Glucose inhibits angiogenesis of isolated human pancreatic islets

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Abstract

Owing to strong interactions between pancreatic islets and the surrounding capillary network, we hypothesized that high glucose concentrations might affect key angiogenesis factors from isolated human islets, thus contributing to β -cell failure in diabetes. Human islets from eight distinct donors were studied following 96 h in culture in the presence of normal (5.5 mmol/l) or high (16.7 mmol/l) glucose concentrations. Similar studies were performed with HUVECs. Human angiogenesis-related genes and corresponding proteins were studied by real-time quantitative PCR (RT-qPCR) and protein arrays respectively. Angiogenesis and proliferation assays were also performed with HUVECs under the same culture conditions. RT-qPCR and proteome analysis of human islets incubated with 16.7 mM/l glucose revealed a significant decrease in pro-angiogenic factors including vascular endothelial growth factor (VEGF) mRNA by 20% and VEGF protein levels by 42% as well as additional proteins such as fibroblast growth factor-4 by 41%, MMP9 by 18%, monocyte chemoattractant protein-1 by 21%, and prolactin by 25%. In contrast, we observed a 17% increase in thrombospondin-1 (*TSP-1*, listed as *THBS1* in the HUGO database) and a 37% increase in angiotensinogen gene expression levels, but neither angiotensin-converting enzyme nor angiotensin II type 1 receptor gene expression was affected. The amounts of anti-angiogenic proteins such as TSP-1 and serpin B5/maspin were also increased by 70 and 98% respectively as well as endostatin by 63%. Angiogenesis assays of HUVECs in the presence of high glucose concentrations revealed a 30% decrease in tree-like tubular network formation. These data suggest that glucose reduces key factors of islet angiogenesis, which might exacerbate β -cell failure.

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Introduction

The pancreas is a complex organ closely associated with an important network of blood capillaries and particularly surrounding the islets of Langerhans. Blood capillaries are important component in the paracrine regulations and exchanges between various tissues involved in glucose metabolism via nutritional or hormonal signals (Ballian & Brunicardi 2007). Angiogenesis constitutes a mechanism where new blood vessels are formed by budding of existing pre-vessels, which can be activated under physiological but also pathological conditions such as inflammatory processes. During diabetes, the angiogenic answer to ischemia differs according to tissue, which is either excessive in certain organs such as the retina with neovascularization, compromising the vision, or defective in other organs such as the heart, determining the severity of ischemia during infarction (Pandya *et al.* 2006). This mechanism is controlled by many pro- or

anti-angiogenic factors, which are in close coordination with one another (Distler *et al.* 2003). In the endocrine pancreas, the local renin–angiotensin systems (RAS) as well as factors involved in angiogenesis are important regulators of islet structure and function mainly through regulation of local blood flow (Leung & Carlsson 2005). Increased insulin requirements promote β -cell replication and neogenesis leading to increase cell size and number as well as the formation of new blood vessels to preserve islet cytoarchitecture and function. Several pieces of evidence suggest abnormal islet angiogenesis in type 2 diabetes. The importance of islet microvessels in β -cell function is also clearly illustrated in type 1 diabetes since survival and optimal engraftment of transplanted islets in the liver depend on graft vascular density and blood flow (Olsson & Carlsson 2006). The present study aimed to investigate the effects of glucose on human β -cell function and endothelial cells.

Materials and methods

Subjects

Human pancreatic islets from eight organ donors (age = 50 ± 9 years; body mass index = 23.6 ± 1.8 kg/m²; purity of islet preparations = $70 \pm 9\%$) have been obtained through the Cell Isolation and Transplantation Centre from the Geneva University Hospital with prior consent for research use.

