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ORIGINAL ARTICLE

Expression and Hypoxic Regulation of the Endothelin System in Endocrine Cells of Human and Rat Pancreatic Islets

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ABSTRACT

Context The success of pancreatic islet transplantation depends largely on the capacity of the islet graft to survive the initial phase immediately after transplantation until revascularization is completed. Endothelin-1 (ET-1) is a strong vasoconstrictor which has been involved in solid organ graft failure but is also known to be a potent mitogenic/anti-apoptotic factor which could also potentially enhance the survival of the transplanted islets.

Objective Characterization of the endothelin system with regard to a potential endothelin agonist/antagonist treatment.

Design Regulated expression of the endothelin system in human and rat pancreatic islets and beta-cell lines was assessed by means of immunohistochemistry, competition binding studies, western blot, RT-PCR, real-time PCR and transplant studies.

Results ET-1, ET_A- and ET_B-receptor immunoreactivity was identified in the endocrine cells of human and rat pancreatic islets. The corresponding mRNA was detectable in rat beta-cell lines and isolated rat and human pancreatic islets. Competition binding studies on rat islets revealed binding sites for both receptor types. ET-1 stimulated the phosphorylation of mitogen-activated protein kinase, which was prevented by ET_A- and ET_B-receptor antagonists. After exposure to hypoxia equal to post-transplant environment oxygen tension, mRNA levels of ET-1 and ET_B-receptor of human islets were robustly induced whereas ET_A-receptor mRNA did not show significant changes. Immunostaining signals for ET-1 and ET_A-receptor of transplanted rat islets were markedly decreased when compared to native pancreatic sections.

Conclusions In pancreatic islets, ET-1 and its receptors are differentially expressed by hypoxia and after transplantation. Our results provide the biological basis for the study of the potential use of endothelin agonists/ antagonists to improve islet transplantation outcome.

INTRODUCTION

The success of clinical islet transplantation for the cure of patients with type 1 diabetes mellitus is limited, despite the recent progress made with an improved islet isolation process or immunosuppressive regimen [1, 2, 3]. A widespread application of this attractive

alternative to standard insulin therapy or pancreas transplantation is limited by donor organ shortage and the requirement of 2-3 donor pancreata per recipient to achieve insulin independence. A key factor for the need of such substantial amounts of donor tissue is early graft loss due to inadequate engraftment early after transplantation. It is suspected that 50-70% of the total transplanted islet mass is lost or non-functional as a result of limited blood supply and subsequent hypoxia, the instant blood-mediated inflammatory reaction (IBMIR), the energy status of the islets, the balance of pro- and anti-apoptotic mediators and graft rejection [4, 5, 6]. Transplanted islets are trapped in the distal branches of the portal system, resulting in local ischemia until neovascularization of the grafted islets has been completed [7].

Local ischemia may trigger the release of vasoactive mediators, of which endothelin-1 (ET-1) and nitric oxide are the most potent in controlling hepatic blood flow. It has been demonstrated that an imbalance in hepatic vasoregulatory gene expression leads to microvascular dysfunction and therefore contributes to ischemia/reperfusion injury in organ transplantation [8, 9]. Among others, endothelins (ET-1, 2 or 3), a family of 21 amino acid residue peptides, are the most potent vasoconstrictors known. ET exerts its biological activity by binding to different cell surface receptors, ET_A or ET_B. Both ET-receptors belong to the G-protein-coupled receptor family which leads to the activation of diverse downstream kinases [10]. Besides vasoconstriction, ET mediation of several other biological activities has been detected, e.g. the production of cytokines, cell growth, cell adhesion and thrombosis, the release of nitric oxide and prostacyclin, the stimulation of insulin release and other functions [10, 11, 12, 13, 14, 15]. ET-1 has also been described as exerting anti-apoptotic effects in the endothelium and cardiomyocytes [16, 17]. Selective ET_A-receptor antagonists have been shown to mitigate hepatic ischemia/ reperfusion injury and to exert beneficial effects in experimental pancreas and liver transplantation by improving microcirculation and reducing tissue injury [18, 19]. Therefore, the use of selective ET_A-receptor antagonists in islet transplantation could potentially improve hepatic microcirculation and counteract the near-ischemic conditions which are generated locally through the embolization of the portal branches by the islet graft. However, treatment with an endothelin receptor antagonist might affect islet cell biology directly, provided that endothelin receptors are also expressed in pancreatic islets. Depending on the receptor subtype expression on islet cells, endothelin may provoke either beneficial or detrimental biological effects which are independent of its vasoactive properties but crucial to the engraftment process [10]. Recent studies on the expression of endothelin and its receptors in pancreatic islets are incomplete and inconclusive [20, 21]. In order to evaluate potential therapeutic strategies in islet transplantation, it is essential to obtain a better understanding of the endothelin system in the endocrine cells of pancreatic islets. The present study focuses on the precise localization of ET-1 and the two main receptors, ET_A and ET_B, in endocrine cells of human and rat pancreatic islets and in islets transplanted into the liver of syngeneic rats. Binding studies with radiolabeled ET-1 and non-labeled competitors specific for either receptor were performed to elucidate the expression of ET_A- and ET_B-receptors. We also studied the functionality of endothelin receptors in isolated rat islets by their ability to induce phosphorylation of p42/44 mitogen-activated protein (MAP) kinase upon stimulation with ET-1. Quantitative polymerase chain reaction (PCR) was performed to study the regulation of endothelin and its receptors under hypoxic conditions at an oxygen tension (1% O_2) comparable to the transplant site in the liver [22]. Our results demonstrate the presence of a functional and hypoxically regulated endothelin system in the endocrine cells of pancreatic islets which suggests a biological role other than the regulation of endothelial function.

SUBJECTS, MATERIALS AND METHODS

Liberase™ and DNAse were obtained from Roche Molecular Biochemicals (Basel, Switzerland). Cell culture media, Hanks' balanced salt solution (HBSS), Roswell Park Memorial Institute 1640 (RPMI 1640), Connaught Medical Research Laboratories 1066 (CMRL 1066), GlutaMax, sodium pyruvate, antibiotic-antimycotic solution were all obtained from Life Technologies, Gibco BRL (now: Invitrogen AG, Basel, Switzerland). ET_A-receptor antagonist (BQ123), ET_B-receptor antagonist (BQ788), and ET_B-receptor agonist (IRL1620) were purchased from Calbiochem (Juro Supply, Switzerland).

Cell Lines and Culture Conditions

Rat beta-cell lines INS-1 (kindly provided by C. Wollheim, Geneva, Switzerland) and RINm5f (American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI 1640 supplemented with 1 mM sodium pyruvate, 10% fetal calf serum (FCS) (PAA, Vienna, Austria), 1 mM GlutaMax and antibiotic-antimycotic solution (100 U/mL penicillin G sodium, 100 U/mL streptomycin sulfate and 0.25 µg/mL amphotericin B as fungizone in 0.85% saline) and 50 µM beta-mercaptoethanol. The cells were incubated in a humidified incubator at 37°C, 5% CO_2/95% air.

