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Short Communication

Insulin enhances vascular cell adhesion molecule-1 expression in human cultured endothelial cells through a pro-atherogenic pathway mediated by p38 mitogen-activated protein-kinase

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Abstract

Aims/hypothesis. Although hyperinsulinaemia in Type 2 diabetes in states of insulin resistance is a risk factor for atherosclerotic vascular disease, underlying mechanisms are poorly understood. We tested the hypothesis that insulin increases monocyte-endothelial interactions, which are implicated in atherosclerosis.
Methods. We treated human umbilical vein endothelial cells with insulin (10^{-10} to 10^{-7} mol/l) for 0 to 24 h. To dissect potentially implicated signal transduction pathways, we treated endothelial cells with known pharmacological inhibitors of two distinct insulin signalling pathways: the phosphatidylinositol-3′-kinase (PI3′-kinase) inhibitor wortmannin (3×10^{-8} to 10^{-6} mol/l), involved in insulin-induced endothelial nitric oxide synthase stimulation, and the p38 mitogen-activated protein (p38MAP) kinase inhibitor SB-203580 (10^{-7} to 2×10^{-6} mol/l). We measured adhesion molecule expression by cell surface enzyme immunoassays and U937 monocytoid cell adhesion in rotational adhesion assays.
Results. At pathophysiological concentrations (10^{-9} to 10^{-7} mol/l), insulin concentration-dependently induced vascular cell adhesion molecule (VCAM)-1 (average increase: 1.8-fold) peaking at 16 h. By contrast, the expression of intercellular adhesion molecule-1 and E-selectin were unchanged. The effect on VCAM-1 was paralleled by increased U937 cell adhesion. In the absence of cytotoxicity, wortmannin significantly potentiated the effect of insulin alone on VCAM-1 surface expression and monocytoid cell adhesion, whereas SB-203580 (10^{-6} mol/l) completely abolished such effects.
Conclusions/interpretation. These observations indicate that insulin promotes VCAM-1 expression in endothelial cells through a p38MAP-kinase pathway, amplified by the PI3′-kinase blockage. This could contribute to explaining the increased atherosclerosis occurring in subjects with hyperinsulinaemia, or in states of insulin resistance, which feature a defective PI3′-kinase pathway. [Diabetologia (2004) 47:532–536]

Keywords Insulin · Atherosclerosis · Adhesion molecules · Endothelial cells

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Abbreviations: VCAM-1, vascular cell adhesion molecule-1 · ICAM-1, intercellular adhesion molecule-1 · PI3′-kinase, phosphatidylinositol 3′-kinase · MAP, mitogen-activated protein · NO, nitric oxide · EIA, enzyme immunoassays

Macro- and microvascular complications are the major causes of morbidity and mortality in Type 1 and Type 2 diabetes [1]. The pathogenesis of accelerated atherosclerosis in diabetes is not known, but several mechanisms have been implicated. Clinical and epidemiological studies have shown associations between hyperinsulinaemia, accompanying states of insulin resistance, and cardiovascular disease. These are partly independent of related risk factors like hypertriglyceridaemia, low HDL concentrations and hypertension [1]. Compensatory hyperinsulinaemia resulting from insulin resistance increases the production of plasminogen activator inhibitor-1 and various growth

factors and cytokines through pathways dependent on mitogen-activated protein(MAP)-kinase. Contrary to the metabolic IRS-1- and phosphatidylinositol 3′-kinase (PI3′-kinase)-dependent branch of insulin signalling, these MAP-kinase dependent pathways appear to be unaffected in insulin resistance [2]. Insulin also promotes the bioavailability of nitric oxide (NO). It does this by increasing the activity and/or mRNA and protein expression of NO synthase-III [3]. Since NO mostly acts anti-atherogenically [4], the mechanisms by which hyperinsulinaemia and insulin resistance possibly cause diabetic atherosclerosis remain controversial.

Interactions between endothelial cells and peripheral blood leucocytes, particularly monocytes and T-lymphocytes, are involved in atherosclerosis. Several families of endothelial-leucocyte adhesion molecules regulate local leucocyte recruitment to the vessel wall. Of these, vascular cell adhesion molecule(VCAM)-1, a member of the immunoglobulin superfamily, is important for monocyte attachment to the arterial wall [5]. As no unequivocal data on the direct pro-atherogenic role of insulin exist, we investigated the actions of insulin on endothelial cells, testing the hypothesis that insulin modulates interactions between leucocytes and endothelial cells.

