Endothelial cell signalling supports pancreatic beta cell function in the rat

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Received: 13 October 2008 / Accepted: 14 July 2009 / Published online: 11 August 2009
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Abstract

Aims/hypothesis The proximity of endothelial cells and beta cells in islets by necessity means that they are exposed to each other's products. Whereas islet endothelial cells require signals from beta cells to function properly, endothelin-1, thrombospondin-1 and laminins, among others, have been identified as endothelial-derived molecules, although their full effects on beta cells have not been explored. We tested the hypothesis that islet endothelial-derived products affect beta cell function.

Methods Endothelial cells from rat islets were proliferated and purified. Endothelium-conditioned culture medium (ECCM) was obtained by maintaining the endothelial cells in culture medium. Islet function was evaluated following exposure of cultured islets to standard culture medium or ECCM. Changes in mRNA levels for key beta cell metabolic enzymes were also measured in islets after ECCM exposure.

Results Glucose-stimulated insulin release and islet insulin content were markedly enhanced by exposure to ECCM. This was at least partly explained by improved mitochondrial function, as assessed by glucose oxidation and an upregulation of the mitochondrial gene for glycerol-3-phosphate dehydrogenase (*mGpdh* [also known as *Gpd2*]), combined with upregulation of the rate-limiting enzyme in the glycolysis, glucokinase, in the islets. The intracellular degradation of insulin was also decreased in the islets. Islet endothelial cells produced laminins, and the positive effects of islet endothelial cells were prevented by addition of a neutralising antibody to the β 1-chain of laminin. Addition of exogenous laminin stimulated islet function.

Conclusions/interpretation This study provides proof of principle that endothelial cells can affect the function of beta cells in their vicinity and that this is at least partially mediated by laminins.

Keywords Endothelial cells · Beta cells · Laminin

Electronic supplementary material The online version of this article (doi:10.1007/s00125-009-1485-6) contains supplementary material, which is available to authorized users.

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Abbreviations

BS-1	<i>Bandeiraea simplicifolia</i>
CM	Culture medium
COX-2	Cyclooxygenase 2
DiI-Ac-LDL	Diiodoacetyl indocarbocyanine acetylated LDL
ECCM	Endothelium-conditioned culture medium
ECM	Endothelial culture medium
ET	Endothelin
HDMEC	Human dermal microvascular endothelial cells
HGF	Hepatocyte growth factor
LM	Laminin
MSC	Mesenchymal stem cells
RBMVEC	Rat brain microvascular endothelial cells

α -SMA	Alpha smooth muscle actin
TSP	Thrombospondin
VE-cadherin	Vascular endothelial cadherin

Introduction

Endothelium has long been considered as a form of plumbing, being important only for transport functions. However, it has in recent years become obvious that endothelial cells produce a number of growth factors, cytokines and other substances that may affect not only the endothelial cells themselves, but also surrounding cells [1]. The importance of endothelial cell signalling for parenchymal cells was first demonstrated for liver and pancreatic organogenesis [2, 3], and growth of adult prostate [4] and liver [5]. Whether endothelial cells may not only affect parenchymal differentiation and growth, but also parenchymal function through paracrine signalling has been less studied [6, 7].

The pancreatic islets in the adult are among the most vascularised of all organs in the body with a unique dense glomerular-like angioarchitecture. This means that in the islet, each beta cell is surrounded by at least one islet endothelial cell, and therefore these cells by necessity are exposed to each other's products [8]. Indeed, the high islet vascular density and the vascular phenotype, with a great number of fenestrations, is induced and maintained by secretion of vascular endothelial growth factor from the beta cells [9–11]. Recently, we and others have shown that endothelial cells signal back and contribute to maintenance [12], and during certain conditions even expansion, of the adult beta cell mass [6, 13–15]. In the present study, we tested the hypothesis that islet endothelial cell products may directly influence beta cell function.

Methods

Animals Adult, inbred male Wistar–Furth rats purchased from Scanbur (Sollentuna, Sweden) were used. The animals had free access to pelleted food and autoclaved tap water. All experimental procedures were approved by the Animal Ethics Committee for Uppsala University.