Cell cultures

Upon isolation, islets were cultured in CMRL 1066 containing 2.5% human serum albumin at 37 °C in a 5% CO₂ incubator. After a short period (1–3 days), human islets (10 000 Islet Equivalents (IEQ)) were shipped to our laboratory and immediately cultivated (1000 IEQ/experimental condition) at 37 °C, 5% CO₂ in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin (P), 0.1 mg/ml streptomycin (S) and 2% HEPES (1 mmol/l). Islets were cultivated for 4 days at 37 °C, 5% CO₂ in the above-described medium containing either 5.5 or 16.7 mmol/l glucose ($n=8$). HUVECs were a gift of Geneva Hospital and were cultivated in endothelial medium (Promocell) containing glucose in the same culture conditions as for human islets. In all experiments, both RNA and proteins were extracted, except for HUVECs where only RNA was extracted. Real-time PCR and/or protein arrays evaluated markers involved in inflammation, and angiogenesis was performed. Media from pancreatic islet cultures were kept frozen and analyzed by IRMA to evaluate insulin secretion. Insulin secretion studies were performed using comparable islet numbers. Aliquots of supernatants were stored at –20 °C until insulin concentrations were measured by a highly specific IRMA (BI-insulin IRMA; Cis-Bio International, Gif sur Yvette, France) that cross-reacts with human proinsulin for <0.0001% and C-peptide for <0.0003%. Insulin levels were normalized to protein concentrations of cell extracts and expressed in mUI/l per prot.

RNA extraction and real-time PCR

We focused our interest on vascular endothelial growth factor-A (*VEGFA*) gene expression and on genes that code for proteins that interfere with the fixation of VEGF to its receptor VEGFR2 (listed as KDR in the HUGO database) such as thrombospondin-1 (TSP-1, THBS1) and tissue inhibitor of metalloproteinase 3 (TIMP3) and ephrin A5 (EFNA5), which is pro-angiogenic and involved in β -cell interactions. Since factors of local RAS may be important regulators of islet structure and function, gene expressions of angiotensinogen (*AGT*), angiotensin receptor 1 (*AT1R*, *AGTR1*), and angiotensin-converting enzyme (*ACE*) were also

studied. To monitor β -cell function and levels of cell apoptosis, we compared levels of gene expressions of insulin (*INS*), nuclear factor κ -B (*NF κ B*), caspase 3 (*CASP3*), the cell survival phosphatidylinositol 3-kinase (*PI3K*)/AKT, and FAS (*FAS*). Levels of transcripts were determined by quantitative RT-PCR using eight independent islet cell preparations. Total RNA was obtained following the method of Chomczynski & Sacchi (1987) using the total RNA extraction kit from Qiagen. RNA quantification was performed using a Nanodrop Spectrophotometer (Labtech Technologies, Palaiseau, France). SYBR Green PCR technique was used to quantify the mRNA level of each gene (Abgene, Courtaboeuf, France). Briefly, cDNA was generated for each sample by reverse transcription of mRNA (1 μ g) using superscript III. PCR was carried out using 2 ng of template cDNA on a Rotor-Gene 6000 Detection System (Corbett Research, Courtaboeuf, France) at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Results from cells cultured at 5.5 mmol/l glucose were used as baseline. Each value was first normalized to the control gene *TBP* to yield a relative abundance. All experiments were performed at least three times with a good agreement among individual experiments. Primer sequences of all selected genes are listed in Table 1.

Proteome analysis

To extend our analysis to more than 50 factors involved in angiogenesis, we then applied our experimental samples to a specific protein array. Proteins were isolated from human islets ($n=3$) cultured under various conditions. Human angiogenesis-related proteins (55 studied) were then studied. Analysis was performed according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). Briefly, antibodies were spotted in duplicate on nitrocellulose membranes. Protein lysates were diluted and mixed with a cocktail of biotinylated antibodies. The sample/antibody mixture was then incubated on the membrane. Antigen–antibody complexes were revealed by its cognate immobilized capture antibody. Streptavidin-HRP and chemiluminescent detection reagents were used for quantification. Array data on developed X-ray films were quantified with software adapted for image analysis (ImageJ; NCBI, Bethesda, MD, USA). Negative control spots were used as background values to normalize each protein spot. We compared the relative changes in the average signal (pixel density) of duplicate spots. Three independent experiments were performed.

