Insulin and insulin-like growth factor-I: two of a kind in the development of cardiovascular disease?

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Chapter 4

Endothelial insulin receptor expression in human atherosclerotic plaques: linking micro- and macrovascular disease in diabetes?


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ABSTRACT

Objective
Exogenous insulin use in patients with type 2 diabetes (DM2) has been associated with an increased risk of cardiovascular events. Through which mechanisms insulin may increase atherosclerotic plaque vulnerability is currently unclear. Because insulin has been suggested to promote angiogenesis in diabetic retinopathy and tumors, we hypothesized that insulin enhances intra-plaque angiogenesis.

Methods
An in-vitro model of pathological angiogenesis was used to assess the potential of insulin to enhance capillary-like tube formation of human microvascular endothelial cells (hMVEC) into a three dimensional fibrin matrix. In addition, insulin receptor expression within atherosclerotic plaques was visualized in carotid endarterectomy specimens of 20 patients with carotid artery stenosis, using immunohistochemical techniques. Furthermore, microvessel density within atherosclerotic plaques was compared between 68 DM2 patients who received insulin therapy and 97 DM2 patients who had been treated with oral glucose lowering agents only.

Results
Insulin, at a concentration of $10^{-8}$M, increased capillary-like tube formation of hMVEC 1.7 fold ($p<0.01$). Within human atherosclerotic plaques, we observed a specific distribution pattern for the insulin receptor: insulin receptor expression was consistently higher on the endothelial lining of small nascent microvessels compared to more mature microvessels. There was a trend towards an increased microvessel density by 20% in atherosclerotic plaques derived from patients using insulin compared to plaques derived from patients using oral glucose lowering agents only ($p=0.05$).

Conclusion
Exogenous insulin use in DM2 patients may contribute to increased plaque vulnerability by stimulating local angiogenesis within atherosclerotic plaques.
INTRODUCTION

Administration of exogenous insulin has significantly improved life expectancy in patients with diabetes. However, epidemiologic surveys have recently suggested that insulin may also increase the risk of cardiovascular events. A post-hoc analysis of the DIGAMI 2 trial revealed that chronic insulin use in patients with diabetes mellitus type 2 (DM2), who survived a myocardial infarction, is associated with increased risk of re-infarction or stroke.\(^1\) Similarly, other studies within large administrative databases also indicated that insulin use is associated with an increased cardiovascular event rate.\(^2-4\) Moreover, increasing dosages of exogenous insulin seem to have an incremental impact on cardiovascular mortality: the higher the cumulative insulin exposure, the higher the risk of dying from cardiovascular disease.\(^5\) Mechanistically, the adverse effects of insulin on cardiovascular outcome have been attributed to adverse actions of insulin on the vessel wall, comprising increased adhesion molecule expression on endothelial cells, increased transendothelial migration of leukocytes\(^6,7\), stimulation of smooth muscle cell (SMC) proliferation,\(^8,9\) and pro-inflammatory effects.\(^10\) If additional mechanisms as to how insulin can increase plaque vulnerability are elucidated, it might become easier to distinguish between patients in whom high insulin doses will increase plaque vulnerability and patients in whom insulin treatment is safe with respect to progression of atherosclerosis.

Angiogenesis within atherosclerotic plaques is involved in progression of atherosclerosis.\(^11\) The first steps of intra-plaque angiogenesis are characterized by migration and proliferation of endothelial cells from pre-existing microvessels. This process results in the formation of a new lumen and is also referred to as capillary-like tube formation. Subsequently, these newly formed capillaries mature into microvessels. Due to disturbed functionality of the endothelial lining of these intra-plaque microvessels, high microvessel density predisposes to an increased risk of intra-plaque hemorrhage and plaque rupture.\(^12,13\) In earlier studies, insulin has been suggested to promote angiogenesis in diabetic retinopathy and tumors.\(^14-16\) However, whether insulin contributes to angiogenesis within atherosclerotic plaques is currently unknown.

