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Insulin potentiates cytokine-induced VCAM-1 expression in human endothelial cells

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Summary

Hyperinsulinemia is an independent risk factor for cardiovascular events and may contribute to cardiovascular disease. Low grade, chronic inflammation has been implicated in the pathogenesis of atherosclerosis. We aimed at determining the impact of pathophysiologically high insulin concentrations on cytokine-induced endothelial activation in human umbilical vein endothelial cells (HUVEC). HUVEC were incubated with insulin (0-24 h) \pm tumor necrosis factor(TNF)- α or lipopolysaccharide (LPS). At pathophysiological/pharmacological concentrations (10^{-9} - 10^{-7} mol/L), insulin selectively induced VCAM-1 expression and potentiated the effects of TNF- α and LPS, effects reverted by the proteasome inhibitor lactacystin. Compared with TNF- α alone, insulin+TNF- α doubled U937 cell adhesion. Insulin markedly increased TNF- α -induced NF- κ B activation and induced phosphorylated I κ B α accumulation. Therefore, hyperinsulinemia enhances cytokine-induced VCAM-1 expression in endothelial cells, thus potentially contributing to detrimental effects of other inflammatory stimuli on atherogenesis.

Key words: Insulin, atherosclerosis, vascular cell adhesion molecule-1, cytokines, type 2 diabetes, insulin resistance.

Introduction

Increased circulating levels of pro-inflammatory cytokines, such as interleukin(IL)-6 [1] and tumor necrosis factor (TNF)- α [2], as well as high levels of acute phase reactants[3], have been found in patients with insulin resistance, obesity and type 2 diabetes. Low-grade, chronic inflammation may play a role in the pathogenesis of insulin resistance in obesity and type 2 diabetes. Indeed, inflammatory cytokines inhibit insulin signaling by impairing the tyrosine kinase activity of both the insulin receptor (IR) and insulin receptor substrate(IRS)-1, thus acting as mediators of insulin resistance[4; 5]. Furthermore, mice lacking TNF receptor function have been shown to develop protection from obesity-induced insulin resistance[6]. Insulin resistance and compensatory hyperinsulinemia are probably causally associated with elevated levels of inflammatory markers and increased risk of cardiovascular disease[7]. Thus, by determining insulin resistance, systemic low-grade chronic inflammation would act as a key pathogenetic factor for cardiovascular disease in type 2 diabetes and the metabolic syndrome[8].

A growing body of evidence now suggests the existence of a bidirectional relationship between hyperinsulinemia and low-grade chronic inflammation, by which hyperinsulinemia might contribute to vascular inflammation, and vascular inflammation leads to insulin resistance and consequent compensatory hyperinsulinemia. Indeed, in humans, plasma concentrations of the pro-inflammatory cytokines IL-6 and TNF- α increase when insulin is administered together with bacterial lipopolysaccharide (LPS)[9]. In human smooth muscle cells[10], in murine glomerular vascular endothelial cells[11] and in human adipose tissue[12] hyperinsulinemia exacerbates inflammation and increases levels of markers of oxidative stress, while on the contrary insulin exerts prevailing anti-inflammatory effect at low, physiological, concentrations[13; 14; 15; 16]. Finally, in macrophages, insulin, at pathophysiologically relevant concentrations, stimulates TNF- α expression and production[17].

We have previously shown that in human umbilical endothelial cells (HUVEC) insulin *per se* increases the expression of vascular cell adhesion molecule-1 (VCAM-1)[18; 19], probably the adhesion molecule most relevant to the development of atherosclerosis[20]. However, the impact of high insulin on the inflammatory response to cytokines is still poorly characterized. In this study we therefore investigated (a) whether insulin promotes endothelial activation in human endothelial cells exposed to pro-inflammatory cytokines; (b) the relevance of nuclear factor(NF)- κ B in insulin-mediated signaling and (c) the functional consequences of the selective modulation of insulin signaling on monocytoid cell adhesion to endothelial cells.

Materials and Methods

Materials – Human recombinant insulin was purchased from Novo-Nordisk Farmaceutici S.p.A., Rome, Italy. TNF- α , endotoxin, polymixin B and lactacystin were purchased from Sigma, St. Louis, MO. The monoclonal antibodies against VCAM-1 (Ab E1/6), E-selectin (Ab H18/7), ICAM-1 (Ab Hu 5/3), and a constitutive and non-cytokine-inducible endothelial antigen (Ab E1/1) were kindly provided by M.A. Gimbrone, Jr., Brigham & Women's Hospital, Boston, MA.

Cell cultures – HUVEC were harvested enzymatically from umbilical cords with Type-II collagenase at 0.1 mg/mL, and cultured as described[21]. Cell viability after treatments was assessed by evaluating several parameters, including cell morphology at phase-contrast microscopy, Trypan blue exclusion, determination of total proteins and of the surface expression of a constitutive and non-cytokine-inducible endothelial cell antigen (E1/1) [22].

