Pro-atherothrombotic effects of leptin in human coronary endothelial cells

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Summary
Adipocytes are nowadays recognised as cells able to produce and secrete a large variety of active substances termed adipokines, which exert direct effects on vascular cells. Among these adipokines, leptin has been proposed to play a role in the pathophysiology of acute coronary syndromes, as well as in increasing cardiovascular risk. At the moment, however, the mechanisms linking leptin to cardiovascular disease are not completely understood. This study investigates the effects of leptin, in a concentration range usually observed in the plasma of patients with increased cardiovascular risk or measurable in patients with acute coronary syndromes, on tissue factor (TF) and cellular adhesion molecules (CAMs) expression in human coronary endothelial cells (HCAECs). We demonstrate that leptin induces transcription of mRNA for TF and CAMs by real-time PCR. In addition, we show that this adipokine promotes surface expression of TF and CAMs that are functionally active since we observed increased procoagulant activity and leucocyte adhesion on cell surface. Leptin effects appear modulated by NF-κB, since L-NAME, Superoxide Dismutase and NF-κB inhibitors suppressed CAMs and TF expression.

Keywords
Adhesion molecules, adipokines, atherothrombosis, leptin, obesity, tissue factor

Introduction
Epidemiological studies have demonstrated that human obesity, is a strong cardiovascular risk factor (1, 2); however, the possible link(s) between these two conditions are not completely understood yet. Indeed, emerging evidence has transformed the current point of view about the adipose tissue, indicating that adipocytes have no longer to be considered only storage cells for fat, but they have to be now recognised as cells able to produce and secrete a large variety of active substances, termed adipokines, with pro-atherogenic and pro-inflammatory effects on vascular cells, which could be, in turn, responsible for the increased cardiovascular risk in obese patients. Among these adipokines, leptin, adiponectin and resistin are of particular interest in cardiovascular pathophysiology (3). In this context, it is of particular interest that obese individuals frequently have elevated plasma levels of leptin (4). Thus it has been recently proposed that this adipokine, involved in the regulation of energy metabolism (5) might be a marker of increased cardiovascular risk, since several actions exerted by leptin on cardiovascular system suggest involvement of this adipokine in the development of cardiovascular disease (1, 6–8).

Atherothrombosis plays a pivotal role in the pathophysiology of cardiovascular disease and of its complications (9). Indeed, leptin has been demonstrated to promote atherosclerosis and thrombosis in apo-E- deficient mice (6). In addition, leptin-dependent platelet aggregation has been suggested as another potential mechanism linking atherothrombosis to obesity (7).

One of the major pathogenetic mechanisms of atherosclerosis is the recruitment of circulating leucocytes onto the vessel wall and their subsequent migration into the subendothelial space (10). This phenomenon appears to be mediated by cellular adhesion molecules (CAMs), usually expressed by endothelial cells only in response to pro-inflammatory stimuli (10–12). Among the CAMs, the inter-cellular adhesion molecule-1 (ICAM-1), the vascular cellular adhesion molecule-1 (VCAM-1) and E-selectin seem to play a pivotal role in mediating leucocyte transmigration into the subendothelial space (13).
On the other hand, the pathophysiology of thrombosis indicates that tissue factor (TF) plays a major role in triggering the formation of intracoronary thrombi following plaque complications (14, 15). In this respect, cells normally exposed to the bloodstream, such as endothelial cells, express TF on their membrane only when activated after exposure to specific stimuli, such as LPS, certain cytokines, and oxygen free radicals (16, 17).

In the present study, we provide support, in vitro, for the hypothesis that leptin, besides being involved in the regulation of energy balance and body weight, might be involved also in the mechanisms responsible for coronary atherosclerotic disease and of its thrombotic complications, promoting both pro-atherosclerotic and pro-thrombotic state in human coronary endothelial cells through induction of CAMs and TF.

