Increased MAPK Activation and Impaired Insulin Signaling in Subcutaneous Microvascular Endothelial Cells in Type 2 Diabetes: The Role of Endothelin-1

Silvia Gogg, Ulf Smith, and Per-Anders Jansson

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 DESIGN AND METHODS—Stromavascular cells from subcutaneous needle biopsies of type 2 diabetic 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 was low, but insulin increased tyrosine phosphorylation of both the insulin receptor and insulin receptor substrate (IRS)-1 and activated protein kinase B (PKB). The IRS-1 protein expression was reduced and the serine phosphorylation of PKB in response to insulin attenuated whereas basal and insulin-stimulated phosphorylation of extracellular signal–related kinase (ERK)1/2 was increased in type 2 diabetes MVEC. Endothelin (ET)-1 mRNA levels were significantly higher in type 2 diabetes cells. The addition of ET-1 increased the phosphorylation of mitogen-activated protein kinase (MAPK), an effect antagonized by the MEK-1 inhibitor PD98059. Furthermore, the endothelin ET_A and ET_B receptor antagonists BQ123 and BQ788 decreased basal MAPK activity in type 2 diabetes MVEC and prevented the ET-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 type 2 diabetes. Our data also support a role of ET-1 for the increased MAPK activity seen in nonstimulated type 2 diabetes MVEC.
Diabetes **58:2238–2245, 2009**

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.

Corresponding author: Silvia Gogg, silvia.gogg@medic.gu.se.

Received 17 July 2008 and accepted 15 June 2009.

Published ahead of print at http://diabetes.diabetesjournals.org on 6 July 2009. DOI: 10.2337/db08-0961.

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; 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 to obtain, validate, and characterize endothelial cells from small human tissue specimens.

We have previously shown that insulin resistance and type 2 diabetes are associated with an impaired insulin signaling because of a reduced expression of the key docking protein insulin receptor substrate (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 because of 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 the two G-coupled protein receptors ET_A and ET_B. Endothelin (ET)-1 is secreted by endothelial cells and produces multiple actions like regulation of vascular tone, tissue remodeling, 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 ET-1 is considered to play a role for this by interfering with the insulin-signaling pathway in the arterial wall (19).

We 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 ET-1 is overexpressed in MVEC from type 2 diabetes donors, leading to increased mitogen-activated protein kinase (MAPK) activity.

RESEARCH DESIGN AND METHODS

Source of adipose tissue and MVEC. Human needle biopsies of the subcutaneous adipose tissue were obtained from the abdominal region of nondiabetic ($n = 12$) and type 2 diabetic ($n = 12$) subjects 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/content/early/2009/06/23/db08-0961/suppl/DC1).

About 3 g of tissue was incubated in sterile Medium199 (Invitrogen, Paisley, U.K.) containing 25 mmol/l HEPES, 4% 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 four 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, after centrifugation (1,300 rpm for 5 min), the pellet was resuspended in endothelial cell growth medium Bulletkit (EGM-2-MV) consisting of endothelial cell basal medium-2 (EBM-2) supplemented with EGM-2-MV SingleQuots containing hEGF, hydrocortisone, FBS, vascular endothelial growth factor, R3-IGF-1, ascorbic acid, and gentamicin sulfate/amphotericin-B (GA-1000) (Clonetics, BioWhittaker, Verviers, Belgium). FGFb was not added to the medium.

The cells obtained were seeded into a 75 cm^2 culture flask and allowed to grow at 37°C and 5% CO_2 in the same media. Before the cells reached confluence, after approximately 1 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).

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 (2.8×10^5 beads/ml) coated with CD31 antibody (PECAM-1). The beads rapidly target and partially coat the endothelial cells expressing the CD31 receptor.

After the incubation, the culture was washed twice with PBS and trypsinized. The cells were collected into a tube and centrifuged for 3 min at 1,300 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), 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 endothelial cell growth medium Bulletkit (EGM-MV2) and maintained at 37°C and 5% CO_2. The cells obtained with this procedure were 99% endothelial cells with typical cobblestone morphology. The endothelial cells were characterized by indirect immunofluorescense 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 h. 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 h. 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 h and washed again.

