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Short communication

Effects of insulin on in vitro vascular cell adhesion molecule-1 expression and in vivo soluble VCAM-1 release

G. De Mattia[1], **M.C. Bravi** [2], **A. Costanzo**[1], **O. Laurenti** [1], **M. Cassone Faldetta** [2], **A. Armiento**[1], **O. De Luca**[1], **C. Ferri**[1]

[1] University "La Sapienza", Andrea Cesalpino Foundation, Rome, Italy
[2] University of L'Aquila, Department of Internal Medicine, L'Aquila, Italy

Abstract

Aims/hypothesis. To evaluate the effects of insulin on vascular cell adhesion molecule-1 expression by cultured human vascular endothelial cells and soluble vascular cell adhesion molecule-1 release in vivo.
Methods. Human vascular endothelial cells derived from umbilical cord veins were incubated with either insulin (from 10^{-6} to 10^{-9} mol/l) or tumour necrosis factor-α (5 ng/ml) for 6 to 24 h. Plasma soluble vascular cell adhesion molecule-1 concentrations were evaluated in 12 non-insulin-dependent diabetic patients (8 men, 4 women, mean age 47.1 ± 7.7 years) and 12 healthy volunteers matched for age, sex and weight (7 men, 5 women, mean age 42.2 ± 7.2 years) before and after a 2-h euglycaemic hyperinsulinaemic clamp.
Results. Transcriptional activities of nuclear factor-ϰB luciferase and vascular adhesion molecule-1 luciferase statistically significantly increased after incubation with tumour necrosis factor-α. By contrast, a slight increment of nuclear factor-ϰB luciferase (mean: 1.8 ± 0.3 fold) but not of vascular cell adhesion molecule-1 luciferase transcriptional activities were detected in cells stimulated with insulin. Soluble vascular cell adhesion molecule-1 concentrations in cell supernatants increased after tumour necrosis factor-α but not insulin stimulation. In vivo, baseline plasma soluble vascular cell adhesion molecule-1 concentrations were higher ($p = 0.03$) in non-insulin-dependent patients (708.7 ± 97.4 μg/l) than controls (632.1 ± 65.2 μg/l) but were not related to fasting insulin concentrations and did not change during insulin infusion.
Conclusion/interpretation. The increased concentrations of circulating soluble vascular cell adhesion molecule-1 indicates that the vascular endothelium is activated in non-insulin dependent diabetic patients. Our in vitro and in vivo findings show that vascular cell adhesion molecule-1 activation cannot be due to hyperinsulinaemia. [Diabetologia (1999) 42: 1235–1239]

Keywords Insulin, adhesion molecules, endothelium, vascular cell adhesion molecule-1, non-insulin-dependent diabetes mellitus.

Although hyperinsulinaemia is an independent risk factor for the development of atherosclerotic lesions [1], mechanisms underlying the atherogenetic actions of insulin are largely unclear. Recent studies have shown that insulin exerts various endothelial effects, such as an increase of endothelin-1 and nitric oxide production [2]. These findings are of particular relevance and suggest insulin might also exert other endothelial effects which, in turn, could trigger and maintain the atherogenetic process.

In this context, vascular cell adhesion molecule(*VCAM*)*-1* is an endothelial adhesin whose up regulation is the most important initiating event in atheroma formation [3]. In spite of this, whether or not insulin might influence *VCAM-1* expression by the human vascular endothelium is notknown.

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Corresponding author: G. De Mattia, MD, Università "La Sapienza", Cattedra di I Clinica Medica, Fondazione Andea Cesalpino, I-00161 Roma, Italy
Abbreviations: VCAM-1, Vascular cell adhesion molecule-1;HUVEC, human umbilical vein endothelial cell; NF-ϰB, nuclear factor-ϰB