In the present study, we hypothesized that insulin enhances intra-plaque angiogenesis. Specifically, we aimed to answer the following questions: 1) Does in-vitro stimulation of human microvascular endothelial cells with insulin enhance capillary-like tube formation? 2) Are insulin receptors (IR) present on endothelial cells within human atherosclerotic plaques? 3) Is microvessel density within atherosclerotic plaques higher in patients with DM2 using insulin than in those using oral glucose lowering agents only?
MATERIALS AND METHODS

1. In-vitro angiogenic sprouting assay

Cell culture
Human microvascular endothelial cells (hMVEC) from foreskin were isolated, cultured and characterized as previously described by van Hinsbergh et al.17 hMVEC were cultured in Medium 199 supplemented with 10% (v/v) heat-inactivated human serum (HuSi), 10% (v/v) heat-inactivated newborn calf serum (NBCSi), 100 IU/ml penicillin, 100 mg/mL streptomycin (pen/strep), 3.75 μg/ml endothelial cell growth factor (ECGF, crude extract from bovine brain), 5 IU/ml heparin and 2 mM L-glutamine. Confluent cells were washed, trypsinized (0.05% trypsin) and seeded at 1:3 density. Cells were cultured on 1% gelatin coated plates at 37°C in a water-saturated atmosphere of 95% air and 5% CO2. hMVEC were used until passage 10. The use of hMVEC was approved by the ethical committee of the VU University Medical Center, Amsterdam, the Netherlands.

Capillary-like tube formation of hMVEC
Human fibrin matrices were prepared with 2 mg/ml fibrinogen (Calbiochem, La Jolla, CA, USA) and 0.1 U/ml thrombin (Organon, Boxtel, the Netherlands) in Medium 199 containing pen/strep. 100 μl mixture was added to the wells of 96-well plates. After clotting at 37°C, the fibrin matrices were soaked with Medium 199 supplemented with pen/strep, 10% HuSi, 10% NBCSi and 2 mM L-glutamin for 16 hr at 37°C to inactivate thrombin. Confluent hMVEC were seeded on the fibrin matrices. Six hours after seeding, the cells were stimulated in serum containing M199 with the combination of 10 ng/mL basic fibroblast growth factor (bFGF) and 10 ng/mL tumor necrosis factor alpha (TNF-α; Sigma Aldrich, St Louis, MO, USA) with or without insulin (bovine, Sigma Aldrich, St Louis, MO, USA) (10⁻⁸-10⁻⁷M). Every second day, the medium was removed and replaced by fresh stimulation medium. Within all experiments each condition was studied in triplicate. After 7-9 days the formation of tubular structures was analyzed by phase-contrast microscopy18 and total tube length of structures of four randomly chosen microscopic fields was measured using a Q-imaging camera connected to a computer with Optimas image analysis software (Media Cybernetics, Bethesda, MD, USA), and expressed as mm/cm².

2. Insulin receptor expression on microvessels within atherosclerotic plaques

Tissue samples
In order to assess IR expression in atherosclerotic plaques we randomly selected 20 human carotid endarterectomy specimens from the vascular tissue bank of the Academic Medical Center, Amsterdam, which contains systematically collected surgical resection specimen of arteries and veins from patients with peripheral- aortic- and carotid artery disease. For this study we used carotid endarterectomy specimens containing atherosclerotic plaques that had been routinely fixed in buffered formalin and were embedded in paraffin. Insulin receptor
expression within the plaques was examined by single and double immunoenzyme staining. Collagen IV expression was assessed in carotid endarterctomy specimen of 6 patients.