Chemicals All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise mentioned.

Islet isolation Islets were isolated by using collagenase digestion followed by separation on a density gradient. Briefly, under deep anaesthesia with sodium pentobarbital (Apoteket, Gothenburg, Sweden; 200 mg/kg i.p.), a

laparotomy was performed and the pancreas exposed. After ligation at the ampulla of Vater, 5 ml collagenase solution (from *Clostridium histolyticum*; F. Hoffman-La Roche, Basel, Switzerland) was injected into the pancreas via the common bile duct. The animal was killed and the pancreas dissected from surrounding tissues, removed and incubated in a water bath for approximately 16 min at 37°C. The islets were separated by a density gradient (Histopaque-1077) centrifugation at 900×g for 22 min. After washing, islets were handpicked and groups of 150 islets were maintained free-floating at 37°C (air/CO₂; 95:5) in culture medium (CM) consisting of RPMI 1640 supplemented with L-glutamine (2 mmol/l), benzylpenicillin (100,000 U/l; Hoffman-La Roche), streptomycin (0.1 g/l; Hoffman-La Roche) and 10% (vol./vol.) FCS for 4–6 days. CM was changed every 48 h.

Isolation of islet endothelial cells Outgrowth of islet stromal cells on a collagen matrix was stimulated using a modification of a previously described protocol [16]. Briefly, 20 hand-picked, apparently clean islets were transferred onto a collagen matrix (1.8 mg collagen type 1/ml; Nutacon, Leimuden, the Netherlands) in a 24 well culture dish. Islets were cultured at 37°C (air/CO₂; 95:5) in 1 ml endothelial CM (ECM), this being CM with 20% (vol./vol.) FCS and with 100 µg endothelial cell growth supplement/ml.

Expanding cells were detached before reaching confluence with 0.25% (wt/vol.) trypsin–EDTA solution for <5 min at room temperature. The suspension was washed twice in CM. The endothelial cells were extracted from the cell suspension by a Dynabead (Dynal Biotech, Oslo, Norway) method previously described [17]. By the use of *Bandeiraea (Griffonia) simplicifolia* (BS-1)-coated Dynabeads, endothelial cells were separated from contaminating cells, generating a purity of >90% [13].

Purity and characterisation of endothelial cell preparations As a control of endothelial cell purity, samples of endothelial cells were trypsinised, centrifuged onto poly-L-lysine-coated glass slides, fixed in 4% (vol./vol.) paraformaldehyde for 2 h, and subsequently dehydrated. The slides were stained to detect endothelium with a rat anti-CD31 antibody (HyCult Biotechnology, Uden, the Netherlands) using the EnVision⁺ system (Dako, Glostrup, Denmark). Antigen retrieval was performed by boiling for 20 min with Target Retrieval Solution (pH 9.0; Dako) in a microwave oven (750 W). The slides were counterstained with haematoxylin. After staining, the fraction of CD31-positive cells was manually counted in a light microscope (×400). Isolated islet endothelial cells were also cultured on collagen-coated glass slides (1.8 mg collagen type 1/ml; Nutacon) and fixed in zinc fixative (Becton Dickinson

Biosciences, San Jose, CA, USA [BD]) for 30 min. After incubation with primary antibodies against CD31 (Chemicon, Chancellors Ford, UK), vascular endothelial cadherin (VE-cadherin; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and alpha smooth muscle actin (α -SMA) the slides were washed and incubated with goat-anti-mouse Alexa 568 (Molecular Probes, Eugene, OR, USA). The slides were then washed, stained with Hoechst, and mounted with Fluoromount-G (Southern Biotechnology, Birmingham, AL, USA). Rat mesenchymal stem cells (MSC) and rat brain microvascular endothelial cells (RBMVEC; Cell Applications, San Diego, CA, USA) were used as negative and positive controls, respectively.