The aim of this study was to investigate the effects of insulin on *VCAM-1* expression by human vascular endothelial cells derived from human umbilical cord veins (*HUVECs*). In this regard, cytokine-induced *VCAM-1* expression is tightly transcriptionally regulated and requires the activation and nuclear translocation of nuclear factor-$\varkappa$B (NF-$\varkappa$B), i.e. a cytoplasmic multisubunit transcription factor which is essential to induce *VCAM-1* promoter gene activation as well as interferon-β regulatory factor-1 [4]. Activation of NF-$\varkappa$B by insulin has been reported in Chinese hamster ovary cells overexpressing wild-type insulin receptors [5]. Thus, in our experiments we used two different promoter-reporter gene constructs (an NF-$\varkappa$B and a *VCAM-1* reporter) to study the effects of insulin on *VCAM-1* transcriptional activation. In addition, because expression of *VCAM-1* associated with membrane by *HUVECs* which have been activated by cytokine is associated with the release of its soluble form, soluble *VCAM-1* concentrations are usually considered as a marker of *VCAM-1* expression [6]. Consequently, we also evaluated the effects of insulin on soluble *VCAM-1* release by cultured *HUVECs* as well as plasma soluble *VCAM-1* concentrations in non-insulin dependent diabetic patients and healthy volunteers, before and after 2-h euglycaemic hyperinsulinaemic clamp studies.

Methods

In vitro studies

Cell cultures. Within 1 h from labour umbilical cord vein was incubated with 0.2 % collagenase II at 37 °C for 30 min. Detached cells were flushed out by rinsing the cord vein thoroughly with medium199. Cells were pelleted and resuspended in Dulbecco's histone acetyl transferase medium before seeding on 35-mm wells (5 ml) containing this medium supplemented with fetal calf serum (15 %), penicillin (100 u/ml), streptomycin (100 μg/ml), L-glutamine (2 mmol/l) and endothelial cell growth factor (10 ng/ml) at 37 °C in a humidified incubator (5 % carbon dioxide in air). After 1 week of incubation, cells were resuspended in medium199 and then seeded again on gelatin-coated 35-mm wells. After 5–6 days, cells reached confluency and the purity of cultures (> 99 % endothelial cells) was evaluated by fluorescence activated cell sorting after labelling with CD31.

Plasmids. The promoter-reporter gene constructs used in these studies included : NF-$\varkappa$B which contains two $\varkappa$B sites from the IgG kappa enhancer upstream of a luciferase encoding cDNA was a kind gift from Dr M. Karin (Dept of Pharmacology, UCSD, San Diego, Calif., USA) and *VCAM*-luciferase, kindly donated by Dr J.M. Redondo (Immunology Unit, Princess Hospital Madrid, Spain), which contains the –519/ + 120 fragment of the VCAM-1 regulatory region upstream of a luciferase encoding cDNA in the pXP2-luciferase plasmid.

Transfections and measurements of reporter gene expression. Human umbilical vein endothelial cells were used after 2 to 4 passages. In particular, 1.8×10^5 *HUVECs* were plated in 35-mm plates the day before transfection and transfected the next day with DNA using a cationic lipid-based reagent (Superfect, Qiagen, Germany). Confluent *HUVEC* monolayers were then stimulated with human recombinant insulin (from 10^{-6} to 10^{-9} mol/l) or human recombinant tumour necrosis factor(TNF)-α (5 ng/ml) (Genzyme, Cambridge, Mass., USA). After 6 and 16 h, cell lysates were then assayed for luciferase activity using the Promega reporter assay system (Promega, Madison Wis., USA). Luciferase activity was measured in duplicate using a Berthold (Schwarzwald, Germany) model LB9501 luminometer.

Measurements of soluble VCAM-1 concentrations in cell supernatants. Human umbilical vein endothelial cell supernatants (50 μl) were taken before and after incubation with insulin (from 10^{-6} to 10^{-9} mol/l) for various times up to 24 h. Soluble *VCAM-1* concentrations were then measured in *HUVEC* supernatants by ELISA (R & D Systems, Minneapolis, Minn., USA).