Isolation o Rat Islets

Male Sprague-Dawley or Lewis rats (225-275 g from Harlan Netherlands B.V., Horst, The Netherlands) were anaesthetized with Isoflurane (Baxter A.G., Volketswil, Switzerland) and subsequently sacrificed by neck dislocation. Islet isolation and purification were performed according to a modified procedure described earlier [23]. In brief, after cannulation of the common bile duct, a 10 mL enzyme solution (Liberase™ 0.17-0.33 mg/mL and DNAse 0.1 mg/mL in HBSS) was instilled for retrograde perfusion of the pancreas. After organ procurement, enzymatic digestion took place for 15-30 minutes at 37°C in a shaking water bath. Following a wash step in HBSS with 10% FCS and filtration through cheese cloth, islets were obtained by centrifugation in a discontinuous density gradient of Histopaque-1077 (Sigma Diagnostics Inc., St. Louis, MO, USA) and HBSS with subsequent hand-picking. Islet purity was generally greater than 95%. Isolated rat islets were cultured in RPMI 1640 as described in *Cell Lines and Culture Conditions* in a humidified incubator at 37°C, 5% CO_2/95% air, unless specified otherwise. For transplantation purposes, isolated Lewis islets were cultured overnight, then washed three times with 10 mL of HBSS and 2% Lewis rat serum and resuspended in washing buffer and placed on ice until transplantation. Experiments under hypoxic conditions (0.8-1.2% O_2) were performed in a hypoxic chamber with an intermediate floodgate system for the hermetic transport of dishes (Coy Laboratory Products, Grass Lake, MI, USA).

Rat Islet Transplantation

Inbred male Lewis rats (150-175 g) were treated with a single injection of streptozotocin (75 mg/kg i.p.). Only rats with non-fasting blood glucose of more than 15 mM for 3 consecutive days were considered diabetic. The rats were anesthetized by sevoflurane inhalation and the abdominal cavity was accessed by midline incision. The portal branches to the left, middle and right liver lobes were temporarily closed with microvascular clamps, after which 400-450 islets in a volume of 150 µL HBSS were injected into the portal vein by a 26-gauge needle, directing the islets to the caudate liver lobes. The clamps were released and the injection needle was removed. Bleeding was stopped with a cotton swap by gentle compression at the site of injection. After closing the abdomen with sutures, the recipient was allowed to recover. The caudate liver lobes were harvested under sevoflurane anesthesia 24 h after transplantation.

Isolation o Human Islets

Human islets were isolated from cadaveric pancreata of heart-beating donors at the

Division of Endocrinology and Diabetes, University Hospital of Zurich, according to the method described previously [24]. Informed consent for organ donation was asked for and obtained by the donors' relatives. The 13 donors (7 females; 6 males) had no previous history of diabetes or metabolic disorders. Their median age was 59 years (range: 40-71 years) and the median body mass index was 25.3 kg/m^2 (range: 19.6-31.5 kg/m^2). Cold ischemia time until perfusion of pancreatic duct with Liberase^{TM} solution averaged 5.5±2.1 h (range: 2.0-9.2 h). Isolated human islets were cultured in CMRL 1066 as described in *Cell Lines and Culture Conditions* for 16-24 h before exposure to hypoxic culture conditions as described above.

Immunofluorescent Stainings

Tissue specimens (human pancreatic biopsies, rat pancreata and livers) were fixed in formalin solution for 24 h, embedded in paraffin and cut into 5 µm sections. After deparaffinization, the slides were placed in 10 mM citrate buffer (pH 6.0) and heated in a microwave oven for 10 minutes (600 W) for antigen retrieval. The blocking of unspecific binding was performed in 10% normal donkey serum in PBS for 30 min. Sections were incubated overnight at 4°C with following primary antibodies: Guinea pig anti-insulin (A0564), dilution 1:100; rabbit anti-glucagon (A0565), dilution 1:100; rabbit anti-somatostatin (A0566), dilution 1:300 (all from DAKO Cytomation, Glostrup, Denmark). Mouse anti-ET-1 (803-001-R100), dilution 1:100; sheep anti-ET_A-receptor (210-507-C250), dilution 1:50; sheep anti-ET_B-receptor (210-506-C250), dilution 1:50 (all from Alexis Corporation, Lausen, Switzerland); rabbit anti-ET_A-receptor (AER-001), dilution 1:100; rabbit anti-ET_B-receptor (AER-002, both Alomone Labs, Jerusalem, Israel), dilution 1:100. For detection, the following secondary antibodies were used: fluorescein-5-isothiocyanate- (FITC)-conjugated rabbit anti-guinea pig IgG (F0233, DAKO Cytomation, Glostrup, Denmark), dilution 1:20; Cy2^{TM}-conjugated donkey anti-rabbit IgG (711-225-152), dilution 1:100; Cy3^{TM}-conjugated donkey anti-mouse IgG (715-165-150); rhodamine-conjugated donkey anti-sheep IgG (713-295-147), dilution 1:100; Cy3^{TM}-conjugated donkey anti-rabbit IgG (711-165-152), dilution 1:100 (all from Jackson Immuno Research Laboratories, West Grove, PA, USA). Binding of ET-1 antibody in the immunoneutralization assay was made visible using the biotin-streptavidin-based Histostain® broad-spectrum kit (Zymed, San Francisco, CA, USA) according to the manufacturer's protocol. Slides were mounted in Hydromount^{TM} (National Diagnostics, Atlanta, GA, USA) and studied with a Zeiss Axioplan® 2 imaging microscope coupled to Axiocam® digital camera and AxioVision® software (version 2.0.5; Zeiss, Feldbach, Switzerland).

Antibody Immunoneutralization

To assess the specificity of ET-1, ET_A-, and ET_B-receptor immunostaining, antibodies were preabsorbed with the corresponding peptide for 2 h at 37°C with a 1,000-fold molar excess of either recombinant ET-1 (155-001-PC01, Alexis Corporation, Lausen, Switzerland) or the specific peptides for ET_A- and ET_B-receptor provided by the supplier (Alomone Labs, JerUSAlem, Israel).

RT-PCR

Total RNA isolation from cells (INS-1, RINm5f) or purified rat or human islets was performed using TriZol reagent (Invitrogen AG, Basel, Switzerland) according to the manufacturer's guidelines. After RNA isolation, reverse transcription was carried out with the Super Script first strand synthesis system (11904-018, Invitrogen AG, Basel, Switzerland) and oligo dT priming. Subsequent PCR amplification was performed with 30-40 cycles of 30 sec at 94°C, 30 sec at 54-60°C and 1 min at 72°C (Taq DNA Polymerase 18038-042, Invitrogen AG, Basel, Switzerland). PCR products were separated on 1% agarose gel, and pictures were taken with a gel documentation system (AlphaImager, Alpha Innotech, San Leandro, CA, USA). Amplification of human beta-actin

served as internal standard and expression levels were shown to be unaffected by the experimental conditions. All primers were designed to be intron-spanning. Primer sequences, PCR fragment length, and amplification conditions of rat ET-1, ET_A-, ET_B-receptor and proinsulin were as follows. ET-1: 5'-atcatctgggtcaacactc-3' (sense), 5'-gaatctcctggctctctg-3' (antisense), 727 bp (annealing: 50°C; 30 cycles). ET_A-receptor: 5'-ttcgtcatggtacccttcga-3' (sense), 5'-gatactcgttccattcatgg-3' (antisense), 546 bp (annealing: 57°C; 40 cycles). ET_B-receptor: 5'-ttcacctcagcaggattctg-3' (sense), 5'-aggtgtggaaagttagaacg-3' (antisense), 474 bp (annealing: 57°C; 40 cycles). Proinsulin: 5'-acaatcatagaccatcagcaagc-3' (sense), 5'-cagttggtagagggagcaga-3' (antisense), 354 bp (annealing: 55°C; 30 cycles). Primers sequences, PCR fragment length, and amplification conditions of human ET-1, ET_A-receptor, ET_B-receptor and beta-actin were as follows. ET-1: 5'-ttctctctgctgtttgtggctt-3' (sense), 5'-ccagcacttcttgtctttttgg-3' (antisense), 338 bp (annealing: 50°C; 27 cycles). ET_A-receptor: 5'-acttcagctttcaaatacattaaca-3' (sense), 5'-ctgcttaagatgttcagtgagggc-3' (antisense), 674 bp (annealing: 50°C; 29 cycles). ET_B-receptor: 5'-ttccaacgccagtctggcgcggtc-3' (sense), 5'-gtcaatactcagagcacatagact-3' (antisense), 423 bp (annealing: 50°C; 30 cycles). Beta-actin: 5'-gatgacccagatcatgtttg-3' (sense), 5'-gagcaatgatcttgatcttc-3' (antisense), 641 bp (annealing: 50°C; 20 cycles).

