

ORIGINAL ARTICLE

HYPOXIA INDUCES DYSFUNCTION AND CELL DEATH OF THE RAT PANCREATIC ISLET

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Abstract The aim of this study was to determine the time course and mechanism of hypoxia-induced pancreatic islet dysfunction. Islets isolated from Sprague Dawley rats were cultured in 1% O₂ (hypoxia). Glucose stimulated insulin secretion (GSIS) was then examined for islets in either static or perfused cultures, followed by an evaluation of mitochondrial activity and islet cell death. Additionally, we examined the effect of culturing previously hypoxic islets for an additional 24 h under normoxia to determine whether the hypoxic effects were reversible and to assess the effects of re-oxygenation on GSIS. In the static islet culture, insulin secretion declined significantly after 24 h. In perfused islets, the area under the curve (AUC) of first-phase GSIS declined significantly after 6 h, while the AUC of second-phase GSIS decreased significantly after 12 h. Mitochondrial activity dropped markedly after 48 h, but cell death assays revealed that apoptosis did not increase in the time period from 6 h to 48 h. However, necrosis increased significantly after 24 h. In the re-oxygenation study, the return to normoxia significantly worsened the decline in GSIS. In conclusion, exposure to hypoxia first causes functional disorder in the islets, followed by cell death due to necrosis rather than apoptosis. Furthermore, re-oxygenation aggravated islet dysfunction.

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Key words: Islet transplantation; islet cell death; mitochondrial activity; hypoxia; re-oxygenation.

原 著

膵ランゲルハンス島に引き起こされる低酸素性機能障害の経時的变化とメカニズム

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抄録 今回我々は低酸素暴露による膵ランゲルハンス島（以下膵島）機能障害の経時変化とその機序を検討した。膵島はSDラットから単離し酸素濃度1%（低酸素）で培養後、インスリン分泌実験（静置法、灌流法）を施行。ミトコンドリア活性と膵島細胞死も測定した。更に低酸素後24時間（以下h）再酸素化し、インスリン分泌実験（灌流法）で評価した。静置法では、24h以降でIns分泌が有意に低下した。灌流法では第1相分泌のAUCが6h以降で、第2相は12h以降で有意に低下した。ミトコンドリア活性は48時間以降で低下が有意となった。アポトーシスに差は無かったが、ネクローシスが24h以降で有意に増加した。再酸素化はインスリン分泌を有意に悪化させた。以上から低酸素暴露はまず機能障害を起こし、その後アポトーシスよりはむしろネクローシスに影響された細胞死を引き起こすと考えられた。また再酸素化は膵島の機能障害を促進させた。

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1. Introduction

The transplantation of pancreatic islets is less invasive than whole-organ transplantation of the pancreas, making islet transplantation a promising therapy for Type 1 diabetes. Recently, islet transplantation had achieved good results through the implementation of the Edmonton protocol^{1, 2}. However, a patient must receive a mean of >10,000 IE/kg to become insulin independent, meaning that two or more donors are currently needed per islet recipient. The fact that patients who retain the function of only 10-20% of the estimated 1,000,000 islets in the pancreas are able to maintain euglycemia suggests that most of the transplanted islets do not engraft and/or fail to engraft sufficiently to become functional. One of the proposed reasons for this failure is the exposure of the islets to hypoxia, which is believed to cause islet injury. In a recent study, it was shown that current techniques for the isolation and perfusion of human islets cause the complete destruction of the islet capillary network³. Islets in the early post-transplantation period only receive oxygen via passive diffusion until revascularization of the islet is complete. Moreover, the pO₂ of the portal circulation is typically only 10-15 mmHg, meaning that an insufficient amount of oxygen is supplied to the islet graft³. Therefore, isolated and purified islets are exposed to long-term hypoxia which is considered to be an important factor for graft dysfunction in the early post-transplantation period. However, there are a few studies that demonstrate the time course of the functional changes that occur within islets exposed to hypoxia^{4, 5}.

In this study, the influence of hypoxia on insulin secretion capacity, mitochondrial activity and islet cell death was assessed *in vitro* using rat islets. In addition, following hypoxic exposure, some islets were cultured in normoxia as a "re-oxygenation test" to assess the effects of re-oxygenation on islet function.