Proliferation and angiogenesis assay

Primary endothelial cells (HUVECs) were cultivated for 4 days in endothelial medium containing either 5.5 or 16.7 mmol/l D-glucose at 37 °C, 5% CO₂. At day 3 of the

Table 1 Sequences of primers used for the RT-qPCR

	Primer sequence (forward)	Primer sequence (reverse)
Genes		
<i>ACE</i>	5'-CCGAAACGAAACCCACTTTG-3'	5'-GAACTGGAAGTGCAGGACAA-3'
<i>AGT</i>	5'-TTCTGCACACCGAGCTGAAC-3'	5'-TGTTGGGTAAGTCTGTGGG-3'
<i>AT1R</i>	5'-AGCCAGCGTCAGTTTCAACC-3'	5'-ACAAGCATTGTGCGTCGAAG-3'
<i>CASP3</i>	5'-CAGATGTCGATGCAGCAAAC-3'	5'-TGGCTCAGAAGCACACAAAC-3'
<i>EFNA5</i>	5'-TACATCTCCTCTGCAATCCC-3'	5'-ACGGTGTCTCTGCTGGTTC-3'
<i>FAS</i>	5'-GCATCTGGACCCCTCCTACCT-3'	5'-CAGTCTGGTTCATCCCCATT-3'
<i>INS</i>	5'-GGGGAACGAGGCTTCTTCTA-3'	5'-CACAATGCCACGCTTCTG-3'
<i>NFκB</i>	5'-GCACCTAGCTGCCAAAGAAG-3'	5'-CATGGCAGGCTATTGCTCATC-3'
<i>PI3K</i>	5'-TGACGCTTTCAAACGCTATC-3'	5'-CAGAGAGTACTCTTGCATTC-3'
<i>TBP</i>	5'-TGGTGTGCACAGGAGCCAAG-3'	5'-TTCACATCACAGCTCCCCAC-3'
<i>TIMP3</i>	5'-CCTTCTGCAACTCCGACATC-3'	5'-ATGTCGGTCCAGAGACACTC-3'
<i>TSP-1</i>	5'-TCCGCAAAGTGAAGAGAG-3'	5'-GTAAGTGAAGTCTGACAGTG-3'
<i>VEGFA</i>	5'-TTGTACAAGATCCGCAGACG-3'	5'-GTCACATCTGCAAGTACGTTTCG-3'

treatment, ^3H thymidine (1 μCi /well) was added to the medium, and cells were incubated overnight at 37 °C, 5% CO_2 . Following treatment cells, plates were kept at -80°C before performing the assay. ^3H thymidine radioactivity was measured in each well by using scintillation spectrometry. Cell proliferation was determined as percent counts compared to control (Gluc 5.5 mmol/l). *In vitro* angiogenesis cell-based assay was performed using a kit (Cayman Chemicals, Ann Arbor, MI, USA). Briefly, HUVECs were cultivated in endothelial medium until confluence before seeding (10^4 cells/well) in 96-well plates coated with extracellular matrix gel. Cells were then cultivated for 4 days in endothelial medium containing 5.5 or 16.7 mmol/l glucose. Following treatments, casein (1/100 v/v) was added to the cells and immediately visualized by using an inverted fluorescence microscope (Zeiss, Le Pecq, France) and FITC filter ($\lambda 520\text{ nm}$). Tubular network was evaluated by using Image J software and expressed as percent of control (Glc 5.5 mmol/l). A stimulator (PMA, 64 nmol/l) and inhibitor (JNJ-10198409, 0.3 $\mu\text{mol/l}$) have been used to validate the assay.

Statistical analyses

All values are presented as mean $\pm$ S.E.M. Comparisons between group means were performed using unpaired *t*-tests as appropriate, and data were considered significant at $P < 0.05$. All variables were normally distributed as determined by Kolmogorov–Smirnov test.

Results

No toxic effects are present in the model used

Human islets were cultured for 4 days in normal or high concentrations of glucose. Insulin secretion was significantly increased by four times at 16.7 mmol/l glucose

when compared to 5.5 mmol/l glucose (55.4 ± 21.4 vs 13.5 ± 11.5 mUI/l per prot from eight independent islet preparations, $P = 0.006$). Similarly, we found that insulin gene expression was significantly increased by 70% ($P = 0.03$) in islets treated with high glucose compared to islets treated with low glucose. Furthermore, we analyzed the expression of *NFκB*, *CASP3*, *FAS*, and cell survival PI3K/Akt pathway when islets were exposed to high versus low glucose concentrations. Under high glucose, no significant difference in gene expression was noticed (data not shown). Altogether, these results exclude the presence of toxic effects following our culture conditions.