Quantitative RT-PCR

Human islet RNA isolation procedure and first strand synthesis was performed as described above. Primers were designed with respect to exon/exon boundaries using Primer Express® version 2.0 (Applied Biosystems, Foster City, CA, USA). Sequence-specific fluorescent TaqMan™ probes were designed for each target. Primer sequences, probe sequence and PCR fragment length, of human ET-1, ET_A-receptor, ET_B-receptor and beta-actin were as follows. ET-1: 5'-gctcgtccctgatggataaag-3' (sense), 5'-agggcttccaagtccatacg-3' (antisense), 5'-tgtgtctacttctgccacctggacatca-3' (probe), 101 bp. ET_A-receptor: 5'-gggatcaccgtcctcaacct-3' (sense), 5'-cacgactccaggaggcaact-3' (antisense), 5'-ctctgtacctgtcaacactaaga-3' (probe), 70 bp. ET_B-receptor: 5'-ggttccaaaatggacagcag-3' (sense), 5'-caccaatcttttgctgtcttg-3' (antisense), 5'-acagccagaaccacagagaccaccc-3' (probe), 189 bp. Beta-actin: 5'-tcgcctttgccgatcc-3' (sense), 5'-cgaagccggccttgc-3' (antisense), 5'-tcgacaacggctccggcatg-3' (probe), 111 bp. Sequence detection of target genes was performed in an ABI Prism 7000 system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's guidelines. The amplification parameters were 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Fluorescence emission from individual PCR tubes and cycle of threshold (C_t) values were collected corresponding to the PCR cycle number at which the fluorescence was detectable above an arbitrary threshold, based on baseline data within cycles 3 to 15. The arbitrary threshold was chosen to ensure that the C_t values were obtained within the exponential phase of PCR. To normalize the differences in the amount of total RNA added to the reaction, amplification of the housekeeping gene beta-actin was performed as an endogenous control. Samples were run in triplicates and differences were calculated as $2^{-((\Delta CtHypoxia) - (\Delta CtNormoxia))}$ whereas ΔC_t represents the differences of C_t-values of the transcript of interest and the endogenous control beta-actin at the respective experimental conditions. No error bars can be computed for the 24 h normoxic reference point (calibrator) due to the delta-delta ct real-time analysis method of always setting the calibrator to a value of 1.

Receptor Binding Assays

Isolated rat islets were dispersed into single cell suspension by mild trypsinization (0.005% trypsin dissolved in PBS with 0.05 mM EDTA) and subsequent mechanical dissociation by pipetting through a Pasteur pipette. Islet cells were taken up in HBSS and 0.3% bovine serum albumin (BSA) and incubated in the presence of 200 pM ^{125}I-ET-1 (IM223, Amersham, Buckinghamshire, United Kingdom) for 2 h at 37°C. the excess

of unlabeled ET-1 (1 μM; 155-001-PC01, Alexis Corporation, Lausen, Switzerland) was used to measure unspecific binding, and total binding was assessed in the absence of unlabeled ET-1. Bound ligand was separated from unbound by centrifugation through mineral oil, after which the cell pellet was measured for radioactivity. Specific binding to ET_A- or ET_B-receptor was determined by competition binding with ET_B- receptor agonist (IRL1620, 1 μM), ET_B- receptor antagonist (BQ788, 1 μM) or ET_A-receptor antagonist (BQ123, 1 μM), respectively.

Phosphorylation of p42/p44 MAP Kinase

Rat islets were isolated and incubated overnight for recovery. The islets were then washed in PBS and resuspended in RPMI 1640 supplemented with 0.5% FCS, 1 mM GlutaMax and antibiotic-antimycotic solution (100 U/mL penicillin, 100 U/mL streptomycin sulfate and 0.25 μg/mL amphotericin B as fungizone in 0.85% saline). Aliquots of 150-200 islets were incubated in the presence of ET_A- (BQ123; 2.2 μM) and/or ET_B- receptor antagonist (BQ788; 0,12 μM) 2 h prior to stimulation with ET-1 (0.01-1 μM). Ten minutes after the addition of ET-1, the islets were immediately lysed in ice cold 50 mM HEPES, pH 7.4, 1% Triton X-100, 4 mM EDTA, 1 mM NaF, 0.1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$; and 1% protease inhibitor cocktail (P8340, Sigma Diagnostics Inc., St. Louis, MO, USA) and protein concentration was determined.

Western Blot

Equal amounts of protein (20-50 μg) were separated by 10% or 15% SDS-PAGE and transferred onto a nitrocellulose membrane (BioRad, Hercules, CA, USA). The membrane was incubated overnight at 4°C with either anti-phospho-p42/p44 MAP kinase antibody (9101) or anti-p42/p44 MAP kinase antibody (9102; both from Cell Signaling Technology Inc., Danvers, MA, USA) diluted 1:1,000 in 2% fat free milk diluent (KPL, Gaithersburg, MD, USA) in TBS/0.1% Tween-20. This was followed by incubation with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG 1:10,000; 170-6515 BioRad, Hercules, CA, USA) for 1 h at room temperature. Signals were visualized by chemiluminescent detection with an enhanced chemiluminescent reagent (ECL, Amersham, Buckinghamshire, United Kingdom) according to the manufacturer's protocol and exposed to imaging film (Hyperfilm ECL, Amersham, Buckinghamshire, United Kingdom). Band intensities were analyzed by densitometry (AlphaImager™, Alpha Innotech Corporation, San Leandro, CA, USA). Phospho-p42/p44 MAP kinase signals were normalized for total p42/p44 MAP kinase.

ETHICS

Rat islets

All procedures and experimental protocols were performed in accordance with Swiss animal protection laws.

Human islets

Informed consent for organ donation was asked for and obtained from the donors' relatives. All studies performed were in accordance with Swiss laws and medical guidelines.

STATISTICS

The results are given as mean and standard deviation (±SD) or median, interquartile, and range. Statistical comparison between the control and the treatment groups was carried out using one-way ANOVA. Dunnett's two-tailed multiple comparison test was applied when more than one group was compared with the control/reference group. The software used was GraphPad Prism 4.00 (GraphPad Software Inc., San Diego, CA, USA). Two-tailed P values of less than 0.05 were considered statistically significant.