Acetylated LDL uptake of MVEC. The cells were incubated in eight-well plastic chamber slides for 48 h. Acetylated LDL labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate (Dil-Ac-LDL) (Biomedical Technologies) was added to the culture medium at a final concentration of 10 μg/ml, and the cells were incubated at 37°C for 4 h. 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, H_2O, and PBS following the manufacturers recommendations. The mix was poured into 12-well culture plates (200 μl/well) and allowed to solidify for 1 h in a humidified atmosphere of 5% CO_2 at 37°C. After 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 h, before adding insulin for 20 min. ET-1, BQ123, BQ788 (Sigma-Aldrich, Stockholm, Sweden), or PD98059 (Calbiochem-Merck Chemicals, Nottingham, U.K.) were added to the culture media for 24 h at the indicated concentrations. When used in combination with other factors, PD98059 was added 1 h before.

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

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 nmol/l 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 h at 4°C as previously reported (21). The proteins were quantified by the bicinchonic acid 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, Foster City, CA) using18s rRNA as endogenous control subjects (23). The gene expression predesigned primer and probe set #Hs00167166_ml was used for eNOS and #Hs00174961_m1 for ET-1 gene analysis (Applied Biosystems).

Human umbilical vein endothelial cells. Human umbilical vein endothelial cells (HUVECs) (ATCC-CRL-1730) were grown in Kaighn's modified F12K media (American *Type Culture* Collection [ATCC]) from LGC Promochem (Boras, Sweden). The media was supplemented with 0.1 mg/ml heparin and 0.03 mg/ml endothelial cell growth supplement, both from Sigma-Aldrich, and 10% FBS (GIBCO, Invitrogen).

Immunoblotting. Crude cell extracts were boiled in Laemmli buffer containing 150 mmol/l dithiothreitol for 5 min. The samples were analyzed by SDS-PAGE (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, U.K.), insulin receptor and eNOS were from Transduction (BD Biosciences, Erembodegem, Belgium), and antiphosphotyrosine (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, U.K.), 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-insulin receptor 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-PAGE (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 means ± SE. 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 nonendothelial cells (CD31 negative) that were recovered with the positive cells. This variation was not related to the subject phenotype (control or type 2 subjects) nor to the defined experimental conditions used. The nonendothelial 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 3×10^3 cells/cm^2. The adherent cultured human MVEC monolayer