To further characterise the isolated endothelial cells, uptake of fluorescently labelled diiodoacetyl indocarbocyanine acetylated LDL (DiI-Ac-LDL; Invitrogen, Lidingö, Sweden) was investigated as endothelial cells have an increased metabolism of Ac-LDL in comparison with supportive stromal cells [18]. Cells cultured on collagen-coated glass slides (1.8 mg collagen type 1/ml; Nutacon) were incubated with 20 μ g/ml DiI-Ac-LDL for 4 h at 37°C. The slides were subsequently washed, fixed in zinc fixative (BD), stained with Hoechst and mounted with Fluoromount G (Southern Biotechnology, Birmingham, AL, USA). Rat MSC and human dermal microvascular endothelial cells (HDMEC; PromoCell, Heidelberg, Germany) were used as negative and positive controls, respectively. The cells were analysed using a confocal microscope (C-1/TE200U; Nikon, Tokyo, Japan).

Endothelium-conditioned CM Dynabead-purified endothelial cells were cultured for 2 days in ECM as described above. At this time point the endothelial cells were in exponential growth, but had not reached confluence. The wells were washed with CM to remove all ECM and fresh CM was then added to the wells. The medium was collected 24 h later and centrifuged for 2 min at 600 g to remove cells. The endothelium-conditioned medium, hereafter termed ECCM, was then stored at –70°C.

ECCM with cyclooxygenase 2 (COX-2) inhibition The endothelial cells were treated as mentioned above, but the CM contained 1 μ mol/l of the COX-2 inhibitor rofecoxib (4-[4-(methylsulfonyl)phenyl]-3-phenyl-2(5H)-furanone) (Kempotec, Middlesbrough, UK) or DMSO as vehicle control. The medium was collected, centrifuged for 2 min at 600×g to remove cells and stored at –70°C.

Islet incubation in ECCM Twenty islets were precultured for 4–7 days in CM, and subsequently cultured in 1 ml of control CM or ECCM in a 24 well culture dish for 24 h. The reason for the pre-culture period was to minimise remnant islet endothelial cells, as studies have shown that

~90% of the intra-islet endothelial cells disappear after 4 days in culture [6, 19].

Glucose-stimulated insulin release Groups of ten islets were transferred in triplicates to glass vials containing 250 μ l Krebs–Ringer bicarbonate buffer supplemented with 10 mmol/l HEPES and 2 mg/ml BSA (MP Biomedicals, Irvine, CA, USA) (hereafter referred to as KRBH buffer). The KRBH buffer contained 1.67 mmol/l D-glucose during the first hour of incubation at 37°C (O₂/CO₂; 95:5). The medium was removed and replaced by 250 μ l KRBH supplemented with 16.7 mmol/l glucose and the incubation continued for a second hour. The medium was retrieved and stored at –20°C. To measure the total insulin content and DNA content, the islets were harvested, pooled in groups of 30, and homogenised by sonication in 200 μ l redistilled water. A fraction of the homogenate was mixed with acid-ethanol (0.18 mol/l HCl in 95% [vol./vol.] ethanol), from which insulin was extracted overnight at 4°C. Samples were then stored at –20°C until insulin and DNA measurement (see below). In separate experiments, soluble laminin (LM)-111 (α 1 β 1 γ 1; according to new LM nomenclature [20]) (30 μ g/ml) (BD), neutralising antibodies to thrombospondin-1 (TSP-1; 1 μ g/ml) (LabVision, Fremont, CA, USA), hepatocyte growth factor (HGF; 1 μ g/ml) (R&D Systems, Minneapolis, MN, USA) or the β 1-chain of LM (18 μ g/ml; Clone 3E5, Catalogue number MAB2041; Millipore, Solna, Sweden), or the endothelin A (ET_A) and endothelin B (ET_B) receptor antagonists BQ-123 (10 μ mol/l) and BQ-788 (1 μ mol/l) (Bachem UK, St Helens, UK), were added to the high-glucose medium.

Insulin measurement After appropriate dilution, insulin concentrations in incubation medium and homogenates were determined by a commercial insulin ELISA (Merckodia, Uppsala, Sweden).

DNA measurement After appropriate dilution, the homogenates were used for DNA measurements by fluorophotometry (PicoGreen dsDNA Quantitation Kit; Molecular Probes).