2. Materials and Methods

All islets were isolated from Sprague Dawley (SD) rats (Charles River, Kingston, USA) by the ductal method and cultured overnight in an untreated 60 mm dish (ATG, Tokyo, Japan) with high glucose (450 mg/dl) Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, California, USA) with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 µg/ml streptomycin. In this study, islets with an estimated diameter of 62.5-156.25 µm were selected using a phase contrast microscope. Following an overnight incubation, we divided the cultured islets into two groups. One group (hypoxia group) was cultured in 1% O₂, 5% CO₂, and 94% N₂, at 37 °C in a multi-gas incubator (APM-30D; ASTEC, Fukuoka, Japan). The other group (normoxia group) was cultured in 95 % air and 5 % CO₂, at 37 °C in a standard incubator (MCO-20A1c; SANYO, Osaka, Japan). The hypoxia and normoxia groups were both cultured for the same period of time. The groups were exposed to their respective conditions for 6 h, 12 h, 24 h and 48 h. In the normoxia group, islets cultured only overnight were defined as the "control islets". Following exposure of both groups and the control islets, intact islets were judged by their shape under a phase contrast microscope and selected. Then, the hypoxia group was compared to the control islets and the normoxia group at each periods of exposure. All experiments were performed in accordance with Guidelines for Animal Experimentation, Hirosaki University.

In this study, we assessed (A) glucose stimulated insulin secretion (GSIS) using the static and perfusion methods, (B) mitochondrial activity, (C) islet cell death and (D) GSIS through the perfusion method in the re-oxygenation group.

(A) GSIS

Glucose-free Hank's balanced salt solution (HBSS) containing 5% FBS was prepared. Additionally, we prepared HBSS with 5% FBS

containing glucose at either 50 mg/dl (Glu50) or 300 mg/dl (Glu300) using a 50% glucose stock. Each GSIS measurement used 20 islets selected as described above.

(A)-1, STATIC METHOD

In this study, we followed the static method procedure of Kudo *et al.*⁶⁾ and Lehmann *et al.*⁷⁾ using a permeable membrane (Cell Culture Insert; Becton, Dickinson and Company, New Jersey, USA) and a 12-well microplate (ATG, Tokyo, Japan). Samples placed in the basket were transferred from well to well to wash samples and/or exchange media. First, we cultured samples for 3 h with Glu50 and washed the sample three times with fresh culture medium. Then, samples were cultured with Glu50 for 30 min to determine a basal level of insulin secretion. Next, samples were cultured with Glu300 for 30 min to determine GSIS. At the end of this procedure, samples were again cultured with Glu50 for 30 min. The supernatants from each of these three incubations were collected, and the insulin concentration in the supernatant was measured. Insulin production was measured by ELISA (Rat Insulin ELISA; Mercodia, Uppsala, Sweden). The results were calculated as an "Insulin Secretion Ratio" (ISR), defined as the ratio of insulin concentration in the Glu50 media to either the Glu300 media or media from the second incubation with Glu50.

(A)-2, PERIFUSION METHOD

We followed the perfusion procedure described previously⁶⁻⁸⁾ using a 0.45 μ m cellulose-acetate membrane (DISMIC; ADVANTEC TOYO, Tokyo, Japan). Islets were placed in the membrane with Glu50 and perfused with Glu50 at a flow rate of 800 μ l per min. During the first 30 min, islets were perfused with Glu50 as a "preparation phase". From 30 to 38 min, the samples were perfused with Glu50 to determine basal levels of insulin secretion. At 38 min, the glucose concentration was increased to 300 mg/dl for a period of 15 min to determine GSIS. Finally, the glucose concentration was

again lowered to 50 mg/dl, and the islets were perfused for a period of 8 min. A sample of the perfusate was collected at several time points to determine the concentration of insulin in the media. Insulin production was measured as above. ISR was calculated as the ratio of the insulin concentration measured after glucose elevation to the insulin concentration measured prior to glucose elevation. In the period perfused with Glu300, we defined the first 5 min as "first-phase secretion" and the latter 10 min as "second-phase secretion". Finally, we calculated the GSIS using the area under the curve (AUC) calculated from the ISR.