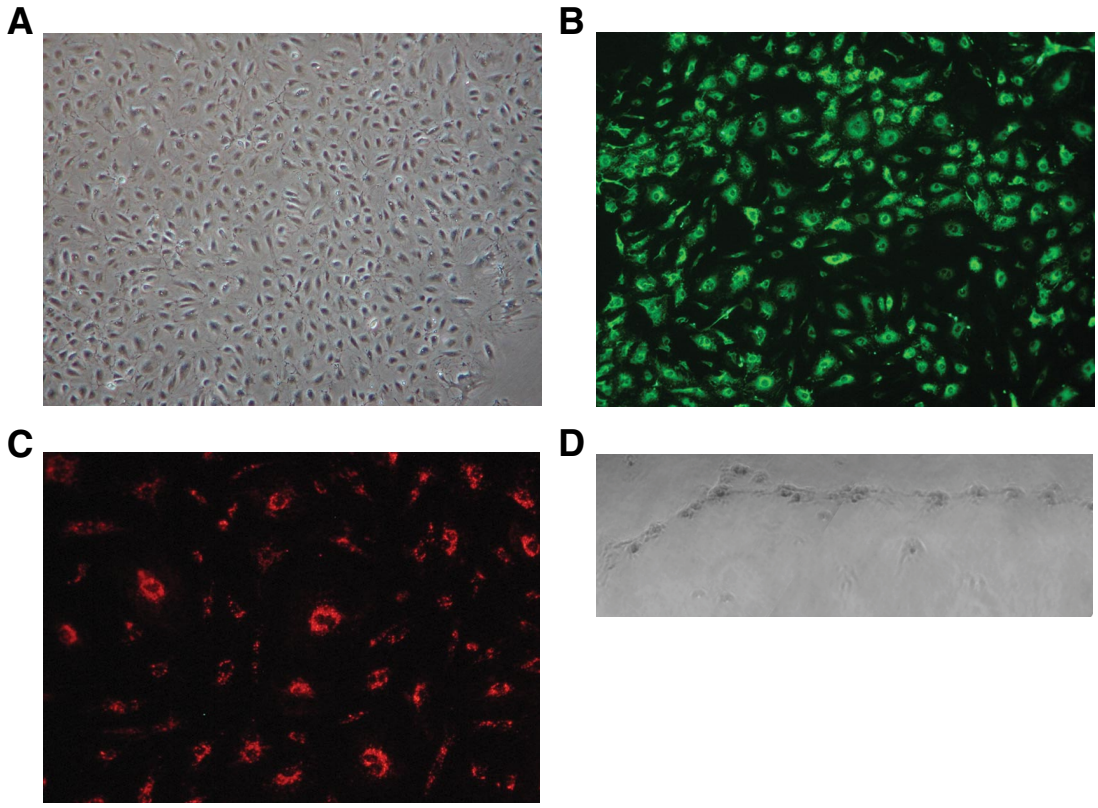

FIG. 1. *A–D*: Characterization of human subcutaneous microvascular endothelial cells (MVEC) in culture. *A*: Confluent MVEC viewed under phase-contrast microscopy exhibit the typical endothelial cobblestone morphology. *B*: Fluorescence microscopy showing positive immunoreactivity for vWF and (C) for Ac LDL uptake. *D*: Phase-contrast micrograph showing tube formation by MVEC grown on collagen. Original magnification *A* and *D* × 40; *B* and *C* × 100. (A high-quality digital representation of this figure is available in the online issue.)

assumed the typical cobblestone morphology, which was maintained throughout the 10–13 passages studied (Fig. 1*A*). 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. 1*B*). In addition, the cells incorporated acetylated LDL, another marker characterizing endothelial cells (Fig. 1*C*).

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. Figure 1*D* shows the morphogenic response of MVEC in collagen and EGM-MV2 media. Morphologic changes and reorganization of the cells were apparent as early as 12 h 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 type 2 diabetes individuals. For comparison, we performed parallel experiments with the HUVEC line, a well-documented endothelial cell line.

Expression of the insulin receptor was low in MVEC compared with HUVEC, but there was a clear insulin-stimulated tyrosine phosphorylation of the receptor in both type 2 and control subjects in MVEC and in HUVECs (Fig. 2).

Because the presence of insulin receptor 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, *right 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 MAPKs extracellular signal–related kinase (ERK)1/2. IRS-1 was clearly the predomi-

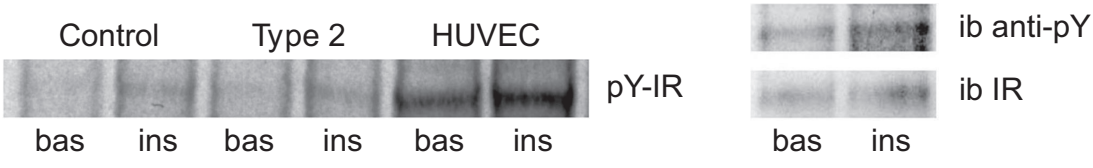

FIG. 2. *Left panel*: Insulin receptor expression and activation by insulin. MVEC from control and type 2 diabetic subjects as well as HUVEC cells were starved for 4 h and incubated in serum free media for 20 min with or without insulin (100 nmol/l). The lysates were subjected to immmunoprecipitation with an anti-IR antibody and the immunoprecipitated proteins were separated on a 7.5% SDS-PAGE, transferred and probed with antiphosphotyrosine (pY99). The figure shows representative immunoblots. *Right panel*: The insulin receptors were immunoprecipitated and immunoblotted with antiphosphotyrosine (pY99) and anti-insulin receptor antibodies. bas, basal; ins, insulin.

