Increased MAPK Activation and Impaired Insulin Signaling in Subcutaneous Microvascular Endothelial Cells in Type 2 Diabetes—role of endothelin-1

Silvia Gogg PhD, Ulf Smith MD and Per-Anders Jansson MD

From The Lundberg Laboratory for Diabetes Research
Center of Excellence for Cardiovascular and Metabolic Research
Department of Molecular and Clinical Medicine,
Sahlgrenska Academy,
University of Gothenburg,
Gothenburg, Sweden

Running title: Endothelial cells in type 2 diabetes

Corresponding author:
Silvia Gogg, PhD
E-mail:silvia.gogg@medic.gu.se

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org

Submitted 17 July 2008 and accepted 15 June 2009.

This is an uncopyedited electronic version of an article accepted for publication in Diabetes. The American Diabetes Association, publisher of Diabetes, is not responsible for any errors or omissions in this version of the manuscript or any version derived from it by third parties. The definitive publisher-authenticated version will be available in a future issue of Diabetes in print and online at http://diabetes.diabetesjournals.org.
**Objective** -To establish a method for isolation and culture of subcutaneous microvascular endothelial cells (MVEC) from small human tissue biopsies to compare gene- and protein expression of insulin signaling molecules in MVEC from insulin-resistant and healthy control subjects.

**Research and Design Methods** - Stromavascular cells from subcutaneous needle biopsies of type 2 diabetic (T2D) and control subjects were expanded in culture and the endothelial cells selected with magnetic immune separation. Western blots and RT-PCR were used for protein- and gene expression assays.

**Results** - At least 99% of the expanded primary MVEC could be characterized as endothelial cells. The expression of insulin receptors (IR) was low but insulin increased tyrosine phosphorylation of both the IR and IRS-1 and activated PKB. The IRS-1 protein expression was reduced and the serine phosphorylation of PKB in response to insulin attenuated while basal and insulin-stimulated phosphorylation of ERK1/2 was increased in T2 MVEC. Endothelin-1 mRNA levels were significantly higher in T2D cells. The addition of endothelin-1 increased the phosphorylation of MAPK, an effect antagonized by the MEK-1 inhibitor PD98059. Furthermore, the endothelin ET\textsubscript{A} and ET\textsubscript{B} receptor antagonists BQ123 and BQ788 decreased basal MAPK activity in T2 MVEC and prevented the endothelin-1-induced activation.

**Conclusions** - We developed a system for isolation and culture of human MVEC from small needle biopsies. Our observations support the concept of “selective” insulin resistance, involving IRS-1 and the PI3kinase pathway, as an underlying factor for a dysregulated microvascular endothelium in T2D. Our data also support a role of endothelin-1 for the increased MAPK activity seen in non-stimulated T2D MVEC.
Methods to culture human vascular endothelium have been available for several decades (1-3). Adipose tissue is a suitable source of endothelial cells because of its rich network of capillaries (4). Subcutaneous fat is relatively easy to access during elective surgery or liposuction and, hence, strategies have been outlined to create an antithrombogenic cell lining in vascular prosthetic devices (5,6). However, aspiration of subcutaneous fat has never been used as a source of microvascular endothelial cells (MVEC) in metabolic research because overgrowth of fibroblasts and other stromal cells has been a substantial problem. A key aim of the present study was to improve the isolation and expansion procedures of human MVEC to enable molecular studies, including insulin signaling and effects, in endothelial cells from subcutaneous needle biopsies obtained from subjects with different clinical phenotypes. Accordingly, we have further developed available methods (7-11) for immunoselection and subsequent culture of MVEC in order to obtain, validate and characterize endothelial cells from small human tissue specimens.

We have previously shown that insulin-resistance and type 2 diabetes (T2D) are associated with an impaired insulin signaling, due to a reduced expression of the key docking protein IRS-1, in human subcutaneous adipose tissue (12) and this is also associated with different aspects of the insulin resistance syndrome including surrogate markers of atherosclerosis (13). An impaired IRS-1 activation by insulin in macrovascular endothelial cells due to a point mutation, is associated with endothelial dysfunction (14).

Endothelins are a family of vasoactive peptides (ET-1, ET-2 and ET-3) that signal through two G-coupled protein receptors ET_A and ET_B. Endothelin-1 is secreted by endothelial cells and produces multiple actions like regulation of vascular tone, tissue remodelling, induction of proliferation, chemotaxis of macrophages, activation of smooth muscle cells and differentiation of fibroblasts (15). Elevated ET-1 plasma levels have also been reported in insulin-resistant states like type 2 diabetes and obesity (16,17). Endothelial dysfunction is a known facet in patients with insulin resistance (18) and endothelin-1 is considered to play a role for this by interfering with the insulin-signaling pathway in the arterial wall (19).

We here provide detailed information on how to isolate and propagate MVEC from needle biopsies of the subcutaneous adipose tissue. We also characterized insulin signaling and provide evidence that endothelin-1 is overexpressed in MVEC from T2D donors leading to increased MAP kinase activity.

RESEARCH DESIGN AND METHODS

Source of adipose tissue and microvascular endothelial cells (MVEC).

Human needle biopsies of the subcutaneous adipose tissue were obtained from the abdominal region of non-diabetic (n=12) and T2D subjects (n=12) under local anesthesia (Xylocain® 10 mg/ml) (Astra Zeneca AB, Södertälje, Sweden), as previously described (12). All subjects were recruited via an advertisement in a local newspaper and their clinical characteristics are shown in Table A1 (available in the online appendix at http://diabetes.diabetesjournals.org).