Methods

Human coronary artery endothelial cells (HCAECs) were purchased from Lonza (Basel, Switzerland). Cells were grown in EGM 2 medium with endothelial cell growth supplement and 10% fetal calf serum and used at passages 2–5. To investigate the effects of leptin on TF expression and activity, cells were enzymatically harvested and counted in a haemocytometer and sub-cultured in 24-well plates at an initial density of about 5 x 10⁴ cell per well, while at confluence, cell density was of about 8.5 x 10⁴ cell per well. For other set of experiments, cells were grown in 100-mm cell plates and, at confluence, cell density was of about 2 x 10⁶ cells per plate. At confluence, cell were starved in serum-free medium for 24 hours (h) and then used in the different sets of experiments. Leptin (Human, purity ≥97%, recombinant, expressed in Escherichia coli, Sigma Chemical Co., St Louis, MO, USA) was used in all studies described. Given the concern surrounding the potential contamination of leptin with endotoxin, we analysed our substance and found the endotoxin level to be <0.125 EU/ml (<12.5 μg/ml). For other set of experiments, cells were grown in 100-mm cell plates and, at confluence, cell density was of about 2 x 10⁶ cells per plate. All media, and water were also tested and endotoxin level found to be <0.125 EU/ml. In addition, in order to avoid a possible bias from contaminating lipopolysaccharide (LPS), all experiments were then repeated in the presence of Polymyxin B (100 μg/ml).

Effects of leptin on TF-mRNA levels

In a preliminary set of experiments, we evaluated the dose-response effects of leptin on TF-mRNA transcription by semi-quantitative polymerase chain reaction (PCR). Then, the effects of leptin on TF-mRNA were investigated by real-time reverse transcription (RT) analysis as previously described (18). Briefly, HCAECs were incubated with leptin (10 ng/ml, concentration chosen on the basis of semi-quantitative results) for 30 minutes (min). Then, cells at each time point used for RNA extraction, were washed with phosphate-buffered saline (PBS) and then fresh medium (EGM 2 containing 0.1% serum) was added. Total mRNA was extracted at baseline, 30, 60 and 120 min after leptin stimulation: TF mRNA levels were examined by RT and PCR by LightCycler (Roche Diagnostics, Basel, Switzerland). In positive control experiments, HCAECs were incubated with LPS (50 μg/ml, Sigma Chemical Co.), for 30 min and then mRNA was extracted at 60 min.

Total mRNA was extracted from cell cultures using TRIzol reagent (GIBCO, Carlshad, CA, USA), according to the manufacturer’s instructions. Reverse transcription was performed using mMLV (GIBCO) and 100 ng of the RNA samples from each culture condition. Samples were run in triplicate in 50 μl reactions by using an ABI PRISM 5700 sequence detector system (Applied Biosystems, Foster City, CA, USA). Samples were incubated at 50°C for 2 min, 95°C for 10 min and then underwent 40 cycles at 95°C for 15 seconds (s) and 60°C for 1 min. Specific oligonucleotides for human GAPDH and human TF were designed on the basis of published sequences using PRIMER EXPRESS Software (Applied Biosystems) and validated for their specificity. SYBR-green chemistry was used to detect fluorescence and an internal standard (Applied Biosystems) was used for quantization of the message. The results were analysed using a comparative method, and the values were normalised to the GAPDH expression and converted into percentage change. Three different experiments were performed for each experimental condition.

Effects of leptin on TF surface expression and activity

Human coronary artery endothelial cells were cultivated as described above. After serum starvation, cells were washed with PBS and then incubated with increasing concentrations of leptin (5, 10 or 20 ng/ml) for 6 h.

TF expression in cell lysates was determined with Western blotting. The samples (30 μg) were treated with SDS-PAGE sample buffer, followed by heating and then subjected to 10% gel. The protein was transferred onto membranes with a semidry transfer unit. TF was detected with a specific antibody (1:100, American Diagnostica Inc, Greenwich, CT, USA). All experiments were performed in triplicate.

TF expression on cell surface was investigated with FACS analysis. After incubation, endothelial cells were detached with 10 mM EDTA in PBS (without trypsin) and stained with FITC-labelled monoclonal antibody (Pharmingen, Franklin Lakes, NJ, USA) against TF, or with the appropriate isotype IgG (phycoerythrin or FITC) as control. Fluorescence intensity of 9000 cells for each sample was quantified by a FACSCalibur analyzer (Becton-Dickinson, Franklin Lakes, NJ, USA).

To evaluate whether leptin-induced TF was functionally active, TF procoagulant activity was determined by a two-step colorimetric assay, based on the ability of TF to promote generation of coagulation FXa, as previously described (17). Briefly, after stimulation with leptin, cells were incubated with 1 nM of recombinant human FVIIa (Novo Nordisk A/S Gentofte, Denmark), followed by 100 nM of purified human factor X (Calbiochem-Novobiochem,
La Jolla, CA, USA) and 5 mM CaCl$_2$ for 15 min at 37°C. A chromogenic substrate, specific for factor X (Cromozym X, Roche Diagnostics, Mannheim, Germany, 0.5 mM) was then added and incubated for 30 min at 37°C. The reaction was stopped by adding 200 μg/ml of sample of a 50% solution of acetic acid and the change in optical density at 405 nm was quantified with a spectrophotometer. Purified factor Xa of known concentration (Sigma Chemical Co.) allowed determination of calibration curves.