Cell surface immunoassays – Assays of cell surface molecules were carried out by cell surface enzyme immunoassays (EIA), using mouse anti-human monoclonal antibodies against VCAM-1 (Ab E1/6), E-Selectin (Ab H18/7), intercellular adhesion molecule(ICAM)-1 (Ab HU5/3), or a constitutive and non-cytokine-inducible endothelial cell antigen (E1/1)[22]. Details of the EIA have been previously reported[22].

Flow cytometry – Flow cytometric analysis of adhesion molecule expression was performed by incubating HUVEC with the mouse anti-human monoclonal antibody E1/6 against VCAM-1 for 60 min at 4° C. After washing, cells were exposed to goat anti-mouse F(ab')₂, labeled with fluoresceine-isothiocyanate (Immunotech, Cremascoli, Milan, Italy) at 4° C. After further washing and fixation in 1% formaldehyde, the cell suspension was passed through a FACScan analyzer (Becton-Dickinson Immunocytometry Systems, Mountain View, CA). Results were plotted as

intensity of fluorescence (arbitrary units, on a logarithmic scale, on the abscissa) vs cell number (on the ordinate, total cells counted: 5×10^3 for each condition).

Immunocytochemistry – Cells grown in 24-well plates (LabTek, Nalgene/Nunc, Naperville, IL, USA) were fixed with cold methanol and then blocked in phosphate-buffered saline (PBS)/3% fetal calf serum (FCS) for 30 min. Cells were incubated overnight at 4° C in PBS/3% FCS with the mouse anti-human monoclonal antibody E1/6 against VCAM-1. After incubation with the primary antibody, cells were washed in PBS/3% FCS, incubated for 1 h with the anti-mouse biotinylated antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and further incubated with EstrAvidin peroxidase (Sigma). Slides were washed, incubated with diaminobenzidine in the dark for 30 min, dehydrated in xylene and ethanol, mounted in balsam, and observed with an inverted microscope.

Measurement of TNF- α release – HUVEC seeded into 24-well culture plates (5×10^5 cells/well) were stimulated with insulin (10^{-9} - 10^{-7} mol/L) for 24 h with or without LPS (0.1 ng/mL). TNF- α release in the supernatants was measured by an enzyme-linked immunosorbent assay (ELISA), using a commercial kit (Bender MedSystems, Vienna, Austria) and following the manufacturer's protocols. A standard curve was prepared from seven TNF- α dilutions. The lower limit of sensitivity was 7.8 pg/mL. The overall inter-assay and intra-assay coefficients of variation were <10%. In each well, residual cells that had been deprived of medium were lysed with ice-cold RadioImmuno Precipitation Assay (RIPA) buffer, and total cellular proteins were measured with a BCA assay (Pierce, Rockford, IL). The TNF- α content in the medium was normalized to cell protein values.

Immunoblotting – Total proteins were isolated from HUVEC in ice-cold RIPA buffer. Nuclear proteins were purified as previously described[21]. Proteins were separated under reducing

conditions and electroblotted onto polyvinylidene fluoride membranes (Immobilon-P; Millipore, Bedford, MA). After blocking, the membranes were incubated overnight at 4° C with primary antibodies against VCAM-1 (Santa Cruz Biotechnologies, Santa Cruz, CA), and with primary antibodies against the constitutive and Ser³²-phosphorylated inhibitor IκBα (Santa Cruz Biotechnology). The blots were developed using a SuperSignal West Pico Chemiluminescent Substrate kit (Pierce). The intensity of each immunoreactive protein band was measured by densitometry.

Adhesion assays – HUVEC grown at confluence in 6-well plates were incubated with 1 mL of 1×10^6 cells/mL U937 cell suspension (American Tissue Culture Collection, Rockville, MD) , under rotating conditions (63 r.p.m.) at room temperature. To test the VCAM-1-dependence of adhesion, some monolayers were treated with the mouse anti-human monoclonal antibody E1/6, directed against VCAM-1. After 10 min, non-adhering cells were removed and monolayers fixed in 1% paraformaldehyde. The number of adhering cells was determined by counting 8 different high-power fields (h.p.f.) using an ocular grid and a 20× objective (0.09 mm² /field). Fields for counting adherent leukocytes were randomly located at half-radius distance from the center of the monolayers.

Northern Analysis – Total RNA was isolated by a single extraction using an acid guanidinium thiocyanate-phenol-chloroform method[23]. Details of Northern analysis have been previously reported[21].