About 3 g of tissue was incubated in sterile Medium199 (Invitrogen, Paisley, UK), containing 25 mmol/l Hepes, 4% bovine serum albumin (BSA), 5.5 mmol/l glucose and 0.8 mg/ml collagenase in a shaking water bath at 37°C. After 30-50 min, the digest was filtered through a sterile nylon mesh (pore size 250 µm) and washed 4 times with fresh medium as described before (20). Two fractions were obtained; the upper fraction
containing the floating isolated adipose cells and the lower fraction containing the stromavascular cells.

The lower fraction was collected into 50 ml Falcon tubes and, following centrifugation (1300 rpm–5 min), the pellet was resuspended in endothelial cell (EC) growth medium Bulletkit (EGM-2-MV) consisting of endothelial cell basal medium-2 (EBM-2) supplemented with EGM-2-MV SingleQuots containing hEGF, hydrocortisone, fetal bovine serum (FBS), VEGF, R3-IGF-1, ascorbic acid, and gentamycin sulfate/amphotericin-B (GA-1000) (Clonetics, BioWhittaker, Verviers, Belgium). FGFb was not added to the medium.

The cells obtained were seeded into a 75 cm² culture flask and allowed to grow at 37°C and 5% CO₂ in the same media. Before the cells reached confluence, after approximately one week, the heterogeneous pool of cells was exposed to a CD31-positive selection as described below.

The isolated adipocytes in the floating fraction were washed four times in fresh Medium 199, resuspended at 2% cytocrit and used as described below.

All subjects gave their written informed consent, and the study was approved by the Ethics Committee of the University of Gothenburg.

**Endothelial cell isolation and culture.** The human MVEC selection was performed with the Dynabeads magnetic CD31 MicroBeads cell sorting system (Invitrogen, Paisley, UK).

The heterogeneous cell population, containing the adipose tissue-derived stromavascular cells, was washed twice with PBS and incubated on a rocking platform (25 revolution/min) during 15 min with 4 ml of PBS/0.2% BSA containing 100 µl of Dynabeads (2.8x10⁵ beads/ml) coated with CD31 antibody (PECAM-1). The beads rapidly target and partially coat the endothelial cells expressing the CD31 receptor.

Following the incubation, the culture was washed twice with PBS and trypsinized. The cells were collected into a tube and centrifuged for 3 min at 1300 rpm. The pellet was resuspended in PBS/0.2% BSA, collected into a 2 ml microcentrifuge tube and placed in a magnet (Dynal MPC-S) (Invitrogen, Paisley, UK), following the manufacturer’s recommended protocol for washings and final extraction. The CD31 negative cells (with no beads attached) were removed during the successive washings.

The positive selected endothelial cells were resuspended in EC growth medium Bulletkit (EGM-MV2) and maintained at 37°C and 5% CO₂. The cells obtained with this procedure were 99% endothelial cells with typical cobblestone morphology. The endothelial cells were characterized by indirect immunofluorescence and acetylated LDL uptake. The cells were used before passage seven.

**Immunocytochemistry of MVEC.** For immunofluorescence, the cells were incubated in eight-well plastic chamber slides (Nunc, A/S, Roskilde, DK) for 48 hours. The cells were washed twice with PBS and fixed for 10 min in methanol. After three rinses in PBS, the cells were incubated with the human primary antibody against von Willebrand Factor (vWF) (DAKO A082) (1:50 dilution in PBS/3% BSA) for 1 hour. The culture was washed three times with PBS and incubated with the fluorescein isothiocyanate-labeled secondary antibody (DAKO F0205) (1:50 dilution in PBS/3% BSA) for 1 hour and washed again.

**Acetylated LDL uptake of MVEC.** The cells were incubated in eight-well plastic chamber slides for 48 hours. Acetylated LDL labeled with 1,1'-dioctadecyl-3,3',3'-tetramethylindo-carbocyanine perchlorate (Dil-Ac-LDL) (Biomedical Technologies, USA) was added to the culture medium at a
final concentration of 10 µg/ml, and the cells were incubated at 37°C for 4 hours. The cells were fixed for 30 min in 3% formaldehyde solution, mounted, and observed by fluorescence microscopy.

**Tube formation.** Rat-tail collagen type I, 10 mg/ml (BD Biosciences, Stockholm, Sweden), was neutralized by the addition of 1N NaOH, H₂O and PBS following the manufacturers recommendations. The mix was poured into 12-well culture plates (200 µl/well) and allowed to solidify for 1 hour in a humidified atmosphere of 5% CO₂ at 37°C. Following solidification, the gels were preincubated with growth medium 30 min before the cells were seeded. The tube formation was monitored by phase contrast microscopy.

**Protein extraction of endothelial cells.** The cultured cells were thoroughly washed with PBS, deprived of serum and EGM-2-MV SingleQuots additions for 4 hours, before adding insulin for 20 min.

Endothelin-1, BQ123, BQ788 (Sigma-Aldrich, Stockholm, Sweden), or PD98059 (Calbiochem-Merck Chemicals Ltd., Nottingham, UK) were added to the culture media for 24 hours at the indicated concentrations. When used in combination with other factors, PD98059 was added one hour before.

The cells were washed with PBS and then lysed in lysis buffer on ice (21). The lysate was vortexed and rocked for 2 hours at 4°C and the proteins quantified by the bicinchonic acid method (BCA) (Pierce, Rockford, USA).