Materials and methods

Materials. Human recombinant insulin was purchased from Novo-Nordisk Farmaceutici, Rome, Italy. SB-203580 and wortmannin were purchased from Sigma, St. Louis, Mo., USA.

Cell cultures. Human umbilical vein endothelial cells were harvested enzymatically from umbilical cords with Type II collagenase 0.1 mg/ml, and propagated as described [6]. Purity of cultures (>90%) was evaluated by von Willebrand factor immunostaining. At confluence, cells were re-plated on 1.5% gelatin-coated flasks and used at 20 000 cells/cm^2. Cells were used within passage 4 after primary cultures. The study was approved by our institutional review board and carried out in accordance with the Declaration of Helsinki as revised in 2000. Informed consent was given by persons donating umbilical cords. Cytotoxicity of treatments was monitored by checking the integrity of the monolayer by light microscopy throughout all experiments and by assessing total protein content (amido-black assay) and expression of the endothelial cell constitutive antigen detected by the E1/1 antibody [6] in selected experiments.

Cell surface immunoassays. Assays of cell surface molecules were done 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-1 (ICAM-1) (Ab HU5/3), or a constitutive and non-cytokine-inducible endothelial cell antigen (E1/1) [6], all provided by M.A. Gimbrone Jr (Brigham & Women's Hospital, Boston, Mass., USA).

EIA were carried out by sequential incubations of cell monolayers with saturating concentrations of specific monoclonal antibodies against the target molecule, biotinylated goat anti-mouse IgG (Vector Labs, Burlingame, Calif., USA), and streptavidin-alkaline phosphatase (Vector). The surface expression of each protein was quantified spectrophotometrically at 410 nm after addition of the chromogenic substrate para-nitrophenylphosphate (Sigma) [6].

Preparation of U937 cells and adhesion assays. We obtained U937 cells from American Tissue Culture Collection (Rockville, Md., USA) and grew them as described [6]. For adhesion assays, HUVEC were grown to confluence in six-well tissue culture plates. Assays were done by adding 1 ml of U937 cell suspensions, at 1×10^6 cells/ml, to each monolayer under rotating conditions (63 rev/min) at 21°C. To test VCAM-1-dependence of adhesion, some monolayers were treated with mouse anti-human monoclonal antibody E1/6 against VCAM-1. After 10 min, non-adhering cells were removed by gentle washing with medium 199, and monolayers fixed with 1% paraformaldehyde. The number of adherent cells was assessed by counting eight different high-power fields (0.09 mm^2 /field). Fields for counting adherent leucocytes were randomly located at half-radius distance from the centre of the monolayers.

Statistical analysis. Two-group comparisons were done by the Student's *t* test for unpaired values. Means of three groups or more were compared by ANOVA, and individual differences, in the case of significant *F* values at ANOVA, tested by Scheffé's multiple contrasts. Significance was set at a probability value of *p* less than 0.05.

Results

Insulin induces VCAM-1 expression through a MAP-kinase-dependent pathway and is subjected to tonic inhibition by the PI3′-kinase-dependent pathway. Insulin stimulation for 16 h induced VCAM-1 expression at cell surface EIA. Expression was about 1.8-fold greater on average than in untreated cells ($p<0.01$). Expression of VCAM-1 peaked at 12 to 16 h, remaining steady for 20 to 24 h and slowly decreasing to near zero at 48 h. This stimulatory effect of insulin was concentration-dependent between 10^{-9} and 10^{-7} mol/l (Fig. 1). Under similar conditions, insulin did not affect surface expression of ICAM-1 and E-selectin or any cytotoxicity.