To evaluate whether leptin-induced expression of TF resulted from de novo synthesis, in another set of experiments, HCAECs were pre-incubated for 120 min with cycloheximide (10 μg/ml), an inhibitor of protein synthesis, or with 5, 6-dichloro-1-β-d-ribofuranosylbenzimidazole (DRB, 10 μg/ml), an inhibitor of DNA transcription, before adding leptin (10 ng/ml). In additional control experiments cells were pre-incubated for 10 min with a mouse monoclonal antibody against human TF (5 μg/ml, American Diagnostica Inc) or with a rabbit polyclonal antibody raised against the human leptin receptor (20 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Positive control experiments included cells incubated for 6 h with LPS (50 μg/ml). Six different experiments were performed for each experimental condition.

As it has been documented that leptin exerts atherogenic effects through the generation of oxidative stress in endothelial cells (19), in another set of experiments, TF activity was evaluated as above but in HCAECs preincubated for 30 min. in the presence of two free radical scavengers, with a different mechanism of action: Superoxide Dismutase (SOD, 500 U/ml), and N-Acetylcysteine (NAC, 30 mM). Furthermore, since it has been recently demonstrated that leptin effects might be mainly due to induction of eNOS in HUVEC (20), in additional control experiments, the cells were preincubated with eNOS inhibitor N-nitro-L-arginine methyl ester (L-NAME, 1 μM) before being stimulated with leptin as above. Positive control experiments included cells incubated for 6 h with LPS (50 μg/ml). Six different experiments were performed for each experimental condition.

Effects of leptin on adhesion molecule expression

Human coronary artery endothelial cells cultivated as above, after starvation, were washed with PBS and then incubated with in-

Effects of leptin on adhesion molecule-mRNA levels

HCAECs were incubated with leptin (10 ng/ml). Total mRNA was extracted at 4 h after leptin stimulation and mRNA levels for ICAM-1, VCAM-1 and E-selectin were examined by RT and PCR by LightCycler (Roche Diagnostics, Basel, Switzerland). In positive control experiments, HCAECs were incubated with LPS (50 μg/ml, Sigma Chemical Co.), for 30 min and then mRNA was extracted at 60 min.

Total mRNA was extracted from cell cultures using TRIzol reagent (GIBCO), according to the manufacturer’s instructions. Reverse transcription was performed using mMLV (GIBCO) and 100 ng of the RNA samples from each culture condition. Samples were run in triplicate in 50 μl reactions by using an ABI PRISM 5700 sequence detector system (Applied Biosystems). The PCR was started at 95°C for 15 min (hot start) to activate the AmpliTaq polymerase, followed by a 45-cycle amplification (denaturation at 94°C for 20 s, annealing at 60°C for 30 s, extension at 72°C for 60 s, and plate reading at 60°C for 10 s). Specific oligonucleotides for human GAPDH and human ICAM-1, VCAM-1 and E-selectin were designed on the basis of published sequences using PRIMER EXPRESS Software (Applied Biosystems) and validated for their specificity. SYBR-green chemistry was used to detect fluorescence and an internal standard (Applied Biosystems) was used to quantify the message. The results were analysed using a comparative method, and the values were normalised to the GAPDH expression and converted into percentage change. Three different experiments were performed for each experimental condition.

Figure 1: A) Effects of leptin on TF-mRNA levels in HCAECs assessed by real-time quantitative PCR. TF mRNA was undetectable at baseline (Base). Leptin (10 ng/ml), caused the time-dependent increase in levels of TF mRNA that peaked at 60 min and started decreasing at 120 min. LPS (50 μg/ml) represents positive control. Each bar represent the mean ± SD of three different experiments. Data are expressed as % change versus control gene represented by GAPDH. B) Dose-response effects of leptin (5, 10, and 20 ng/ml for 6 h) on TF protein levels in HCAECs, evaluated by Western blot analysis. Leptin caused a dose-response increase of TF protein. Tubulin served as loading control. Graphs are summary of data from three separate experiments (mean ± SD; * = p< 0.005 vs. Base); Insert shows result of a representative experiment.
creasing concentrations of leptin (5, 10, or 20 ng/ml) or with LPS for 12 h. Cells were then detached with 10 mM EDTA in PBS (without trypsin) and stained with R-phycoerythrin–labelled monoclonal antibody against VCAM-1 (CD106), with R-phycoerythrin-cyanin 5-labelled monoclonal antibody against ICAM-1 (CD54), with phycoerythrin-labelled monoclonal antibody against E-Selectin or with the appropriate isotype IgG (phycoerythrin or FITC) as control. Fluorescence intensity of 9,000 cells for each sample was quantified by a FACSCalibur analyzer (Becton-Dickinson). All experiments were performed in triplicate.