Electrophoretic mobility (gel) shift assay – Nuclear proteins from stimulated or unstimulated HUVEC were purified as previously described[21]. Protein concentration was determined by the Bio-Rad Laboratories Protein Assay (Bio-Rad Laboratories Inc, Hercules, CA). The double-stranded synthetic oligonucleotides NF-κB motif (5'-AGT TGA GGG GAC TTT CCC AGG C-3'

and 5'-CCT GGG AAA GTC CCC TCA ACT-3') was labeled with [γ - 32 P]-ATP. Binding reactions containing 10 μ g of crude nuclear extract were performed using the electrophoretic mobility gel shift assay core system (Promega, Madison, WI) according to the manufacturer's protocol.

Statistical Analysis – Two-group comparisons were performed by the Student's t-test for unpaired values. Comparisons of means of ≥ 3 groups were performed by analysis of variance (ANOVA), and the existence of individual differences, in case of significant F values at ANOVA, tested by Scheffé's multiple contrasts. Comparisons of distribution of fluorescence intensities at flow cytometry were performed by the Kolgomorov-Smirnov statistics with the aid of the CellQuestTM software package from Becton Dickinson, 1997.

Results

Insulin, at pathophysiological concentrations, potentiates cytokine-induced VCAM-1 expression in human umbilical endothelial cells.

Insulin, in a concentration-dependent manner, *per se* induced VCAM-1 surface expression (**Fig. 1, A** and **Table 1**), but also potentiated the effects of TNF- α and LPS. Quiescent endothelial cells co-incubated with pathophysiologically relevant concentrations of insulin (10^{-9} - 10^{-8} mol/L) and low concentrations of TNF- α (0.1 ng/mL) had an average 3.9-fold increase in VCAM-1 expression ($p < 0.001$ vs untreated cells), which was greater than the stimulation by insulin alone (2 fold) or TNF- α alone (2.4-fold, * $p < 0.001$ vs untreated cells) (**Fig. 1, A**). In the absence of any cytotoxic effect (**Online Supplement Figure**), the addition of insulin to TNF- α -treated cells produced a maximum of 1.4-fold increase in VCAM-1 surface expression (** $p < 0.05$, insulin + TNF- α - vs TNF- α -treated cells). Similar at-least-additive and concentration-dependent effects of insulin were obtained when a higher TNF- α concentration (1 ng/mL) was used (**Fig. 1, B**).

The co-stimulatory effect of insulin and TNF- α seen at cell surface EIA was paralleled by an increase of the total cellular content of VCAM-1, as assessed by immunoblotting (**Fig. 1, E**). Consistently, flow cytometry demonstrated that HUVEC treated with insulin for 16 h showed not only a significant increase in mean fluorescent intensity for VCAM-1 compared with untreated cells, but also an expression greater than that obtained after co-incubation with TNF- α 1 ng/mL (**Table 1**). We also observed an at-least-additive and concentration-dependent effect on VCAM-1 expression by adding increasing concentrations of insulin (10^{-9} - 10^{-8} mol/L) to low concentrations of LPS (0.1 ng/mL). The maximum induction of VCAM-1 expression with insulin and LPS co-administration was 2.3-fold vs basal untreated cells, whereas it was 1.7-fold vs LPS-treated cells (# $p < 0.05$), and 1.4-fold with LPS alone vs untreated cells (* $P < 0.05$) (**Fig. 1, C**). Immunocytochemistry on endothelial cell monolayers further confirmed the effect of insulin + TNF- α on VCAM-1 surface expression. (**Fig. 1, D**). Since a robust costimulatory effect of insulin

was seen at a concentration of TNF- α of 1 ng/mL, further experiments were performed with this concentration of TNF- α .

Ruling out possible cytolytic or cytostatic effects of TNF- α and LPS [24], phase-contrast microscopy revealed no morphologic signs of injury to human endothelial cells due to TNF- α (1 ng/ml, 24 h) in these experiments in the presence or absence of insulin (10^{-9} - 10^{-8} mol/L, 24 hours) (**Online Supplement Figure**). Similarly, LPS (0.1 ng/mL given for 24 h) or TNF- α (1 ng/ml given for 24 h) or insulin alone (10^{-9} - 10^{-8} mol/L given for 24 h) did not reduce cell number or protein content of endothelial cultures, or expression of the constitutive surface endothelial protein E1/1 (**Online Supplement Table**).

To rule-out that endotoxin contamination contributed to the effect of insulin on VCAM-1 expression, the LPS inactivator polymixin-B sulfate (PMBS) was added in a parallel set of experiments. PMBS completely abrogated LPS-induced VCAM-1 expression, but had no effect on insulin \pm TNF- α -induced VCAM-1 expression (data not shown).