Glucose impairs angiogenesis and reduced the expression of key genes and proteins from isolated human islets

To test whether glucose had the capacity to modulate the number of endothelial cells, indirect immunofluorescence studies were performed. As shown in Fig. 1, the number of CD31-positive cells (in red) was notably reduced when islets were incubated in media containing high glucose concentrations (16.7 mmol/l). The mean $\pm$ S.D. percentage of CD31+ cells among islet cells decreased from $9.39\% \pm 4.3$ to $5.22\% \pm 2.4$ during four independent experiments ($P < 0.05$). This observation clearly indicated that glucose may have profound effects on human endothelial cells and islet microcirculation. Understanding the molecular basis of these effects was achieved by the study of key pathways. As shown in Fig. 2, islets cultured at 16.7 mmol/l glucose had a 20% reduction in *VEGF* gene expression when compared to 5.5 mmol/l glucose ($P = 0.05$). Strikingly, this was associated with a 17% increase in *TSP-1* and a 37% increase in *AGT* gene expression ($P = 0.04$ and $P = 0.01$ respectively). Interestingly, neither *ACE* nor *AT1R* gene expression was affected

Figure 1 Immunostaining of human islets cultivated in medium containing 5 mmol/l (A) or 16.7 mmol/l (B) glucose. Islets were labeled with FITC anti-insulin and Cy3-conjugated anti-CD31 antibodies.

by high glucose concentrations (1.41 ± 0.48 vs 1.34 ± 0.39 and 0.90 ± 0.18 vs 0.94 ± 0.22 , $P=NS$ respectively). No difference in gene expression was found for TIMP3 and EFNA5 when islets were treated with high glucose compared to low glucose (2.74 ± 0.92 vs 2.75 ± 0.91 , 2.10 ± 0.88 vs 2.43 ± 0.98 , and 2.12 ± 0.88 vs 2.14 ± 0.50 , $P=NS$ respectively).

We also performed proteome analysis of islets cultured at different glucose conditions as shown in Fig. 3. Results presented in Table 2 using three independent islet preparations indicated that exposition to high glucose concentrations impaired levels of multiple pro-angiogenic proteins and in contrast increased the expression of several anti-angiogenic factors. In line with our gene expression analyses, we observed a significant 42% decrease of VEGF protein expression ($P=0.002$) and a 70% increase of TSP-1 protein expression that reached statistical significance ($P=0.01$). Several additional factors involved in endothelial cell migration

and division were significantly reduced such as fibroblast growth factor-4 (FGF4) by 41% ($P<0.001$) and hepatocyte growth factor (HGF) by 21% ($P<0.05$). In addition, expression of proteins involved in extracellular matrix integrity such as MMP9 was reduced by an average of 18% ($P=0.02$). Levels of monocyte chemoattractant protein-1 (MCP-1) were also significantly reduced by 21% ($P<0.05$). Interestingly, we found that among growth factors, prolactin expression was decreased by 25% in the presence of 16.7 mmol/l glucose ($P=0.01$). On the other hand, several anti-angiogenic factors involved in endothelial dysfunction relative to their migration and their ability to form vascular tubes were significantly increased such as insulin-like growth factor-binding protein-1 (IGFBP1) by 63% ($P=0.003$), maspin, a protein member of serpin family by 98% ($P<0.05$), endostatin by 63% ($P<0.05$), and endoglin by 31% ($P<0.05$). Levels of vasohibin increased by 38% without reaching statistical significance ($P=0.07$).

Figure 2 VEGF, INS, TSP-1, and AGT gene expression results adjusted to TBP mRNA levels of human islets cultured with 5.5 or 16.7 mmol/l glucose (4 days) from eight independent islet cell preparations. * $P \leq 0.05$.

Figure 3 Comparison of protein array data of human islets cultured at 5.5 mM/l (A) or 16.7 mM/l (B) glucose. Average pixel density of the duplicate spots corresponding to VEGF protein (circled) was reduced by 56% in a representative experiment.

