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Response of Encapsulated Rat Pancreatic Islets to Hypoxia

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Hypoxia contributes to encapsulated pancreatic islet graft failure. To gain insight into the mechanisms that lead to hypoxia-induced graft failure, encapsulated islet function, vitality, and cell replication were assessed after 2 and 5 days of hypoxic (1% O₂) and normoxic (20% O₂) culture. The mRNA expression levels of Bcl-2, Bax, inducible nitric oxide synthase (iNOS), and monocyte chemoattractant protein 1 (MCP-1) were assessed, as well as the amount of nitrite and MCP-1 in the culture medium. Hypoxia was associated with loss of encapsulated islet function and vitality, but not with an increase in islet cell replication. Loss of vitality was due to necrosis, and only modestly due to apoptosis. Hypoxia was not associated with changes in the Bcl-2/Bax mRNA ratio, but it did increase the expression of iNOS and MCP-1 mRNA. The increased mRNA levels were, however, not associated with elevated concentrations of nitrite nor with elevated levels of MCP-1 protein. The increased iNOS mRNA levels imply a role for NO in the completion of cell death by hypoxia. The increased MCP-1 mRNA levels suggest that encapsulated islets in vivo contribute to their own graft failure by attracting cytokine-producing macrophages. The discrepancy between iNOS mRNA and nitrite is explained by the longer half-life of NO during hypoxia. MCP-1 protein levels are underestimated as a consequence of the lower number of vital cells in combination with a higher proteolytic activity due to necrosis. Thus, strategies to eliminate hypoxia may not only improve islet function and vitality, but may also reduce the attraction of macrophages by encapsulated islets.

Key words: Hypoxia; Islets of Langerhans; Encapsulation; Cell death; Replication; Monocyte chemoattractant protein 1 (MCP-1)

INTRODUCTION

Microencapsulation of pancreatic islets may avoid the need for permanent immunosuppressive drug therapy and open the perspective of xenotransplantation in the treatment of insulin-dependent diabetes mellitus. The commonly applied alginate poly-L-lysine alginate microcapsules provide protection against antibodies and immune cells, while allowing diffusion of insulin and glucose across the capsule membrane. Inherent to the use of immunoprotective capsules is that revascularization of the islets of Langerhans cannot occur. Absence of blood supply causes attrition and hypoxia, which will lead to metabolic exhaustion and cell death. This explains why, in spite of successful transplantation experiments in several animal models, encapsulated islet graft survival generally has been limited to periods of several months (12, 24,35). Graft failure is a gradual process associated with decreasing islet function, decreasing vitality, and an increased proliferation of all islet cells (10).

The aim of the present study was to specifically determine the effect of hypoxia on encapsulated islets. To

this end, islet function, islet vitality, and islet cell replication rates of encapsulated islets were assessed during normoxic and hypoxic culture. In relation to islet vitality, apoptosis was also studied by measuring the expression of the antiapoptotic gene Bcl-2 and the proapoptotic gene Bax. Bcl-2 is a known inhibitor of hypoxia-induced cell death (31–33). To determine whether hypoxia-induced cell death was mediated through nitric oxide (NO) (18, 31), the expression of inducible nitric oxide synthase (iNOS) gene and nitrite formation as a measure of NO production were measured during culture. The effect of hypoxia on the expression of monocyte chemoattractant protein 1 (MCP-1), a factor that may contribute to graft failure by attracting cytokine-producing macrophages, was assessed on both RNA and protein level.

MATERIALS AND METHODS

Design of the Study

Encapsulated islets were cultured for 2 and 5 days under hypoxic (1% O₂, i.e., ~7.5 mmHg) or normoxic (20% O₂, i.e., ~150 mmHg) conditions. On days 2 and 5, islet

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function, islet vitality, and islet cell replication were assessed as well as the mRNA expression of Bcl-2, Bax, iNOS, and MCP-1. NO formation was estimated by measuring the amount of nitrite during culture. The amounts of secreted MCP-1 protein, lactate, and insulin were also assessed in the culture medium.

Animals and Islet Isolation

Male inbred Albino Oxford rats (Harlan CPB, Zeist, The Netherlands) weighing 297 ± 17 g (weight \pm SD, $n = 12$) served as islet donors. All animals received care in compliance with the guidelines of the local Animal Care and Use Committee following National Institutes of Health guidelines.