**Preparation of adipose cells-protein and RNA extraction.** The isolated human adipocytes, as described above, were distributed into plastic vials (12-15% cell suspension) and incubated with 100 nM insulin for 15 min. The cells were rapidly separated by centrifugation through silicone oil and suspended in lysis buffer. The lysate was vortexed and rocked for 2 hours at 4°C as previously reported (21). The proteins were quantified by the BCA method. RNA was extracted according to Chirgwin et al (22).

**RNA extraction of endothelial cells.** The cultured cells were deprived of serum and EGM-2 MV SingleQuots additions and insulin was added, when specified, for 24 h. Total cellular RNA was extracted with RNeasy (Quiagen Gmbh, Hilden, Germany). The mRNA expression was analyzed with the ABI PRISM 7900 sequence detection system (TaqMan, Applied Biosystems, USA) using 18s ribosomal RNA as endogenous control (23). The gene expression pre-designed primer and probe set #Hs00167166_ml was used for eNOS and #Hs00174961_m1 for endothelin-1 gene analysis (Applied Biosystems, USA).

**HUVEC cells.** HUVEC cells (ATCC-CRL-1730) were grown in Kaighn’s modified F12K media (ATCC) from LGC Promochem (Boras, Sweden). The media was supplemented with 0.1 mg/ml heparin, 0.03 mg/ml endothelial cell growth supplement, both from Sigma-Aldrich, and 10% FBS (GIBCO, InVitrogen, Paisley, UK).

**Immunoblotting.** Crude cell extracts were boiled in Laemmli buffer containing 150 mM dithiothreitol for 5 min. The samples were analyzed by SDS-polyacrylamide gel electrophoresis (7.5 or 10%). Proteins were transferred from the gel to nitrocellulose sheets, blocked in 5% fat-free milk and probed with the different primary antibodies according to the manufacturer’s recommendations. IRS-1 and MAPK antibodies were from Upstate Biotechnology (Millipore, Solna, Sweden), p-PKB, PKB and p-MAPK were from Cell Signaling (New England Biolabs, Hertfordshire, UK), IR and eNOS were from Transduction (BD Biosciences, Erembodegem, Belgium) and anti-phosphotyrosine (pY99) was from Santa Cruz Biotechnology (SDS, Falkenberg, Sweden). The proteins were detected by
enhanced chemiluminescence using horseradish peroxidase-labeled secondary antibodies (Amersham Biosciences, Buckinghamshire, UK), and the intensity of the bands was quantitated with a laser densitometer (Molecular Dynamics, Sunnyvale, CA).

**Immunoprecipitations.** Cell lysates (60 µg) were immunoprecipitated with anti-IR antibody according to the recommendation of the manufacturers. Subsequently, the immune complexes were precipitated with Protein A/G Plus-Agarose (Santa Cruz Biotechnology) for 90 min at 4°C. The immunoprecipitates were washed three times with lysis buffer. The samples were analyzed by SDS-polyacrylamide gel electrophoresis (7.5 %) and immunoblotted as described above.

**cGMP measurements.** Cellular cGMP was measured by the cGMP Enzyme-immunoassay Biotrak System (Amersham Biosciences) following the manufacturer’s recommendations.

**Statistical analysis.** Data analyses are presented as mean ±SEM. Student’s t-test was used to test for statistical significance.

**RESULTS**

**Cell recovery of the CD31 isolation method.** At least 99% of the primary MVEC generated by the CD31 extraction showed a positive staining for vWF and LDL uptake. However, there was a certain variation between the subjects regarding the number of non-endothelial cells (CD31 negative) that were recovered with the positive cells. This variation was not related to the subject phenotype (control or type 2) nor to the defined experimental conditions used. The non-endothelial cells were easy to distinguish from the MVEC in culture and were easily removed by performing a new CD31 extraction in a subsequent passage.

MVEC proliferation was slow and sensitive to seeding densities. A suitable density of cells was around 3x10^3 cells/cm^2.

The adherent cultured human MVEC monolayer assumed the typical cobblestone morphology, which was maintained throughout the 10-13 passages studied (Fig.1a). Later passages were associated with reduced growth and distorted cell borders. The cells never formed thick aggregates and maintained their contact inhibition.

**von Willebrand Factor and Ac-LDL.** The MVEC were positive for the endothelial-specific vWF immunostaining (Fig.1b). In addition, the cells incorporated acetylated LDL; another marker characterizing endothelial cells (Fig.1c).

**Endothelial cell tube formation.** The ability to form a capillary/tube-like network is a specialized function of vascular endothelial cells. We characterized the subcutaneous MVEC by their ability to form capillary-like structures. Fig.1d shows the morphogenic response of MVEC in collagen and EGM-MV2 media. Morphological changes and reorganization of the cells were apparent as early as 12 hours after initiation of the culture.

**Insulin signaling pathway and MAPK activation.** We investigated the responsiveness to insulin of the adipose tissue-derived MVEC from healthy and T2D individuals. For comparison, we performed parallel experiments with the HUVEC cell line; a well-documented endothelial cell line.

Expression of the insulin receptor (IR) was low in MVEC as compared to HUVEC but there was a clear insulin-stimulated tyrosine phosphorylation of the receptor in both type 2 and control MVEC and in HUVEC cells (Fig.2).

Since the presence of IR in MVEC is a controversial issue we wanted to further validate the presence of insulin receptors by immunoprecipitating the insulin receptors and immunoblotting for both phosphotyrosine and receptor protein. As shown (Fig.2, lower panel) insulin receptors are indeed present in MVEC albeit at a low level.
Both main docking proteins for insulin, IRS-1 and IRS-2, were expressed as well as the downstream signaling proteins PKB/Akt and the MAPKinas (MAPK) ERK 1/2. IRS-1 was clearly the predominant protein while IRS-2 was only expressed at a low level (data not shown).