Inflammatory mediators such as interferon- γ and LPS are known to increase the secretion of TNF- α from several cell types, including macrophages [25], monocytes [26] and smooth muscle cells [27]. In HUVEC, LPS has been demonstrated however not to induce TNF secretion [26]. In macrophages, insulin by itself has been shown to increase gene and protein expression of TNF- α [17]. To test whether insulin – alone or in combination with LPS – induces TNF- α secretion in HUVEC, which might account, in an autocrine manner, for the potentiation by insulin of the effects of other agents, we measured TNF- α protein mass by ELISA in the cell medium of HUVEC treated with insulin (10^{-9} - 10^{-7} mol/L) in the presence or absence of LPS (0.1 ng/mL). Treatment of HUVEC with insulin alone or in the presence of LPS for 16 h did not end up in any detectable production of TNF- α (TNF- α concentrations always below the detection limit of our assay (7.8 pg/mL)).

Insulin, at pathophysiological concentrations, increases cytokine-induced monocytoic cell adhesion to human umbilical endothelial cells.

Because the induction of VCAM-1 on the endothelium is known to support monocyte adhesion, we examined the functional consequences of endothelial treatment with insulin in terms of monocytoic (U937) adhesion to endothelial cells, using a rotational adhesion assay. In agreement with data on VCAM-1 surface expression, treatment of quiescent endothelial cells with insulin (10^{-8} mol/L) alone for 16 h produced a 2.8-fold increase in U937 cell adhesion compared with control, unstimulated endothelial cells (**Fig. 2, panels A and C, conditions a and b**). Stimulation of endothelial cells with TNF- α (1 ng/mL) substantially increased U937 cell adhesion, and this effect was further potentiated by the addition of insulin (2-fold increase in U937 cell adhesion compared with TNF- α alone, **Fig. 2, panels A and C, conditions d and e**). This insulin- and cytokine-induced adhesion was markedly suppressed (by about 70%) by a 30 min pre-treatment of endothelial cells with the blocking anti-VCAM-1 monoclonal antibody E1/6, at saturating concentrations (**Fig. 2, panels A and C, conditions c and f**).

Insulin, at pathophysiological concentrations, induces and co-induces VCAM-1 mRNA at Northern analysis.

We observed increased mRNA levels in cells treated with 10^{-8} mol/L insulin \pm TNF- α (1 ng/mL), consistent with the observed increase of VCAM-1 protein expression (**Fig. 2, panels B and D**). In untreated cells, VCAM-1 mRNA was not detectable, while in cells treated with insulin alone substantial levels of mRNA were detected (albeit, compared with stimulations in the presence of TNF- α , with longer exposure times and a double intensifying screen) ($p < 0.05$). Compared with TNF- α alone, the addition of insulin to TNF- α increased levels of VCAM-1 mRNA by 1.6 fold ($p < 0.05$), as determined by densitometry of Northern blots at 4-6 h in two different experiments. Overall, these results indicate that increased VCAM-1 surface levels after insulin \pm cytokine stimulation occurs at a pre-translational levels, while the absence of VCAM-1 mRNA levels in

control conditions and their presence after stimulation with insulin alone indirectly argues for an induction of transcription.

Insulin, at pathophysiological concentrations, increases cytokine-induced NF- κ B activation and the phosphorylated form of I κ B- α in the cytosol of human umbilical endothelial cells

Insulin treatment markedly increased TNF- α -induced activation of NF- κ B (**Fig. 3 A**, lanes 3 and 4). The specificity of the NF- κ B DNA-protein complex was verified by successful competition with unlabeled (cold) NF- κ B oligonucleotide and by the lack of any binding reaction with a mutated oligonucleotide (not shown). In addition, there was no significant and specific binding with the unlabeled (cold) oligonucleotide (**Fig. 3**, lane 5), or by omitting the nuclear protein extracts or the oligonucleotide probe (not shown), as well as by using nuclear protein extracts from unstimulated HUVEC (**Fig. 3**, lane 1). Insulin treatment further increased TNF- α -induced cellular accumulation of Ser³²-phosphorylated I κ B- α in a concentration-dependent manner (**Fig. 4 B**). Treatment with insulin was thus shown to promote I κ B- α phosphorylation, thus accounting for NF- κ B translocation into the nucleus.

We next examined the effect of the proteasome inhibitor lactacystin (10 μ mol/L) on insulin-induced VCAM-1 expression in the presence of TNF- α . In the absence of any cytotoxicity, treatment of HUVEC with lactacystin significantly decreased TNF- α -induced VCAM-1 expression (* = $p < 0.05$ vs TNF-treated cells, **Fig. 4 A**), and this effect was further potentiated in HUVEC co-treated with insulin and TNF- α (# = $p < 0.001$, **Fig. 4 A**). Taken together, these results demonstrate that NF- κ B plays a key role in mediating the pro-inflammatory effects of high insulin in cytokine-induced human endothelial cells.