(B) MITOCHONDRIAL ACTIVITY

We assessed the mitochondrial activity using the metabolic indicator, Alamar Blue, (Serotec, Oxford, UK) to reveal the effects of hypoxia on the islets. Alamar Blue assay is a metabolic redox indicator that changes color and fluorescence in response to alternations in cellular metabolic activity. We followed the procedure of Li X *et al.*⁹⁾, using 20 islets (selected as above) for each sample and DMEM (without phenol red) in a 96-well plate. Islets were cultured for 4 h, and we measured the reduction of Alamar Blue by a colorimetric method at 570 nm and 600 nm absorbance. After three washes with fresh medium, islets were cultured under normoxia or hypoxia for various time periods. At the end of the treatment, islets were cultured for another 4 h, and the reduction of Alamar Blue was measured again. To analyze the results, the percent difference in the reduction of Alamar Blue between the hypoxia and normoxia groups was calculated according to the manufacturer's instructions. The percent difference in the indicator's reduction following various exposure times was also compared.

(C) ISLET CELL DEATH

In this study, we evaluated islet cell death using an ELISA assay (Cell Death Detection ELISA^{PLUS}; Roche, Mannheim, Germany).

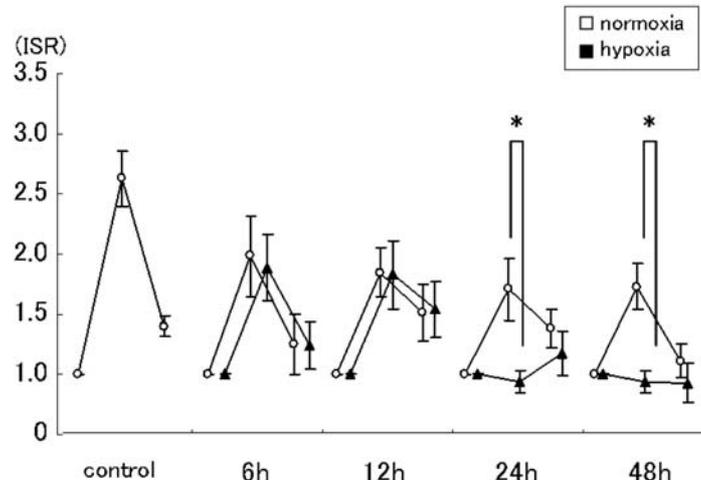


Figure 1 Glucose stimulated insulin secretion using the static method. This figure indicates the time-course of “Insulin Secretion Ratio” (ISR). The secretion pattern of insulin changed after 24 h. By comparing ISRs for Glu300, ISR was seen to decline significantly after 24 h. The count for control islets is 130. The counts of both groups are the same for each period of exposure. The counts for 6 h, 12 h, 24 h and 48 h are 12, 18, 24 and 12, respectively.

During cell death, endogenous endonucleases cleave double stranded DNA, generating mono- and oligonucleosomes, where the DNA is tightly complexed with the core histones. This assay is based on a quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies directed against DNA and histones. Apoptosis and necrosis can be measured simultaneously with this assay. Following an overnight incubation, 10 islets were selected per sample and placed in a sterilized microtube. The tubes were cultured under normoxic and hypoxic conditions, and then centrifuged (1100 rpm, 10 min, 4°C). The supernatant was collected to evaluate islet cell necrosis and stored in a refrigerator. The pelleted islets were resuspended in lysis buffer and centrifuged again (1100 rpm, 10 min, 4°C). The supernatant from the second centrifugation step was collected to evaluate islet cell apoptosis. These supernatants, used in determining necrosis or apoptosis, were transferred into a coated 96-well microplate. Following the manufacturer’s instructions, the absorbance of the supernatants was measured at 405 nm with a reference wavelength of 409 nm. Based on the absorbance data, the “enrichment

factor (EF)”, the ratio of the sample’s enrichment of mono- and oligo-nucleosomes released into the cytoplasm to those of the unexposed islets, for both apoptosis and necrosis were calculated.

(D) RE-OXYGENATION TEST

Following exposure for periods of 6 h, 12 h and 24 h, islets were cultured for 24 h in normoxia as a “re-oxygenation” step. After re-oxygenation, GSIS was evaluated using the perfusion method.

(E) STATISTICS

Data are presented as the mean±standard error (SE). Statistical significance was calculated using one-factor ANOVA or two-factor factorial ANOVA. For the analysis of GSIS and mitochondrial activity, Dunnett’s test for multiple comparisons was applied. For the analysis of cell death and re-oxygenation, Sheffe’s F-test for multiple comparisons was applied. Values of P less than 0.05 were considered to be significant.