Similar to our previous observations in human adipocytes (12) IRS-1 protein expression was markedly decreased in MVEC from T2D subjects (Fig.3). This reduction was also reflected by the impaired activation and serine phosphorylation of the downstream protein PKB/Akt in type 2 MVEC while PKB/Akt protein expression was unchanged (Fig.4, upper panel). This observation was confirmed in four separate immunobLOTS quantified by scanning and shown in Fig.4 (bottom panel). A pronounced insulin-stimulated activation of PKB was also seen in the HUVEC cells (Fig.4, upper panel).

The MAPK, ERK1/2, had an increased phosphorylation in the basal state in MVEC from T2D subjects (Fig.5, upper panel). Insulin further increased the MAPK phosphorylation in control MVEC cells (p=0.057) while there was no effect of insulin (p=0.18) in the type 2 cells, probably masked by the high basal MAPK/ERK activation. This observation was confirmed in five separate experiments measuring the p-MAPK/MAPK volume ratios (Fig.5, bottom panel) supporting that the major change was due to an increased phosphorylation of the MAPK in the basal state.

**Effects of insulin on eNOS activation.** Surprisingly, there was also a very low protein expression of eNOS in the MVEC and, as expected, in the isolated adipose cells from the same individuals compared to HUVEC (Fig.6, top). The gene expression of eNOS was also almost undetectable in the MVEC, from both control and T2D subjects, while there was a clear expression of both the gene and protein in HUVEC cells (Fig.6, bottom panel). Insulin had no effect on the expression of eNOS mRNA in MVEC while the levels were significantly increased in HUVEC. In order to further pursue the ability of insulin to activate eNOS we incubated the cells with or without the hormone for 1, 3, 5, 15 or 20 min and measured phosphorylation of ser1177 and thr495. Since no activation was found, we investigated the effect of insulin on cyclic GMP formation. However, insulin did not increase cyclic GMP levels in human cultured MVEC from subcutaneous adipose tissue (data not shown). Thus, we conclude that eNOS is not an important target for insulin in these cells, at least under the culture conditions used.

**Endothelin-1 expression.** As shown in Fig.7 endothelin-1 mRNA levels were significantly higher in cells from T2D individuals, both when compared to cells from the control group as well as to HUVEC cells, while there was no significant difference between the control group and HUVEC cells. Adding insulin (100 nM) for 24 hours did not increase endothelin-1 expression in any of the three groups studied (Fig.7).

To further explore its role, we added endothelin-1 (100 nM) to the culture medium and found that it increased ERK 1/2 phosphorylation in control MVEC (Fig.8, upper panel). The addition of the ET<sub>A</sub> (BQ123) and ET<sub>B</sub> (BQ788) receptor antagonists (20µM) as well as the MEK-1 inhibitor PD98059 (50 µM) prevented this effect of endothelin-1 (Fig. 8, upper panel). It should be noted that both ET-1 receptor antagonists were required since both A and B receptors were present in MVEC (data not shown).

To examine if the increased MAPK activation in the non-stimulated state in MVEC from T2D individuals was due to the increased endothelin, we incubated the cells with both ET<sub>A</sub> and ET<sub>B</sub> receptor antagonists. As shown in Fig.8 (middle and bottom
Endothelial cells in type 2 diabetes

panels), the increased basal MAPK activity in MVEC from type 2 diabetic subjects was normalized by the ET-1 receptor antagonists. This was also seen with the MEK-1 inhibitor PD98059. Taken together, our results show that endothelin-1 is increased in MVEC from T2D individuals and that this activation can account for the increased MAPK activation seen in the basal state.

DISCUSSION

This study describes an improved method for the primary culture of an apparently pure population of MVEC obtained from a subcutaneous needle biopsy (around 3 g), using CD31 extraction. In general, the purification steps had to be performed at least twice to obtain a pure cell population. The cells displayed the typical cobblestone morphology, positive immunostaining for the vWF, uptake of acetylated LDL as well as capacity to form a capillary/tube-like network on a collagen gel. The usefulness of our procedure is illustrated by the fact that the MVEC can be expanded and used to characterize gene expression and hormone signaling. This opens up new possibilities to study molecular mechanisms in MVEC in relation to the clinical phenotype including endothelial dysfunction.

We focused on characterizing insulin signaling and action in the MVEC since these cells are considered to be important target cells for insulin. We have previously shown that IRS-1 protein expression, like GLUT-4, is reduced in the adipose cells in insulin resistance and is, indeed, a biomarker of the insulin resistance syndrome (IRS) (12). Moreover, a group of subjects exhibiting low adipocyte IRS-1 expression displayed early signs of atherosclerosis, including an increased intima-media thickness (IMT) (13) and an increased vascular stiffness as measured with pulse tonometry (24) compared to subjects with “normal” adipocyte IRS-1 expression. Animal studies have also shown that, in the vasculature of obese Zucker rats, insulin resistance affects the PI3Kinase pathway, but not other pathways of insulin signaling including the MAPK pathway (25). This “selective” insulin resistance would provide insights into the mechanisms for the increased shedding of cellular adhesion molecules in insulin-resistant subjects (26), the enhanced vasoconstrictory effect of insulin (27) and the pathophysiology of microvascular complications in diabetes (28).