RESULTS

Immunohistochemical Studies of the Endothelin System on Rat and Human Islets

To determine the presence of ET-1 and its receptors ET_A and ET_B on human and rat

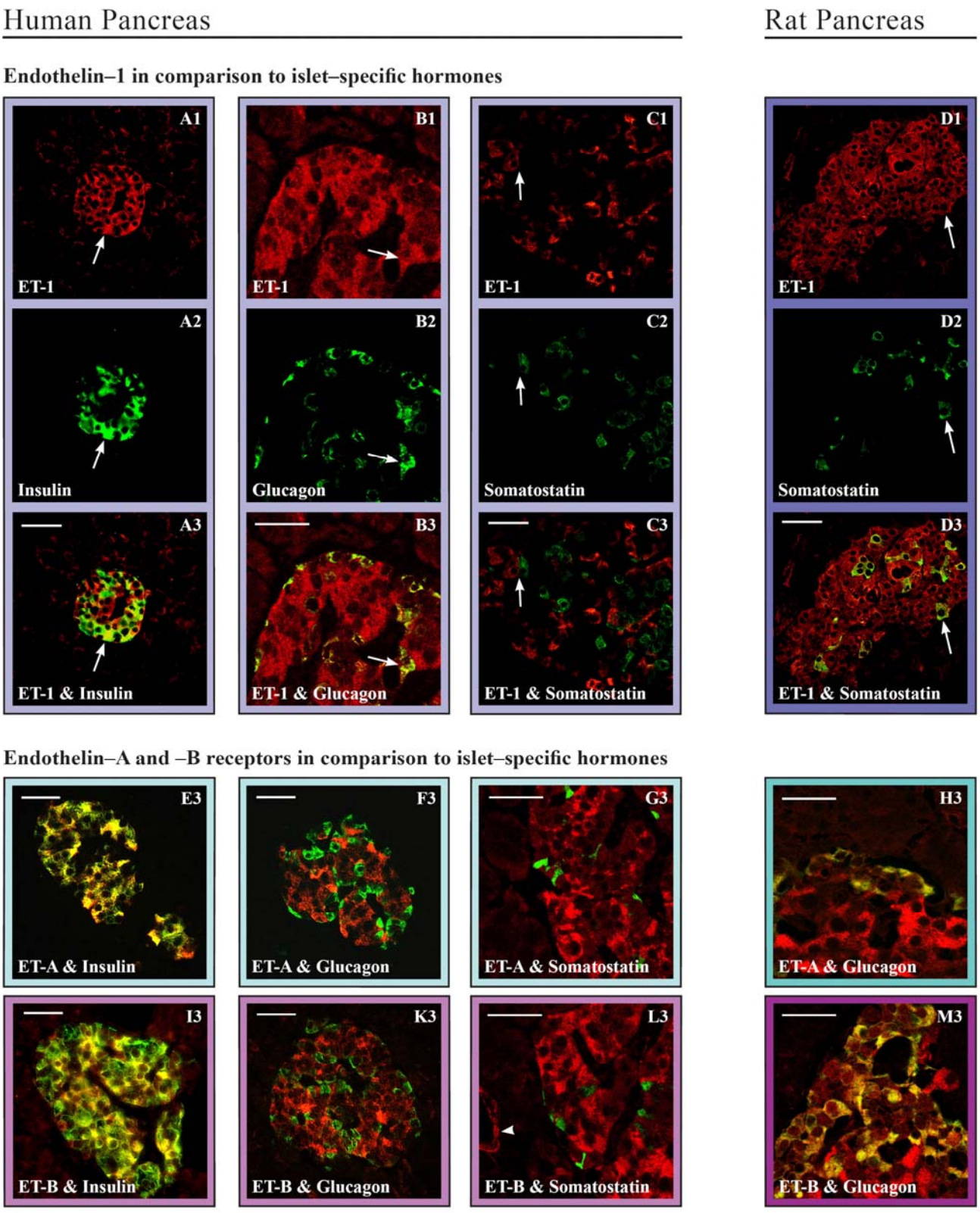

Figure 1. Expression of endothelin-1 (ET-1) and its corresponding receptors ET_A and ET_B in comparison to islet-specific hormones in human and rat pancreatic sections. Positive staining for components of the endothelin system are shown in red whereas islet specific hormones are visualized in green. The yellow color in the merged pictures (A3-M3) represents the co-expression of indicated antigens within individual cells. Arrows designate identical cells within the corresponding column. Rat sections are only shown where immunofluorescence staining differs from human sections. L3, arrow head: positive immunostaining of ET_B-receptor in vascular endothelium. Bars = 50 µm.