To understand mechanisms underlying the effects of insulin on VCAM-1 expression, we used pharmacological inhibitors (wortmannin and SB-203580) to investigate (i) the PI3′-kinase-Akt pathway, which is primarily involved in the metabolic effects of insulin, and (ii) the MAP-kinase pathway, which contributes to insulin's mitogenic effects (Fig. 1b). In the absence of any cytotoxicity, the inhibition of PI3′-kinase activity by 30 min of pretreatment with 10^{-7} mol/l wortmannin potentiated the effect of insulin on VCAM-1 surface expression at EIA [average increase: 2.2± 0.3-fold, compared with untreated cells, $p<0.01$, more ($p<0.05$) than in cells not treated with the PI3′-kinase inhibitor] (Fig. 1). Wortmannin alone had no stimulating effect. However, 30-min pretreatment with 10^{-6} mol/l

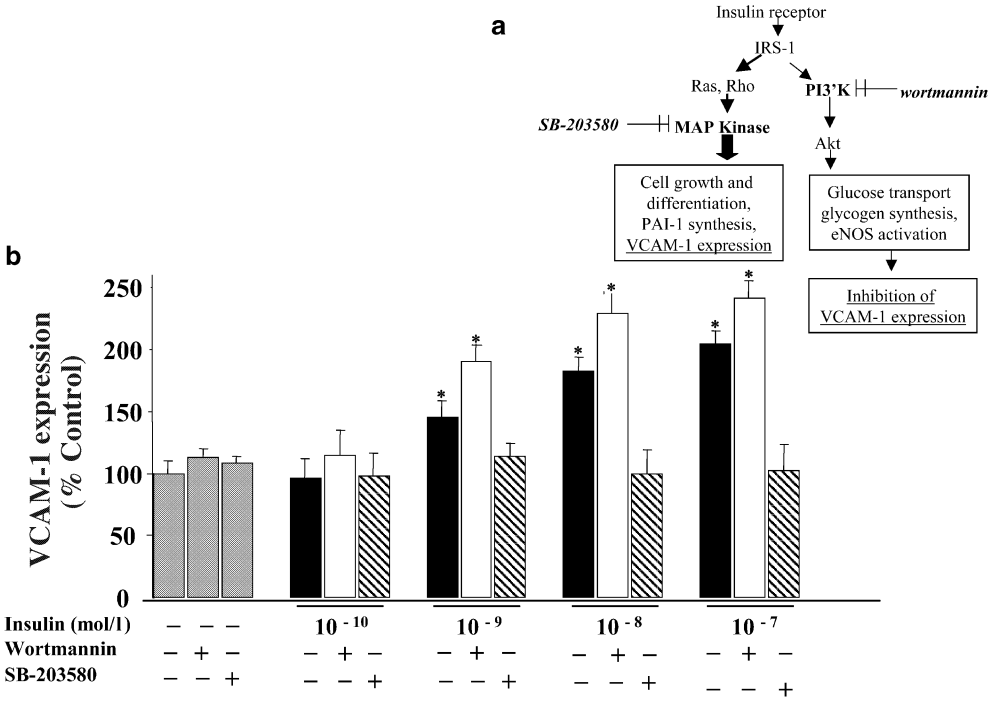

Fig. 1a, b. The opposing effects of p38MAP-kinase and PI3′-kinase block on insulin-induced surface expression of VCAM-1 in endothelial cells. Confluent serum-starved HUVEC were pretreated (**a**) with the p38MAP-kinase inhibitor SB-203580 (10^{-6} mol/l) or the PI3′-kinase inhibitor wortmannin (10^{-7} mol/l) for 30 min, followed by addition of increasing insulin concentrations (10^{-11} to 10^{-7} mol/l) for 16 h over an additional 0–24 h. After incubation, VCAM-1 expression was assessed by cell-surface EIA and expressed in milliunits of optical density. Results are expressed as percent of unstimulated control, and each concentration point is the mean ± SD from three experiments, each consisting of eight replicates per condition. *p<0.05 vs unstimulated control. The inset (**b**) shows a scheme of the pro- and the anti-atherogenic insulin pathways, with the site of action of known inhibitors. PI3′K, phosphatidylinositol 3′-kinase; MAP, mitogen-activated protein; PAI-1, plasminogen activator inhibitor-1; VCAM-1, vascular cell adhesion molecule-1; eNOS, endothelial nitric oxide synthase; EIA, enzyme immunoassays

SB-203580, a specific p38MAP-kinase inhibitor, abolished the stimulatory effects of insulin on VCAM-1 surface levels (Fig. 1).