Discussion

We had previously demonstrated that insulin, at pathophysiologically relevant concentrations, exerts pro-inflammatory, pro-atherogenic effects on endothelial cells by enhancing VCAM-1 expression and monocyte-endothelial interactions[18; 19]. The present findings now (a) demonstrate the ability of insulin to act in concert with other pro-inflammatory stimuli in enhancing the surface expression of VCAM-1 on endothelial cells; (b) provide a mechanistic explanation of the observed effects. This is – to the best of our knowledge – the first report showing the additive effects of insulin and pro-inflammatory cytokines on endothelial activation.

We used insulin concentrations between 10^{-9} and 10^{-7} mol/L, in the range of binding and activation of insulin receptors in endothelial cells[28]. Such concentrations, equivalent to 139-13,900 μ U/mL, span from pathophysiological to pharmacological levels of insulinemia. Concentrations of 10^{-9} and 10^{-8} mol/L are indeed attainable in fasting and post-prandial states of individuals with insulin resistance[29], indicating the plausibility, with all the limitations of extrapolating from *in vitro* findings, that these observations are applicable to *in vivo* settings where hyperinsulinemia occurs.

TNF- α and LPS are potent inflammatory mediators, known to synergize in the upregulation of endothelial adhesion molecule expression during immune and inflammatory processes[30]. TNF- α has been shown to be a mediator of insulin resistance in infection, tumor cachexia and obesity[4; 5]. Indeed TNF- α is known to impair tyrosine kinase activity of both insulin receptor (IR) and IR substrate-1 (IRS-1), with subsequent blocking of the IRS-1/phosphatidyl inositol(PI)-3 kinase association, through multi-site phosphorylations of serine/threonine residues as a consequence of the activation of multiple serine/threonine protein kinases or the inactivation of serine/threonine phosphatases[4]. In addition, TNF- α , through serine/threonine or tyrosine phosphorylations, has been shown to enhance the activity of mitogen-activated protein (MAP) kinases[31], particularly p38MAP kinase[32]. Furthermore, insulin has been recently shown to stimulate TNF- α production

in macrophages, by regulating the expression of TNF- α mRNA through the ERK signalling pathway[17]. All these interactions, together with the known ability of these primary cytokines to activate the gene expression of adhesion molecules through NF- κ B activation, provide ample biological rationale for the observed at least additive interaction between insulin and inflammatory cytokines or LPS, here for the first time formally demonstrated. The ability of insulin, alone or in combination with low concentrations of TNF- α , to promote VCAM-1 expression in our system appears related, at least in part, to the ability of insulin to increase steady-state levels of mRNA, in turn due to insulin ability to activate transcription factors traditionally implicated in VCAM-1 transactivation, such as NF- κ B.

In conclusion, mimicking hyperinsulinemia and the simultaneous exposure to inflammatory mediators *in vitro* we have shown here the promotion of pro-atherogenic actions of insulin in endothelial cells. Favoring the pro-atherogenic properties of inflammatory cytokines on the endothelium may represent a major pathogenetic mechanism involved in increased atherogenesis and the promotion of vascular disease in patients with type 2 diabetes and the metabolic syndrome.

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Legends to figures

Fig. 1. Costimulatory effects of insulin and TNF- α or LPS on VCAM-1 surface expression in endothelial cells.

Subconfluent HUVEC were starved in 96-well plates by growth factor withdrawal and reduction of serum (down to 2%) for 20 h, and then stimulated with insulin (10^{-10} - 10^{-8} mol/L) for 16 h (**Panels A-C**) with or without TNF- α 0.1 ng/mL (**A**), or TNF- α 1 ng/mL (**B**) or LPS 0.1 ng/mL (**C**), added to the medium at the same time as insulin. VCAM-1 expression at EIA is expressed as percent of control in the absence of insulin, and values are the mean \pm S.D. from five experiments, each consisting of 8 replicates per condition. * = $p < 0.05$ vs unstimulated control; ** = $p < 0.05$ vs TNF- α , and # = $p < 0.05$ vs LPS. **Panels D** shows the immunocytochemical analysis of the effect of insulin and TNF- α on the surface expression of VCAM-1 in endothelial cells. HUVEC were grown in 24-well plates, starved by growth factor withdrawal and reduction of serum (down to 2%) for 20 h, and stimulated with insulin (10^{-9} to 10^{-8} mol/L) for 16 h. TNF- α 1 ng/mL was added to the medium at the same time as insulin. Immunocytochemistry with a non-immune IgG revealed no staining (not shown). Scale bar = 20 μ m. **Panel E**, immunoblotting with an antibody against VCAM-1 of the total cellular proteins extracted from subconfluent serum-starved HUVEC stimulated with insulin (10^{-9} - 10^{-8} mol/L) for 16 h, in the presence or absence of TNF- α 1 ng/mL. Blots are representative of 3 separate experiments.