3. Results

GSIS by the static method

The results of the static method GSIS are shown in Figure 1. For islets cultured in hypoxia, the ISR in Glu300 appeared to decline after 24 h (upper figure), so the ISRs were compared

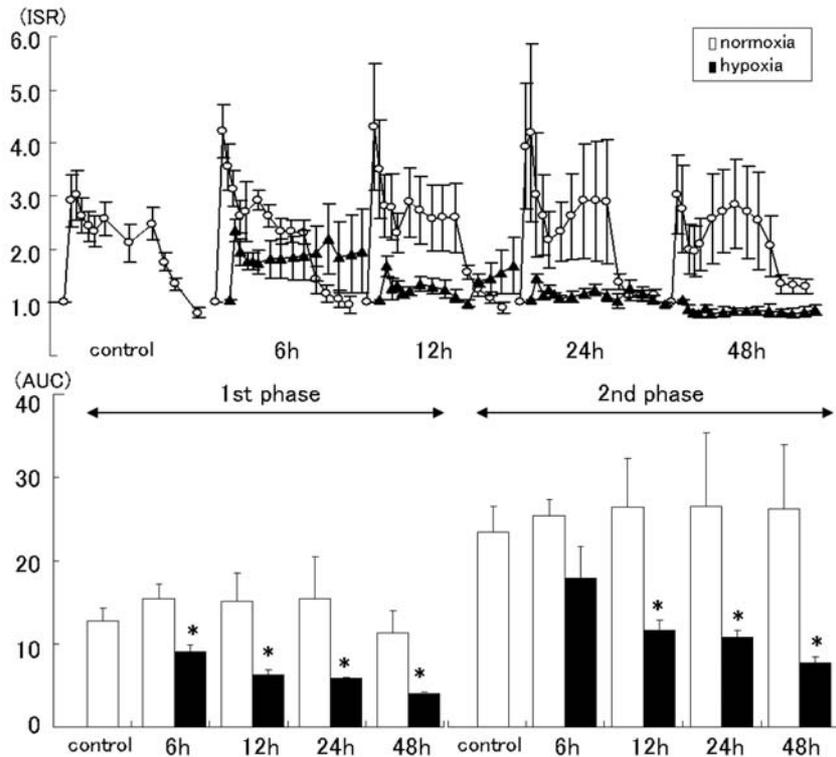


Figure 2 Glucose stimulated insulin secretion using the perfusion method.

The upper figure indicates the dynamic change and time course of “Insulin Secretion Ratio” (ISR). ISR in Glu300 is divided into two phases (first-phase and second-phase), as described in the Materials and Methods section. The lower figure shows the comparison of area under the curve (AUC) for each phase. In the first-phase assessment, AUC had already significantly decreased after 6 h exposure. In the second-phase assessment, AUC was significantly decreased after 12 h of exposure. The count for each group was six.

statistically (lower figure). No interaction was found with a two-factor factorial ANOVA. Subsequently, Dunnett's test for multiple comparisons was applied to the normoxia and hypoxia groups. While ISR did not change in the normoxia group (control: 2.63 ± 0.23 , 6 h: 1.98 ± 0.34 , 12 h: 1.85 ± 0.21 , 24 h: 1.70 ± 0.25 , 48 h: 1.73 ± 0.19), the hypoxia group showed a significant decline in ISR after 24 h and 48 h exposure (control: 2.63 ± 0.23 , 6 h: 1.89 ± 0.28 , 12 h: 1.82 ± 0.28 , 24 h: 0.94 ± 0.09 , 48 h: 0.94 ± 0.09).

GSIS by the perfusion method

The results of the perfusion GSIS are shown in Figure 2. In hypoxic islets, insulin secretion appeared to already be declining after 6 h of exposure (upper figure). First-phase insulin secretion and second-phase insulin secretion were compared statistically (lower figure). No

interaction was found with a two-factor factorial ANOVA following assessment of both phases. Dunnett's test for multiple comparisons was then applied to normoxia and hypoxia. In the normoxia group, there were no significant changes in either phase of insulin secretion (in first-phase, control: 12.67 ± 1.66 , 6 h: 15.38 ± 1.73 , 12 h: 15.07 ± 3.48 , 24 h: 15.39 ± 5.03 , 48 h: 11.29 ± 2.75 ; or in second-phase, control: 23.47 ± 3.19 , 6 h: 25.44 ± 1.91 , 12 h: 26.48 ± 5.95 , 24 h: 26.64 ± 8.77 , 48 h: 26.31 ± 7.76). In contrast, the hypoxia group's first-phase insulin secretion had already declined significantly after 6 h of exposure (control: 12.67 ± 1.66 , 6 h: 8.97 ± 0.82 , 12 h: 6.28 ± 0.60 , 24 h: 5.73 ± 0.25 , 48 h: 3.96 ± 0.26 ; $p < 0.05$) and second-phase insulin secretion also decreased markedly after 12 h of exposure (control: 23.47 ± 3.19 , 6 h: 17.84 ± 3.97 , 12 h: 11.60 ± 1.23 , 24 h: 10.77 ± 0.88 , 48 h: 7.67 ± 0.71 ; $p < 0.05$).