Glucose oxidation rate Islet glucose oxidation rates were determined according to a previously described method [21]. Briefly, triplicates of ten islets were transferred to glass vials containing 100 μ l KRBH supplemented with D-[U-¹⁴C]glucose (GE Healthcare Life Sciences, Chalfont St Giles, UK) and non-radioactive D-glucose to a final glucose concentration of 16.7 mmol/l and a specific radioactivity of 18.5×10⁶ Bq/mmol. The ¹⁴CO₂ produced was captured with Hyamine. After incubation for 90 min at 37°C (O₂/CO₂; 95:5), the oxidation was terminated by injection of 100 μ l 0.05 mmol/l antimycin A into the vials. The ¹⁴CO₂ generated by cell metabolism was released by

the addition of 100 μ l 0.4 mmol/l NaH_2PO_4 (pH 6.0) during a 120 min incubation. The radioactivity in the samples was then measured by liquid scintillation counting.

Islet (pro)insulin biosynthesis and total protein biosynthesis Groups of ten islets were incubated in duplicate for 2 h in 100 μ l Krebs–Ringer buffer containing 2 mg/ml BSA, 16.7 mmol/l glucose and 1.85×10^9 Bq/mmol L-[3,4,5- ^3H] leucine (American Radiolabeled Chemicals, St Louis, MO, USA) at 37°C in (air/ CO_2 ; 95:5). After incubation the islets were washed in Hanks' balanced salt solution containing 10 mmol/l non-radioactive leucine, and then sonicated in 200 μ l redistilled water. The islet homogenates were frozen before immunoprecipitation.

Insulin degradation Islet intracellular degradation of insulin was determined using a pulse-chase method. The islet proteins were labelled by maintaining about 150 islets in the presence of 5.5 mmol/l glucose and 1.5 GBq/l L-[3,4,5- ^3H]leucine (American Radiolabeled Chemicals), yielding a specific radioactivity of 3.3 GBq/mmol in the medium during the 72 h incubation. The islets were then washed twice in non-radioactive medium, and duplicate groups of 15 islets were homogenised by sonication (22 kHz, 50 W) for 10 s in 200 μ l of a 50 mmol/l glycine buffer pH 8.8 containing 2.5 g/l BSA. The remaining islets were divided into two aliquots, which were incubated for 24 h without radioactivity in CM or ECCM. After the chase period, duplicates of 15 islets from both groups were homogenised by sonication in 200 μ l glycine–BSA–buffer, and 100 μ l portions of the incubation medium were also retrieved. The islet homogenates and incubation medium were frozen before immunoprecipitation.

Immunoprecipitation Portions of 10 μ l from the islet homogenates and the 100 μ l medium samples from the islet incubations were mixed with 100 μ l of a 50 mmol/l glycine buffer supplemented with 2.5 g/l BSA and 1 ml/l Triton X-100, and in duplicates supplied with 10 μ l of guinea pig anti-human insulin serum (Fitzgerald Industries International, Concord, MA, USA) or 10 μ l normal guinea pig serum (Harlan Sera-Lab, Loughborough, UK). The immune binding was allowed to proceed for 1 h at room temperature. Subsequently 50 μ l rProtein-A-Sepharose Fast Flow suspended in glycine–BSA–Triton X-100 buffer to a concentration of 110 ml/l was added and the material was tumbled for 15 min at room temperature and then centrifuged for 3 min at 4,000 $\times$ g. The Sepharose sediment with the anti-insulin–insulin complex was washed twice with 500 μ l glycine–BSA–Triton X-100 buffer and finally resuspended in 500 μ l 1 mol/l acetic acid with 2.5 g/l BSA. After addition of 4 ml Ultima Gold scintillation fluid, the radioactivity was determined by a liquid-scintillation

spectrometer (Wallac System 1400; Turku, Finland) using external standardisation. Non-specific binding was corrected for by subtracting the radioactivity bound by normal guinea pig serum from that precipitated by immune serum. For the analysis of total protein biosynthesis rate, two 10 μ l aliquots were taken from the water homogenate samples of sonicated islets and transferred to Eppendorf tubes. Glycine solution (250 μ l) was added to dilute the homogenate and 250 μ l of 10% (wt/vol.) trichloroacetic acid was added to precipitate the proteins. The samples were mixed vigorously and centrifuged (10 min at 600 g). The supernatant fraction was removed and the pellets dissolved in 2×250 μ l of 0.15 mol/l NaOH and transferred to scintillation vials before 4 ml Ultima Gold scintillation liquid was added and the samples counted in a liquid scintillation counter.