Fig. 2. Effect of insulin and TNF- α on monocytoid cell adhesion to endothelial cells and on endothelial VCAM-1 steady-state mRNA levels.

Panel A: The effects of insulin and/or TNF- α treatment of HUVEC on the adhesion of U937 in a rotational adhesion assay. HUVEC were starved by growth factor withdrawal and reduction of serum (down to 2%) for 20 h, and then either left untreated (**a**) or treated with 10^{-8} mol/L insulin (**b**), 1 ng/mL TNF- α (**d**), or a combination of both (**e**) for 16 h. In **c**, HUVEC were incubated with

10^{-8} mol/L insulin + the anti-VCAM-1 monoclonal antibody E1/6; in **f**, HUVEC were incubated with 10^{-8} mol/L insulin + 1 ng/mL TNF- α + the anti-VCAM-1 monoclonal antibody E1/6. The increased adhesion with insulin alone (**b**) or in addition with TNF- α (**e**) is due to VCAM-1, since markedly suppressed in the presence of saturating concentrations of the blocking anti-VCAM-1 antibody for 30 min before the assay (**c** and **f**). Photographs represent randomly chosen high-power fields at half-radius distance from the center of the well in one of 3 comparative experiments with similar design. Quantitative data of U937 cell adhesion to endothelial cells are presented in panel **C**. Numbers of adhering U937 cells were expressed as percent of unstimulated control; each point is the mean \pm S.D. of adhering cells from 3 experiments, each consisting of 8 counts per condition. * = $p < 0.05$ vs unstimulated control; ** = $p < 0.05$ vs TNF- α 1 ng/mL; # = $p < 0.05$ vs insulin 10^{-8} mol/L; *** = $p < 0.05$ vs insulin + TNF- α .

Panel B: The effect of insulin and TNF- α on the expression of VCAM-1 mRNA in endothelial cells. **a:** ethidium bromide-staining of total RNA; and **b:** Northern analysis of VCAM-1 mRNA in insulin- and TNF- α -treated and untreated HUVEC. Cells were starved by growth factor withdrawal and reduction of serum (down to 2%) for 20 h, then incubated with insulin (10^{-8} mol/L), TNF- α (1 ng/mL) and a combination of both, for 6 h. The signal resulting from cell treatment with insulin is compared with control without insulin. The blots from TNF- α -untreated cells were obtained with a longer exposure time and a double intensifying screen compared with the conditions of TNF- α treatment. The images are representative of 2 independent experiments. Densitometric analyses are presented in panel **D** as Optical Density pixels, and are proportional to VCAM-1 mRNA in comparisons of conditions with and without insulin. * = $p < 0.01$ vs control (no insulin, no TNF- α); # = $p < 0.01$ vs TNF- α alone.

Fig. 3 The effect of insulin on NF- κ B activation in TNF- α -stimulated endothelial cells.

A: Electrophoretic mobility gel shift assay (EMSA), showing the effect of TNF- α \pm insulin on NF- κ B activation. EMSA was performed by mixing the 32 P-oligonucleotide encoding for the NF- κ B consensus sequence in the VCAM-1 promoter with nuclear proteins from HUVEC treated with insulin (10^{-9} - 10^{-8} mol/L) for 16 h before TNF- α stimulation (1 ng/mL added to HUVEC during the last 15 min of insulin treatment). Electrophoretic runs with nuclear protein extracts from HUVEC treated with insulin + TNF- α 1 ng/mL or TNF- α alone are shown in lanes 3-4 and 2 respectively. The electrophoretic run with unlabeled (cold) oligonucleotide is shown in lane 5, while the run with nuclear protein extracts from unstimulated HUVEC is shown in lane 1.

B: The densitometry plot of the NF- κ B band from the autoradiogram. Results are representative of 3 separate experiments

Fig. 4. Effects of the proteasome inhibitor lactacystin on VCAM-1 surface expression induced by TNF- α in the presence or absence of insulin in endothelial cells, and effects of TNF- α , with or without insulin, on the phosphorylated and total cellular levels of I κ B- α .

Panels A and B: VCAM-1 surface expression, at EIA, in cells pretreated with lactacystin (10 μ mol/L for 30 min), and then co-incubated with insulin (10^{-9} to 10^{-7} mol/L) and TNF- α for 16 h. * = $p < 0.05$ vs non-insulin-stimulated control; # = $p < 0.001$ vs non-lactacystin treated cells. Results are expressed as mU of optical density, and each concentration point is the mean \pm S.D. from 3 experiments, each consisting of 8 replicates per condition.