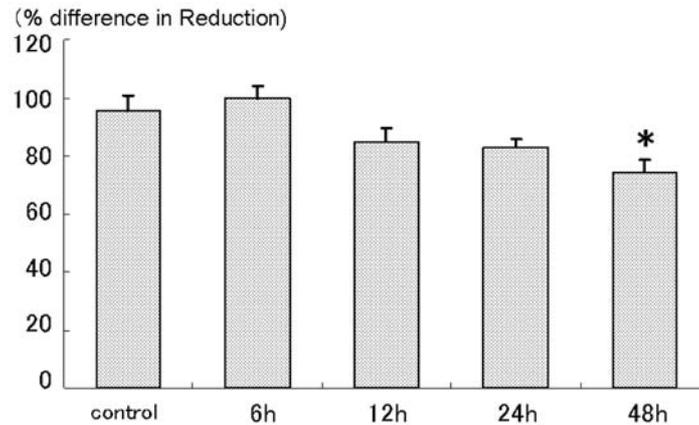


Figure 3 Alamar Blue assay.

The “percent difference in reduction” is calculated as the ratio of the amount of reduction in normoxia to the amount observed in hypoxia for each period. Sample absorbance was measured at 570 and 600 nm. Mitochondrial activity declined gradually. After 48 h, the decline in activity reached significance. The count for each group was six.

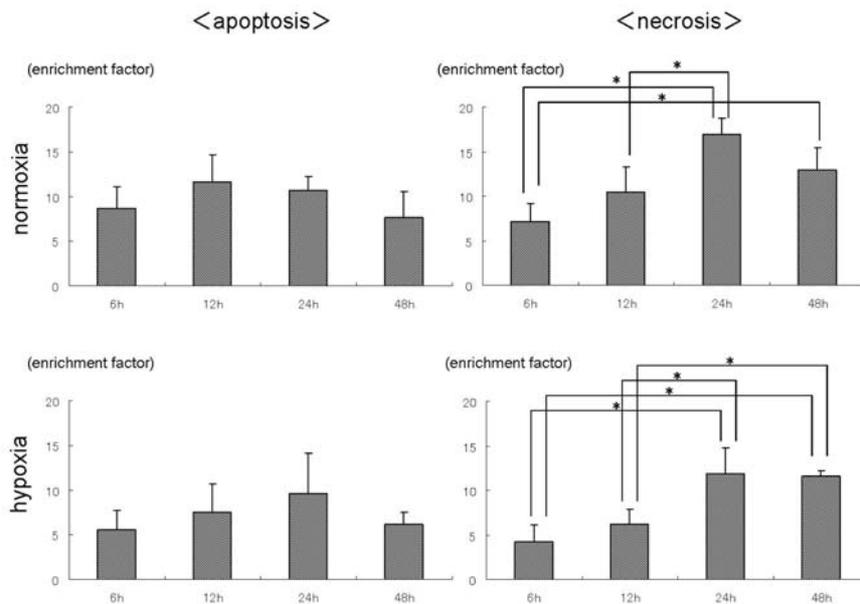


Figure 4 Cell death assays.

The necrosis enrichment factor was calculated as the ratio of the absorbance of the sample supernatant to that of the control (0 h). Sample absorbance was measured at 405 nm. The formula for apoptosis was similar to necrosis, except that the sample from the islet pellet was used. Necrosis, under both normoxic and hypoxic conditions, increased significantly as the exposure periods were prolonged, while there was no significant difference in apoptosis. In comparing the oxygenation states, necrosis during hypoxia was significantly less than during normoxia after a 12 h exposure. The count of each group was six.

