold in leptin-stimulated PMN, and 1.79 ± 0.28-fold in leptin-stimulated PBMC compared to unstimulated control cells (p<0.004 and p<0.001 respectively). Similarly, immunocytochemical staining for TF antigen in PMN of the same donors using the same antibody showed a strong immunosignal in all leptin-stimulated cells compared to untreated cells (Fig. 4B), while in PBMC leptin increased the proportion of TF-stained cells from 5.00 ± 0.40% to 11.00 ± 0.70% (p<0.01 compared to untreated cells – data not shown). These results were also confirmed by immunoblotting the cellular lysates from the above experiments for TF antigen (Fig. 4C).

JAK2-dependent signal transduction pathways are involved in mediating the effect of leptin on TF activity

In order to begin to clarify the intracellular signal transduction pathways involved in the observed increase of TF production after leptin stimulation, PMN and PBMC from healthy individuals were incubated with the JAK2 inhibitor AG490. Compared to the supernatants from unstimulated cells, supernatants of leptin-stimulated PMN (Fig. 5A, bar 2) and PBMC (Fig. 5B, bar 2) showed a significant decrease in the mPT compared to untreated cells (control), whereas this decrease was inhibited in leptin-stimulated cells also treated with AG490 (PMN Fig. 5A, bar 3; PBMC, Fig. 5B, bar 3, p<0.001 compared to leptin-treated cells). Flow cytometry analysis confirmed the inhibition of leptin-induced TF expression (data not shown).

Since leptin signalling downstream of JAK2 activation may involve PI3 kinase as well as MAP kinases, these molecules were also functionally examined by using specific inhibitors. Our analysis revealed that PI3 kinase may also be involved in the leptin-induced TF production since co-incubation with LY294002 (50 μM) abolished the effects of leptin on the mPT, both in PMN (Fig. 5A, bar 4; p<0.001, compared to leptin-stimulated cells, bar 2) and PBMC (Fig. 5B, bar 4; p<0.001 compared to stimulated cells, bar 2). On the other hand, PD98059 appeared not to have an effect in the leptin-induced TF expression, since stimulation with leptin in the presence of PD98059 (25 μM) continued to significantly reduce the mPT similarly to leptin only stimulated cells (Fig. 5A and B, bar 5; p=ns compared to leptin-treated cells).

TF expression after leptin stimulation is mediated by TNFα

Having shown that leptin stimulation causes TF expression, we tested if this action is direct or through the release of another mediator. It has been shown previously [36] that leptin stimulation can produce TNFα and IL-6 in monocytes. Both IL-6 and TNFα are able to induce TF in cell lines [19], while stimulated PMN can express TNFα [36,37]. Therefore, in the present study, PMN and PBMC were co-incubated with leptin and either 0.20 μg/μl final concentration anti-TNFα, or 0.01 μg/μl anti-IL-6. The functional analysis revealed that in cells co-incubated with leptin and anti-TNFα, there was no functional TF produced (Fig. 5A and B, bar 6) while addition of anti-IL-6 with leptin showed no changes to the mPT (data not shown). These results were confirmed by flow cytometry analysis (representative data in Fig. 4A) and real-time RT-PCR. More specific, co-incubation with leptin and anti-TNFα reduced the leptin-induced MFI from 3.94 ± 0.49 to 2.01 ± 0.23-fold (p<0.001 compared to leptin-stimulated cells) in PMN, and from 1.79 ± 0.28 to 1.17 ± 0.11-fold in PBMC (p<0.006 compared to leptin-stimulated cells) in 6 healthy donors. At the same time, 2−ΔΔCT analysis of TF expression at the mRNA level in PMN co-incubated with anti-TNFα and leptin decreased from 4.58 ±0.40 to 2.30 ± 0.30-fold, p<0.001 compared to leptin stimulation alone (average ΔCt from 9.40 ±1.43 to 7.92 ±0.29). Similar TF mRNA expression pattern was observed in PBMC (data not shown).

Discussion

In the present study, we described a novel role of leptin as an inducer of functional TF in human primary neutrophils, suggesting that the adipocytokines may contribute to thrombosis by modulating the extrinsic coagulation cascade. Additionally, increased TF expression in PMN after stimulation with leptin was demonstrated functionally, as well as at the mRNA and protein level. Similar results were observed in PBMC. We further demonstrated that inhibition of JAK-2 and PI3 kinase signalling completely abolished TF expression in primary leukocytes from healthy individuals, and that the effect of leptin is probably mediated by TNFα secretion.