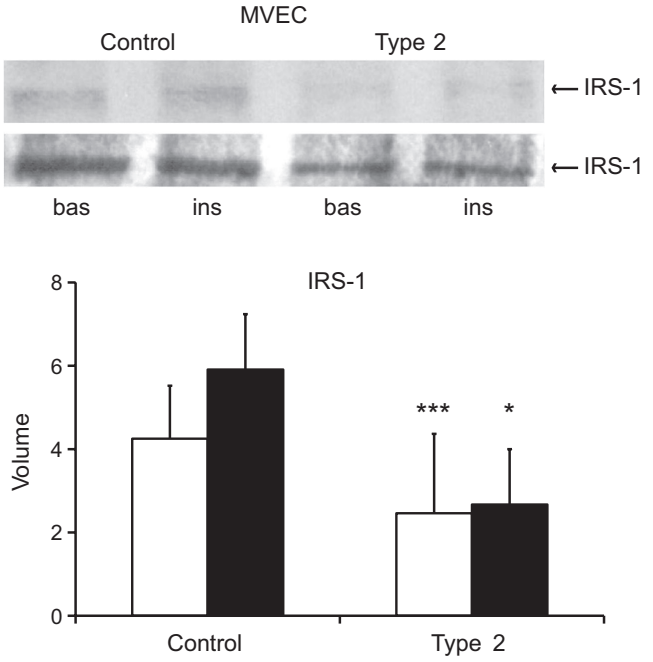

FIG. 3. MVEC from control and type 2 diabetic subjects were starved for 4 h and incubated for 20 min with or without insulin (100 nmol/l). The lysates were separated on SDS-PAGE, transferred, and analyzed by immunoblotting. The *upper panel* shows representative blots of the IRS-1 protein. The bands 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 with respective control subjects. bas, basal, (□); ins, insulin, (■).

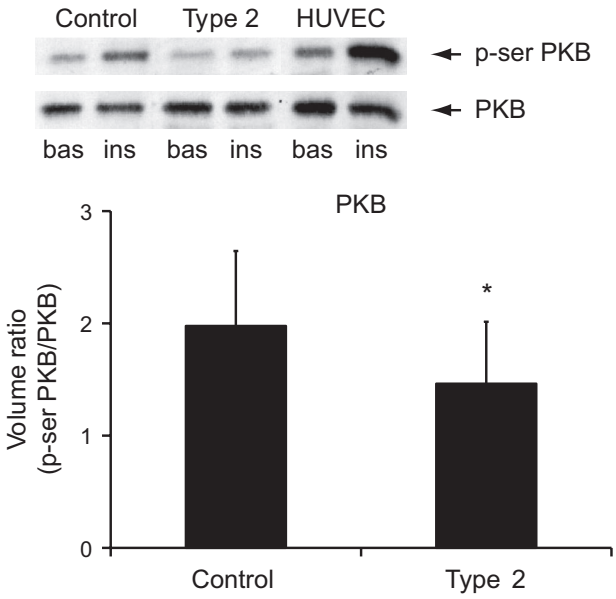

FIG. 4. MVEC from different control ($n = 4$) and type 2 diabetic ($n = 4$) subjects as well as HUVEC cells were starved for 4 h and incubated for 20 min with or without insulin (100 nmol/l). The lysates were separated on a 10% SDS-PAGE and analyzed by immunoblotting with antiphosphoserine (p-ser) PKB/Akt 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 with control subjects. bas, basal; ins, insulin, (■).

nant 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 type 2 diabetic subjects (Fig. 3).