In vivo studies

This study was conducted in 12 non-insulin-dependent diabetic outpatients (8 men and 4 women, mean age 47.1 ± 7.7 years) in good metabolic control. The latter was achieved and maintained by diet and glibenclamide, 5 mg twice daily. Exclusion criteria were: age under 25 and over 55 years, pregnancy, use of birth control pills, personal history of cerebrovascular or cardiovascular diseases, concomitant diseases, allergic diatesis, hypertension, obesity, smoking, microalbuminuria, hyperlipidaemia, serum creatinine more than 100 μmol/l, overt atherosclerotic lesions, acute recent (< 3 months) diseases. A group of 12 healthy volunteers (7 men and 5 women, mean age 42.2 ± 7.2 years) served as a control.

After informed consent had been given and 2 weeks on a weight-maintaining diet, blood samples for measurements of plasma soluble *VCAM-1* concentrations were drawn from an antecubital vein. The samples were taken after the subjects had been in a supine position and an intravenous catheter had been inserted in a hand vein for 1 h. The vein was cannulated in retrograd fashion and the catheter kept pervious by saline infusion (0.2 ml/min). On the same occasion, blood samples for routine haematochemical check, plasma glucose and insulin concentrations were taken.

After baseline blood samplings, non-insulin-dependent diabetic patients and control subjects underwent euglycaemic hyperinsulinaemic clamp studies. Blood samples for plasma soluble *VCAM-1* concentrations were taken at 60 and 120 min during insulin infusion and then after 60 min recovery. Throughout the test, plasma glucose concentrations were measured at 5-min intervals and maintained at euglycaemic concentrations by a variable infusion of 20 % D-glucose solution.

Plasma soluble VCAM-1 concentrations were assessed by ELISA (R & D Systems). Plasma insulin concentrations were assessed by RIA (Ares-Serono, Milan, Italy). Plasma glucose concentrations were measured by the glucose oxidase method.

Statistical analysis. Differences among groups were tested for significance by one-way analysis of variance followed by the Bonferroni's test and the Newman-Keuls test for pairwise comparisons. Multiple comparisons were analysed by the analysis of variance and then by analysis for adjusting the significance level. Linear regression and correlation were used to evaluate relations between variables. Descriptive variables were tested for significance by the chi–squared method. A p

value less than 0.05 was considered less than significant. All data are means ± SD. In vitro data represents the mean of four different experiments.

Results

In vitro data. To determine whether insulin induces a NF-ϰB driven reporter gene expression, *HUVECs* were transiently transfected with an NF-ϰB-luciferase reporter and then treated with increasing concentrations of insulin for 6 or 16 h. Cells stimulated by TNF-α were used as a control.

Insulin induces only a 1.8 ± 0.3-fold increase in NF-ϰB transcription activities in cultured *HUVECs* (Fig. 1A). When the effect of insulin on the *VCAM-1* reporter gene construct was tested, insulin was unable to induce transcriptional induction of the reporter (Fig. 1B). Further, insulin was also unable to increase soluble *VCAM-1* concentration in *HUVEC* supernatants at all times up to 24 h (Fig. 1C). As expected, TNF-α strongly increased NF-ϰB transcription activities (mean increment: 13.2 ± 1.4–fold), *VCAM-1* reporter gene construct (mean increment: 6.2 ± 1.0–fold), and soluble *VCAM-1* release in *HUVEC* supernatants (control *HUVECs*, 220 ± 15 μg/l after 24 h; TNF-α HUVECs stimulated by: 1375 ± 95 μg/l after 24 h).

In vivo data. The general characteristics of the study group are given in Table 1. As is shown, non-insulin-dependent diabetic patients had good metabolic control but had manifested higher fasting glucose ($p < 0.0001$), insulin ($p < 0.001$) concentrations and glycated haemoglobin ($p < 0.0001$) values than healthy subjects.

The baseline concentration of soluble *VCAM-1* was higher ($p = 0.03$) in non-insulin-dependent diabetic patients (708.7 ± 97.4 μg/l) than control subjects (632.1 ± 65.2 μg/l). Circulating soluble *VCAM-1* concentrations were not related to other variables. No statistically significant changes in plasma soluble *VCAM-1* concentrations were observed during the clamp studies (Fig. 1D).