The MVEC showed a low, but similar, expression of the insulin receptor and its tyrosine phosphorylated form in type 2 diabetic patients and control subjects, whereas IRS-1 protein expression and phosphorylation by insulin were markedly attenuated in T2D patients. Consequently, serine phosphorylation of PKB/Akt was impaired in agreement with an impaired signaling through the PI3Kinase pathway. However, we were unable to observe any difference in eNOS expression and phosphorylation in response to insulin between the groups due to a very low expression of eNOS in the MVEC. This was unexpected but low eNOS expression in MVEC has been reported before by some (29), but not all studies with MVEC (11). However, our findings were corroborated by measuring cGMP production which also was extremely low.

An intriguing finding was the increased non-stimulated (basal) as well as insulin-stimulated phosphorylation of ERK1/2 in cells from diabetic subjects. This observation fits with the known imbalance of insulin action in the microvasculature often referred to as pathway-selective insulin resistance in obesity and diabetes (25,26,30). The increased phosphorylation of ERK1/2 in diabetes may be an important reason for the decreased IRS-1 protein expression since MAPK phosphorylate IRS-1 on serine residues leading to an increased degradation of the protein (31). Interestingly,
basal ERK1/2 phosphorylation is also increased in adipose cells in T2D (32) and IRS-1 protein is also decreased (33). The finding of an increased ERK1/2 phosphorylation together with decreased IRS-1 in MVEC cultured for up to seven passages in vitro suggests a genetic cause and/or that the MVEC release factors which activate the cells.

DNA methylation and post-translational histone modifications are epigenetic mechanisms that are known to influence mammalian gene expression. T2D shows a progressive development with increasing age and environmental sensitivity. These characteristics make this disease suitable for epigenetic changes but it is only recently that we have started to understand the role of these epigenetic mechanisms. Therefore, it is possible that epigenetic mechanisms, due to high glucose concentrations or other factors (34,35), may lead to a long-term activation of genes in the T2D MVEC. However, further studies are needed to address this point.

Interestingly, endothelin-1 expression was significantly increased in the MVEC from diabetic subjects when compared to both non-diabetic subjects and HUVEC cells. In fact, there was no difference between the non-diabetic and HUVEC cells further corroborating the increase in the diabetic cells. Increased circulating endothelin-1 levels have also been reported in T2D (36; 37) and this finding is consistent with the increased ERK-1/2 activation seen since these kinases have been associated with an increased endothelin expression (30).

In our study, the increased endothelin-1 expression was not altered by the presence of insulin and, thus, is more likely a consequence of other activators. The nature of these upstream factors is a current focus of research.

Addition of endothelin-1 to the culture media increased MAPK phosphorylation in control MVEC. This effect was prevented by PD98059 showing that ERK1/2 is a target for endothelin-1 in MVEC. Importantly, the increased non-stimulated (basal) MAPK activation in MVEC from T2D subjects was completely antagonized by the addition of the endothelin-1 receptor antagonists BQ123 and BQ788. The use of both antagonists was required since MVEC were found to express both the ETA and ETB receptors. These results support the concept that the increased endothelin-1 expression plays a major role for the increased basal MAPK activation seen in MVEC of T2D subjects.

Endothelin-1 was recently shown to impair insulin signaling in adipose cells by decreasing the tyrosine phosphorylation and expression of IRS-1 (37,38). Thus, it is also possible that the reduced IRS-1 protein in adipose cells from T2D subjects is due to the increased endothelin-1 and MAPK activation as discussed above. If this is indeed the case, it would imply that the endothelial cells can directly cross-talk with the adipose cells and modulate their insulin sensitivity and action. It should also be added that only ERK1/2 were increased in the MVEC since p38 phosphorylation was not increased (data not shown).

There has been much discussion recently about the putative presence of insulin and/or IGF-I receptors, or chimeric receptors, in endothelial cells (39,40). We have also found that the MVEC express IGF-1 receptors (data not shown). Thus, we can not exclude that signaling downstream the IGF-1 receptor is more important than that downstream the insulin receptor (IR). In fact, previous studies have suggested that IGF-1 is more important than insulin for the generation of NO via the cognizant receptor or via hybrid receptors in endothelial cells (39,40). Nevertheless, our data show that the IR was expressed and activated in the MVEC and a discussion about the relative quantity of each receptor and/or
the presence of hybrid receptors is beyond the scope of this study.

It could be argued that subcutaneous MVEC are not representative cells for studies of NO-signaling. This may to some extent be true because endothelial cells derived from the microvasculature can in many ways differ from macrovascular endothelial cells (e.g., Bovine Aorta and Human Umbilical Vein). Indeed, tissue-specific expression patterns in different MVEC suggest that they are distinct cell types involved in the local regulation of their respective organs (41). Our observation that ERK1/2 is activated in T2D MVEC, like in the adipose cells (32), highlights the potential of these cells to be involved in both adipose tissue expansion through angiogenesis (42) as well as adipose cell insulin sensitivity and action.

In conclusion, we have developed a procedure whereby the subcutaneous MVEC can be isolated and cultured from small tissue specimens in human subjects. Using this technique, we find that MVEC from T2D patients have a reduced IRS-1 protein expression and an impaired insulin-stimulated PKB/Akt activation, whereas activation of ERK1/2 was increased in the basal state. This activation of ERK1/2 can be accounted for by the increased endothelin-1 levels in MVEC from T2D subjects. These observations support the concept of “selective” insulin resistance as an underlying factor for a dysregulated microvascular endothelium in T2D. Furthermore, our results are consistent with the possibility that the endothelial cells can cross-talk with the parenchymal cells and, thereby, be important modulators of insulin sensitivity and action.