**Single immunohistochemistry for IR and IGF-1 receptor**
Paraffin tissue sections of 4 mm thickness were dewaxed in xylene and re-hydraded via graded alcohols. Endogenous peroxidase activity was blocked with methanol + 0.3% peroxide (20 min, room temperature). Heat-induced epitope retrieval (HIER) for tissue pretreatment was applied using Tris-EDTA pH9.0 (20 min at 98°C) in a Pretreatment Module (Thermo/LabVision, Fremont, CA). For single stainings we used IR antibody (Clone CT-3, 1mg/ml, 60 minutes incubation at room temperature) and IGF-1 receptor antibody (clone Rb IgG, 0.25mg/ml, overnight incubation at 4°C). Both antibodies were purchased from Millipore/Chemicon, Temecula, CA, USA. A three-step polymer detection system was applied (ImmunoLogic, Duiven, Netherlands), using a post antibody blocking step (15 min, room temp) followed by an anti-rabbit/mouse/rat horseradish peroxidase (HRP)-conjugated polymer. HRP activity was visualized in brown using Bright DAB (ImmunoLogic). Negative controls consisted of concentration-matched isotype controls (Dako).

**Double Immunohistochemistry**
Immuno double staining combining IR antibody in red with SMA, CD31, CD3 or CD68 antibodies in blue was performed with the sequential double alkaline phosphatase (AP) method as previously described and summarized in supplemental table 1.19 In brief: immunostaining started with the detection of SMA, CD31, CD3 or CD68 antibody with an appropriate Bright Vision anti-mouse or anti-rabbit alkaline phosphatase (AP)-conjugated polymer (ImmunoLogic, Duiven, Netherlands). AP activity was visualized in blue using Vector Blue (Vector Labs, Burlingame, CA). To abolish cross-reaction between first and second primary mouse antibody, a 10 minute heat step (98°C) was performed in between, using Tris-EDTA pH9.0 to remove the first set of immunoreagents, but leaving the blue AP reaction product unchanged. IR mouse antibody was next detected with anti-mouse AP-conjugated polymer and visualized in red using Vector Red (Vector Labs). No nuclear counterstain was applied.

Immuno double staining combining IR antibody in brown with Collagen IV or CD34 antibodies in red was performed as described in supplemental table 1. First, IR antibody was detected with a Bright Vision anti-mouse HRP-conjugated polymer and visualized with Bright DAB. Secondly, after a heat-step, CD34 antibody was detected with Bright Vision anti-mouse AP-conjugated polymer and visualized in red using Vector Red. Collagen type IV antibody required pepsin digestion treatment after this second heat-step (supplemental table 1). The DAB reaction product at the end of the first staining sequence ensures effective shielding, preventing cross-reaction of the collagen type IV antibody and subsequent Bright Vision anti-mouse AP-conjugated polymer with the first staining sequence.20-21 A weak hematoxylin (1:10 diluted, 2 min) nuclear counterstain was applied. Controls consisted of half-double staining experiments, omitting one of the primary antibodies.

All sections were organically mounted with VectaMount (Vector).
Spectral imaging
Double staining slides were analyzed with the Nuance spectral imaging system (Caliper Life Sciences/ Cambridge Research Instrumentation, Woburn, MA, USA). Spectral imaging data cubes were taken from 420-720 nm at 20 nm intervals and analyzed with the Nuance™ 2.8 software. Spectral libraries of single brown (DAB), single-red (Vector Red), single-blue (Vector Blue), and single-hematoxylin were obtained from the control slides. The resulting library was applied to the double stained slides for spectral unmixing into individual component images, representing the localization of each of the reaction products. Fluorescent-like images composed of pseudo-colors showing co-localization, as well as an exclusive image of co-localization, were created with the Nuance 3.0 software.