This reduction was also reflected by the impaired activation and serine phosphorylation of the downstream protein, protein kinase B (PKB)/Akt, in MVEC from type 2 diabetic subjects, whereas 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 MAPKs ERK1/2 had an increased phosphorylation in the basal state in MVEC from type 2 diabetic subjects (Fig. 5, *upper panel*). Insulin further increased the MAPK phosphorylation in control subject MVECs ($P = 0.057$) while there was no effect of insulin ($P = 0.18$) in the type 2 diabetic subject 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 because of 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 with HUVEC (Fig. 6, *top*). The gene expression of eNOS was also almost undetectable in the MVEC, from both control and type 2 diabetic subjects, while there was a clear expression of both the gene and protein in HUVECs (Fig. 6, *bottom panel*). Insulin had no effect on the expression of eNOS mRNA in MVEC while the levels were significantly in-

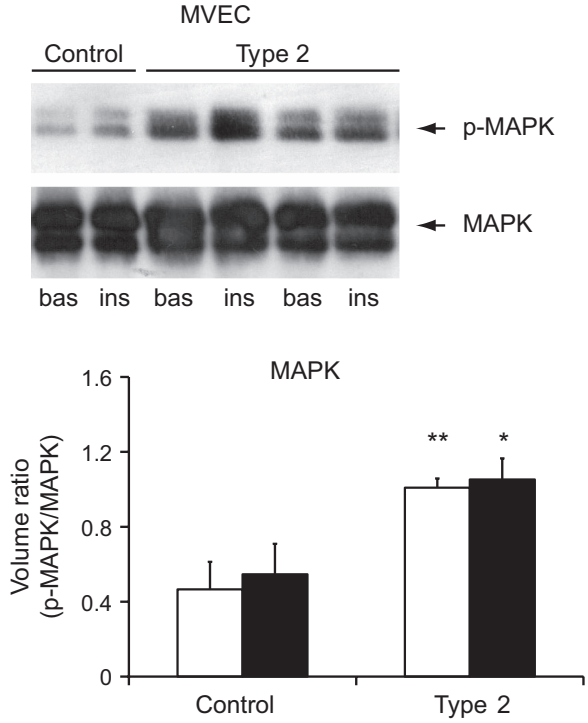

FIG. 5. *Upper panel*: MVEC from different control ($n = 5$) and type 2 diabetic ($n = 5$) subjects were starved for 4 h and incubated for 20 min with or without insulin (100 nmol/l). The lysates were separated on a 10% SDS-PAGE, transferred, and probed with phosphospecific tyrosine/threonine MAPK (ERK1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

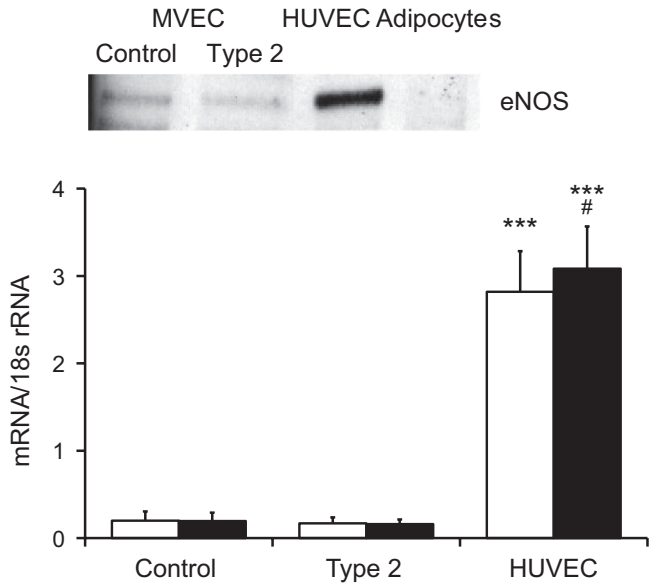

FIG. 6. *Upper panel*: MVEC from different control subjects and type 2 diabetic subjects as well as HUVEC cells were starved for 4 h and lysates separated on a 10% SDS-PAGE, transferred and probed with anti-eNOS antibody. Protein from isolated adipocytes from one of the same subjects was included as control. *Lower panel*: eNOS gene expression. MVEC from control and type 2 diabetic subjects as well as HUVEC cells were starved and incubated with or without insulin (100 nmol/l) 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 with MVEC (control or type 2 subjects), #$P = 0.03$ for the effect of insulin to increase eNOS mRNA levels compared with basal HUVEC cells. Basal (□); insulin (■).

creased in HUVEC. 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 Ser^{1177} and Thr^{495}. Because 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.