ACKNOWLEDGMENTS

This study was supported by grants from the Swedish Medical Research Council (K2005-72X-15389-01A), (K2008-55X-15358-04-3) and (K2007-54X-03506-36-3), the Novo Nordisk Foundation, the Swedish Diabetes Association, the Torsten and Ragnar Söderbergs Foundations, the Inga-Britt and Arne Lundberg Foundation, The Foundation for Strategic Research and the European Community as part of the project EUGENE2, which is supported by the European Commission (Contract LSH MCT 2004-512013)
REFERENCES
1. Jaffe EA, Nachman RL, Becker CG, Minick CR: Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest 52:2745-2756, 1973
2. Gimbrone MA, Jr.: Culture of vascular endothelium. Prog Hemost Thromb 3:1-28, 1976
3. Davison PM, Bensch K, Karasek MA: Isolation and growth of endothelial cells from the microvessels of the newborn human foreskin in cell culture. J Invest Dermatol 75:316-321, 1980
4. Kern PA, Knedler A, Eckel RH: Isolation and culture of microvascular endothelium from human adipose tissue. J Clin Invest 71:1822-1829, 1983
5. Williams SK, Wang TF, Castrillo R, Jarrell BE: Liposuction-derived human fat used for vascular graft sodding contains endothelial cells and not mesothelial cells as the major cell type. J Vasc Surg 19:916-923, 1994
6. Koyama M, Satoh K, Yoshida H, Suzuki S, Koie H, Takamatsu S: Surface coverage of vascular grafts with cultured human endothelial cells from subcutaneous fat tissue obtained with a biopsy needle. Thromb Haemost 76:610-614, 1996
7. Jackson CJ, Garbett PK, Nissen B, Schrieber L: Binding of human endothelium to Ulex europaeus I-coated Dynabeads: application to the isolation of microvascular endothelium. J Cell Sci 96 (Pt 2):257-262, 1990
8. Hewett PW, Murray JC: Immunomagnetic purification of human microvessel endothelial cells using Dynabeads coated with monoclonal antibodies to PECAM-1. Eur J Cell Biol 62:451-454, 1993
9. Scott PA, Bicknell R: The isolation and culture of microvascular endothelium. J Cell Sci 105 (Pt 2):269-273, 1993
10. R. Bicknell. Endothelial Cell Culture—Handbooks in Practical Animal Cell Biology, Cambridge University Press, Cambridge, UK (1996).
11. Hutley LJ, Herington AC, Shurety W, Cheung C, Vesey DA, Cameron DP, Prins JB: Human adipose tissue endothelial cells promote preadipocyte proliferation. Am J Physiol Endocrinol Metab 281:E1037-1044, 2001
12. Carvalho E, Jansson PA, Axelsen M, Eriksson JW, Huang X, Groop L, Rondinone C, Sjostrom L, Smith U: Low cellular IRS 1 gene and protein expression predict insulin resistance and NIDDM. Faseb J 13:2173-2178, 1999
13. Jansson PA, Pellme F, Hammarstedt A, Sandqvist M, Brekke H, Caidahl K, Forsberg M, Volkman R, Carvalho E, Funahashi T, Matsuzawa Y, Wiklund O, Yang X, Taskinen MR, Smith U: A novel cellular marker of insulin resistance and early atherosclerosis in humans is related to impaired fat cell differentiation and low adiponectin. Faseb J 17:1434-1440, 2003
14. Federici M, Pandolfi A, De Filippis EA, Pellegrini G, Menghini R, Lauro D, Cardellini M, Romano M, Sesti G, Lauro R, Consoli A: G972R IRS-1 variant impairs insulin regulation of endothelial nitric oxide synthase in cultured human endothelial cells. Circulation 109:399-405, 2004
15. Remuzzi G, Perico N, Benigni A: New therapeutics that antagonize endothelin: promises and frustrations. Nat Rev Drug Discov 1:986-1001, 2002
16. Caballero AE, Arora S, Saouaf R, Lim SC, Smakowski P, Park JY, King GL, LoGerfo FW, Horton ES, Veves A: Microvascular and macrovascular reactivity is reduced in subjects at risk for type 2 diabetes. Diabetes 48:1856-1862, 1999
17. Ferri C, Bellini C, Desideri G, Baldoncini R, Properzi G, Santucci A, De Mattia G: Circulating endothelin-1 levels in obese patients with the metabolic syndrome. Exp Clin Endocrinol Diabetes 105 Suppl 2:38-40, 1997
18. Goldfine AB, Beckman JA, Betensky RA, Devlin H, Hurley S, Varo N, Schonbeck U, Patti ME, Creager MA: Family history of diabetes is a major determinant of endothelial function. J Am Coll Cardiol 47:2456-2461, 2006
19. Jiang ZY, Zhou QL, Chatterjee A, Feener EP, Myers MG, Jr., White MF, King GL: Endothelin-1 modulates insulin signaling through phosphatidylinositol 3-kinase pathway in vascular smooth muscle cells. Diabetes 48:1120-1130, 1999
20. Smith U, Sjostrom L, Bjornstorp P: Comparison of two methods for determining human adipose cell size. J Lipid Res 13:822-824, 1972