3. Microvessel density within atherosclerotic plaques
In order to compare microvessel density in atherosclerotic plaques from DM2 patients on insulin therapy with plaques from DM2 patients without insulin therapy we selected human endarterectomy specimens from the ATHERO-EXPRESS (ATHEROsclerotic plaque EXPRESSion) study. This is an ongoing longitudinal multicenter study in the Netherlands which enrolls patients undergoing carotid endarterectomy. All patients undergoing carotid endarterectomy in the participating centers (St. Antonius Hospital Nieuwegein and University Medical Center Utrecht) were considered eligible for inclusion. The medical ethics board of the participating hospitals approved the study and all participants provided written informed consent. The definition of diabetes was restricted to those cases requiring medical treatment. We analyzed 165 patients with carotid artery stenosis and concomitant DM2. Sixty-eight of them had been on insulin therapy and 97 had not been on insulin therapy. Plaque characteristics were determined as previously described. For semi-quantitative analyses of collagen, macrophage, SMC and calcification content, two categories were made (no/minor and moderate/heavy).

All sections were stained for CD34 using a mouse anti-CD34 monoclonal antibody (1μg/ml; Immunotech, Marseille, France) as primary antibody. Subsequently, sections were stained with Powervision poly AP-anti-mouse IgG [Immunologic, Duiven, The Netherlands], developed in New Fuchsin Alkaline Phosphatase Substrate and counterstained with hematoxylin. Using a magnification of x400, CD34-positive microvessels were counted in three areas of the plaque with the highest microvessel density (so called hotspots). Subsequently, the average microvessel density per hotspot was calculated for each plaque.

Statistical analysis
Data of in-vitro experiments are presented as mean +/- SEM. Statistical differences in capillary-like tube formation were determined by paired t-test, using Prism 4 (GraphPad, San Diego, CA, USA). Patients’ and plaque characteristics are presented as mean +/- SD (for normally distributed parameters), median (IQR) (for not-normally distributed parameters), or as percentage. Statistical differences between groups for normally distributed continuous variables were calculated using an unpaired t-test. For not-normally distributed parameters Mann-Whitney U
test and for categorical data a χ²-test was used. Data were analyzed using SPSS 16.0 for Windows (SPSS Inc, Chicago, Illinois). P values below 0.05 were considered statistically significant.

RESULTS

1. In-vitro angiogenic sprouting assay

*Insulin enhances capillary-like endothelial tube formation*

To determine whether insulin has a stimulatory effect on the formation of microvessels, we used an in-vitro angiogenesis assay. Human microvascular endothelial cells (hMVEC) seeded on a 3D fibrin matrix were stimulated with bFGF and TNF-α for 7 to 9 days to induce capillary-like tube formation. Addition of insulin (in a range of 10⁻⁹M–10⁻⁷M) to the medium increased capillary-like endothelial tube formation dose dependently (figure 1A and 1B). Insulin alone did not induce capillary-like tube formation. Combining the results of 11 separate experiments (using 3 different hMVEC donors) revealed that stimulating with 10⁻⁸M insulin combined with bFGF and TNF-α significantly increased capillary-like tube formation 1.7 fold compared to stimulation with bFGF and TNF-α alone (figure 1c).

Figure 1. In-vitro angiogenic sprouting assay.

hMVEC were cultured on top of a 3D fibrin matrix in M199 supplemented with 10% human serum and 10% NBCSi. Capillary-like tube formation was induced by stimulating hMVEC with 10 ng/mL TNF-α and 10ng/mL bFGF alone (control) or in combination with different doses of insulin. A. Representative phase contrast pictures after 7 days of culture showing increased capillary-like tube formation in the presence of insulin. Scale bar is 0.5mm. B/C. Capillary-like tube formation quantified with Optimas software. B: Results of a single experiment using one donor (each condition in triplicate) showing that insulin increases capillary-like tube formation dose dependently. C. Combined results of 11 experiments using 3 donors. † p=0.06; * p<0.01 ** p<0.001 compared with control (c).
Increased insulin receptor expression on small microvessels within human atherosclerotic plaques

In total we studied IR expression in 20 carotid endarterectomy specimens containing atherosclerotic plaques (stage III to VI, according to the American Heart Association classification). All sections showed positive staining for IRs, especially on microvessels within the plaque. More in detail, we found a specific distribution pattern with regard to IR expression, consisting of increased expression on small microvessels compared with larger microvessels (figure 2). These IR positive microvessels were located predominantly at the border between the media and the lipid core, or in the lipid core. Additional stainings for insulin like growth factor (IGF)-1 receptors did not reveal such a distribution pattern on microvessels as observed for the IR. IGF-1 receptors were expressed both on small and large microvessels and also on other cell types within atherosclerotic plaques (data not shown).