Insulin increases monocytoid cell adhesion to cultured endothelial cells. Because ICAM-1 was not induced, we examined functional consequences of endothelial treatment with insulin in terms of monocytoid (U937) adhesion to endothelial cells. In agreement with data on VCAM-1 surface expression, treatment of endothelial cells with insulin alone (10^{-8} to 10^{-7} mol/l) for 16 h produced a concentration-dependent increase in U937 cell adhesion (average fold increase 2.3±0.42, compared with untreated cells, p<0.01) (Fig. 2A, a–c). Addition of wortmannin (10^{-7} mol/l), which by itself had no stimulating effect (Fig. 2A, e vs a), potentiated insulin's effect on U937 cell adhesion [average fold induction 4.95±0.91, compared with untreated cells (Fig. 2A, f and h vs a, p<0.01), and 2.15±0.50, compared with insulin treated-cells (Fig. 2A, f and h vs b–c, p<0.05)]. Addition of the p38MAP-kinase inhibitor SB-203580 abolished insulin's effects (Fig. 2A, g and i vs b and c). Adhesion induced by insulin with or without wortmannin was suppressed by around 70% by 30 min pretreatment of endothelial cells with the blocking anti-VCAM-1 monoclonal antibody E1/6, at saturating concentrations (Fig. 2A, d, j, p<0.01).

Discussion

We found that insulin, at concentrations pathophysiologically relevant for insulin resistance states [7], selectively promotes expression of VCAM-1 in human cultured endothelial cells through a MAP-kinase-dependent pathway. We also showed that inhibition of the PI3′-kinase pathway enhances insulin's effect on VCAM-1 expression, uncovering an alternative, opposing, intracellular signal transduction pathway, which could, if prevalent, abrogate insulin's effect on VCAM-1.

Because insulin increases NO bioavailability [3], reduced adhesion molecule expression after insulin treatment was expected. Contrary to expectations, we found that insulin-induced expression of one particular adhesion molecule, VCAM-1, which might partly explain the increased atherosclerosis in conditions of insulin resistance. These observations complement previous findings on acquisition of a pro-atherogenic endothelial cell profile after inhibition of the PI3′-kinase pathway [8].