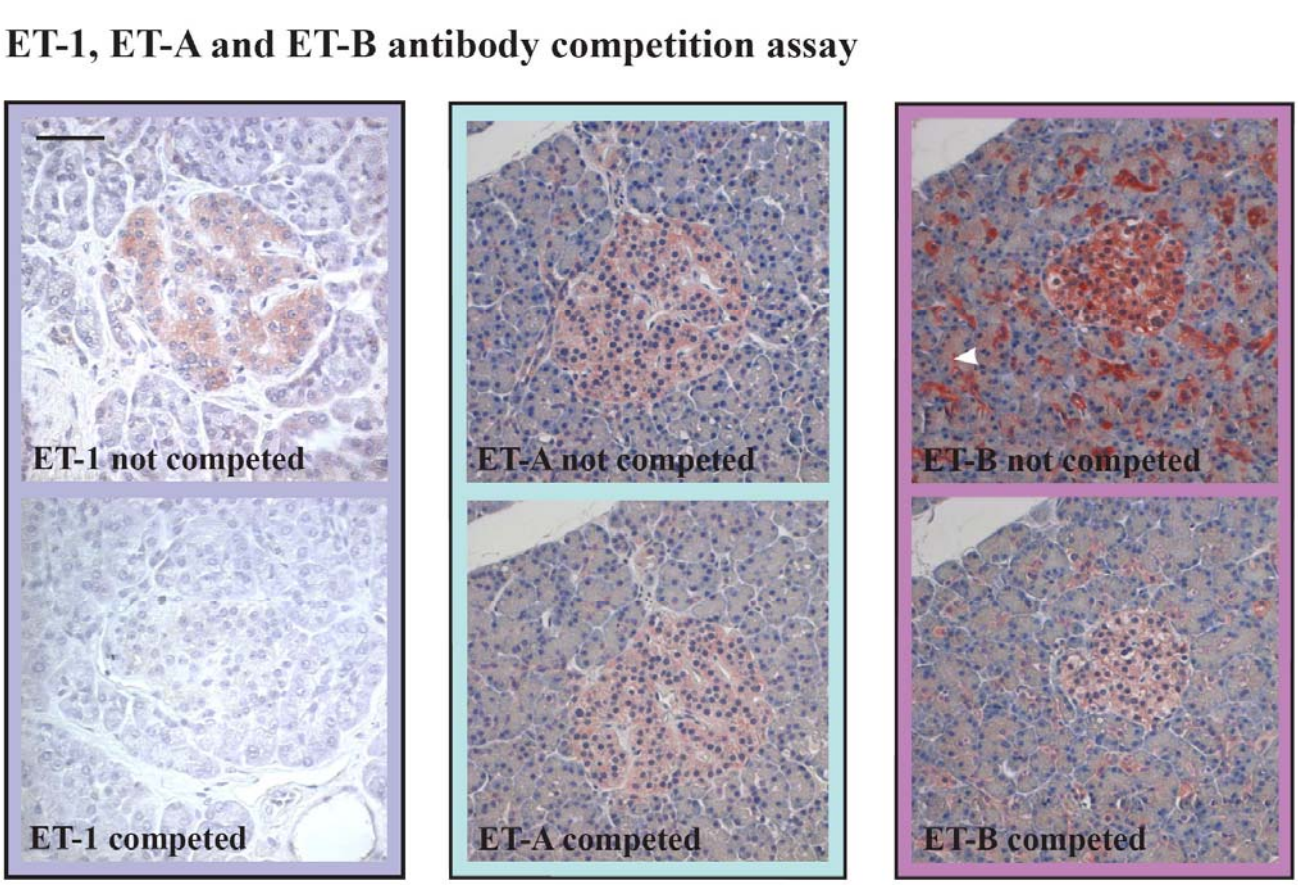

Figure 2. Competition assay: specificity of antibodies used. Preabsorption of ET-1 and ET_B-receptor antibody with a 1,000-fold molar excess of the corresponding peptide confirms the specificity of the respective antibodies. Competition with the ET_A-receptor-specific peptide was unsatisfactory; an unspecific staining by this antibody cannot be excluded. However, both ET_A-receptor antibodies derived from different suppliers were consistent in terms of their staining pattern in pancreatic islets. Bar = 50 µm; same magnification in all pictures.

pancreatic islets, co-staining studies with islet-specific hormones were performed on whole pancreatic sections. In human pancreatic beta-cells, all three components of the endothelin system (ET-1, ET_A- and ET_B-receptor) were detected whereas alpha-cells were only positive for ET-1 (Figure 1). Somatostatin-expressing delta-cells were negative for all three components. Interestingly, co-staining patterns differed in the two species. As opposed to human pancreatic islets, the alpha-cells of rat islets were also positive for ET_B-receptor and weakly positive for ET_A-receptor while delta-cells showed immunoreactivity for ET-1 (not shown). Figure 2 summarizes the differences in species-specific co-expression patterns of endothelin and its receptors with islet-specific hormones. In order to confirm the specificities of the antibodies, competition immunoassays were performed (Figure 3). ET-1 and ET_B-receptor immunoreactivity was no longer detectable after preabsorption of the antibody with a 1,000-fold molar excess of the corresponding peptide. Since competition with the ET_A-receptor specific peptide was unsatisfactory, an unspecific staining by this antibody could not be excluded. However, both ET_A-receptor antibodies derived from different suppliers were consistent in terms of their staining pattern in pancreatic islets.

	Beta cells, Insulin		Alpha cells, Glucagon		Delta cells, Somatostatin	
	Human	Rat	Human	Rat	Human	Rat
Endothelin–1	✓	✓	✓	✓	×	✓
Endothelin–A	✓	✓	×	(✓)	×	×
Endothelin–B	✓	✓	×	✓	×	×

Figure 3. Overview of the localization of the endothelin system in comparison to islet-specific hormones. Co-localization is indicated by a check mark (✓: positive; (✓): weakly positive), no co-localization by a cross (×: negative). Species-specific differences in co-localization are emphasized by grey boxes.

ET-1 Binding Studies on Isolated Rat Islets

To demonstrate that ET_A- and ET_B-receptor immunoreactivity represents the expression of ligand binding endothelin receptors, ET-1 binding assays were performed with dispersed rat islet cells. Maximal specific binding of radiolabeled ET-1 was 52.2±11.6 fmol/mg protein with an IC50 value of 0.37 nM, indicating the presence of high-affinity binding sites (Figure 4a). Maximal inhibition of radioligand binding by an excess of the specific ET_A-receptor antagonist BQ123 (51±21%, n=3) was comparable to those of the specific ET_B-receptor agonist IRL1620 (45±22%, n=3; P=0.742) and ET_B-receptor antagonist BQ788 (62±11%, n=3**;** P=0.346) (Figure 4b). The inhibitory capacity of these ligands in the range of 40-60% suggests that pancreatic islets express both ET_A- and ET_B-receptors in the ratio of roughly 1:1. Ligand binding in the presence of both antagonists (BQ123/BQ788; 22±13%, n=3; P=0.173 *vs.* BQ123) could not be inhibited completely which might indicate the presence of a third type of endothelin-binding receptor.

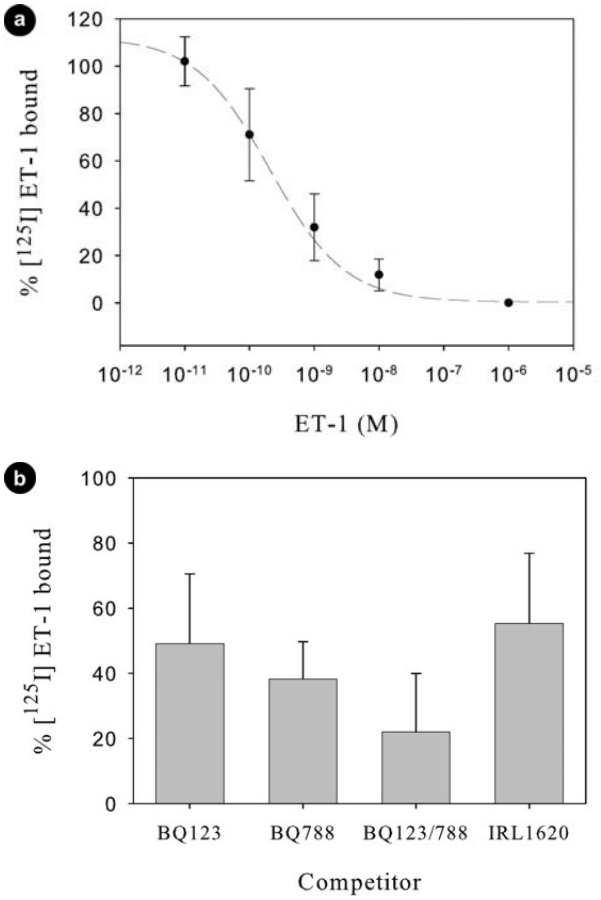

Figure 4. ET-1 binds to high affinity binding sites on dispersed rat islet cells. **a.** The cells were incubated for 2 h with ^{125}I-ET-1 (200 pM), and increasing concentrations of unlabeled ET-1. Measurements were carried out in triplicate, three experiments were performed. **b.** Competition binding of ^{125}I-ET-1 (200 pM) in the presence of 10 µM ET_A-receptor antagonist BQ123, ET_B-receptor antagonist BQ788 or ET_B-receptor agonist IRL1620. Data are mean±SD from three independent experiments.

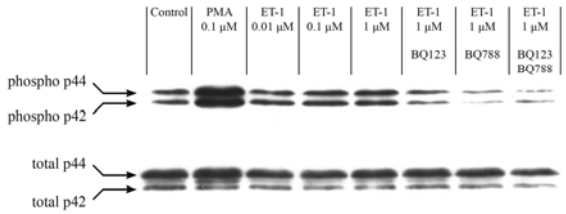

Figure 5. ET-1 induces phosphorylation of p42/p44 MAP-kinase in rat islet cells through the activation of ET_A- and ET_B-receptors. Rat islets were stimulated either with phorbol 12-myristate-13-acetate (PMA) or ET-1 for 10 min and phosphorylation of p42/44 mitogen-activated protein kinase was determined by Western Blot. Pre-incubation with ET_A-receptor antagonist (BQ123) or ET_B-receptor antagonist (BQ788), either alone or in combination, reduces phosphorylation of p42/44 MAP kinase by ET-1. Reprobing with antibody directed against total p42/44 MAP kinase serves as a loading control. One out of three similar experiments is shown.