Gene expression analysis Isolation of total RNA was performed with an RNeasy Micro Kit (Qiagen, Hilden, Germany) and all the samples were treated with RNase-free DNase (Qiagen). Conversion of total RNA to cDNA was performed with reverse transcription system (Promega, Madison, WI, USA) using oligo (dT)₁₅ primers. The Light-Cycler System (Hoffman-La Roche), and detection with SYBR Green (SYBR Green JumpStart Taq ReadyMix), was used to amplify and analyse generated cDNA. TATA box binding protein (GenBank accession number D01034; CyberGene, Stockholm, Sweden), 5'-ACC CTT CAC CAA TGA CTC CTA TG-3', 5'-ATG ATG ACT GCA GCA AAT CGC-3', was used as housekeeping gene. PCR amplifications were performed in a total volume of 10 μ l, containing 1 μ l of cDNA, 1 μ l of each primer (Tebu-Bio, Roskilde, Denmark or MWG Biotech, Edersberg, Germany) (see Electronic supplementary material [ESM] Table 1), 4 μ l CYBR Green JumpStart Taq ReadyMix and RNase-free water added to the final volume. Each RT-PCR run started with a denaturation at 94°C for 10 s. Cycling variables were 94°C, 10 s; 57°C, 15 s; and 72°C, 8 s.

Statistics For multiple comparisons, repeated measurements of ANOVA with Bonferroni's post hoc test was used. When only two groups were compared, Student's paired or unpaired *t* test was applied as appropriate.

Results

Purity and characteristics of endothelial cell preparations CD31-staining was used to characterise the endothelial cells purified by BS-1-coated Dynabeads. Evaluation by manual counting showed that the cultured sorted cells contained $89 \pm 6\%$ ($n=6$) of cells positive for the endothelial cell marker CD31. The purified isolated cell preparations were further validated as endothelial cells not only by

positive staining for a second CD31 antibody (Fig. 1a), but also for VE-cadherin (Fig. 1b) and by induced uptake of DiI-Ac-LDL (Fig. 1c), as were HDMEC (Fig. 1a–c). Moreover, the vast majority of the isolated cells and RBMVEC were negative for the smooth muscle cell marker α -SMA (Fig. 1d) while MSC were positive for α -SMA (Fig. 1d) but negative for all the endothelial cell markers (Fig. 1a–c).

Insulin release The basal insulin release at 1.67 mmol/l glucose did not differ between islets that had been exposed to ECCM or CM, whereas the response in insulin release to 16.7 mmol/l glucose was augmented in ECCM-exposed islets (Fig. 2a). Control islets and ECCM-incubated islets had similar DNA contents (92.5 ± 1.8 ng DNA per ten islets vs 93.7 ± 5.4 ng DNA per ten islets; $n=6$ in both groups). Addition of neutralising antibodies against TSP-1, HGF or receptor antagonists to ET_A and ET_B could

not prevent the augmentative effect of ECCM on glucose-stimulated insulin release (Fig. 2b, c). Likewise, there was no difference in insulin release from islets that had been exposed to ECCM with addition of a COX-2 inhibitor, compared with islets exposed to ECCM with DMSO as vehicle control. However, addition of a function-inhibiting antibody towards the $\beta 1$ -chain of LM neutralised the positive effects of ECCM on glucose-stimulated insulin release (Fig. 2c). Moreover, there was an increase in insulin release from islets incubated in CM with an addition of LM-111 when compared with islets incubated in control CM (Fig. 2d).