ET-1 expression. As shown in Fig. 7, ET-1 mRNA levels were significantly higher in cells from type 2 diabetes

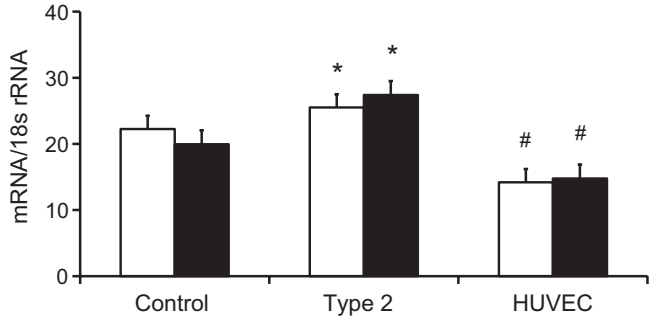

FIG. 7. ET-1 gene expression. MVEC from different control ($n = 8$) and type 2 diabetic ($n = 8$) subjects as well as HUVEC cells were starved and incubated with or without insulin (100 nmol/l) for 24 h. The RNA was extracted as described in RESEARCH DESIGN AND METHODS and the ET-1 gene expression quantified 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 with respective control subjects, #$P < 0.05$ compared to type 2 MVEC. Basal (□); insulin (■).

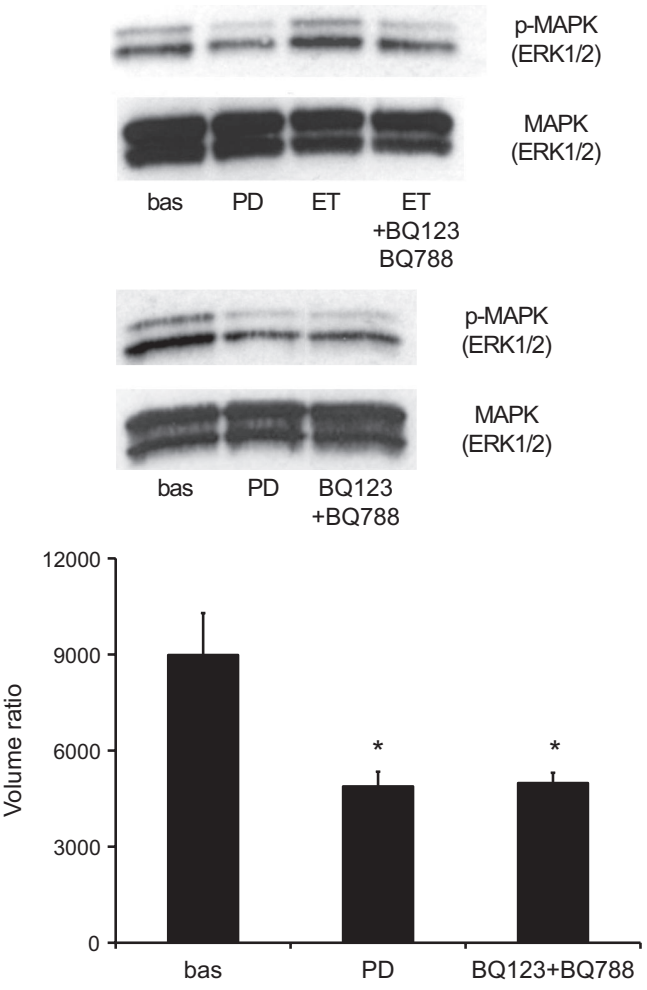

FIG. 8. MVEC were starved for 4 h and incubated as shown with ET-1 (100 nmol/l), PD98059 (50 μmol/l), or the ET-1 receptor antangonists BQ123 and BQ788 (20 μmol/l) for 24 h. The cell lysates from control (*upper panel*) and type 2 diabetic subjects (*middle panel*) were separated on a 10% SDS-PAGE, transferred 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 ± SE of scanned data from four separate experiments, *$P < 0.05$ compared with basal. bas, basal; PD, PD98059.