To evaluate whether leptin-induced expression of CAMs resulted from de novo synthesis, in another set of experiments, HCAECs were pre-incubated for 120 min with cycloheximide (10 μg/ml), an inhibitor of protein synthesis, or with 5, 6-dichloro-1-β-d-ribofuranozylobenzimidazole (DRB, 10 μg/ml), an inhibitor of DNA transcription, before adding leptin (10 ng/ml).

To study whether leptin-induced CAMs were functionally active, in another set of experiments we investigated the effects of leptin stimulation on leukocyte adhesion on HCAECs. Briefly, peripheral blood leukocytes were separated from blood of healthy volunteers by Histopaque density gradient centrifugation. Then, leukocytes were washed twice, re-suspended in pre-warmed EGM-2 medium, and finally were added (1.5 x 10^6/ml) to confluent monolayer of HCAECs that had been grown in 24-well plates and stimulated with leptin as above. In control experiments, leukocyte adherence was evaluated in endothelial cells incubated with an antibody against leptin receptor or, alternatively, with a nonsense/control antibody, before being stimulated with the adi-

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**Figure 2:**

A) Dose-response effects of leptin (5, 10, and 20 ng/ml for 6 h) on TF expression in HCAECs determined by FACS analysis. Leptin induced a dose-response increase in TF expression. LPS represents positive control experiments. B) Control experiments performed by pre-incubating endothelial cells with cycloheximide (CE), DRB, with a mouse monoclonal antibody directed against human TF (anti-TF), with a rabbit polyclonal antibody raised against the human leptin receptor (anti-L-Rc), with pyrrolidine dithiocarbamate ammonium (PDTC), or with Bay 11–7082. C and D) Effects of leptin on TF activity in HCAECs, determined by a two-step colorimetric assay based on the ability of TF/FVIIa to promote generation of coagulation FXa. TF activity reflects results observed for TF expression, confirming that TF was functionally active. Each bar represent the mean ± SD of six different experiments. * = p < 0.005 vs. unstimulated, control cells. ** = p<0.005 vs. leptin (Lep)
pokine. Finally, leukocyte adherence was evaluated in cells incubated with the antibody against leptin receptor but not stimulated with leptin. After incubation for 60 min at 37°C, HCAECs were washed with pre-warmed EGM-2 to remove non-adherent leukocytes while adherent cells were manually counted with a phase-contrast microscope. All experiments were performed in triplicate.

Effects of leptin on NF-κB activation

To investigate whether expression of TF, ICAM-1, VCAM-1 and E-selectin induced by leptin involved activation of the NF-κB pathway, we employed a sensitive multiwell colorimetric assay (21) using a TransAM NF-kappaB Kit (Active Motif; Carlsbad, CA, USA). After starvation, HCAECs were washed with PBS and incubated with different doses of leptin (5, 10, or 20 ng/ml) for 30 min. Cells incubated with 50 μg/ml of LPS served as positive control. Control experiments included cells pre-incubated with pyrroline dithio carbamate ammonium (PDTC, 100 μM), or with Bay 11–7082 (5 μM), two inhibitors of NF-κB activation with a different mechanism of action, for 60 min before stimulation with leptin (10 ng/ml). HCAECs were scraped and centrifuged for 10 min at 1,500 revolutions/min. The pellet was resuspended in 100 μl of lysis buffer, and the lysate was centrifuged for 20 min at 15,000 rpm. Supernatant constituted the total protein extract. Cell extracts (5 μg) from each sample were incubated in 96-well plates coated with NF-κB consensus double-stranded oligonucleotide sequence (5’-AGTTGAGGGGACTTTCCCAGGC-3’) for 1 h and then with primary NF-κB antibody (1:500) for 1 h, and subsequently with peroxidase-conjugated secondary antibody (1:1,000) for 1 h at room temperature. After colorimetric reaction, optical density was read at 450 nm. For competition assays, the cell extracts were incubated with 22-bp double-stranded DNA, either wild-type or mutated. All experiments were performed in triplicate.