Mitochondrial activity and cell death

The results of Alamar Blue assay are shown in Figure 3. In this assay, the data are expressed as a ratio of the normoxia group to the hypoxia group in each exposure period. The percent difference in reduction of the indicator declined gradually out to 48 h of exposure, at which

point the decline was found to be significant by both the one-factor ANOVA and Dunnett’s test (control: 95.52 ± 5.03 , 6 h: 99.71 ± 4.60 , 12 h: 84.58 ± 5.11 , 24 h: 82.78 ± 3.19 , 48 h: 74.06 ± 4.43 ; $p < 0.05$). The results of the Cell Detection ELISA^{PLUS} are shown in Figure 4. The figure indicated in right rank shows an assessment necrosis, while the

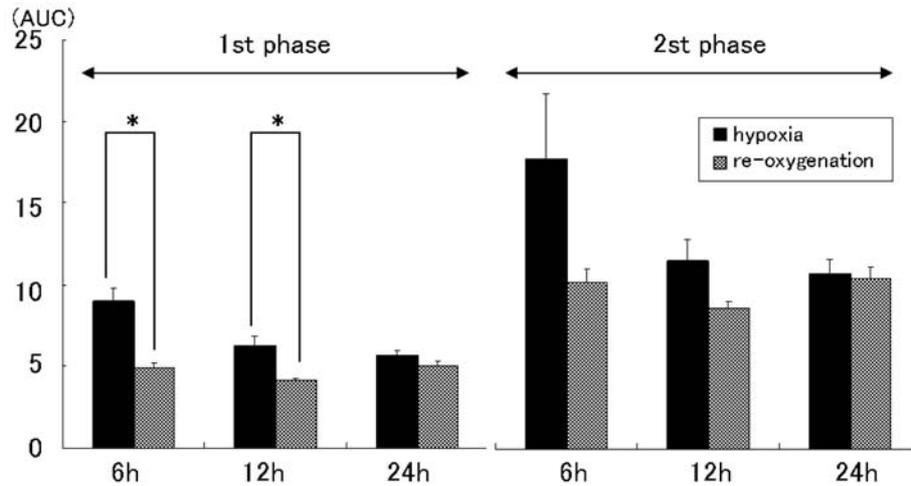


Figure 5 Re-oxygenation test.

The glucose stimulated insulin secretion test was performed using the perfusion method and the results were analyzed by area under the curve (AUC) calculating the "Insulin Secretion Ratio". The AUC of the first-phase showed that the deterioration of glucose stimulated insulin secretion was significantly worse at 6 h and 12 h. In contrast, there was no significant difference in the AUC of the second-phase. The count of the hypoxia group is described above. In the re-oxygenation groups, the counts for the 6 h, 12 h and 24 h groups were eight, eight and six, respectively.

left shows the amount of apoptosis observed. In both analyses, the data were expressed as the degree of apoptosis and necrosis compared to the control islets (enrichment factor, or EF). There was no interaction detected by a two-factor factorial ANOVA for either type of cell death. And Sheffe's F-test for multiple comparisons was applied to analyze the degree of apoptosis and necrosis. Examining apoptosis revealed no significant difference between the normoxia and hypoxia groups (normoxia, 6 h: 8.74 ± 0.98 , 12 h: 11.64 ± 1.22 , 24 h: 10.64 ± 0.66 , 48 h: 7.67 ± 1.19 ; hypoxia, 6 h: 5.56 ± 0.89 , 12 h: 7.54 ± 1.27 , 24 h: 9.65 ± 1.82 , 48 h: 6.19 ± 0.56). The assays of necrosis showed that EF increased significantly after 24 h exposure (normoxia, 6 h: 7.19 ± 0.78 , 12 h: 10.38 ± 1.16 , 24 h: 16.96 ± 0.71 , 48 h: 12.92 ± 1.05 ; hypoxia, 6 h: 4.19 ± 0.79 , 12 h: 6.21 ± 0.67 , 24 h: 11.85 ± 1.19 , 48 h: 11.54 ± 0.26 ; $p < 0.05$). Additionally, the EF of the normoxia group was significantly higher than the hypoxia group at the 24 h time point ($p < 0.05$).

