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^{-6} 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.

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1. 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

Abbreviations: 3D, three-dimensional; AHERO-EXPRESS, AHEROsclerotic plaque EXPRESSION; bFGF, basic fibroblast growth factor; DM2, type 2 diabetes mellitus; EGF, endothelial cell growth factor; hMVEC, human microvascular endothelial cells; HuSi, heat-inactivated human serum; IR, insulin receptor; NBCSi, heat-inactivated newborn calf serum; SMA, smooth muscle cell actin; SMC, smooth muscle cell; TNF-α, tumor necrosis factor alpha.

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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 (IRs) 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?

2. Materials and methods

2.1. In vitro angiogenic sprouting assay

2.1.1. 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), 1% (v/v) heat-inactivated newborn calf serum (NBCSi), 100 IU/ml penicillin, 100 μg/ml streptomycin (pen/strep), 3.75 μg/ml endothelial cell growth factor (ECGF, crude extract from bovine brain), 5 μg/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.

2.1.2. 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-glutamine for 16 h 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 tumour necrosis factor alpha (TNF-α; Sigma Aldrich, St Louis, MO, USA) with or without insulin (bovine, Sigma Aldrich, St Louis, MO, USA) (10⁻⁹–10⁻⁷ M). 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 microscopy [18] 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.

2.2. Insulin receptor expression on microvessels within atherosclerotic plaques

2.2.1. 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 endarterectomy specimen of 6 patients.

2.2.2. Single immunohistochemistry for IR and IGF-1 receptor

Parafﬁn tissue sections of 4 μm thickness were dewaxed in xylene and re-hydrated 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 pH 9.0 (20 min at 98 °C) in a Pretreatment Module (Thermo/LabVision, Fremont, CA). For single stainings we used IR antibody (Clone CT-3, 1 μg/ml, 60 min incubation at room temperature) and IGF-1 receptor antibody (clone Rb IgG, 0.25 μg/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, the 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).

2.2.3. Double Immunohistochemistry

Immunodouble 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, the Netherlands). AP activity was visualized in blue using Vector Blue (Vector Labs, Burlingame, CA). To abolish cross-reaction between first and second primary mouse antibodies, a 10 min heat step (98 °C) was performed in between, using Tris–EDTA pH 9.0 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).

2.2.4. 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 to 720 nm at 20 nm intervals and analyzed with the NuanceTM 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 [22]. 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 [23]. 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 [23]. 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 [24]. 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 (1 µg/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 400×, 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 [25].

3.1. 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’s U test and for categorical data a χ²-test were used. Data were analyzed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL). p values below 0.05 were considered statistically significant.

4. Results

4.1. In vitro angiogenic sprouting assay

4.1.1. 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 7–9 days to induce capillary-like tube formation. Addition of insulin (in a range of 10⁻⁵–10⁻⁷ M) to the medium increased capillary-like endothelial tube formation dose dependently (Fig. 1A and B). 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 10⁻⁸ M 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 (Fig. 1C).

4.2. Insulin receptor expression on microvessels within atherosclerotic plaques

4.2.1. 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 (stages III–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 (Fig. 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).

4.2.2. 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 (Fig. 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.
Fig. 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 10 ng/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 0.5 mm. (B and 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).

Fig. 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.
4.2.3. 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 (Fig. 4).

4.3. Microvessel density within atherosclerotic plaques

4.3.1. 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...
Fig. 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 and G) and IR and collagen IV (D and J). The black squares indicate from which part of the section the spectral imaging pictures in the second and third column are derived. (B and 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 and 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.

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 Section 2. As depicted in Fig. 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 Fig. 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).

### Table 1b

<table>
<thead>
<tr>
<th>Plaque phenotype</th>
<th>Oral agents n = 97</th>
<th>Insulin n = 68</th>
<th>p-Value</th>
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</thead>
<tbody>
<tr>
<td>Fibrous (%)</td>
<td>44.3</td>
<td>32.4</td>
<td>0.22</td>
</tr>
<tr>
<td>Fibro-atheromatous (%)</td>
<td>30.9</td>
<td>42.6</td>
<td></td>
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<tr>
<td>Atheromatous (%)</td>
<td>24.7</td>
<td>25.0</td>
<td></td>
</tr>
<tr>
<td>Lipid core &gt; 40% (%)</td>
<td>25.8</td>
<td>30.9</td>
<td>0.47</td>
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<td>Moderate/heavy collagen (%)</td>
<td>82.3</td>
<td>83.8</td>
<td>0.80</td>
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<td>Moderate/heavy macrophages (%)</td>
<td>60.4</td>
<td>64.7</td>
<td>0.58</td>
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<tr>
<td>Moderate/heavy SMC</td>
<td>71.1</td>
<td>72.1</td>
<td>0.90</td>
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<tr>
<td>Moderate/heavy calcifications (%)</td>
<td>52.6</td>
<td>63.2</td>
<td>0.17</td>
</tr>
<tr>
<td>Macrophages (% of plaque area)</td>
<td>0.49 (0.1–1.3)</td>
<td>0.70 (0.2–1.2)</td>
<td>0.39</td>
</tr>
<tr>
<td>SMCs (% of plaque area)</td>
<td>2.1 (1.0–3.7)</td>
<td>1.4 (0.4–3.7)</td>
<td>0.21</td>
</tr>
<tr>
<td>Luminal thrombus (%)</td>
<td>45.4</td>
<td>45.6</td>
<td>0.98</td>
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<tr>
<td>Intraplaque hemorrhage (%)</td>
<td>22.7</td>
<td>25.4</td>
<td>0.33</td>
</tr>
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</table>

### 5. 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 neovascularization 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 neovascularization, disruption of IR signaling, using antisense oligonucleotides against insulin receptor substrate-1, resulted in regression of corneal neovascularization [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 (>10⁻⁸ M) also activates IGF-1 receptors [29], 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 [30]. In fact, many intra-plaque microvessels show poor endothelial integrity, with membrane blebs, intracytoplasmic vacuoles, poorly formed endothelial cell junctions and basement membrane detachment [13]. Consequently, via these microvessels, erythrocytes and leukocytes can leak into the atherosclerotic plaque, resulting in increased inflammation and intra-plaque hemorrhage [12,31]. 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 heterogeneous 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 [11] 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.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2012.01.035.

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