B: Immunoblottings with antibodies against Ser 32 -phosphorylated (**upper panels**) or non-phosphorylated I κ B- α (**bottom panels**) in total proteins extracted from HUVEC incubated with insulin (10^{-9} - 10^{-7} mol/L) for 16 h, with (**B**) subsequent incubation with 0.1 ng/mL TNF- α for 15 min.

Table 1: The co-induction of VCAM-1 expression by insulin and TNF- α at flow cytometry

Treatment	% gated (a)	fluorescence intensity (b)
untreated	1.07 \pm 0.05	5.11 \pm 1.53
insulin 10 ⁻⁹ mol/L *	2.08 \pm 0.7	9.22 \pm 2.18
insulin 10 ⁻⁸ mol/L *	4.22 \pm 1.2	15.6 \pm 3.2
TNF- α 1 ng/mL *	20.27 \pm 4.2	115.8 \pm 11
insulin 10 ⁻⁹ mol/L + TNF- α 1 ng/mL **	32.87 \pm 5.3	148.7 \pm 14.3
insulin 10 ⁻⁸ mol/L + TNF- α 1 ng/mL **	38.97 \pm 8.3	154.5 \pm 18.1

Subconfluent serum-starved HUVEC were treated with TNF- α and/or insulin (10⁻⁹-10⁻⁸ mol/L) for 16 h. Cells were then immunostained for VCAM-1 or FITC-conjugated IgG isotype control and submitted to flow-cytometry. Values (a) are mean \pm S.D. of % cells expressing VCAM-1; values (b) are mean \pm S.D. of units of fluorescence intensity. Results are representative of three independent experiments. * = p<0.05 vs unstimulated control; ** = p<0.05 vs TNF- α .