In additional experiments, the protein levels of IκB-α were determined by Western blot. A total of 20 μg of cytoplasmatic proteins was separated on a 15% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked in washing solution with 5% non-fat dried milk for 30 min at 37°C, then it was incubated first with 1 μg/ml of primary antibody overnight at 4°C and then with a peroxidase-conjugated secondary antibody for 30 min at 37°C. The bands were detected by calorimetric. All experiments were performed in triplicate.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Differences between groups were determined by a one-way ANOVA followed by a Student’s t-test with Bonferroni’s correction. A p-value < 0.05 was considered statistically significant.

Results

Effects of leptin on TF mRNA levels

Leptin effects of TF-mRNA transcription were investigated by RT analysis. TF mRNA levels were very low in unstimulated HCAECs, as expected (22). Leptin (10 ng/ml), caused a time-dependent increase in TF mRNA levels, as compared to unstimulated cells. The peak of TF mRNA levels was observed at 60 min, while at 120 min, TF mRNA levels started decreasing (Fig. 1A). In experiments repeated in the presence of polymyxin B, leptin had similar effects on mRNA transcription (data not shown).

Effects of leptin on TF surface expression and activity

HCAECs were incubated with increasing concentrations of leptin (5, 10, or 20 ng/ml) for 6 h, and then processed to evaluate TF protein levels with Western blot analysis and TF expression on cell surface with FACS analysis. Western blot showed that TF protein was
undetectable at baseline, in unstimulated cells. TF was barely detectable in cells stimulated with leptin at low doses (5 ng/ml) and progressively increased in a dose-response manner (Fig. 1B). TF expression measured on cell surface with FACS was almost undetectable in unstimulated HCAECs. Leptin, caused progressive increase of TF expression, in a dose-dependent fashion (Fig. 2A). Similarly, TF procoagulant activity, determined by a two-step colorimetric assay, based on the ability of TF to promote generation of coagulation factor Xa, was almost undetectable at baseline, and progressively increased after stimulation with increasing leptin doses (Fig. 2C). In experiments repeated in the presence of polymyxin B, leptin had similar effects on TF expression and activity (data not shown).

In additional experiments, pre-incubation with cycloheximide (10 μg/ml), an inhibitor of protein synthesis, or with DRB (10 μg/ml), an inhibitor of DNA transcription, significantly inhibited TF expression as well as TF procoagulant activity, suggesting that leptin is able to induce de novo synthesis of TF and these new TF molecules are then expressed in an active form on the cell surface. Control experiments, performed by pre-incubating cells with a mouse monoclonal antibody directed against human TF, confirmed that only TF molecules have been detected by FACS analysis, and that procoagulant activity measured was really due to TF expression on cell surface after leptin induction (Fig. 2B and D). In additional control experiments performed in cells pre-incubated with an antibody able to block leptin receptor, leptin did not exert any ef-