The influences of re-oxygenation

The findings from the perfusion GSIS in the re-oxygenation experiment are shown in

Figure 5. In our analysis of the first-phase, an interaction was found by two-factor factorial ANOVA, and Sheffe's F-test for multiple comparisons was applied to all groups. Re-oxygenation aggravated the decline in GSIS caused by hypoxia at 6 h and 12 h (re-oxygenation, 6 h: 4.93 ± 0.32 , 12 h: 4.14 ± 0.12 , 24 h: 5.06 ± 0.28 , see above for hypoxia group; $p < 0.05$). In contrast, analysis of the second-phase revealed no interaction through two-factor factorial ANOVA. Sheffe's F-test for multiple comparisons was applied to all groups for second-phase insulin secretion, and no significant difference was found between the hypoxia and re-oxygenation groups (re-oxygenation group, 6 h: 10.17 ± 0.88 , 12 h: 8.64 ± 0.38 , 24 h: 10.49 ± 0.73).

4. Discussion

In the early post-transplantation period, the main cause of islet loss and dysfunction has been considered to be exposure to a hypoxic environment³. Several studies were conducted to uncover the causes of islet dysfunction^{3, 5, 10-13}, along with clinical trials to test a strategy for

reducing hypoxic exposure^{9, 14-19}). Results thus far have been insufficient to explain why islets fail, and preventing islet loss and dysfunction in grafts remains a challenging problem.

In this study, we showed that exposing rat islets to hypoxia led to insufficient insulin secretion. Insulin secretion significantly declined after 24 h of exposure to hypoxia through the static GSIS protocol. Using the perfusion method, first-phase insulin secretion was found to decline significantly after 6 h of exposure to hypoxia, while second-phase insulin secretion dropped markedly after 12 h of hypoxic exposure. Mitochondrial activity decreased gradually, reaching significance after 48h of hypoxia. Our studies of islet cell death showed that islet apoptosis failed to change significantly after 6 h, regardless of oxygen state in contrast, necrosis increased significantly as the exposure period was prolonged. Moreover, after 12 h of exposure, the levels of necrosis for hypoxic islets were significantly less than those of normoxic islets. Furthermore, re-oxygenation after exposure to hypoxia worsened islet dysfunction.

GSIS measured by the static method declined after 24 h of hypoxia. This impairment of insulin secretion might be explained by the loss of islet cells due to necrosis. On the other hand, using the perfusion method, the decrease in the first-phase of insulin secretion after 6 h of hypoxia, which was followed by a decrease in the second-phase of insulin secretion after 12 h of hypoxia, could not be explained by the assessment of mitochondrial activity or islet cell death. Thus, neither of those mechanisms may be involved in the islet dysfunctions observed. Other factors influencing islet cell function may include a change in the responsiveness of the islet to glucose and/or insulin production. Furthermore, re-oxygenation may be harmful, possibly due to low-levels of antioxidant enzymes and the associated increase of oxidative stress on the pancreatic islets^{17, 20}). These results indicate that exposure to hypoxia first causes

functional disorder in the islets, followed by cell death due to necrosis rather than apoptosis. Exposure to normoxia following hypoxia worsens islet dysfunction, in addition to causing further islet loss. We therefore hypothesize that the functional insufficiency of grafts, despite huge numbers of transplanted islets, is caused not only by islet loss but also by a decrease in the insulin secretion of the surviving islets. In other words, a huge number of islets are needed to achieve euglycemia following the decrease in insulin secretion from islets that survive hypoxia and re-oxygenation. For transplantation to be successful using a small number of islets, it will be essential to find ways to circumvent islet dysfunction and/or islet cell death due to hypoxic exposure, as well as re-oxygenation, which mediates further deterioration. Both challenges will need to be addressed simultaneously. Furthermore, it was unexpected that the degree of necrosis was higher in normoxic islets as compared to hypoxic islets. We cannot explain the mechanisms behind this at this time. However, this phenomenon might be related to the adaptation of the islet cells to hypoxia.

A limitation of this study is that our procedures were only performed *in vitro*, so we were unable to determine the effects of the immune system or blood flow on the islets. A second limitation is that the rise in oxygen concentration during the re-oxygenation test was unnaturally rapid and would not be experienced during islet transplantation. In addition, we could not confirm whether the effect of re-oxygenation was due to time in culture or to the re-oxygenation itself. Further studies of re-oxygenation that utilize a gradual restoration of normoxia may be needed to assess the effects of re-oxygenation more precisely.

We have demonstrated the time course of the functional and physiological changes that occur when islets are exposed to hypoxia. In conclusion, exposure to hypoxia diminished both

graft mass and function. The knowledge obtained in our study provides a useful basis for further research on the establishment and optimization of treatments that can simultaneously cope with the decreases in graft mass and function. Additionally, using the perfusion method, we observed a decrease in first-phase GSIS that preceded the deterioration of second-phase GSIS. This is similar to the deterioration of GSIS seen in early type 2 diabetes mellitus. The destruction or deterioration of the microvascular system of the islet may thus be a common pathological mechanism leading to the characteristic pattern of GSIS decline.