Islets were isolated as previously described (41). Briefly, the donor pancreas was removed after infusion of 10 ml sterile Krebs-Ringer-HEPES solution (KRH, pH 7.4) containing 10% bovine serum albumin (BSA), through the bile duct. The pancreas was chopped and digested using a two-stage incubation of two times 10 min at 37°C with successively 1.0 and 0.7 mg/ml collagenase (Boehringer P). Islets were separated from exocrine tissue by centrifugation over a discontinuous dextran gradient (38) and further purified by hand picking. Islets were cultured overnight in nontreated petri dishes (Greiner, Alphen a/d/ Rijn, The Netherlands) in portions of 100 islets per 25 cm² in CMRL 1066, containing 10% fetal calf serum (FCS, Gibco), 8.3 mmol/L glucose, 10 mmol/L HEPES, and 1% penicillin/streptomycin, at 37°C in humidified air containing 5% CO₂.

Islet Encapsulation

Alginate (3.4% w/v, 60% mannuronic acid; Keltone LV, Kelco International, London, UK) was mixed with cultured islets to a concentration of 1500 islets/ml. Microencapsulation was performed as described elsewhere (8), except that droplets were incubated in CaCl₂ (100 mmol/L, pH 7.4) for at least 5 min but no more than 7 min. CaCl₂ was then replaced with KRH and the islet-containing alginate beads (725–775 μ m in diameter) were kept in KRH until all beads were generated. All beads were pooled and washed for 1 min with KRH containing 2.5 mmol/L CaCl₂. PLL (poly-L-lysine-HCl) coating (molecular weight 22,000; Sigma) was achieved by incubating for 10 min with 0.1% PLL. Unbound PLL was washed away with Ca²⁺-free KRH (135 mmol/L NaCl) before applying the outer alginate layer by 10-min incubation in 10 times diluted alginate solution. Three successive washings with Ca²⁺-free KRH removed the excessive alginate.

Encapsulated islets were cultured in a 48-well culture plate (tissue culture treated, Costar®, Cambridge, MA), for 2 and 5 days at 37°C in humidified air with either 5% CO₂, 20% O₂, and 75% N₂ (normoxia) or 5% CO₂,

1% O₂, and 94% N₂ (hypoxia). Each well contained 90 encapsulated islets suspended in 400 μ l of CMRL 1066 containing 5% normal rat serum (Gibco BRL), 8.3 mM glucose, 10 mM HEPES, and 1% penicillin/streptomycin.

Glucose Challenge Test

Encapsulated islets were tested for their glucose-induced insulin response in three separate samples of 20 islets each. The islets were preincubated for 45–50 min in 2 ml Krebs-Ringer-bicarbonate (KRB, pH 7.4), gassed with 95% O₂, and 5% CO₂, containing 0.25% BSA and 2.75 mM glucose. The in vitro insulin secretion was then assessed by three consecutive incubations of 45 min in KRB containing (i) 2.75 mM glucose, (ii) 16.5 mM glucose with 0.1% isobutylmethylxanthine (IBMX, Sigma), and again (iii) 2.75 mM glucose. At the end of each incubation, the KRB was completely removed and frozen for insulin determination by a radioimmunoassay for rat insulin (Linco, Ede, The Netherlands). The insulin secretory responses of 20 encapsulated islets during 45 min were expressed as nanograms of insulin per milliliter.

Islet Vitality Staining

Encapsulated islet vitality was assessed by discriminating between apoptotic, necrotic, and vital cells. To this end, encapsulated islets were stained with 20 μ g/ml Hoechst 33258 (bisbenzimidazole, Sigma) and 10 μ g/ml propidium iodide (PI; Sigma) in PBS for 10 min at 37°C, and scored as described by J. Saldeen (29). Islet cells with Ho (Hoechst)-positive round nuclei were regarded as viable. Round nuclei were regarded as necrotic if both Ho and PI positive. Highly condensed or fragmented nuclei, HO positive or both Ho and PI positive, were identified as apoptotic cells. Counting was executed by an independent examiner who scored 700–800 cells at a 400 \times magnification (Leica DMLB/DC 300F) per experiment. Scores should be regarded as estimations, because superposition of cells makes it difficult sometimes to determine the true cellular status.