**Figure 4:** A) Effects of leptin on mRNA levels of VCAM, ICAM and E-selectin in HCAECs assessed by real-time quantitative PCR. CAMs-mRNA was undetectable at baseline (Base) in unstimulated cells. Leptin (10 ng/ml), caused the increase in levels of CAMs-mRNA evaluated at 4 h. LPS (50 μg/ml) represents positive control. Each bar represent the mean ± SD of three different experiments. Data are expressed as % change versus control gene represented by GAPDH. B) Dose-response effects of leptin (5, 10, and 20 ng/ml for 12 h) on adhesion molecules expression in HCAECs, determined by FACS analysis. Leptin induced expression of ICAM-1, VCAM-1 and E-selectin on cell surface. C) Pre-incubation with cycloheximide (CE), an inhibitor of protein synthesis, or with DRB, an inhibitor of DNA transcription, significantly inhibited CAMs expression. Similarly, pyrrolidine di-thio carbamate ammonium (PDTC), Bay 11–7082, two inhibitors of NF-κB activation, the free radical scavenger superoxide dismutase (SOD), the inhibitor of eNOS, L-NAME, significantly decreased the expression of ICAM-1, VCAM-1 and E-selectin. D) Effects of leptin stimulation on leukocyte adhesiveness on HCAECs. Leptin caused a significant increase of cell-adherent leukocytes. In cells pre treated with rabbit polyclonal antibody raised against the human leptin receptor (anti L-Rc) this phenomenon was not observed. Pre-treatment with an antibody not directed against the leptin receptor did not affect the number of adherent cells (Ctrl Ab). When HCAECs were treated with the leptin receptor antibody and then not stimulated with leptin, the number of cell-adherent leukocytes was similar to that observed at baseline. Each bar represents the mean ± SD of six experiments.* = p<0.005 vs. unstimulated control cells; ** = p<0.005 vs. leptin (Lep).
Figure 5: A) Effects of leptin on NF-κB activation. Cells exposed to increasing concentrations of leptin (5, 10, and 20 ng/ml) or LPS (positive control), exhibited increased DNA binding activity of NF-κB. The NF-κB DNA binding capacity was effectively competed by the wild-type consensus oligonucleotide, but not mutated oligonucleotide. PDTC, Bay 11–7082, SOD and L-NAME significantly reduced NF-κB activation induced by leptin. Graphs are summary of data from four separate experiments (mean ± SD; * = p< 0.005 vs. Base; # = p<0.005 vs. Lep 10 mg/ml). B) Activation of IκB. IκB could be detected at baseline, in unstimulated cells. Any change in IκB-α levels were observed after cell pretreatment with PDTC, Bay 11–7082 and SOD. Graphs are summary of data from three separate experiments (mean ± SD); Insert shows result of a representative experiment. C) IκB levels in HCAECs pre incubated with L-NAME.
Figure 6: Proposed signalling mechanism by which leptin induces TF and CAM expression on HCAEC surface. Interaction between leptin and its receptor on HCAECs drives up-regulation of eNOS which, in turn, causes \( O_2^- \) production. These highly reactive species activates the enzyme I\( \kappa B \) kinase (IKK). This, in turn, phosphorylates the I\( \kappa B \)-\( \alpha \) protein, with consequent dissociation and degradation of I\( \kappa B \)-\( \alpha \) by the proteosome. NF-\( \kappa B \) can then translocate into the nucleus where it activates the transcription of specific genes, encoding for TF and CAMs.

Effects of leptin on adhesion molecule-mRNA levels

Levels of mRNA for ICAM-1, VCAM-1 and E-selectin were examined by RT analysis. In unstimulated HCAECs, levels of mRNA were undetectable. Leptin (10 ng/ml), caused a significant increase in mRNA levels of adhesion molecules, as compared to unstimulated cells (Fig. 4A). In experiments repeated in the presence of polymyxin B, leptin had similar effects on mRNA transcription (data not shown).

Effects of leptin on ICAM-1 and VCAM-1 expression

HCAECs expressed very low basal levels of ICAM-1, VCAM-1 and E-selectin. Incubation with increasing leptin concentrations caused the expression of the three adhesion molecules on cell surface (Fig. 4B). In additional experiments, pre-incubation with cycloheximide (10 \( \mu \)g/ml), an inhibitor of protein synthesis, or with DRB (10 \( \mu \)g/ml), an inhibitor of DNA transcription, significantly inhibited CAMs expression suggesting that leptin is able to induce de novo synthesis of these molecules, which are then transported to the cell surface. Similarly, pre-treatment of endothelial cells with pyrrolidine di-thio carbamate ammonium (PDTC, 100 \( \mu \)M), or with Bay 11–7082 (5 \( \mu \)M), two inhibitors of NF-\( \kappa B \) acti-
vation, for 60 min before stimulation with leptin (10 ng/ml), significantly decreased the expression of ICAM-1, VCAM-1 and E-selectin (Fig. 4C). In cells preincubated with the free radical scavenger SOD, and with the inhibitor of eNOS, L-NAME, leptin did not induce CAMs expression. (Fig. 4C). In the presence of polymyxin B, leptin had similar effects on CAMs expression (data not shown).

Interestingly, additional experiments, designed to evaluate whether CAMs expressed on HCAECs were functionally active, showed a significant increase of cell-adherent leukocytes in HCAECs stimulated with leptin. Similar results could be observed in HCAECs pre-treated with an antibody not directed against the leptin receptor and then stimulated with leptin as above. As expected, in cells pre-treated with an antibody against the leptin receptor, this phenomenon was not observed. In control experiments, in which HCAECs were treated with the leptin receptor antibody and then not stimulated with leptin, the number of cell-adherent leukocytes was similar to that observed at baseline (Fig. 4D).