References

- 1) Ryan EA, Lakey JR, Paty BW, et al. Successful islet transplantation: Continued insulin reserve provides long-term glycemic control. *Diabetes* 2002;51:2148-2157.
- 2) Shapiro AM, Ricordi C, Hering BJ, et al. International trial of the Edmonton protocol for islet transplantation. *N Engl J Med*. 2006;28:1318-1330.
- 3) Emamaullee JA, Shapiro AM Factors influencing the loss of beta-cell mass in islet transplantation. *Cell Transplantation* 2007;16:1-8.
- 4) Keith E, Dionne, Clark K, Colton, Martin L, Yarmush Effect of hypoxia on insulin secretion by isolated rat and canine islets of langerhans. *Diabetes* 1993;42:12-21.
- 5) Per-Ola Carlsson, Anne Kiuru, Astrid Nodin, et al. Microdialysis measurements demonstrate a shift to nonoxidative glucose metabolism in rat pancreatic islets transplanted beneath the renal capsule. *Surgery* 2002;132:487-494.
- 6) Kudo T, Wu J, Ogawa Y, et al. Novel mechanism of chronic exposure of oleic acid-induced insulin release impairment in rat pancreatic beta-cells. *J Pharmacol Exp Ther*. 2006;318:1203-1210.
- 7) Lehmann R, Zuellig RA, Kugelmeier P, et al. Superiority of small islets in human islet transplantation. *Diabetes* 2007;56:594-603.
- 8) Spinas GA, Laffranchi R, Francoys I, et al. The early phase of glucose-stimulated insulin secretion requires nitric oxide. *Diabetologia* 1998;41:292-299.
- 9) Li X, Chen H, Epstein PN. Metallothionein protects islets from hypoxia and extends islet graft survival by scavenging most kinds of reactive oxygen species. *J Biol Chem*. 2004;279:765-71.
- 10) Moritz W, Meier F, Stroka DM, et al. Apoptosis in hypoxic human pancreatic islets correlates with HIF-1alpha expression. *FASEB J*. 2002;16:745-747.
- 11) de Groot M, Schuurs TA, Keizer PP, et al. Response of encapsulated rat pancreatic islets to hypoxia. *Cell Transplantation* 2003;12:867-875.
- 12) Giuliani M, Moritz W, Bodmer E, et al. Central necrosis in isolated hypoxic human pancreatic islets: evidence for postisolation ischemia. *Cell Transplantation* 2005;14:67-76.
- 13) Miao G, Ostrowski RP, Mace J, et al. Dynamic production of hypoxia-inducible factor-1alpha in early transplanted islets. *Am J Transplant*. 2006;6:2636-2643.
- 14) Wang J, Cao Y, Chen Y, et al. Pancreatic beta cells lack a low glucose and O2-inducible mitochondrial protein that augments cell survival. *Proc Natl Acad Sci U S A*. 2006;103:10636-10641.
- 15) Hara Y, Fujino M, Adachi K, et al. The reduction of hypoxia-induced and reoxygenation-induced apoptosis in rat islets by epigallocatechin gallate. *Transplant Proc*. 2006;38:2722-2725.
- 16) Lepore DA, Shinkel TA, Fiscaro N, et al. Enhanced expression of glutathione peroxidase protects islet beta cells from hypoxia-reoxygenation. *Xenotransplantation*. 2004;11:53-59.
- 17) Stagner JI, Parthasarathy SN, Wyler K, et al. Protection from ischemic cell death by the induction of cytoglobin. *Transplant Proc*. 2005;37:3452-3453.
- 18) Hughes SJ, Davies SE, Powis SH, et al. Hyperoxia improves the survival of intraportally transplanted syngeneic pancreatic islets. *Transplantation* 2003;75:1954-1959.
- 19) Sakai T, Li S, Tanioka Y, et al. Intraperitoneal injection of oxygenated perfluorochemical improves the outcome of intraportal islet transplantation in a rat model. *Transplant Proc*. 2006;38:3289-92.
- 20) Tiedge M, Lortz S, Drinkgern J, et al. Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. *Diabetes* 1997;46:1733.