Discussion

Our study showed increased circulating *VCAM-1* concentrations in adult non-obese, non-dyslipidaemic, normotensive, non-insulin-dependent diabetic patients. It failed to shown, however, any relation between *VCAM-1* regulatory mechanisms and hyperinsulinaemia. Indeed, although non-insulin-dependent diabetic patients were greatly hyperinsulinaemic, no correlations were found between plasma insulin and soluble *VCAM-1* concentrations at fast as well as during 2-h euglycaemic insulin infusions. Further, incu-

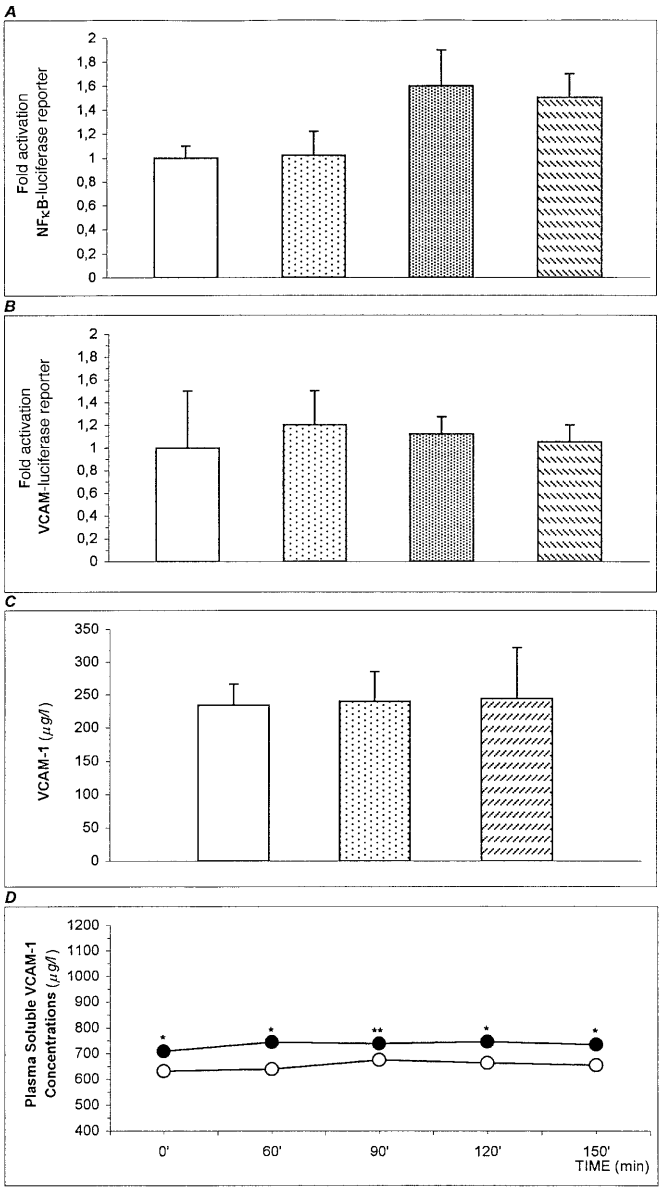

Fig. 1. A Nuclear factor-ϰB transcriptional activity modifications after stimulation with insulin. Data are means ± SD of four different experiments. **B** Effects of insulin on luciferase activity in cultured human vascular endothelial cells. Data are means ± SD of four different experiments. **C** Bars show soluble VCAM– 1 concentration (means ± SD of four experiments) in supernatants from cultured HUVECs incubated alone or with insulin over 24 h. **D** Effect of 2-h euglycaemic hyperinsulinaemia on plasma soluble vascular cell adhesion (VCAM)-1 concentrations in 12 non-insulin-dependent diabetic patients and 12 control subjects. Standard deviations were omitted for clarity. At all times, plasma soluble VCAM-1 concentrations were higher in non-insulin-dependent diabetic patients than controls ($_{*}$ p = 0.03; $_{**}$ p = 0.04). Panels A, B and C: □ control; ▨ Insulin 10^{-6} ; ▨ Insulin 10^{-7}; ▨ Insulin 10^{-9} ; Panel D: ● non-insulin-dependent diabetic patients ○ control subjects