A large body of evidence suggests a crosstalk between inflammatory and thrombotic pathways [38]. TF is the primary trigger of thrombosis in vivo through the extrinsic coagulation pathway and a number of inflammatory mediators such as TNFα, IL-6, IL-1 and complement anaphylatoxins, have been demonstrated to induce functional TF in cell lines, in animal and human disease models [19,21]. Adipose tissue has been recognised as an active endocrine organ and a source of several inflammatory mediators [2]. Obesity results in predisposition to a variety of chronic disorders including atherosclerosis, and inflammation is known to play a leading role in atherosclerosis [39]. It is thus assumed that adipocytokines including leptin may represent a direct link between these conditions.

In humans, obesity is frequently characterised by elevated plasma levels of leptin combined with central leptin resistance [5], and it is associated with an elevated risk of arterial and venous thrombosis [12]. Plasma leptin levels are also associated with cardiovascular atherosclerosis [3], while elevated plasma leptin was suggested to predict acute (atherothrombotic) cardiovascular
events [9]. Although leptin appears to promote platelet aggregation [11], its possible procoagulant effects on other blood cell populations (e.g. PMN) are still unknown. Despite the debate [40,41] regarding whether PMN produce functional TF or not, more recent reports [20–22,42] indicate that PMN do participate in the production of TF. It is thus of interest to investigate the crosstalk between PMN and leptin as a coagulopathy mechanism.

Acute coronary syndromes (ACS) are usually triggered by rupture or erosion of an atheromatous plaque thus exposing TF from subendothelial tissues to blood and activating the coagulation cascade causing thrombus formation [38]. Elevated levels of TF are observed in patients with cardiovascular risk factors [43]. Given the pivotal role of thrombus formation in ACS, the role of leptin as an inducer of TF-driven coagulation could represent a possible link between obesity and thrombosis. This scenario is further strengthened by the clinical observation that weight loss reduces TF in morbidly obese patients [18]. Our study now helps extend and further supports our RT-PCR findings that these cells possess a functional longer form of the leptin receptor. Although inhibition with PD98059 had no effects in our experiments, Napoleone et al. [27] showed the involvement of MAPK in leptin signalling in monocytes using the same inhibitor. This discrepancy could probably be due to differences in cell culture protocols (i.e. incubation time and different monocytes concentration). In humans with antiphospholipid syndrome it has been demonstrated that, NF-κB, p38 kinase and ERK-1 play an important role in TF expression by monocytes [44]. Furthermore, in this study we also show the involvement of the JAK2 as an additional pathway in the triggering of TF in both cell types. While several signalling mechanisms have been suggested for TF expression, their detailed description has yet to be determined.

It has been shown previously that leptin promotes TNFα and IL-6 production in monocytes [29] and activates leukocytes via TNFα [19]. Studies have shown that PMN are also able to produce TNFα [35,37]. TNFα and IL-6 are known to induce TF expression in endothelial cell lines as well [19]. Our results show that both PMN and PBMC treated together with leptin and pharmacological doses of neutralising anti-TNFα antibody resulted to the inhibition of TF induction by the adipokine. Our findings thus suggest that leptin-mediated TF expression in leukocytes is mediated through the secretion of TNFα but not IL-6. However, it is not yet clear if TNFα alone is responsible for TF induction after leptin stimulation, or if leptin has in part a direct involvement as well. Although previous findings [19] showed that leptin activates PMN via the release of TNFα from monocytes, herein we demonstrated the involvement of PMN-derived TNFα for TF expression in high purity leptin-treated neutrophils, indicating thus the pivotal role of these inflammatory cells in thrombosis.

This crosstalk of leptin and TF was in consistence with a recent study [27] showing the induction of TF by isolated monocytes after leptin stimulation. Moreover the additional information offered by our study regarding the contribution of neutrophils through the JAK2 pathway and the involvement of TNFα, reinforce the theory concerning the role of leptin in thrombosis via the extrinsic coagulation system. It is thus of great importance that future studies in humans concentrate on the possible mechanisms and molecules that could interfere with leptin’s action against TF.

Our findings can be summarised as follows: Leptin appears to be capable of triggering the extrinsic coagulation cascade by upregulating the expression of TF in human peripheral neutrophils and PBMC. These effects appear to be mediated by the leptin receptor and the JAK2-P13 kinase intracellular signalling pathway, and they may require, at least
in part, the presence of TNFα. The contribution of PMN as a source for circulating TF via leptin suggests a possible novel link between obesity, inflammation and thrombosis.

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