ET-1 Stimulates Phosphorylation of p42/p44 MAP kinase in Isolated Rat Islets

ET-1 has been shown to activate p42 and p44 MAP kinases through a protein kinase C (PKC)-dependent pathway [25]. To test whether the ligand binding capacity in pancreatic islet cells also translates into activation of an ET-1 relevant signaling pathway, phosphorylation of MAP kinases upon stimulation with ET-1 was analyzed. While phorbol 12-myristate-13-acetate (PMA), another known activator of MAP kinase, stimulated phosphorylation of p42/p44 by more than 4-fold, the maximal response to treatment with ET-1 was at the most 2.5-fold (Figure 5). The relatively small response of ET-1 on p42/p44 phosphorylation may originate from relatively high basal MAP kinase activity in freshly isolated rat islets. This observation is consistent with a previous

report, describing sustained activation of MAP kinase during the first 24 h after the isolation procedure [26]. However, pre-incubation with the selective ET_A or ET_B-receptor antagonist (BQ123 or BQ788) blocked the ability of ET-1 to induce phosphorylation of MAP kinase p42/p44 completely. When combined, BQ123 and BQ788 reduced the activation of MAP kinase even below the basal value of untreated control islets (43±4%, n=3 *vs.* 100%**;** $P<0.001$). This suggests that ET-1 expressed in pancreatic islets may act in an autocrine fashion and stimulate MAP kinase activation. Thus, the results strongly indicate that, in isolated rat islets, both ET_A- and ET_B-receptors are expressed and are biologically active.

mRNA of Endothelin and Its Receptors in Beta-cell Lines and Isolated Rat Islets

To substantiate our immunohistological observations that all three components of the endothelin system are expressed in pancreatic beta-cells, we aimed to demonstrate the presence of ET-1, ET_A- and ET_B receptor mRNA in rat beta-cell lines by means of a reverse transcriptase polymerase chain reaction (RT-PCR). For all the PCR reactions, intron-spanning primer pairs were chosen to exclude the amplification of genomic DNA. DNA-fragments of ET-1, ET_A-receptor and ET_B-receptor from both INS-1 and RINm5f cells could be amplified, though at quite different intensities (Figure 6). Even though no quantitative assessment was performed, mRNA expression levels from rat islets were comparable to RINm5f cells, but clearly

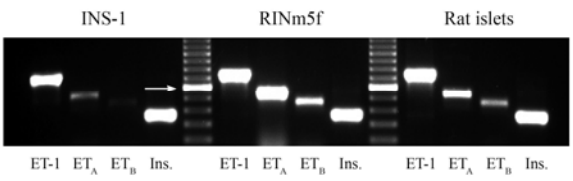

Figure 6. ET-1, ET_A-, and ET_B-receptor mRNA are expressed in rat islets and rat beta-cell lines. RT-PCR from isolated total RNA from freshly isolated rat islets and rat beta-cell lines (INS-1, RINm5f) was performed with intron-spanning primers specific for ET-1 (727 bp), ET_A- (546 bp), ET_B-receptor (474 bp) and proinsulin (354 bp). For reference, a 100 bp-ladder DNA size marker is shown (arrow: 600 bp).

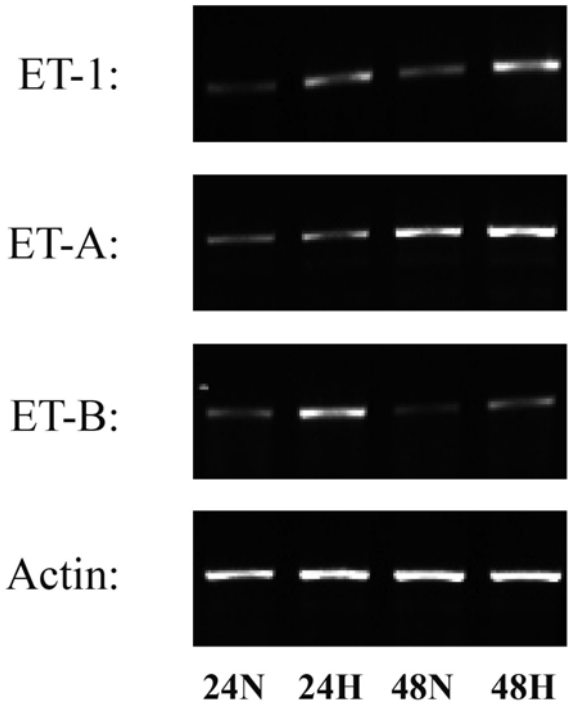

Figure 7. Semi-quantitative assessment of hypoxic regulation of ET-1, ET_A-, and ET_B-receptor mRNA transcript levels in human isolated islets. RNA from isolated human islets incubated at either normoxic (21% O_2) or hypoxic (1% O_2) culture conditions for 24 and 48 h was reversed transcribed and amplified with specific intron-spanning primers for either ET-1, ET_A-, or ET_B-receptor. PCR cycle numbers were chosen to guarantee linear amplification. Amplification of the housekeeping gene beta-actin served for normalization.

stronger when compared to the levels obtained from INS-1 cells. Different mRNA expression levels of the components of the endothelin system may simply reflect the property of the particular beta-cell line. On the other hand, it is conceivable that intraislet endothelium also contributes to the signals generated by RT-PCR.

Islet transplantation is inherently correlated to prolonged periods of ischemia as a consequence of the transient avascular state of the grafted tissue. It has previously been demonstrated that partial oxygen tension in the transplant site in the liver may be as low as 1% O_2 as compared to 5-6% O_2 in the native pancreas [22]. Therefore regulation of ET-1, ET_A- and ET_B-receptor transcript levels in purified isolated human islets after exposure to hypoxia (1% O_2) was investigated by means of semi-quantitative and quantitative (real-time) RT-PCR (Figures 7