21. Gogg S, Smith U: Epidermal growth factor and transforming growth factor alpha mimic the effects of insulin in human fat cells and augment downstream signaling in insulin resistance. J Biol Chem 277:36045-36051, 2002
22. Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ: Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294-5299, 1979
23. Heid CA, Stevens J, Livak KJ, Williams PM: Real time quantitative PCR. Genome Res 6:986-994, 1996
24. Sandqvist M, Nyberg G, Hammarstedt A, Klintland N, Gogg S, Caidahl K, Ahren B, Smith U, Jansson PA: Low adipocyte IRS-1 protein expression is associated with an increased arterial stiffness in non-diabetic males. Atherosclerosis 180:119-125, 2005
25. Jiang ZY, Lin YW, Clemont A, Feener EP, Hein KD, Igarashi M, Yamauchi T, White MF, King GL: Characterization of selective resistance to insulin signaling in the vasculature of obese Zucker (fa/fa) rats. J Clin Invest 104:447-457, 1999
26. Kim JA, Montagnani M, Koh KK, Quon MJ: Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms. Circulation 113:1888-1904, 2006
27. Eringa EC, Stehouwer CD, van Nieuw Amerongen GP, Ouwehand L, Westerhof N, Sipkema P: Vasoconstrictor effects of insulin in skeletal muscle arterioles are mediated by ERK1/2 activation in endothelium. Am J Physiol Heart Circ Physiol 287:H2043-2048, 2004
28. Hadjadj S, Pean F, Gallois Y, Passa P, Aubert R, Weekers L, Rigalleau V, Baudueau B, Bekherraz A, Roussel R, Dussol B, Rodier M, Marechaud R, Lefebvre PJ, Marre M: Different patterns of insulin resistance in relatives of type 1 diabetic patients with retinopathy or nephropathy: the Genesis France-Belgium Study. Diabetes Care 27:2661-2668, 2004
29. Aljada A, Dandona P: Effect of insulin on human aortic endothelial nitric oxide synthase. Metabolism 49:147-150, 2000
30. Rask-Madsen C, King GL: Mechanisms of Disease: endothelial dysfunction in insulin resistance and diabetes. Nat Clin Pract Endocrinol Metab 3:46-56, 2007
31. Aguirre V, Werner ED, Giraud J, Lee YH, Shoelson SE, White MF: Phosphorylation of Ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action. J Biol Chem 277:1531-1537, 2002
32. Carlson CJ, Koterski S, Sciotti RJ, Poccard GB, Rondinone CM: Enhanced basal activation of mitogen-activated protein kinases in adipocytes from type 2 diabetes: potential role of p38 in the downregulation of GLUT4 expression. Diabetes 52:634-641, 2003
33. Rondinone CM, Wang LM, Lonroth P, Wesslau C, Pierce JH, Smith U: Insulin receptor substrate (IRS) 1 is reduced and IRS-2 is the main docking protein for phosphatidylinositol 3-
kinase in adipocytes from subjects with non-insulin-dependent diabetes mellitus. *Proc Natl Acad Sci U S A* 94:4171-4175, 1997
34. El-Osta A, Brasacchio D, Yao D, Pocai A, Jones PL, Roeder RG, Cooper ME, Brownlee M: Transient high glucose causes persistent epigenetic changes and altered gene expression during subsequent normoglycemia. *J Exp Med* 205:2409-2417, 2008
35. Matouk CC, Marsden PA: Epigenetic regulation of vascular endothelial gene expression. *Circ Res* 102:873-887, 2008
36. Ak G, Buyukberber S, Sevinc A, Turk HM, Ates M, Sari R, Savli H, Cigli A: The relation between plasma endothelin-1 levels and metabolic control, risk factors, treatment modalities, and diabetic microangiopathy in patients with Type 2 diabetes mellitus. *J Diabetes Complications* 15:150-157, 2001
37. Irving RJ, Noon JP, Watt GC, Webb DJ, Walker BR: Activation of the endothelin system in insulin resistance. *QJM* 94:321-326, 2001
38. Wilkes JJ, Hevener A, Olefsky J: Chronic endothelin-1 treatment leads to insulin resistance in vivo. *Diabetes* 52:1904-1909, 2003
39. Chisalita SI, Arnqvist HJ: Insulin-like growth factor I receptors are more abundant than insulin receptors in human micro- and macrovascular endothelial cells. *Am J Physiol Endocrinol Metab* 286:E896-901, 2004
40. Nitert MD, Chisalita SI, Olsson K, Bornfeldt KE, Arnqvist HJ: IGF-I/insulin hybrid receptors in human endothelial cells. *Mol Cell Endocrinol* 229:31-37, 2005
41. Chi JT, Chang HY, Haraldsen G, Jahnsen FL, Troyanskaya OG, Chang DS, Wang Z, Rockson SG, van de Rijn M, Botstein D, Brown PO: Endothelial cell diversity revealed by global expression profiling. *Proc Natl Acad Sci U S A* 100:10623-10628, 2003
42. Rupnick MA, Panigrahy D, Zhang CY, Dallabrida SM, Lowell BB, Langer R, Folkman MJ: Adipose tissue mass can be regulated through the vasculature. *Proc Natl Acad Sci U S A* 99:10730-10735, 2002
FIGURE LEGENDS