2. Insulin receptor expression on microvessels within atherosclerotic plaques

Insulin receptors co-localize with endothelial cells on small microvessels

To assess whether IRs were indeed present on endothelial cells of microvessels, double immuno-staining was performed. We found a marked co-localization of IRs with CD31 positive (endothelial) cells, especially on small microvessels (figure 3b and c). Co-localization of IRs with other components within the atherosclerotic plaque was less evident. Macrophages (characterized by CD68) showed focal, minimal IR expression, whereas T-cells (characterized by CD3) showed no IR expression. Immuno-enzyme staining for IRs and smooth muscle actin (SMA) revealed a variable expression of IRs on smooth muscle actin positive cells; insulin receptors were predominantly present on myofibroblastic cells which were located in the intima (figure 3k, 3l) and not on smooth muscle cells in the media layer (figure 3n, 3o).

Insulin receptors are mainly present on nascent, collagen IV negative, microvessels

We further characterized the IR expressing microvessels within human atherosclerotic plaques by staining sections for collagen IV, which is considered a surrogate marker of vessel maturity as it delineates the endothelial basement membrane. We found that microvessels that expressed IRs had in general no co-expression of collagen IV. In contrast, IR negative microvessels broadly showed clear collagen IV expression (figure 4).

3. Microvessel density within atherosclerotic plaques

Microvessel density is higher in atherosclerotic plaques from DM2 patients that use insulin than in plaques from DM2 patients that use only oral glucose lowering agents.

To determine whether insulin use in DM2 patients is associated with local angiogenesis within atherosclerotic plaques, microvessel density was analyzed within carotid endarterectomy specimens obtained from DM2 patients using insulin (n=68) or oral glucose-lowering agents only (n=97) (ATHERO-EXPRESS biobank). No statistically significant differences in cardiovascular risk factors were observed between these two groups (table 1a). Additionally, we did not find statistically significant differences in atherosclerotic plaque characteristics (table 1b). For each
patient microvessel density was quantified by calculating the average number of microvessels in three hotspot areas within the plaque, as explained in the methods section. As depicted in figure 5, we found a clear trend towards an increased microvessel density in plaque tissues of insulin users compared with those using oral glucose lowering agents only (average number per hotspot: 8.3 (± 0.5) and 10.0 (±0.8) respectively; median: 7.3 (IQR 5.0-10.3) and 9.2 (IQR 6.4-11.7) respectively; p=0.05).

When we grouped all 165 patients into quartiles according to their microvessel density, we observed a linear-by-linear association between microvessel density and the presence of intra-plaque hemorrhages (supplemental figure 1). The percentage of intra-plaque hemorrhages was three times higher among patients in the highest microvessel density quartile compared with patients in the lowest microvessel density quartile (p-for trend<0.01). However, we did not find statistically significant differences in the percentage of intra-plaque hemorrhages between patients using insulin and patients using oral glucose lowering agents (29.4% versus 22.7%, p=0.33, table 1b).

Figure 2. Insulin receptor expression within human atherosclerotic plaque.
Representative photomicrographs of insulin receptor expression (IR), in brown, in human atherosclerotic plaque. A. Numerous IR positive microvessels within a foam cell rich area of the lipid core; B. Growth of IR positive small microvessels (SV) out of the media into the lipid core. The larger microvessel (LV) is IR negative; C. Small IR positive vessels (SV) sprouting off larger vessels (LV) that are less positive for the IR.
Figure 3. Co-localization of insulin receptors with different cell type markers within human atherosclerotic plaques.