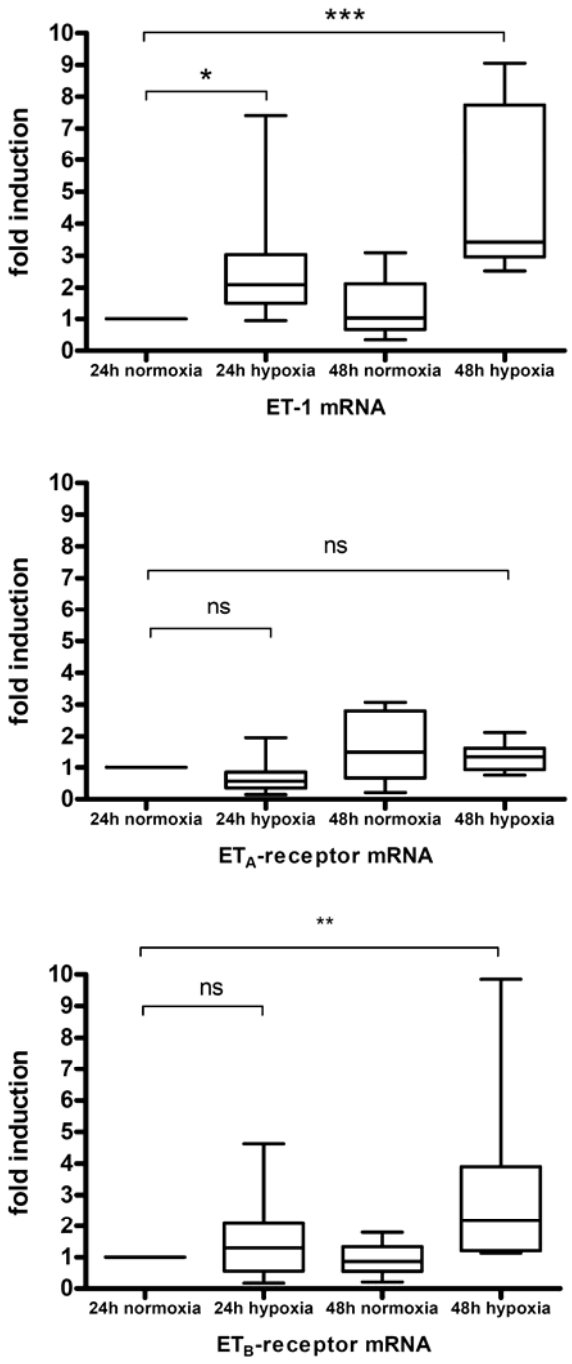

Figure 8. Quantitative RT-PCR analysis of ET-1, ET_A-, or ET_B-receptor mRNA levels in human islets incubated for 24 h and 48 h at normoxic (21% O_2) or hypoxic (1% O_2) conditions. Results are box (interquartile range; median value is shown within the box) and whisker (minimal and maximal values) plots from 11 human islet isolations. No variability can be computed for the 24 h normoxic reference point (calibrator) due to the delta-delta ct real-time analysis method of always setting the calibrator to a value of 1.

P values resulted from the comparison of 24 and 48 h hypoxia *vs*. 24 h normoxic conditions

Number of observations: 24 h normoxia (n=11); 24 h hypoxia (n=11); 48 h normoxia (n=8); 48 h hypoxia (n=8)

*P<0.05; **P<0.01, ***P<0.001

and 8). Hypoxic ET-1 expression levels were increased by 2.3-fold after 24 h (P<0.05) and 4.7-fold after 48 h (P<0.001) (average values; Figure 8). ET_B-receptor mRNA levels were increased by 3.3 fold (P<0.01) after 48 h of hypoxia as compared to normoxia. There was a tendency towards upregulation after 24 h of hypoxia which did not reach statistical significance. In contrast to ET-1 and ET_B-receptor, transcript levels of ET_A-receptor did not show significant changes upon hypoxic exposure.

These data indicate that hypoxia induces the expression of ET-1 and ET_B-receptor transcript levels whereas ET_A-receptor transcription levels are slightly reduced.

Immunohistochemical Studies of the Endothelin System in Rat Islets Before and After Transplantation

To investigate whether hypoxic regulation of the endothelin system also occurs *in vivo*, we performed immunohistochemical staining of ET-1 and its receptors (ET_A and ET_B) in islet grafts one day after intrahepatic (lobus caudatus) transplantation into syngeneic normoglycemic rats. At this stage, islets have been assumed to experience hypoxic conditions due to their avascular state. Similar to our *in vitro* data of hypoxic islets and compared to pancreatic sections, ET_B-receptor expression in transplanted islets appeared to be increased while ET_A-receptor immunoreactivity was completely lost (Figure 9). Immunostaining for ET-1 was no longer detectable which is discordant with the hypoxic upregulation of rat islet ET-1 transcript levels *in vitro* and could be attributed to transplant site microenvironmental factors other than hypoxia.

DISCUSSION

Endothelins have been shown to be involved in the physiology and pathophysiology of the vascular system, heart, lungs, kidney, brain, liver and various other tissues [10]. Endothelins mediate diverse biological effects in an autocrine or paracrine fashion which can either be beneficial or detrimental. With

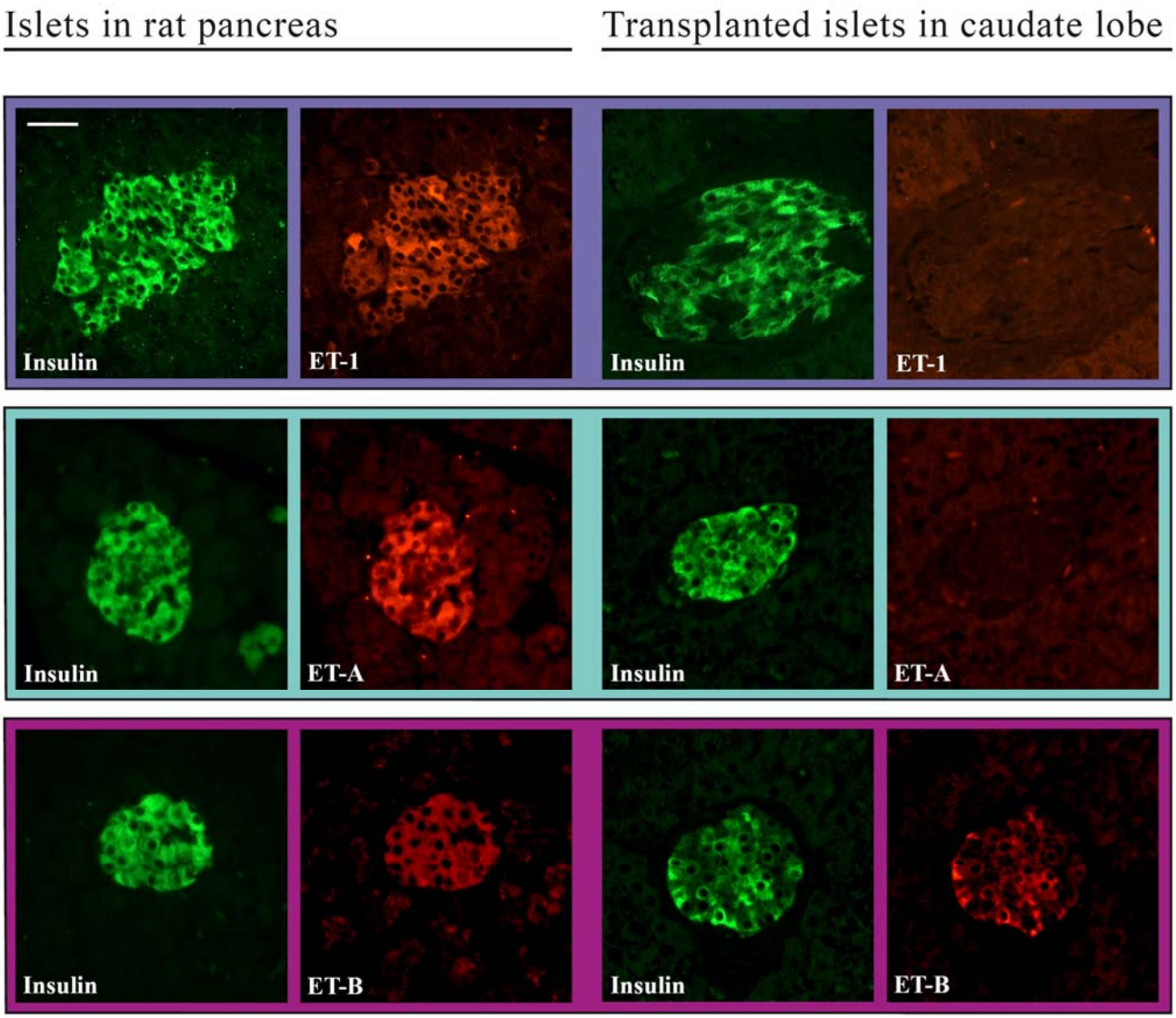

Figure 9. Immunohistochemical staining of the endothelin system in rat islets before and after transplantation. The two left columns: pancreatic sections with insulin in green and the endothelin system in red. The two right columns: engrafted islets in liver sections with insulin in green and the endothelin system in red. Bar = 50 μm; same magnification in all pictures.