Immunohistochemical Staining of BrdU

5-Bromo-2-deoxy uridine (BrdU) labeling of the islets cells was performed by incubating the encapsulated islets with 10 μ mol/l BrdU (Boehringer Mannheim) for 24 h. BrdU-labeled islets were fixed in 2% paraformaldehyde, washed overnight with PBS containing 6% sucrose, and dehydrated with acetone for 1 h prior to glycolmethacrylate (GMA) embedding (all incubations at 4°C). Sections of 2 μ m were treated with 0.1% trypsin (0.1 M Tris-HCl, pH 7.8 + 0.1% CaCl₂) for 15 min at room temperature, stained with anti-BrdU (goat anti-BrdU with nuclease, Amersham, Uppsala, Sweden), and

finally incubated with a secondary antibody conjugated with peroxidase (rabbit anti-goat PO, DAKO, Glostrup, Denmark). Peroxidase activity was demonstrated by applying 3-amino-9-ethylcarbazole (AEC) containing hydrogen peroxidase. Hematoxylin was used for staining the cell nuclei. Per condition, approximately 1000 intact cells were counted. Necrotic and apoptotic cells were excluded from counting, as well as weakly stained cells, because cell repair instead of cell replication could be responsible for staining the cell nuclei. BrdU-positive cells showed a dark red staining of the nucleus. The number of BrdU-positive cells was expressed as the percentage of the total number of BrdU-negative and BrdU-positive cells. BrdU-negative cells were either hematoxylin blue or colorless as a result of the nuclease activity during the first antibody incubation step.

RT-PCR Analysis

Microcapsules were removed from the islets before analysis by shearing the encapsulated islets through a pipette, after a 30-min incubation step with 0.5 mM EDTA and 0.25% trypsin at 37°C (7). Batches of 60 decapsulated islets were washed three times with cold PBS and pellet frozen (−80°C). Total RNA was isolated using a Strataprep® Total RNA microprep kit (Stratagene, CA, USA) according to manufacturer's protocol. cDNA synthesis included an initial step in which RNA is incubated with T₁₁VN oligos (0.5 µg) for 10 min at 70°C. Subsequently, first-strand buffer, dNTPs (final conc. 1 mM), DTT (final conc. 10 mM), 200 U M-MLV reverse transcriptase (all from Gibco BRL), and 20 U recombinant RNasin® ribonuclease inhibitor (Promega) were added and incubated for 50 min at 37°C, after which the reaction was inactivated by a 15-min incubation at 70°C. Because only low amounts of total RNA can be isolated from 60 islets, we standardized RT-PCR by always using one third of the retrieved RNA in the cDNA synthesis step. cDNA (2 µl) was amplified by PCR in a buffer consisting of 0.2 mM dNTPs, 1.5 mM MgCl₂, 1× PCR buffer, and 1 U Taq DNA polymerase (all from Gibco BRL). Primer pairs (0.5 µM each) were added after 3 min at 94°C (hot start). PCR was performed on a Mastercycler Gradient apparatus (Eppendorf, Germany) and cycles consisted of 94°C for 40 s, a primer pair-specific annealing temperature (see below) for 40 s, and 72°C for 40 s. For each gene, the number of cycles was selected to allow amplification within the linear range. The primer sequences, their annealing temperature, the number of cycles, and their respective PCR fragment lengths were: β-actin (253 bp), 24 cycles, 55°C, 5'-AACACCCCAGCCATGTACG-3', 5'-ATGTCACGCACGATTTCCC-3'; Bcl-2 (446 bp), 33 cycles, 58°C, 5'-GCTACGAGTGGGATACTGGAGA-3', 5'-AGTCATCCACAGAGCGATGTT-3' (30); Bax (300 bp),

30 cycles, 52°C, 5'-AGGATGATTGCTGATGTGGATAC-3', 5'-CACAAAGATGGTCACTGTCTGC-3' (30); iNOS (308 bp), 32 cycles, 57°C, 5'-GACTGCACAG AATGTTCCAG-3', 5'-TGGCCAGATGTTCTCTATT-3' (5); MCP-1 (306 bp), 28 cycles, 57°C, 5'-TTCACAG TTGCTGCCTGTAGC-3', 5'-GTGCTGAAGTCCTTAG GTTGA-3'. Each RNA sample was verified for absence of genomic DNA contamination by performing RT-PCR reactions in which the addition of the enzyme reverse transcriptase was omitted. Ethidium bromide-stained agarose gels were scanned on Image Master® VDS (Pharmacia Biotech, Upsala, Sweden) using LISCAP software. PCR abundance was quantified using Divercity One™ 1.3 DOS-version (pdi Inc., NY, USA)

Culture Sample Assessments

Medium culture samples were collected after 2 and 5 days of culture and frozen (−20°C). MCP-1 protein was measured by an Enzyme Immunometric Assay Kit (Titerzyme® EIA, Assay Designs Inc.). A polyclonal antibody to rat MCP-1 immobilized on a microtiter plate was used to bind the rat MCP-1 in the sample. The MCP-1 was subsequently incubated with a Ho-labeled antibody against MCP-1. Addition of a substrate generates a yellow color that was measured at 450 nm on a microplate fluorescence reader (TL 600, Bio-Tek Instruments Inc.).