individuals, both when compared with cells from the control group as well as with HUVECs, while there was no significant difference between the control group and HUVECs. Adding insulin (100 nmol/l) for 24 h did not increase ET-1 expression in any of the three groups studied (Fig. 7).

To further explore its role, we added ET-1 (100 nmol/l) to the culture medium and found that it increased ERK1/2 phosphorylation in control subject MVEC (Fig. 8, *upper panel*).

The addition of the ET_A (BQ123) and ET_B (BQ788) receptor antagonists (20 μmol/l) as well as the MEK-1 inhibitor PD98059 (50 μmol/l) prevented this effect of ET-1 (Fig. 8, *upper panel*). It should be noted that both ET-1 receptor antagonists were required because both A and B receptors were present in MVEC (data not shown).

To examine if the increased MAPK activation in the nonstimulated state in MVEC from type 2 diabetes individuals was because of the increased endothelin, we incubated the cells with both ET_A and ET_B receptor antagonists. As shown in Fig. 8 (*middle* and *bottom*

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 ET-1 is increased in MVEC from type 2 diabetes 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 because these cells are considered to be important target cells for insulin. We have previously shown that IRS-1 protein expression, like GLUT4, is reduced in the adipose cells in insulin resistance and is, indeed, a biomarker of the IRS (12). Moreover, a group of subjects exhibiting low adipocyte IRS-1 expression displayed early signs of atherosclerosis, including an increased intima-media thickness (13) and an increased vascular stiffness as measured with pulse tonometry (24) compared with 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 type 2 diabetic 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 because of 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 that also was extremely low.

An intriguing finding was the increased nonstimulated (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 type 2 diabetes (32), and IRS-1 protein is also decreased (33). The finding of an increased ERK1/2 phsophorylation 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 that activate the cells.

DNA methylation and posttranslational histone modifications are epigenetic mechanisms that are known to influence mammalian gene expression. Type 2 diabetes 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, because of high glucose concentrations or other factors (34,35), may lead to a long-term activation of genes in the type 2 diabetes MVEC. However, further studies are needed to address this point.

Interestingly, ET-1 expression was significantly increased in the MVEC from diabetic subjects when compared with both nondiabetic subjects and HUVECs. In fact, there was no difference between the nondiabetic and HUVECs, further corroborating the increase in the diabetic cells. Increased circulating ET-1 levels have also been reported in type 2 diabetes (36,37), and this finding is consistent with the increased ERK1/2 activation seen since these kinases have been associated with an increased endothelin expression (30).

In our study, the increased ET-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 ET-1 to the culture media increased MAPK phosphorylation in control subject MVEC. This effect was prevented by PD98059 showing that ERK1/2 is a target for ET-1 in MVEC. Importantly, the increased nonstimulated (basal) MAPK activation in MVEC from type 2 diabetic subjects was completely antagonized by the addition of the ET-1 receptor antagonists BQ123 and BQ788. The use of both antagonists was required because MVEC were found to express both the ET_A and ET_B receptors. These results support the concept that the increased ET-1 expression plays a major role for the increased basal MAPK activation seen in MVEC of type 2 diabetic subjects.

ET-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 type 2 diabetic subjects is because of the increased ET-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 cannot exclude that signaling downstream the IGF-1 receptor is more important than that

downstream the insulin receptor. In fact, previous studies have suggested that IGF-1 is more important than insulin for the generation of nitric oxide (NO) via the cognizant receptor or via hybrid receptors in endothelial cells (39,40). Nevertheless, our data show that the insulin receptor 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 type 2 diabetes 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 type 2 diabetic 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 ET-1 levels in MVEC from type 2 diabetic subjects. These observations support the concept of "selective" insulin resistance as an underlying factor for a dysregulated microvascular endothelium in type 2 diabetes. 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).