regard to intrahepatic islet transplantation, pre- and/or post-operative treatment with endothelin receptor antagonists would potentially improve graft perfusion and function, similar to the beneficial effects described in an experimental model involving transplantation of a pig pancreas or hepatic ischemia/reperfusion in rats [19, 27]. However, this treatment could also affect the islet graft in a negative fashion due to the blockade of the antiapoptotic effects of the endothelin system [16, 17]. The present study was performed to determine whether the endothelin system is present in the endocrine cells of pancreatic islets and to assess its regulation by hypoxia and in a transplant setting. Our results provide the necessary biological basis for the study design of endothelin agonist/antagonist treatment before and/or after islet transplantation and its putative impact on graft viability and function.

We were able to show the presence of ET-1, ET_A- and ET_B-receptors in endocrine cells of human and rat pancreata by immunohistochemical staining. In human pancreatic beta-cells, all three components of the endothelin system (ET-1, ET_A- and ET_B-receptor) can be detected whereas alpha cells were only positive for ET-1. Somatostatin-expressing delta-cells were negative for all three components which is in agreement with a

previous study [28]. Interestingly, in contrast to human pancreatic islets, the alpha-cells of rat islets were also positive for ET_B-receptors, weakly positive for ET_A-receptors, and delta-cells showed immunoreactivity for ET-1. Such species-specific differences in expression levels of the components of the endothelin system have been described elsewhere [29, 30]. Therefore, species-dependent differences in the biological activities of the endothelin system need to be anticipated when drawing conclusions from animal experimentation for human biology. Preclinical experiments could involve transplanting human islets into non-obese diabetic severe combined immunodeficiency (NOD-SCID) mice which might provide a possible solution for the study of human islet biology in a transplant setting, but brings up the problems of xenotransplantation. As no perfect experimental model exists, biological findings need to be confirmed in human experimentation.

Endothelin receptor expression on pancreatic islet cells was confirmed by binding studies with radiolabeled ligand and competition by unlabeled ET-1 or receptor-specific antagonists. The binding capacity for ET-1 is comparable to other tissues such as the liver or heart, both of which have also been shown to express ET-receptors on parenchymal cells [31, 32]. Both the relatively high ET-1 binding capacity in islet tissue and the presence of ET_A- and ET_B-receptor mRNA in beta-cell lines (RINm5f, INS-1) strongly supports the immunohistochemical finding of non-endothelial expression of the two receptor subtypes in pancreatic islets. ET_A- and ET_B-receptors are expressed at a ratio of nearly 1:1 based on competition binding experiments with the specific antagonists BQ123 or BQ788, respectively. The presence of both receptor subtypes in isolated islets was confirmed by the ability of either of the antagonists to prevent ET-1-stimulated phosphorylation of p42/p44 MAP kinases, both of which are downstream mediators of endothelin signaling [33]. The finding of endothelin receptor expression on endocrine cells of pancreatic islets supports previous observations by which endothelin stimulates insulin secretion in isolated islets [34, 35]. It was suggested that stimulation of insulin secretion was mediated indirectly through the activation of glucagons-producing alpha-cells [21]. However, the evidence shown for ET-receptor expression on beta-cells and the ability of endothelin to elicit a raise in intracellular Ca^{2+} suggests a direct insulinotropic effect of endothelin on pancreatic beta-cells [36, 37].

Endothelins do not only regulate vascular homeostasis [38], but also mediate cell proliferation [39, 40] and are involved in the promotion or prevention of apoptosis [17, 41]. Several biological properties of endothelin may therefore also have a direct impact on islet cell survival and function after intrahepatic transplantation. For example, in adipocytes or rat lung epithelial cells, ET-1 reduces tumor necrosis factor-alpha (TNF-alpha)-induced expression of inducible nitric oxide synthase (iNOS) [42, 43]. TNF-alpha in combination with other proinflammatory cytokines is released either through the activation of Kupffer cells or by islet resident macrophages known to promote beta-cell dysfunction and apoptotic beta-cell death via synthesis of nitric oxide [44, 45]. ET-1 mediates cytoprotection through the inhibition of iNOS, which is especially relevant in tissue inflammation, considering the fact that proinflammatory cytokines stimulate the expression of this hormone [46, 47].

Another beneficial function of ET-1 could be the ability to stimulate angiogenesis through enhanced expression of the vascular endothelial growth factor (VEGF) [48, 49]. ET-1 stabilizes hypoxia-inducible factor-1-alpha (HIF-1-alpha) which then promotes the transcription of the VEGF gene. Both factors, VEGF and HIF-1-alpha, are expressed in islet cells and could therefore not only stimulate islet revascularization but also provide cytoprotection from hypoxia-related graft injury [50, 51]. The majority of the above-described biological activities of ET-1 will be potentially advantageous to islet survival and engraftment, especially in the post-transplantation period where near-ischemia

may be prevalent in the islet graft [52, 53]. *In vitro*, hypoxia stimulates the expression of ET-1 and ET_B-receptor mRNA whereas ET_A-receptor steady state transcript levels are slightly reduced. This could be confirmed only partially in our syngeneic rat islet transplantation model where ET_B-receptor staining intensity seemed to be increased while ET_A-receptor and ET-1 immunoreactivity was strongly reduced. This discrepancy between *in vitro* and *in vivo* regarding the level of ET-1 expression may be explained by the more complex biology present in a transplantation setting. A very similar regulation pattern of the endothelin system was also found in a rat liver model of 1 h ischemia followed by 6 h of reperfusion [54]. While, in the liver, the described specific regulation pattern of the endothelin system may warrant a balanced action of vasodilators and vasoconstrictors under ischemic conditions, its involvement in ischemic islets remains speculative. It is conceivable that, in transplanted islets, altered expression of the endothelin system reflects a biological response by which cytoprotection is promoted through upregulation of the ET_B-receptor and microcirculation is improved by downregulation of ET-1.

In summary, our study clearly demonstrates the presence of ET-1 and its ET_A and ET_B receptors in non-endothelial, hormone-producing cells of pancreatic islets. Its oxygen-dependent expression strongly suggests a rescue mechanism by which islet cells adapt to hypoxic conditions. These findings are of importance with respect to a potential therapeutic application of ET-1 antagonists in islet transplantation as has been used successfully in other organ transplant models. Further investigation is needed to shed light on the question as to whether the potential benefits of antagonizing endothelin in clinical islet transplantation outweigh the potent angiogenic and cytoprotective effect of endothelin demonstrated in other tissues.

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Keywords Anoxia; Endothelins; Ischemia; Islets of Langerhans Transplantation; Receptors, Endothelin

Abbreviations ET: endothelin; FCS: fetal calf serum; HBSS: hanks balanced salt solution; HIF: hypoxia-inducible factor; MAP: mitogen activated protein; PCR: polymerase chain reaction; PMA: phorbol 12-myristate-13-acetate

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