Nitrite formation was determined by use of the Griess reaction (13). An equal volume of the Griess reagent was mixed with 50 µl of medium and incubated for 10 min at room temperature. The absorbance at 540 was measured on an ultramicroplate reader (EL 808, Bio-Tek Instruments Inc.). Values were calculated from a standard curve ranging from 0.5 to 6.9 µg/ml.

Single samples of medium were used for lactate determination by use of a YSI 2300 Stat Plus (Salm & Kipp bv) analyzer. Insulin accumulation during culture was determined in 2000 times diluted duplicate medium samples by use of a radioimmunoassay, as mentioned above.

Statistical Analysis

Results were evaluated using a paired Student *t*-test with a two-tailed distribution.

RESULTS

Islet Function

Encapsulated islet function was assessed by measuring the insulin production of encapsulated islets during culture and by a glucose challenge test after 2 and 5 days of culture. On day 2, equal amounts of insulin had accumulated in the culture medium of hypoxic (5.4 ± 1.7 ng/ml) and normoxic (5.4 ± 0.4 ng/ml) cultured islets. On day 5, different amounts of insulin were found in the

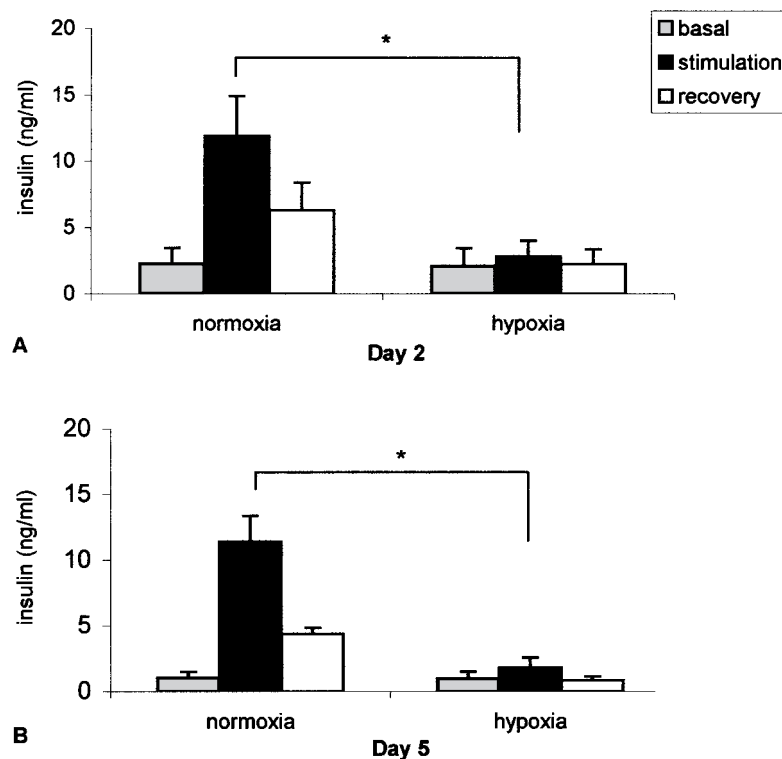


Figure 1. The insulin secretion (ng/ml) of encapsulated islets during a glucose challenge test after 2 (A) and 5 (B) days of culture under normal (normoxia, 20% O₂) and low (hypoxia, 1% O₂) oxygen tension ($n = 6$). * $p < 0.05$, two-tailed Student t -test.

culture medium. A significant increase in the amount of insulin was found for normoxic cultured islets (towards 13.5 ± 1.1 ng/ml), but not for hypoxic cultured islets (6.4 ± 1.3 ng/ml). Anaerobic metabolic activity was determined by measuring the amount of lactate in the culture medium, which was found to be significantly lower during normoxia (2.8 ± 0.4 and 5.3 ± 0.9 mM) compared with hypoxia (4.1 ± 0.4 and 7.7 ± 0.4 mM) after 2 and 5 days, respectively.