Table 1. General characteristics of the study group

Variable	Non-insulin dependent diabetic patients (n = 15)	Control subjects (n = 10)	$P <$
Sex (male/female)	8/4	7/5	NS
Fasting glucose (mmol/l)	5.3 ± 0.4	4.3 ± 0.3	0.0001
Fasting insulin (pmol/l)	138.6 ± 48.2	87.2 ± 11.4	0.001
Glycated haemoglobin (%)	6.1 ± 0.3	4.5 ± 0.7	0.0001
Body mass index (kg/m^2)	24.0 ± 0.2	23.2 ± 0.4	NS
Duration of disease (years)	4.8 ± 1.9	–	–
Serum cholesterol (mmol/l)	4.2 ± 0.5	3.9 ± 0.7	NS
Serum triglycerides (mmol/l)	1.6 ± 0.2	1.4 ± 0.2	NS
Systolic blood pressure (mm Hg)	118.8 ± 6.1	115.6 ± 5.1	NS
Diastolic blood pressure (mm Hg)	79.6 ± 6.1	78.4 ± 4.9	NS

bation of *HUVECs* with insulin (from 10^{-6} to 10^{-9} mol/l) for various times up to 24 h was not able to increase NF-$\varkappa$B and VCAM-1 transcriptional activities and soluble *VCAM-1* secretion.

Our in vitro data do not completely agree with recent findings showing a 3.5–fold NF-$\varkappa$B induction in Chinese hamster ovary cells after insulin stimulation [5]. Ovary cell transfectants stably overexpressing wild-type insulin receptors were, however, used in that study [5]. Thus, discrepancies between our and previous data [5] simply reflects differences in cellular systems, i.e. in insulin sensitivity between *HUVECs* and transfected ovary cells. Accordingly, NF-$\varkappa$B was not stimulated by insulin in parental, i.e. untransfected and therefore not overexpressing insulin receptors, Chinese hamster ovary cells [5]. Our data seems also to be in contrast with recent data showing that NF-$\varkappa$B induced *mdr1 b*, a membrane-bound P-glycoprotein, expression in rat hepatoma cells stimulated by insulin [7]. After insulin stimulation NF-$\varkappa$B alone was not, however, sufficient to induce *mdr1 b* transcriptional activation [7]. Thus, other transcriptional factors must be activated by insulin in rat hepatoma cells to induce *mdr1 b* transcription.

In our in vivo findings, the increased concentration of circulating soluble *VCAM-1* indicated that membrane-bound *VCAM-1* was up regulated and the vascular endothelium was activated in non-insulin-dependent diabetic patients. Despite these latter patients being greatly hyperinsulinaemic, insulin was not responsible for the observed endothelial activation, as indicated by the lack of effects of insulin infusion on plasma soluble *VCAM-1* concentrations. Consistent with this interpretation, increased plasma soluble *VCAM-1* concentrations, that were unrelated to fasting insulin concentrations, have already been described in non-insulin-dependent [8] and insulin-dependent diabetic patients [9]. Further, plasma soluble *VCAM-1* negatively correlated with intraerythrocytic glutathione concentrations and decreased after *N*-acetyl-L-cysteine was given orally [8]. Therefore, the current findings support the previous hypothesis [8] that an early up regulation of *VCAM-1* is present in non-insulin-dependent diabetic patients due to increased oxidative stress rather than hyperinsulinaemia.

Our study shows that plasma soluble *VCAM-1* concentrations are increased in non-insulin-dependent diabetic patients without complications and in good metabolic control. In addition, we clearly showed that insulin was not able to induce *VCAM-1* up regulation in vitro and hyperinsulinaemia was not responsible for the increased soluble *VCAM-1* release in vivo. Thus, other mechanisms must be active in non-insulin-dependent diabetic patients without complications and these lead to earliest endothelial changes. In this context, circulating concentrations of soluble *VCAM-1* but not of other endothelial adhesion molecules, i.e. intercellular adhesion molecule-1, E-selectin and P-selectin and thrombomodulin correlated with the extent and severity of atherosclerosis in humans [10].

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