**Effects of leptin on NF-κB activation**

To investigate whether expression of TF, ICAM-1, VCAM-1 and E-selectin induced by leptin involved activation of the NF-κB pathway, we employed a sensitive multi-well colorimetric assay. As shown in Figure 4A, cells exposed to leptin concentrations exhibited increased DNA binding of NF-κB in a dose-dependent fashion. The NF-κB DNA binding capacity was effectively competed by the wild-type consensus oligonucleotide but not mutated oligonucleotide. Pre-treatment of cells with the two inhibitors of NF-κB activation, PDTC or with Bay 11–7082, significantly reduced the activation of this nuclear transcriptional factor. Interestingly, L-NAME and SOD significantly prevented leptin-induced NF-κB activation (Fig. 5A). As described above, this phenomenon was associated with the parallel reduction of leptin-induced TF expression/activity as well as of CAMs expression. No significant adverse effects of the two inhibitors of NF-κB activation on cell viability as assessed by MTT assay were detected (data not shown).

To evaluate the correlation of NF-κB activation with changes of the expression of IκB, levels of this factor were determined by Western blot analysis. As expected, in unstimulated cells, baseline level of IκB was detected. Conversely, IκB levels decreased after leptin stimulation. Any significant change in IκB-α levels, as compared with unstimulated cells, was observed after cell pre-treatment with PDTC, Bay 11–7082 (Fig. 5B), or with L-NAME and SOD (Fig. 5B and C). These changes in IκB-α levels were coincident with changes of NF-κB levels described above.

**Discussion**

The main findings of the present study are: a) Leptin induces mRNA transcription for TF, VCAM-1, ICAM-1 and E-selectin and de novo synthesis of functionally active TF and CAMs in HCAEC in culture; b) these leptin-induced phenomena seem to be mediated by eNOS-related production of oxygen free radicals through activation of NF-κB pathway.

Emerging evidence has changed the current view about adipose tissue, indicating that adipocytes should no longer be considered only for their role in fat storage, but also as secretory cells capable of influencing the activity of other cell systems. In fact, they are able to produce and secrete a large variety of active substances with pro-atherogenic effects on vascular cells, known as adipokines, which include leptin, adiponectin and resistin (2, 3). Indeed, several epidemiological studies have clearly demonstrated that human obesity, a disease in which adipose tissue is largely represented, is a strong cardiovascular risk factor causally involved in the development of cardiovascular disease (1, 23). However, the possible pathophysiological link(s) existing between these two clinical entities have not been completely investigated yet.

Among adipokines, leptin seems to exert several peripheral effects, independently of its role in the regulation of energy balance and body weight (24). In fact, leptin receptors have been identified on several cell types (25), including HCAECs (26). In addition, sev-
eral experimental reports have clearly indicated a close relationship existing among leptin, platelets and arterial thrombosis (6, 7) supporting the hypothesis that leptin might be involved in the pathophysiology of acute coronary syndromes. Indeed, several clinical studies have underlined that increased plasma levels of leptin are associated with increased risk of developing myocardial infarction (27) and stroke (28). Also, increased serum leptin concentrations have been measured in patients with ST-elevated myocardial infarction (29). Finally, a large prospective study on leptin and cardiovascular risk performed on West of Scotland Coronary Prevention Study (WOSCOPS) population, confirmed that leptin is an independent predictor of coronary events (30). More recently, this adipokine has been indicated as a powerful prognostic marker of future cardiovascular events, in patients with angiographically documented coronary atherosclerosis (31).

In the present study we have demonstrated, using a cell culture model, that leptin induces TF expression in HCAEC in a dose-dependent fashion and that this phenomenon appears to be mainly related to the synthesis of new TF molecules, since cycloheximide and DRB, an inhibitor of protein synthesis and mRNA transcription, respectively, completely inhibited the leptin effects on TF expression.

Of particular pathophysiological interest was the finding that these newly formed TF molecules were functionally active, as demonstrated by the parallel increase in TF-procoagulant activity, which was detectable on the surface of stimulated cells. This observation might have important pathophysiological consequences, considering that endothelial cells are at the interface between the vessel wall and circulating blood; thus, leptin induces a “procoagulant” phenotype in coronary endothelial cells.

In addition, we have demonstrated that leptin causes the expression of CAMs such as ICAM 1, VCAM 1 and E-selectin on endothelial cell surface. These leptin-induced CAMs are functionally active since they caused leukocyte adhesion on cell surface. These CAMs are responsible for the recruitment and transmigration of leukocytes in the subendothelial space and are known to be directly involved in the pathogenesis of atherosclerosis mediating many of the early stages of the disease (10–13). In addition, serum concentrations of CAMs is strongly correlated with coronary artery disease (32) and with the risk of cardiovascular events (33).