The first column shows endarterectomy specimens stained with Elastica-van Gieson. The white rectangles indicate from which part of the plaques the immunohistochemical images of the second column are derived. In the second column IR is shown in red (using Vector Red) and CD31, CD68, CD3 and SMA are shown in blue (using Vector Blue). With use of spectral imaging individual layers were unmixed and co-localization of the different layers is depicted in the third column. b,c: Marked co-localization of IR with endothelium (CD31), especially on small microvessels; e,f: minimal co-localization of IR with macrophages (CD68); h,i: Hardly any co-localization of IR with T-cells (CD3); k,l: Marked co-localization of IR with SMA on myofibroblast cells located in the intima; n,o: No co-localization of IR with SMA on smooth muscle cells in the media layer. b, c, h, i, n, o images were taken from the media layer of atherosclerotic plaques. e, f, k, l images were taken from the intima layer of atherosclerotic plaques. White bar is 1mm; Black bar is 0.05mm.
Figure 4. Insulin receptor expression on immature microvessels within human atherosclerotic plaques.
The first column shows representative photographs of double immunoenzyme staining for IR and CD34 (a, g) and IR and collagen IV (d, j). The black squares indicate from which part of the section the spectral imaging pictures in the second and third column are derived. b, c, Marked co-localization of insulin receptors with endothelial cells on a small microvessel; a larger microvessel is insulin receptor negative. In picture e, this small microvessel is not surrounded by collagen IV, whereas the larger microvessel has positive collagen IV staining. h, i, k The microvessels in h. and i. that show co-localization of insulin receptors with endothelial cells are mostly not surrounded by collagen IV(k). In pictures f and l, HE overview photographs of endarterectomy specimen are depicted. The black arrows indicate in which part of the plaque the double immunoenzyme stainings were performed. Scale bar in a, d, g and j = 0.1 mm; Scale bar in f and l = 0.5 mm.
Table 1a. Patients’ characteristics

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<th>Oral agents n=97</th>
<th>Insulin n=68</th>
<th>p-value</th>
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<td>Mean age, yrs</td>
<td>65.9 ± 9.0</td>
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<td>Gender, % male</td>
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<td>Mean BMI, kg/m2</td>
<td>27.9 ±4.1</td>
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<td>Treated hypertension, %</td>
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<td>Smoking, %</td>
<td>33.7</td>
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<td>Positive family history of CVD, %</td>
<td>55.3</td>
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Table 1b. Atherosclerotic plaque characteristics

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<th>Oral agents n=97</th>
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<td>Plaque phenotype</td>
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<td>Fibrous, %</td>
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<td>Lipid core &gt;40%, %</td>
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<td>Moderate/heavy collagen, %</td>
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<td>Moderate/heavy macrophages, %</td>
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<td>Moderate/heavy SMC</td>
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<td>72.1</td>
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<tr>
<td>Moderate/heavy calcifications</td>
<td>52.6</td>
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<td>Macrophages, % of plaque area</td>
<td>0.49 (0.1-1.3)</td>
<td>0.70 (0.2-1.2)</td>
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<td>SMCs, % of plaque area</td>
<td>2.1 (1.0-3.7)</td>
<td>1.4 (0.4-3.7)</td>
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<td>Luminal thrombus, %</td>
<td>45.4</td>
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<td>Intraplaque hemorrhage, %</td>
<td>22.7</td>
<td>29.4</td>
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</table>
Chapter 4

Insulin receptor expression in atherosclerotic plaques.

Figure 5. Microvessel density in atherosclerotic plaques from DM2 patients.
Atherosclerotic plaques from 68 DM2 patients who use insulin and 97 DM2 patients who do not use insulin were analyzed for the presence of microvessels. The average microvessel density of 3 hotspots was calculated within each plaque. * p=0.05

DISCUSSION

In the present study we showed that insulin enhances capillary-like tube formation of human microvascular endothelial cells. Secondly, we demonstrated that IRs are highly expressed on endothelial cells of nascent microvessels within human atherosclerotic plaques. Finally, we found a trend towards higher intra-plaque microvessel density among DM2 patients using insulin than among those using only oral glucose lowering agents. These findings imply that insulin may stimulate angiogenesis within atherosclerotic plaques.