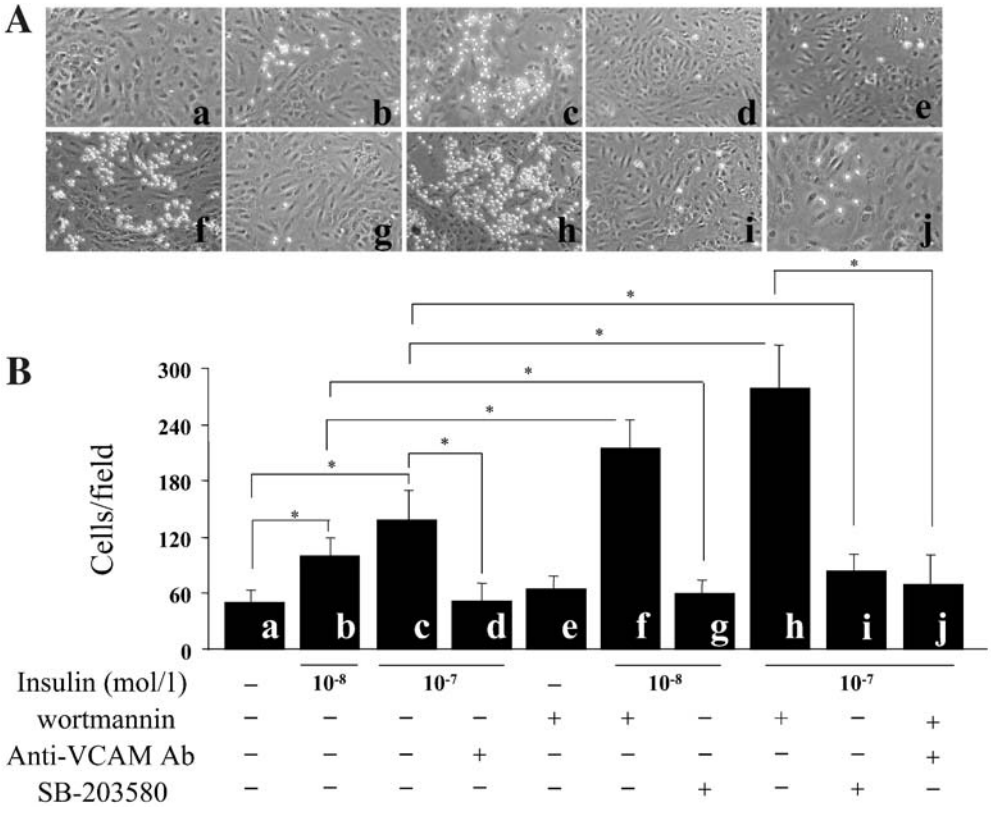

Fig. 2A, B. Insulin +/- wortmannin treatment of HUVEC increases, but the p38MAP-kinase inhibitor SB-203580 inhibits the insulin-induced adhesion of U937 cells in a rotational adhesion assay. HUVEC (**A**) were either untreated (**a**), or treated (**b**, **c**) with insulin alone for 16 h (10^{-8} to 10^{-7} mol/l), or treated with 10^{-7} mol/l wortmannin (**f**, **h**) or with 10^{-6} mol/l SB-203580 (**g**, **i**) for 30 min before insulin was given. HUVEC were also incubated with 10^{-7} mol/l insulin + anti-VCAM-1 IgG (**d**), with 10^{-7} mol/l wortmannin alone (**e**), with 10^{-7} mol/l insulin + 10^{-7} mol/l wortmannin + anti-VCAM-1 IgG (**j**). The increased adhesion with insulin alone (**b**, **c**) or in insulin plus wortmannin (**f**, **h**) is due to VCAM-1, since this was suppressed by saturating concentrations of the blocking anti-VCAM-1 monoclonal antibody E1/6 for 30 min before the assay (**d**, **j**). (**A**) Photographs are randomly chosen high-power fields taken at half-radius distance from the centre of the well in one of three comparative experiments of similar design, showing U937 monocytoid cell adhesion to endothelial cells. Quantitative data are reported in (**B**) expressing the number of U937 cells adhering within a high-power field (0.09 mm^2). Each measurement is the mean ± SD of adhering cells from three experiments, each consisting of eight counts per condition. *$p<0.05$ between groups joined by brackets. VCAM, vascular cell adhesion molecule

Oxidised and/or minimally modified LDL [9] or the AGE found in diabetes [6] are thought to be primary triggers of atherogenesis, before monocyte infiltration in the intima has occurred. None of these, however, are selective inducers of VCAM-1, a molecule clearly more relevant to atherosclerosis than E-selectin or ICAM-1 [5]. Thus, insulin seems to be unique in its ability to induce VCAM-1 selectively.

Previous reports have shown that insulin can increase rolling and adhesion of monocytes to endothelial cells when pretreated with the PI3′-kinase inhibitor wortmannin, but the dependence of this on VCAM-1 had not been tested [8]. One report also described activation of the transcription factor nuclear factor-κB by insulin (consistent with our findings), but not the transcriptional activation of VCAM-1 in endothelial cells exposed to insulin [10], the latter possibly because lower-sensitivity techniques were used. Using a rotational adhesion assay we found that insulin per se (or even more together with PI3′-kinase inhibition by wortmannin) increases monocytoid cell adhesion, an effect related to the increase in VCAM-1 since largely inhibited by the blocking anti-VCAM-1 E1/6 antibody.

In Type 2 diabetes insulin resistance is associated with impaired activation of the IR-IRS-PI3′-kinase pathway, but with normal activation of the MAP-kinase pathway [2]. Recent studies suggest that activations of *Raf*/extracellular signal-regulated kinase and p38MAP-kinase pathways are required for various mitogenic actions of insulin [11]. In agreement with these findings, we report that blocking p38MAP-kinase signalling abolishes insulin's stimulatory effect on VCAM-1 expression. Conversely, inhibition of the PI3′-kinase pathway potentiates this effect, mimicking in vitro the in vivo situation of insulin resistance.

In conclusion, the role found by us for insulin in priming VCAM-1 expression and monocyte-endothelial interactions through activation of the MAP-kinase pathway provides further evidence that hyperinsuli-

naemia is an important mediator in initiation and progression of atherosclerosis in states of insulin resistance.

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