Figure 1 shows the basal insulin secretion, the stimulated insulin secretion, and the recovered insulin secretion of encapsulated islets during a glucose challenge test after 2 and 5 days of culture. Encapsulated islets cultured under normoxic condition responded adequately to both low and high concentrations of glucose. Encapsulated islets cultured under hypoxic conditions did show adequate basal insulin releases, but did not respond to the glucose load (i.e., no stimulation).

Islet Vitality

Encapsulated islet vitality was determined by estimating the number of vital, necrotic, and apoptotic cells after staining with Hoechst and PI. The vitality score is expressed as the percentage vital cells, which was found

to be low after 2 days of hypoxia ($71 \pm 7\%$) compared with normoxia ($86 \pm 5\%$), and even lower after 5 days of hypoxia ($62 \pm 9\%$). The loss of vitality was associated with a substantial increase in necrosis ($p < 0.05$ on both days 2 and 5, Table 1) and a slight increase in apoptosis ($p < 0.05$ only on day 2).

To gain insight in the molecular mechanism that leads to cell death, mRNA expression levels of the anti-apoptotic Bcl-2, the proapoptotic Bax, and iNOS, which is associated with both necrosis and apoptosis, were as-

Table 1. Islet Vitality

	Day 2		Day 5	
	Normoxia	Hypoxia	Normoxia	Hypoxia
Vital	86 ± 5	$71 \pm 8^*$	96 ± 0	$62 \pm 9^*$
Necrosis	12 ± 6	$23 \pm 8^*$	3 ± 0	$35 \pm 10^*$
Apoptosis	2 ± 1	$6 \pm 2^*$	1 ± 0	4 ± 2

The percentage of vital, necrotic, and apoptotic cells in encapsulated islets after 2 and 5 days of hypoxic (1% O₂) and normoxic (20% O₂) culture ($n = 6$).

* $p < 0.05$, two-tailed Student t -test.

sessed. The expression level of both genes remained similar after 2 days, but was lower for hypoxic islets compared with normoxic islets for Bcl-2 ($p < 0.05$) and tended to be lower for Bax ($p < 0.10$) on day 5 (Table 2). These decreases did not affect the Bcl-2/Bax ratio, which remained identical for both conditions after 2 and 5 days of culture (Table 2). The expression of iNOS mRNA had significantly increased after 2 days of hypoxia, but returned to a level similar to normoxic culture on day 5 (Fig. 2A). The NO production was measured by determining the nitrite formation during culture. Unexpectedly, the amounts of nitrite found in medium of hypoxic cultured islets were similar to the amounts of nitrite found in medium of normoxic cultured islets (Fig. 2B).

Islet Cell Replication

To test whether hypoxia affects the proliferation of islet cells, the islet cell replication during 24 h was assessed. The replication rate is the number of BrdU-positive cells expressed as the percentage of the total number of BrdU-negative and BrdU-positive cells. Because necrotic and apoptotic cells were excluded from counting, these values represent the percentage of replicating cells of the vital part of the islets. No significant differences in the encapsulated islet cell replication rates were found after 2 days ($5.8 \pm 1.9\%$ and $4.3 \pm 1.7\%$ per 24 h) and after 5 days ($2.1 \pm 0.4\%$ and $3.0 \pm 0.9\%$ per 24 h) of normoxic and hypoxic culture, respectively.

MCP-1 in Response to Hypoxia

To determine the relation between hypoxic stress and islet factors that may induce pericapsular overgrowth by macrophages in vivo, the MCP-1 production of encapsulated islets was measured. MCP-1 mRNA expression was determined by RT-PCR, and MCP-1 protein levels in the culture medium were assessed by EIA. RT-PCR results showed that the expression of MCP-1 mRNA was elevated under hypoxic stress after 2 days (2.6-fold, not

significant) and after 5 days (1.8-fold, $p < 0.05$) of culture (Fig. 3A). MCP-1 protein levels (Fig. 3B), however, were similar for encapsulated islets under normoxic and hypoxic culture, with a tendency to be lower for hypoxic islets rather than higher ($p < 0.10$ on both days).

DISCUSSION

Hypoxia is regarded as one of the main causes of encapsulated islet graft failure. The present study is an analysis of encapsulated islets during hypoxic stress in vitro, in which we show that hypoxia leads to dysfunction and loss of vitality, but not to increased proliferation of the islet cells.