The engagement of insulin signaling in angiogenesis is supported by several in-vivo studies. Mice lacking IRs on their endothelium had a profound reduction in retinal neovascularisation following hypoxia compared with control mice, indicating a crucial role for insulin in inducing angiogenesis.26 In line, skin of mice injected with insulin subcutaneously for 5 days showed longer vessels with more branches compared with skin injected with saline. 27 Finally, in patients with progressive corneal neovascularisation, disruption of IR signaling, using antisense oligonucleotides against insulin receptor substrate-1, resulted in regression of corneal neovascularisation.28 We now provide consistent data to show that insulin may also increase angiogenesis within atherosclerotic plaques.

It has already been shown that insulin can increase tube formation of endothelial cells on a two-dimensional Matrigel.15,27 However, we used a model of angiogenic sprouting, in which capillary-like tube formation occurs in a three-dimensional fibrin matrix and results in the formation of true lumen-like structures.18 This model shows similarities with sprouting of new blood vessels at sites of chronic inflammation, as occurs in atherosclerotic plaques. Since insulin at supraphysiological concentrations (≥10⁻⁸M) also activates IGF-1R receptors29, it could be speculated whether the observed increased capillary-like tube formation following
insulin stimulation was (in part) mediated through IGF-1 receptors. However, we also found effects of insulin at concentrations that only IRs are activated ($10^{-9}$M insulin), pointing towards a direct effect of insulin on the IR.

Subsequently, we showed that insulin receptors are present on endothelial cells of microvessels within human atherosclerotic plaques. Interestingly, IRs were found to be present mainly on those microvessels lacking collagen IV. Collagen IV is a component of the basement membrane which is formed during maturation of capillary-like tubes. Microvessels that do not express collagen IV therefore fall into the category of nascent microvessels, as opposed to the collagen IV positive more mature microvessels. The increased IR expression on the endothelium of nascent microvessels within atherosclerotic plaques is consistent with the concept that IRs contribute to further outgrowth of these microvessels into atherosclerotic plaques. Since we did not reveal such a specific distribution pattern for the IGF-1 receptor, insulin signaling is more likely to be involved in intra-plaque angiogenesis than IGF-1 signaling.

Consistent with the literature, we found that patients with higher intra-plaque microvessel density were more common to have intra-plaque hemorrhages. The presence of more microvessels within atherosclerotic plaques has been associated with increased plaque vulnerability and is an independent predictor of future cardiovascular events. In fact, many intra-plaque microvessels show poor endothelial integrity, with membrane blebs, intracytoplasmic vacuoles, poorly formed endothelial cell junctions and basement membrane detachment. Consequently, via these microvessels, erythrocytes and leukocytes can leak into the atherosclerotic plaque, resulting in increased inflammation and intra-plaque hemorrhage. Together with increased release of proteolytic enzymes and matrix breakdown this makes the plaque more vulnerable. Although we did not find significant differences in the percentage of intra-plaque hemorrhages between patients using insulin and patients using oral glucose lowering agents only, the increased microvessel density found in patients using insulin can be expected to be associated with an increased cardiovascular risk.

It should be acknowledged that insulin may only enhance angiogenesis, when pre-existing vessels are already present within the plaque. Since microvessels are mainly present in more advanced plaques, it could be hypothesized that insulin use might increase angiogenesis especially in patients with advanced atherosclerosis. In these patients it may be reasonable to reconsider the advantages and disadvantages of high insulin doses.