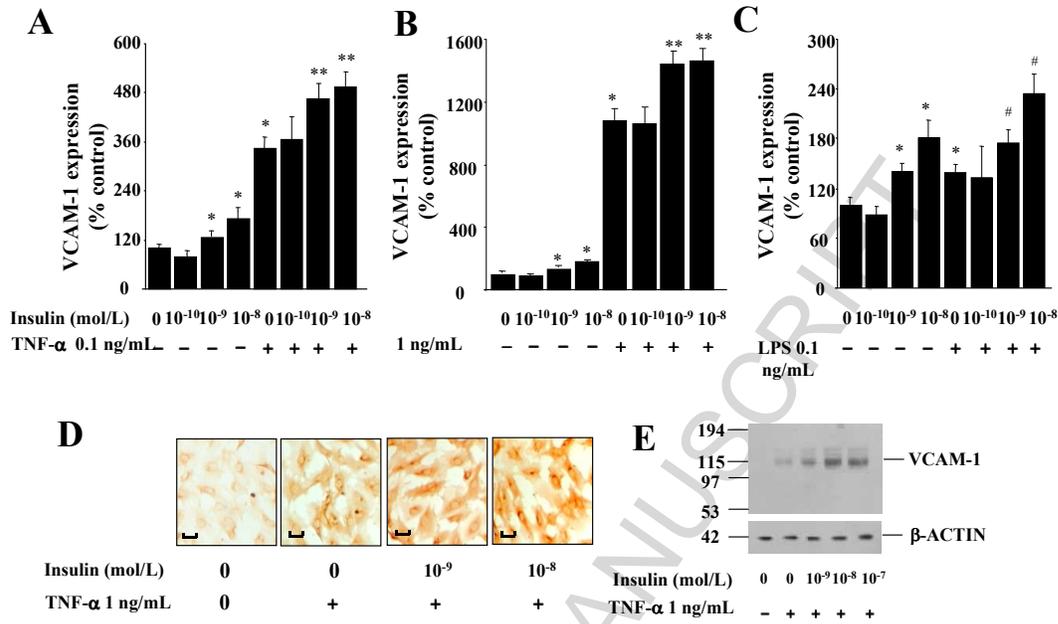


Fig. 1

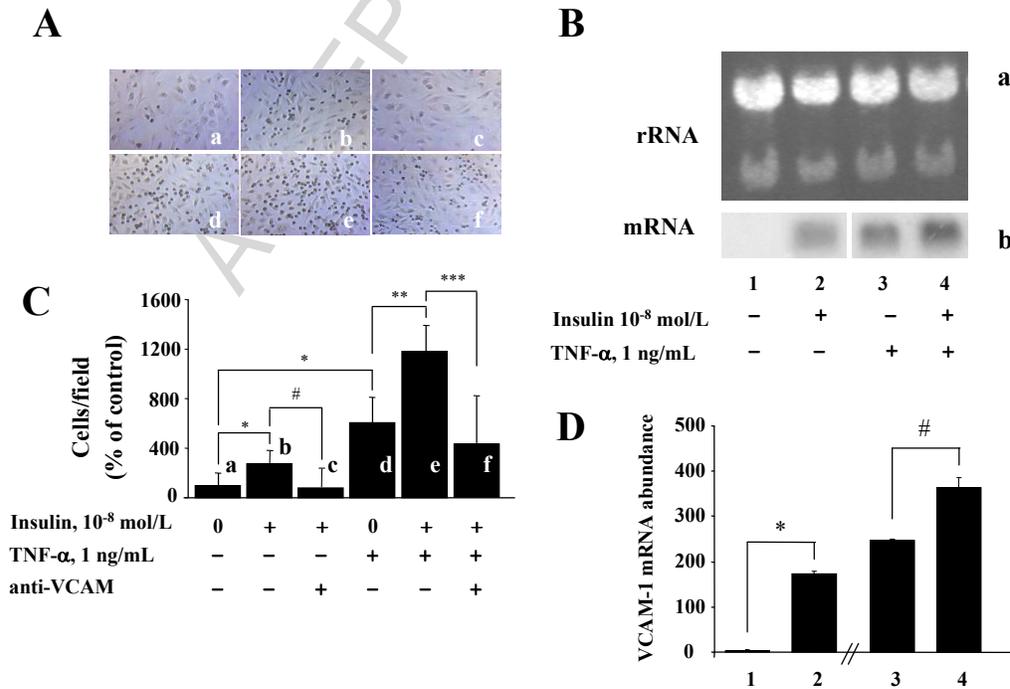


Fig. 2

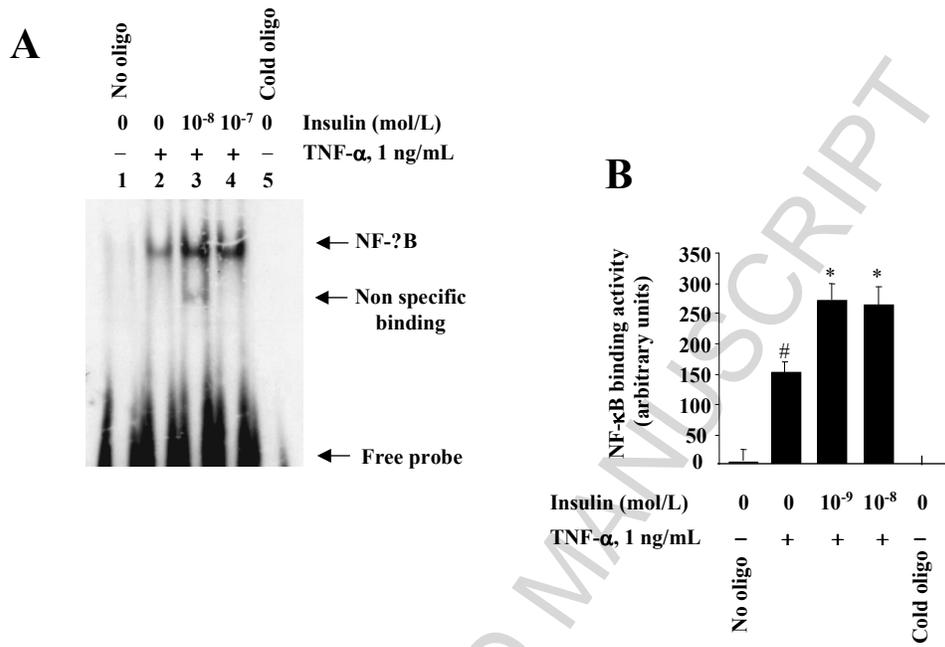


Fig. 3

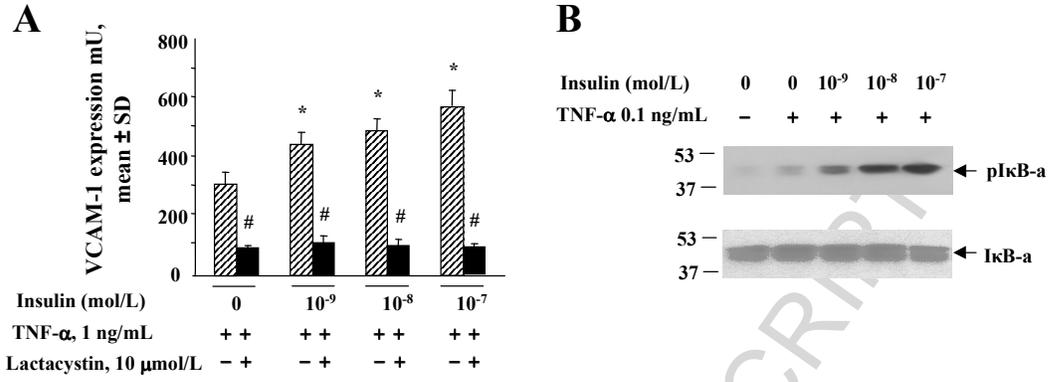


Fig. 4