Encapsulated islet function, as expressed in insulin secreting capacity, was severely affected by hypoxia. Glucose challenge of hypoxic cultured islets did not result in elevated insulin secretion levels, and also the basal insulin secretion was affected. Although the basal levels were identical for hypoxic and normoxic encapsulated islets, the amount of insulin on day 5 was lower in the hypoxic than in the normoxic culture medium. These findings are consistent with those of Dionne et al., who found the second-phase insulin secretion rate during perfusion to be almost completely eliminated at a pO_2 of 10 mmHg ($\approx 1.3\%$ kPa $\approx 1.3\%$ O_2), while the basal secretion rate was only slightly reduced (11). The maintenance of basal insulin secretion capacity can be explained by the lower oxygen consumption of islets during the basal insulin secretion phase (3,26,40). In the present study, the stimulated insulin secretion level was reduced to a basal level already after 2 days, while it decreased gradually over a period of 2–3 months in a previous in vivo study from our laboratory (10). The differences between these in vivo and the present in vitro results may be explained by the difference in the degree of hypoxia. The pO_2 in the peritoneal transplantation site was found to be in the order of 26–77 mmHg (≈ 3.5 – 10% O_2) (16,17,19,27), which is higher than the level of hypoxia applied here (1% $O_2 \approx 7.5$ mmHg). We chose severe hypoxia (1% O_2) because the pO_2 in the peritoneum is low, and may locally even approximate zero according to Zimmerman et al. (44). It is reasonable to assume that the oxygen tension in the encapsulated islets is even lower than the 1% O_2 in the overlying gas phase. As a consequence, the core of the islets is likely to be anoxic rather than hypoxic, which gives an explanation to the high loss of vitality and the high loss of function.

The reduced vitality was mainly associated with necrosis and to a lesser extent to apoptosis. The cell sensitivity for apoptosis did not change via Bcl-2 or Bax, because their ratio was not affected by hypoxia. The individual expression levels, however, were reduced for both Bcl-2 and Bax to half of the initial value after 5

Table 2. mRNA Expression Levels

	Day 2		Day 5	
	Normoxia	Hypoxia	Normoxia	Hypoxia
Bcl-2	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	$0.3 \pm 0.1^*$
Bax	1.0 ± 0.2	0.8 ± 0.2	1.1 ± 0.3	$0.6 \pm 0.1^\dagger$
Bcl-2/Bax	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.2

Expression levels of Bcl-2 mRNA, Bax mRNA, and their ratio, as assessed by semiquantitative RT-PCR ($n = 5$).

*Significantly lower compared with normoxia ($p < 0.05$, two-tailed Student *t*-test).

†Significantly lower compared with normoxia ($p < 0.10$, two-tailed Student *t*-test).