Glucose impairs tubular network formation but not proliferation of HUVECs

As shown in Fig. 4A and B, we observed that high glucose concentrations as compared to normal glucose levels induced a 30% decrease in tree-like tubular network formation ($P=0.03$, $n=4$). As expected, the angiogenic inhibitor (JNJ-10198409) significantly reduced tubular density by 80% ($P=0.001$), but angiogenic activator (PMA) did not increase tubular

Table 2 Regulation of protein expression of angiogenic factors from human islets treated with high glucose (16.7 mmol/l, 4 days) determined by protein array analysis from three independent islet preparations. Results expressed as percent of control islets (glucose 5.5 mmol/l 4 days) reached statistical significance

	% decrease	Anti-angiogenic markers	% increase
Pro-angiogenic markers			
FGF4	41 ± 17 [†]	Endoglin	31 ± 40*
HGF	21 ± 17*	Endostatin	63 ± 41*
MCP-1	21 ± 22*	IGFBP1	63 ± 24 [†]
MMP9	18 ± 18*	Maspin	98 ± 64*
Prolactin	25 ± 16 [†]	TSP-1	70 ± 66*
VEGF	42 ± 20 [†]	Vasohibin	38 ± 43

* $P<0.05$ and [†] $P<0.01$.

density ($P=NS$). However, we found that proliferation of HUVECs measured by using ³H thymidine incorporation was not influenced by glucose as compared to normal glucose levels (3065 ± 1100 vs 2496 ± 729 c.p.m. (high versus low glucose, $n=3$, $P=NS$)).

Discussion

The effects of glucose on endothelial cells are heterogeneous depending on tissues, underlying many chronic diabetic complications. However, at the pancreatic level, little is known on the specific effects of glucose on islet endothelium. The islet vascular system is critical for intercellular interactions and β -cell function. Using isolated human islets, we found that chronic exposition to high glucose levels impairs several important steps of islet angiogenesis. No direct toxic effect was noticed even if we cannot exclude an apoptotic effect of glucose on endothelial cells surrounding the islets.

Isolation of pancreatic islets by enzymatic digestion disrupts the islet vascular connection and microvascular networks. The reconstitution of islet endothelial cells requires adequate culture conditions and growth factors, and is an interesting model to explore islet angiogenesis *in vitro*. VEGF and cognate receptors are critical for angiogenesis. VEGFA is the most important hypoxia-driven mediator that regulates blood vessel growth (Neufeld *et al.* 1999) with differential roles attributed to VEGFR1 and -R2 (Shibuya 2006). We have therefore focused our research on VEGF and VEGF-related genes both at the mRNA and protein levels. Already at 16.7 mmol/l glucose, VEGFA gene expression was reduced by 20% and protein levels by 42% in our experiments. VEGF synthesis was shown to be regulated by glucose using fibroblasts of db/db mice as compared to wild-type mice (Lerman *et al.* 2003). Interestingly, mice with disrupted *Vegfa* gene specifically in β cells had reduced islet vascular density glucose intolerance and impaired insulin secretion (Iwashita *et al.* 2007). However, insulin secretion in this model was also enhanced *in vitro*, thus indicating that VEGFA was not implicated in β -cell function. We observed that several factors involved in endothelial cell migration or extracellular matrix were down-regulated by glucose. Interestingly, a significant reduction of MMP9 expression was noticed. MMP9 is a zinc-dependent endopeptidase involved in angiogenesis through VEGFA accessibility (Bergers *et al.* 2000) and tumor growth (Memtsas *et al.* 2009). In addition, expression of MCP-1 was reduced. The CC chemokine MCP-1/CCL2 has both inflammatory and pro-angiogenic effects. It mediates recruitment of mononuclear cells, modulates monocyte and lymphocyte phenotype, and is known to increase collateral vessel formation during limb ischemia (Shireman 2007), and has an

Figure 4 Upper panel: tree-like tubular network in HUVECs treated with 5.5 mmol/l (A) or 16.7 mmol/l glucose (B), angiogenesis activator PMA (C), or inhibitor JNJ (D). Lower panel: tube density expressed as percent of control (5.5 mmol/l glucose). * $P < 0.05$; # $P < 0.001$.