Insulin content The total islet insulin content was increased in islets that had been exposed to ECCM (Fig. 3a). This augmentative effect of ECCM on insulin content could not be prevented by addition of neutralising antibodies to TSP-1 or HGF or receptor antagonists to ET_A and ET_B (Fig. 3b, c).

Fig. 1 Characterisation of isolated islet cells. EC, RBMVEC or HDMEC, and MSC stained for CD31 (red) (a), VE-cadherin (red) (b), DiI-Ac-LDL (red) (c) and α -SMA (red) (d). Nuclei stained with Hoechst (blue). Scale bars, 50 μ m

Fig. 2 Glucose-stimulated insulin release. Black bars, 1.67 mmol/l glucose; hatched bars 16.7 mmol/l glucose. The islets were cultured for 4–7 days in CM or ECCM. **a** The insulin release in response to 16.7 mmol/l glucose was enhanced in islets incubated in ECCM compared with CM prior to experiments. **b** With or without receptor antagonists (antag) to ET_A , ET_B or neutralising antibody (ab) to thrombospondin-1. **c** With or without neutralising antibodies to LM or HGF. In additional experiments the COX-2 inhibitor rofecoxib or DMSO as vehicle was added to the ECCM. **d** With or without an addition of LM-111 (30 μ g/ml). All values are means $\pm$ SEM for 12 paired experiments in **a** and six paired experiments in **b–d**. * p <0.05 and ** p <0.01 compared with corresponding CM group; $^\dagger p$ <0.05 compared with ECCM

Fig. 3 Total islet insulin content after glucose-stimulated insulin release. **a** Islets cultured in ECCM contained more insulin than islets cultured in CM. **b** With or without receptor antagonists (antag) to ET_A , ET_B or neutralising antibody (ab) to thrombospondin-1. **c** With or without neutralising antibodies to LM or HGF. In additional experiments the COX-2 inhibitor rofecoxib or DMSO as vehicle was added to the ECCM. **d** With or without addition of LM-111 (30 μ g/ml). All values are means $\pm$ SEM for 12 paired experiments in **a** and six paired experiments in **b–d**. * p <0.05 and ** p <0.01 compared with corresponding CM group; $^\dagger p$ <0.05 compared with ECCM

Addition of a COX-2 inhibitor, when compared with islets exposed to ECCM with DMSO as vehicle control, did not affect islet insulin content. However, neutralisation of the LM β 1-chain in the ECCM prevented its positive effects on islet insulin content (Fig. 3c). Moreover, there was an increase in insulin content from islets incubated in CM with addition of LM-111 when compared with control CM (Fig. 3d).

Glucose oxidation rate When exposed to 16.7 mmol/l glucose, the islets cultured in ECCM had a 60% increase in glucose oxidation rate compared with islets cultured in CM (Fig. 4).

Islet (pro)insulin biosynthesis and total protein biosynthesis The (pro)insulin biosynthesis did not differ between the islets that had been cultured in CM compared with ECCM. Similar results were observed irrespectively of whether the (pro)insulin biosynthesis per se was measured (no data shown) or was expressed as a ratio of total protein biosynthesis (Fig. 5a).

Insulin degradation Islet intracellular degradation of insulin was determined using a pulse-chase method including a 72 h pulse with [3 H]leucine and a subsequent 24 h chase in CM or ECCM. After the chase period the content of radioactively labelled insulin was measured and calculated as a percentage of the radioactive insulin in the islets retrieved immediately after the pulse incubation. Lacking insulin was regarded as degraded intracellularly, since no insulin degradation occurs in the incubation media at the present conditions. The islets incubated in CM during the chase period degraded 57% of the intracellular insulin,

Fig. 4 Glucose oxidation rate. The islets were cultured for 4–7 days in CM and then 24 h in CM or ECCM. The glucose oxidation rate at 16.7 mmol/l glucose was higher in the islets that had been cultured in ECCM compared with the islets that had been cultured in CM. Values are means $\pm$ SEM for six paired experiments. * p <0.05 compared with CM