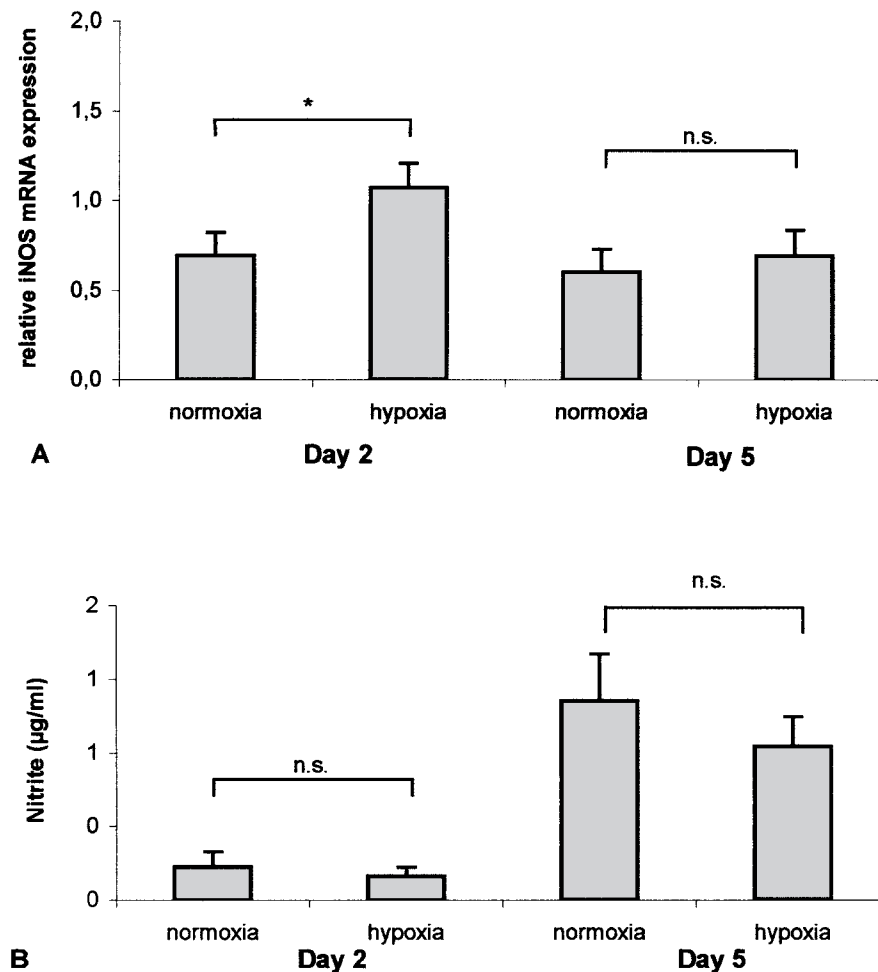


Figure 2. The mRNA expression of iNOS in encapsulated islets after 2 and 5 days of normoxic (20% O₂) and hypoxic (1% O₂) culture (A, **p* < 0.05, two-tailed Student *t*-test, *n* = 6). (B) The amounts of nitrite (ng/ml) in the culture medium produced by encapsulated islets after 2 and 5 days of hypoxic and normoxic culture (n.s., no significant difference).

days of hypoxia. Because Bcl-2 family members are more expressed in the centrally located beta cells than in the peripherally located alpha cells (14), the observed reduction of Bcl-2 and Bax expression may well reflect the decrease in the number of beta cells as a result of central necrosis. Hypoxia is reported to induce both necrosis and apoptosis, and the proportion of these two modes depends on the cell type, the duration, and the severity of the hypoxic stress (4,31). Moritz et al. recently reported complete disintegration of the islet core after 48 h of hypoxia (1% O₂), and they found hypoxia to be particularly related to apoptosis after 6 h (22). It is thus possible that in the present study also apoptosis was the predominant mode of cell death during the first hours of hypoxia. Because apoptotic cells can become necrotic if they are not engulfed by phagocytes, it remains unclear to what extent the high percentage of ne-

crolosis after 48 h was directly induced by hypoxia, or a secondary product of hypoxia-induced apoptosis.

The massive cell death during hypoxia, which consisted mainly of necrosis, was associated with an increase of iNOS mRNA on day 2, which implies a role for NO in the completion of cell death. The observed discrepancy between the high iNOS mRNA and the low nitrite levels as a measure for NO production in vitro has been described before (1,2,39). Low oxygen tension increases the half-life of NO, which enhances the chance of NO to react with scavengers, or to escape from the medium by partitioning in the overlying gas phase (1,2). This means that despite the low levels of nitrite measured, high levels of NO may have been present in the medium.

Increased islet cell proliferation after transplantation has been repeatedly reported and is regarded as a func-