No potential conflicts of interest relevant to this article were reported.

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 1973;52:2745–2756
2. Gimbrone MA, Jr. Culture of vascular endothelium. Prog Hemost Thromb 1976;3:1–28
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 1980;75:316–321
4. Kern PA, Knedler A, Eckel RH. Isolation and culture of microvascular endothelium from human adipose tissue. J Clin Invest 1983;71:1822–1829
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 1994;19:916–923
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 1996;76:610–614
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 1990;96(Pt 2):257–262
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 1993;62:451–454
9. Scott PA, Bicknell R. The isolation and culture of microvascular endothelium. J Cell Sci 1993;105(Pt 2):269–273
10. R. Bicknell. *Endothelial Cell Culture—Handbooks in Practical Animal Cell Biology*. Cambridge University Press, Cambridge, U.K., 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 2001;281:E1037–E1044
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 1999;13:2173–2178
13. Jansson PA, Pellme F, Hammarstedt A, Sandqvist M, Brekke H, Caidahl K, Forsberg M, Volkmann 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 2003;17:1434–1440
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 2004;109:399–405
15. Remuzzi G, Perico N, Benigni A. New therapeutics that antagonize endothelin: promises and frustrations. Nat Rev Drug Discov 2002;1:986–1001
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 1999;48:1856–1862
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 1997;105(Suppl):38–40
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 2006;47:2456–2461
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 1999;48:1120–1130
20. Smith U, Sjostrom L, Bjornstorp P. Comparison of two methods for determining human adipose cell size. J Lipid Res 1972;13:822–824
21. Gogg S, Smith U. Epidermal growth factor and transforming growth factor α mimic the effects of insulin in human fat cells and augment downstream signaling in insulin resistance. J Biol Chem 2002;277:36045–36051
22. Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 1979;18:5294–5299
23. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. Genome Res 1996;6:986–994
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 2005;180:119–125
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 1999;104:447–457
26. Kim JA, Montagnani M, Koh KK, Quon MJ. Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms. Circulation 2006;113:1888–1904
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 2004;287:H2043–H2048
28. Hadjadj S, Pean F, Gallois Y, Passa P, Aubert R, Weekers L, Rigalleau V, Bauduceau 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 2004;27:2661–2668

29. Aljada A, Dandona P. Effect of insulin on human aortic endothelial nitric oxide synthase. Metabolism 2000;49:147–150
30. Rask-Madsen C, King GL. Mechanisms of Disease: endothelial dysfunction in insulin resistance and diabetes. Nat Clin Pract Endocrinol Metab 2007;3:46–56
31. Aguirre V, Werner ED, Giraud J, Lee YH, Shoelson SE, White MF. Phosphorylation of Ser^{307} in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action. J Biol Chem 2002;277:1531–1537
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 2003;52:634–641
33. Rondinone CM, Wang LM, Lonnroth 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 1997;94:4171–4175
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 2008;205:2409–2417
35. Matouk CC, Marsden PA. Epigenetic regulation of vascular endothelial gene expression. Circ Res 2008;102:873–887
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 2001;15:150–157
37. Irving RJ, Noon JP, Watt GC, Webb DJ, Walker BR. Activation of the endothelin system in insulin resistance. Q J Med 2001;94:321–326
38. Wilkes JJ, Hevener A, Olefsky J. Chronic endothelin-1 treatment leads to insulin resistance in vivo. Diabetes 2003;52:1904–1909
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 2004;286:E896–E901
40. Nitert MD, Chisalita SI, Olsson K, Bornfeldt KE, Arnqvist HJ. IGF-I/insulin hybrid receptors in human endothelial cells. Mol Cell Endocrinol 2005;229:31–37
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 2003;100:10623–10628
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 2002;99:10730–10735