Fig. 5 **a** Islet (pro)insulin biosynthesis expressed as percentage of total protein biosynthesis. The islets were cultured for 4–7 days in CM and then 24 h in CM or ECCM. The (pro)insulin biosynthesis in response to 16.7 mmol/l glucose did not differ between the groups. **b** Islet intracellular insulin degradation. Insulin degradation was determined using a pulse-chase method including a 72 h pulse with [3 H]leucine and a subsequent 24 h chase in CM or ECCM. The islets cultured in ECCM degraded less insulin compared with islets cultured in CM. Values are means $\pm$ SEM for six experiments. * p <0.05 compared with CM

whereas the islets incubated in ECCM only degraded 41% of the intracellular insulin (Fig. 5b).

Gene expression There was no difference in mRNA expression of genes encoding glucose transporter 2 (*Slc2a2*), pyruvate carboxylase (*Pcx* [also known as *Pc*]) or pancreatic and duodenal homeobox 1 (*Pdx1*) in islets that had been cultured in ECCM compared with CM. However, islets cultured in ECCM had increased mRNA expression of glucokinase gene (*Gck*) when compared with islets cultured in control CM (Fig. 6a). Likewise, the

Fig. 6 Relative mRNA levels for islet *Gck* (**a**) and *mGPDH* (**b**) after 4 days of culture in CM and then an additional 24 h culture in CM or ECCM. Values are means $\pm$ SEM for six experiments. * p <0.05 compared with CM

mRNA expression for the gene for glycerol-3-phosphate dehydrogenase (*mGpdh* [also known as *Gpd2*]) was increased in islets cultured in ECCM compared with CM (Fig. 6b).

In order to investigate whether LM is indeed expressed in the endothelial cells, the mRNA expression of genes encoding LM chains $\alpha 4$ (*Lama4*), $\alpha 5$ (*Lama5*), $\beta 1$ (*Lamb1*) and $\beta 2$ (*Lamb2*) were measured in endothelial cells and whole islets. All tested LM chains displayed markedly higher mRNA expression in endothelial cells compared with whole islets (Fig. 7).

There was no difference in mRNA expression of insulin gene (*Ins1*) in islets cultured with CM with addition of LM-111 compared with islets cultured in CM alone ($n=6$; data not shown).

Discussion

The present study demonstrates that endothelial cells can be important not only for transport functions by providing nutrients and oxygen to parenchymal cells, but also for paracrine functional support. In our investigations of rat pancreatic islets exposed or not to islet endothelial cell

products, we observed that the insulin release was enhanced by more than 40% in response to 16.7 mmol/l glucose and that the insulin content was increased by 35% when the islets had been exposed to ECCM compared with CM. At least part of the augmented insulin release after endothelial cell product exposure was explained by increased mitochondrial activity as assessed by glucose oxidation. This was also reflected in an upregulation of the mitochondrial gene *mGpdh*. Also the rate limiting enzyme in the beta cell glycolysis, glucokinase, was upregulated.

It has previously been shown that vascular basement membrane proteins, particularly LMs, promote insulin gene expression in mouse beta cells [7]. However, in the present study in the rat no such effect could be observed after exposure to LM-111. Moreover, despite the fact that both insulin release and total insulin content were increased in the islets that had been cultured in ECCM, we could see no increase in (pro)insulin biosynthesis. This suggests that any raised expression of the insulin gene was not translated to protein levels. Analysis of the medium after exposure to ECCM (data not shown) indicated that the increased insulin content did not result from an effect of inhibited insulin release to the medium during the culture in ECCM. Instead, we observed decreased intracellular insulin degradation after culture of islets in ECCM.