The present study has several limitations. Firstly, the endarterectomy specimens that were used for microvessel density analysis were derived from a heterogenous group of DM2 patients, with different duration of diabetic disease and treatment. This excessive variation cannot be correlated or corrected for as parameters which could provide information about the severity of diabetes, like HbA1c, onset of diabetes and insulin dosage are lacking.

A second limitation of our study is that we do not provide direct evidence that insulin can enhance intra-plaque angiogenesis in-vivo. Unfortunately, a suitable animal model in which this question can be directly tested in-vivo is currently lacking. In most atherosclerotic animal models intra-plaque microvessels are scarce which makes it difficult to assess influence of insulin on
Intra-plaque angiogenesis. Because in-vivo imaging modalities for real time imaging of human plaque angiogenesis are improving, it may become feasible in the future to study the effect of insulin on intra-plaque angiogenesis directly in humans by using a prospective study design.

In conclusion, high insulin receptor expression on nascent microvessels within atherosclerotic plaques is likely to be involved in stimulating intraplaque angiogenesis, and thereby links microvascular and macrovascular disease in type 2 diabetes. However, whether insulin therapy increases plaque vulnerability and subsequent cardiovascular risk needs to be assessed by future in-vivo studies.
REFERENCE LIST


Supplemental figure 1. Percentage of intra-plaque hemorrhage by microvessel density quartiles.
The presence of intra-plaque hemorrhages was analyzed in plaques of all 165 DM2 patients and grouped according to microvessel quartiles: low (n= 42), moderately low (n=45), moderately high (n=37), high (n=41).
### Supplemental table 1. Double immunohistochemistry procedures.

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<th>First antibody</th>
<th>Concentration</th>
<th>Incubation</th>
<th>Visualisation</th>
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<td>TrisEDTA pH9.0 10 min</td>
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<td>TrisEDTA pH9.0 20 min</td>
<td>CD3, 3 Rb SP7</td>
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<td>60 min RT</td>
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<td>CD31, 1 JC70A</td>
<td>1.03 mg/ml</td>
<td>60 min RT</td>
<td>Vector Blue</td>
<td>TrisEDTA pH9.0 10 min</td>
<td>IR, 2 CT-3</td>
<td>1.0 mg/ml</td>
<td>60 min RT</td>
<td>Vector Red</td>
</tr>
<tr>
<td>TrisEDTA pH9.0 20 min</td>
<td>CD68, 1 PG-M1</td>
<td>0.08 mg/ml</td>
<td>60 min RT</td>
<td>Vector Blue</td>
<td>TrisEDTA pH9.0 10 min</td>
<td>IR, 2 CT-3</td>
<td>1.0 mg/ml</td>
<td>60 min RT</td>
<td>Vector Red</td>
</tr>
<tr>
<td>TrisEDTA pH9.0 20 min</td>
<td>IR, 2 CT-3</td>
<td>0.05 mg/ml</td>
<td>60 min RT</td>
<td>DAB+</td>
<td>TrisEDTA pH9.0 10 min</td>
<td>CD34 1 Q8End10</td>
<td>0.1 mg/ml</td>
<td>60 min RT</td>
<td>Vector Red</td>
</tr>
<tr>
<td>TrisEDTA pH9.0 20 min</td>
<td>IR, 2 CT-3</td>
<td>0.05 mg/ml</td>
<td>60 min RT</td>
<td>DAB+</td>
<td>Coll IV 0 PHM-12 + CIV22</td>
<td>n.a.*</td>
<td>60 min RT</td>
<td>Vector Red</td>
<td></td>
</tr>
</tbody>
</table>

1 Dako, Glostrup, Denmark  
2 Millipore/Chemicon, Temecula, CA, USA  
3 Thermo/labvision, Runcorn, UK  
IR= Insulin receptor β-subunit; IGF1-R= Insulin-like growth factor 1 receptor α-subunit; SMA= Smooth muscle cell α-actin; RT=Room temperature. n.a. concentration of stock solution not available, *work solution diluted 1:1000. ** work solution diluted 1:600