Studies dealing with the potential effects of leptin provided evidence that promotion of oxidative stress is a fundamental principle of leptin’s mode of action (19). In addition, a more recent study has demonstrated that leptin does not directly stimulate oxygen free radicals release from the endothelium. Specifically, it might act through upregulation of eNOS expression accompanied by a significant increase of O$\textsuperscript{2-}$ production (20). In line with this observation, we have indirectly demonstrated that leptin-induced TF and CAMs expression in HCAECs is mediated by eNOS through production of oxygen free radicals and that the transcription factor NF-kB is the potential link in modulating these phenomena. Indeed eNOS inhibition by L-NAME significantly reduced NF-kB activation as well as TF and CAMs expression. Similarly, SOD, a scavenger for the oxygen free radicals O$\textsuperscript{2-}$, prevented leptin-induced activation of this transcription factor, as well as its effects on TF and CAMs. In contrast, these results were not observed in cells treated with NAC, a scavenger for peroxide. Moreover, leptin effects on TF and CAMs expression were significantly reduced by PDTC and Bay 11–7082, two inhibitors of NF-kB activation with a different mechanism of action.

NF-kB is present in an inactive form in the cytoplasm of many cells such as lymphocytes, monocytes, endothelial and smooth muscle cells. It seems to be activated by several stimuli including the redox status of cells (34); it is activated during the atherosclerotic process and it might be responsible for the expression of inflammatory proteins that actively participate in the process, leading to plaque disruption and acute coronary events (35). Specifically, binding sites for this transcription factor are contained in the promoter for TF (36) and for adhesion molecules (37, 38). Interestingly, NF-kB has been demonstrated to be activated in the unstable plaques of patients with acute coronary syndromes (39, 40).

Thus, taken together, the finding observed in the present study that leptin induces TF and CAMs expression via activation of NF-kB might explain, at least in part, why this adipokine is a powerful prognostic marker of future cardiovascular events in patients with angiographically documented coronary atherosclerosis (31).

The relationship existing between leptin and atherothrombosis has been recently investigated (41–44). In particular, Kralisch et al. have shown that infranatants obtained from human adipocytes were able to induce up-regulation of monocytes adhesion on HUVEC and HCAECs (41). However, they did not find that leptin resulted in up-regulation of adhesion molecule in HUVEC and did not investigate the effects of leptin on HCAECs. Similarly, Skilton et al. have investigated the potential role played by leptin in inducing the expression of adhesion molecule in HUVEC, but they demonstrated that neither leptin nor insulin altered cell adhesion molecule expression or monocyte adhesion to endothelial cells (42). Thus, the present report is the first one that demonstrates a direct proatherogenic effect of leptin in a cell population never studied in this pathophysiological context before, such as HCAECs.

The effects of leptin on TF expression have been recently investigated by other groups (43, 44). In particular, it has been demonstrated that this adipokine induces expression of functional TF in human peripheral blood mononuclear cells suggesting a possible mechanism that might contribute to the cardiovascular risk associated with obesity. However, in those studies, the authors investigated the effects exerted by leptin at pharmacological concentrations or, alternatively, by amount of leptin usually measurable in plasma of obese individuals.

In the present study, we have pointed out our attention on the effects exerted by leptin concentrations significantly lower that those used in previous studies. These concentrations were chosen, to better define the pathophysiological mechanisms by which leptin might be involved in atherothrombosis, since previous studies have found that mean serum leptin levels in patients with acute coronary syndromes were about 12 ng/ml (45) and that the range usually observed in plasma of patients with increased cardiovascular risk was about 10 ng/ml (31). Again, the observed leptin effects on TF expression, might explain, at least in part, why, in patients
with acute myocardial infarction and admissional plasma leptin levels 214 ng/ml were observed higher failure rates of thrombolytic therapy as compared to those with lower leptin levels (46). Finally, in line with these pathophysiological aims, we have directed our attention on a cell population never investigated before in this context, and directly involved in the pathophysiology of acute coronary syndromes such as endothelial cells from the coronary district.

In conclusion, the present study, although in vitro, describes a close relationship between leptin, atherosclerosis and thrombosis, providing support to the view that this adipokine, besides being involved in the pathophysiology of obesity, might play a relevant role as an active mediator linking obesity to atherothrombotic disease.

References