In an attempt to find the factors responsible for the positive effects of ECCM on beta cell function, a number of different neutralising antibodies or receptor antagonists were tested to see if they could remove these effects from the ECCM. The rationale for the selection of the interventions was based on previous observations by us and others. Tsp-1 is a natural inhibitor of angiogenesis expressed in islet endothelium, which we have recently observed is important for beta cell differentiation in TSP-1-deficient mice [22]. ET-1 is a potent vasoconstrictor derived from endothelial cells, which is known to stimulate insulin release [23]. HGF was recently shown by us to be expressed by islet endothelial cells and to stimulate beta cell proliferation [13]. Likewise, there are indications that beta cells do not form their own basement membrane, but rely on endothelial cells to form a vascular basement membrane containing LMs next to the beta cell [14]. LM-111 has been demonstrated to promote differentiation of pancreatic precursor cells into beta cells in vitro [24] and to promote glucose-stimulated insulin secretion in vitro [15, 25, 26]. Prostaglandins exert a number of effects on islet beta cells, although mostly negative [27, 28].

The LM $\beta 1$ -chain was recently observed to be the perhaps most important basement membrane component that promotes proliferation in beta cells through interaction with $\alpha 6$ - and especially $\beta 1$ -integrins on the beta cell surface [14]. In our studies, an antibody towards the $\beta 1$ -chain of LM was the only factor investigated that could

Fig. 7 Endothelial and whole-islet relative mRNA levels for genes for LM chain $\alpha 4$ (*Lama4*) (a), $\alpha 5$ (*Lama5*) (b), $\beta 1$ (*Lamb1*) (c) and $\beta 2$ (*Lamb2*) (d). Values are means $\pm$ SEM for six experiments. *** $p < 0.001$ compared with whole islets

neutralise the positive effects of ECCM on islet insulin content and glucose-stimulated insulin release. The positive effect of LM was further investigated by adding LM-111 to islets in CM, and these islets displayed a pronounced increase in insulin content and glucose-stimulated insulin release compared with control islets. The results from the RT-PCR studies showed that islet endothelial cells had markedly higher mRNA levels of LM chains $\alpha 5$ and $\beta 1$ than whole islets. LM chains $\alpha 4$ and $\beta 2$ were also more expressed in islet endothelial cells compared with whole islets. At least part of the beneficial effect of islet endothelial cells on beta cells can therefore be attributed to LMs. At least some of the expression of LMs in whole islets probably reflects residual endothelial content. It has earlier been shown that LM-411 and LM-511 are the major LM isoforms in vascular basement membranes [29]. In addition, the peri-islet capsule seems to be composed of LM chains $\alpha 2$, $\beta 1$ and $\gamma 1$, suggesting the presence of LM-211 [30]. At least in humans, LM-421 and LM-521 also seem to be present in the islet basement membranes [31].

Previous studies aiming to study the importance of endothelial paracrine support for the beta cell function have used PDX1-CRE:VEGF^{fl/fl}, VEGF^{fl/fl}, RIP-CRE:VEGF^{fl/wt} or RIP-CRE:VEGF^{fl/fl} [7, 11, 32]. These studies have provided conflicting results, showing both decreased and augmented beta cell function in the VEGF-deficient islets. Nevertheless, the influence of VEGF deficiency per se for beta cell function, as well as of the likely change of phenotype in islet endothelial cells after loss of VEGF exposure, in these models remains to be determined. Moreover, beta cell function is likely to be affected by the decreased transport capacities caused by the low vascular density and decreased endothelial fenestrations in these islets.

In conclusion, the present study points to an important supportive role for islet endothelial cells in beta cell function that is most probably mediated by LMs. The insufficient revascularisation of islets following transplantation of pancreatic islets [33, 34] may therefore cause further consequences than impaired oxygen transport [35, 36] and contribute to islet dysfunction. Likewise, loss of islet endothelial cells, as recently reported in long-term experimental type 2 diabetes [37], may contribute to progressive beta cell failure.

Acknowledgements The skilled technical assistance of A. Nordin is greatly acknowledged. This study was supported by grants from the Swedish Research Council (55X-15043), the Juvenile Diabetes Research Foundation, the EFSD/JDRF/Novo Nordisk Research Programme 2006, the Swedish Diabetes Association, the Swedish Juvenile Diabetes Fund, the Novo Nordisk Insulin Fund, the Anér Foundation, the Åke Wiberg Foundation, the Wallenberg Foundation and the Family Ernfrors Fund.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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