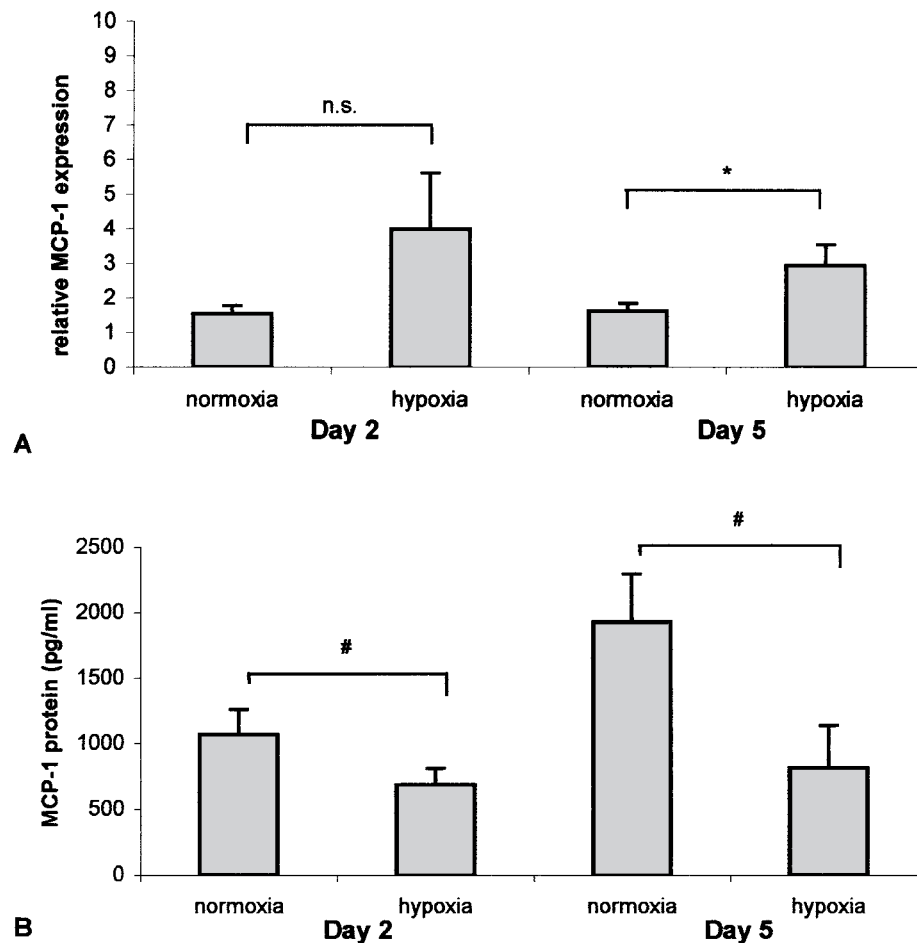


Figure 3. The mRNA expression of MCP-1 in encapsulated islets (A) and the amount of MCP-1 protein (pg/ml) in the culture medium (B) after 2 and 5 days of hypoxic (1% O₂) and normoxic (20% O₂) culture ($n = 5$). * $p < 0.05$, # $p < 0.10$, two-tailed Student t -test.

tional compensation for an insufficient islet mass (10,21, 23,36). Increased proliferation may also be the result of signals released by necrotic cells (20). However, although hypoxic cultured islets became necrotic, hypoxic and normoxic cultured islets showed similar replication rates in the present study. The proliferation rates of both hypoxic and normoxic cultured islets in the present study (2–6%) are in the same range as the proliferation rates observed after transplantation in a previous *in vivo* study (4–6%), which were increased compared with the proliferation rate in the native pancreas (10). High replication rates *in vitro* have been observed before and may well be explained by the amount of glucose in the culture medium. Sjöholm et al. showed that rat islets had a replication rate of 3.0% if cultured with 3.3 mM glucose and 5.8% if cultured with 11.1 mM glucose (34). The initial glucose concentration was 8.3 mM in the present and in a previous study from our laboratory, where free and encapsulated islet cell replication rates were found

to be 6.1% and 6.5%, respectively, after 2 days of culture (10). This is also in line with the results of Hayek et al., who showed that human islet cell replication was 4.7% after 3 days of culture at 5.5 mM glucose (15).

Hypoxia was associated with an increase in the mRNA expression level of MCP-1, but this increase was not detected on a protein level. On the contrary, the amount of MCP-1 protein tended to be lower in the culture medium of hypoxic compared with normoxic islets. This apparent contradiction can be readily explained, because the number of vital cells able to produce MCP-1 is considerably lower during hypoxic culture. If we correct the amount of secreted MCP-1 in line with the vitality scores, it appears that at least equal amounts of MCP-1 were produced under hypoxic and normoxic conditions. The MCP-1 production is probably even higher with hypoxia because a higher proteolytic activity due to necrosis may well be responsible for an underestimation of the actual amount of MCP-1 protein. A high

MCP-1 mRNA expression can be understood as a signal of islets to promote angiogenesis to solve the lack of oxygen (28). In a transplantation setting, however, the chemotactic activity of MCP-1 may contribute to graft failure by attracting cytokine-producing macrophages (25).

Thus, hypoxia is a crucial factor in encapsulated islet graft failure. It leads to loss of islet function and vitality, but not to pronounced proliferation of islet cells. Obviously, strategies to reduce the hypoxic stress on islets are required for successful transplantation. One approach is to improve the blood supply by the use of a prevascularized implantation site (9). It may not only improve graft function by eliminating hypoxia, but it may also diminish the chemotactic process of attracting peritoneal macrophages that contribute to graft failure through pericapsular overgrowth and the production of cytokines. Another approach would be to increase the islet resistance against hypoxia, either by using hypoxia-resistant piscine islets from tilapia, a tropical teleost fish (42,43), or by, for example, ischemic preconditioning (6,37). Conceptually, these approaches may very well be combined.

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