important role in infarct healing and post-infarction remodeling (Xia & Frangogiannis 2007). Using rat islets, it has been suggested that MCP-1 secretion could be induced by IL1 during an early phase of inflammatory response perhaps linked to islet isolation (Ehres *et al.* 2009). This was not confirmed using human islets (Welsh *et al.* 2005). In our experiments, islets were studied at least 6 days after isolation. The 21% reduction of MCP-1 protein in the presence of high glucose concentrations is therefore against an inflammatory response, and is concordant with the hypothesis that glucose impairs angiogenesis. Reduction of prolactin expression by high glucose is an interesting feature. Recent reports have demonstrated that prolactin enhanced human β -cell viability (Yamamoto *et al.* 2008) as well as β -cell proliferation through the PI3K signaling pathway (Hügl & Merger 2007). During pregnancy, both pancreatic β cells and islet endothelial cell display a highly reproducible physiological proliferation that requires the expression of prolactin receptor (Huang *et al.* 2009). From all these observations, reduction of prolactin levels by glucose is important to consider for both islet and endothelial cell functions.

In contrast, expression of anti-angiogenic factors was increased such as TSP-1 and AGT. TSP-1 is a secreted glycoprotein, member of the serine protease inhibitor family that binds to the extracellular matrix, inhibits NO signaling, and modulates vascular cell behavior with inhibition of angiogenesis (Isenberg *et al.* 2008). TSP-1 $-/-$ mice had increased retinal vascular density (Wang *et al.* 2003), and inhibition of TSP-1 improved vascular engraftment of pancreatic islets (Olerud *et al.* 2008). We also found that serpin B5/maspin known to block VEGF/b-FGF-induced vascular endothelial cell migration and to inhibit angiogenesis (Bailey *et al.* 2006) was increased. Additional proteins were also increased by glucose such as endostatin and vasohibin. Endostatin

inhibits migration and promotes apoptosis specifically in vascular endothelial cells via multiple pathways, notably by reducing expression of the anti-apoptotic protein BCL-2 as well as growth-associated factors (Dhanabal *et al.* 1999, Shichiri & Hirata 2001). Endostatin also inhibits phosphorylation of ERK-1 and -2 via the VEGF and FGF pathways and inhibits PEDF production (Shichiri & Hirata 2001), and down-regulates c-myc, which is a protein necessary for endothelial cell migration (Skovseth *et al.* 2005). Vasohibin is an endothelium-derived negative feedback regulator of angiogenesis expressed by endothelial cells (Watanabe *et al.* 2004).

Altogether, these results lead us to the hypothesis that elevated glucose could reduce angiogenesis late markers such as VEGF and enhance key markers involved in inhibition of endothelial cell migration and tubular network formation. This was clearly suggested by the reduction of tree-like tubular networks in HUVECs when cultured in high glucose conditions. Results from our study showing alterations of islet angiogenesis could further lead to a progressive loss of β -cell function. However, reduced VEGF expression and pro-angiogenic factors did not impair the capacity of our islets to mount a strong insulin response to glucose *in vitro*. Nevertheless, our experiments were performed during short culture periods, and we cannot exclude that reduction in islet angiogenesis could be detrimental to β -cell function *in vivo* in the long term. Targeting islet microendothelium might improve β -cell function. Indeed, Shao *et al.* (2006) and Lacraz *et al.* (2009) have recently shown the beneficial effects of calderstan (an AT1R blocker) and IL1Ra treatments on β -cell function and morphology together with an improvement in islet vascularization in db/db mice and GK rats.

In conclusion, we found that glucose interferes with islet angiogenesis key factors, a condition that

predisposes to dysfunction of blood capillary system surrounding pancreatic islets. Modifications of islet microendothelium after chronic exposure to high glucose concentrations may apply for islet dysfunction for both type 1 and 2 diabetes as well as for islet revascularization during transplantation settings. It seems important to determine whether some anti-diabetic drugs or molecules used in the clinics might also be beneficial to the endothelium in order to preserve β -cell function.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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