Fig. 1 a, b, c and d. Characterization of human subcutaneous microvascular endothelial cells (MVEC) in culture. a) Confluent MVEC viewed under phase-contrast microscopy exhibit the typical endothelial cobblestone morphology. b) Fluorescence microscopy showing positive immunoreactivity for vWF and c) for Ac LDL uptake. d) Phase-contrast micrograph showing tube formation by MVEC grown on collagen. Original magnification: A and D x 40; B and C x 100.

Fig. 2. Upper panel—Insulin receptor expression and activation by insulin. MVEC from control and type 2 diabetic subjects as well as HUVEC cells were starved for 4 h and incubated in serum free media for 20 min with or without insulin (100 nM). The lysates were subjected to immunoprecipitation with an anti-IR antibody and the immunoprecipitated proteins were separated on a 7.5% SDS-PAGE, transferred and probed with anti-phosphotyrosine (pY99). The figure shows representative immunoblots.

Lower panel—The insulin receptors were immunoprecipitated and immunoblotted with anti-phosphotyrosine (pY99) and anti-insulin receptor antibodies.

Fig. 3. MVEC from control and type 2 diabetic subjects were starved for 4 h and incubated for 20 min with or without insulin (100 nM). The lysates were separated on SDS-PAGE, transferred and analyzed by immunoblotting. The upper panel shows representative blots of the IRS-1 protein. The bands on top correspond to a long 10% gel and the bands under to a short 7.5% gel. These gels were loaded with different amounts of protein.

The bars in the graph below represent the means of scanned data from three control and three type 2 subjects *p=0.05 and ***p<0.001 compared to respective control.

Fig. 4. MVEC from different control (n=4) and type 2 diabetic (n=4) subjects as well as HUVEC cells were starved for 4 h and incubated for 20 min with or without insulin (100 nM). The lysates were separated on a 10% SDS-PAGE and analyzed by immunoblotting with anti-phosphoserine (p-ser) PKB/Akt or anti-PKB/Akt antibodies. Bottom panel: The graph shows the p-ser PKB/PKB volume ratio from four separate experiments quantified by scanning. *p=0.04 compared to control.

Fig. 5. Upper panel: MVEC from different control (n=5) and type 2 diabetic (n=5) subjects were starved for 4 h and incubated for 20 min with or without insulin (100 nM). The lysates were separated on a 10% SDS-PAGE, transferred and probed with phosphospecific tyrosine/threonine MAPK (ERK1 and ERK2) or MAPK antibodies. Bottom panel: The graph shows the p-MAPK/MAPK volume ratio. The results are means ±SEM of scanned data from five separate experiments *p=0.02, **p=0.01 compared to respective control.

Fig. 6. Upper panel: MVEC from different control and type 2 diabetic subjects as well as HUVEC cells were starved for 4 h and lysates separated on a 10% SDS-PAGE, transferred and probed with anti-eNOS antibody. Protein from isolated adipocytes from one of the same subjects was included as control. Bottom panel: MVEC from control and type 2 diabetic subjects as well as HUVEC cells were starved and incubated with or without insulin (100nM) for 24 h. The RNA was extracted as described in Research Design and Methods and the eNOS gene expression
quantified by TaqMan RT-PCR. The results, expressed as mRNA/18s rRNA ratio, are the means of data from five (MVEC) or six (HUVEC) experiments. ***p<0.001 compared to MVEC (control or type 2), # p=0.03 for the effect of insulin to increase eNOS mRNA levels compared to basal HUVEC cells.

Fig.7. MVEC from different control (n=8) and type 2 diabetic (n=8) subjects as well as HUVEC cells were starved and incubated with or without insulin (100nM) for 24 h. The RNA was extracted as described in Research Design and Methods and the endothelin-1 gene expression quantified by TaqMan RT-PCR. The results, expressed as mRNA/18s rRNA ratio, are the means of data from eight (MVEC) or five (HUVEC) experiments. * p <0.05 compared to respective control, # p< 0.05 compared to type 2 MVEC.

Fig.8 MVEC were starved for 4 h and incubated as shown with endothelin-1 (ET-1) (100nM), PD98059 (50µM) or the ET-1 receptor antagonists BQ123 and BQ788 (20µM) for 24 h. The cell lysates from control (upper panel) and type 2 diabetic subjects (middle panel) were separated on a 10% SDS-PAGE, transferred and probed for phosphospecific tyrosine/threonine MAPK (ERK1 and ERK2) and reprobed with MAPK protein. The figure shows representative immunoblots.

The bottom graph shows the p-MAPK /MAPK volume ratio as means ±SEM of scanned data from four separate experiments, *p<0.05 compared to basal.
Figure 1

A

B

C

D

Figure 2

| Control | Type 2 | HUVEC |
|---------|--------|-------|
| bas     | ins    | bas   |

pY-IR

| bas | ins |

ib anti-pY

| bas | ins |

ib IR
Endothelial cells in type 2 diabetes

Figure 3

![Image showing IRS-1 expression in Control and Type 2 MVEC under basal (bas) and insulin (ins) conditions.](image)

Volume

Control | Type 2
---|---
Basal | 4 | 6
Insulin | 2 | 4

Note: ** indicates a significant difference at p < 0.001 and * indicates a significant difference at p < 0.05.

Figure 4

![Image showing p-ser PKB expression in Control, Type 2 HUVEC, and bas and ins conditions.](image)

Volume ratio (p-ser PKB/PKB)

Control | Type 2
---|---
Basal | 2 | 3
Insulin | 1 | 2

Note: * indicates a significant difference at p < 0.05.
Figure 5

**MAPK**

| Control | Type 2 |
|---------|--------|
| bas     | bas    |
| ins     | ins    |

**Volume ratio** (p-MAPK/MAPK)

Control Type 2

Type 2

Figure 6

**eNOS**

| MVEC | HUVEC | Adipocytes |
|------|-------|------------|
| Control | Type 2 |

**eNOS gene expression**

Control Type 2 HUVEC

**MVEC**

**HUVEC**

**Adipocytes**
**Figure 7**

Endothelin-1 gene expression

![Bar graph showing mRNA/18s rRNA expression levels for Control, Type 2, and HUVEC conditions.](image)

**Figure 8**

![Western blot images showing p-MAPK (ERK1/2) and MAPK (ERK1/2) levels under different conditions: basal (bas), PD, ET, ET +BQ123, and BQ788.](image)

![Bar graph showing volume ratio for basal (bas), PD, and BQ123+BQ788 conditions.](image)