Transplant International

Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Metabolic aspects of neonatal rat islet hypoxia tolerance

Ayman Hyder, Christiane Laue and Jürgen Schrezenmeir

Department of Physiology and Biochemistry of Nutrition, Max Rubner Institute, and Clinical Research Center, Innovation and Technology centre, Kiel, Germany

Keywords
adult pancreatic islets, glucose metabolism, hypoxia, insulin, neonatal pancreatic islets.

Correspondence
Prof. J. Schrezenmeir, Department of Physiology and Biochemistry of Nutrition, Max Rubner Institute, Hermann-Weigmann-Strasse 1, D-24103, Germany. Tel.: +49 431-2390331; Fax: +49 431 6092472; e-mail: juergen.schrezenmeir@gmx.de

Received: 7 May 2009
Revision requested: 3 July 2009
Accepted: 17 July 2009

doi:10.1111/j.1432-2277.2009.00943.x

Summary

Sensitivity of pancreatic islets to hypoxia is one of the most important of the obstacles responsible for their failure to survive within the recipients. The aim of this study was to compare the *in vitro* hypoxia tolerance of neonatal and adult rat islet cells and to study the glucose metabolism in these cells after exposure to hypoxia. Islet cells from both age categories were cultured in different hypoxic levels for 24 h and insulin secretion and some metabolites of glucose metabolism were analysed. Glucose-stimulated insulin secretion decreased dramatically in both cell preparations in response to the decrease in oxygen level. The reduction of insulin secretion was more detectable in adult cells and started at 5% O_2, while a significant reduction was obtained at 1% O_2 in neonatal cells. Moreover, basal insulin release of neonatal cells showed an adaptation to hypoxia after a 4-day culture in hypoxia. Intracellular pyruvate was higher in neonatal cells than in adult ones, while no difference in lactate level was observed between them. Similar results to that of pyruvate were observed for adenosine triphosphate (ATP) and the second messenger cyclic adenosine monophosphate (cAMP). The study reveals that neonatal rat islet cells are more hypoxia-tolerant than the adult ones. The most obvious metabolic observation was that both pyruvate and lactate were actively produced in neonatal cells, while adult cells depended mainly on lactate production as an end-product of glycolysis, indicating a more enhanced metabolic flexibility of neonatal cells to utilize the available oxygen and, at the same time, maintain metabolism anaerobically.

Introduction

Glucose-induced insulin secretion is determined by signals generated from the glycolytic pathway and in the mitochondria, and depends on glucose metabolism itself that initiates these signals. Glucose metabolism in insulin-secreting beta cells is mostly aerobic and depends on the presence of oxygen to be perfect. In the native pancreas, islets of Langerhans are highly oxygenated through an extensive islet vascular glomerular-like network of capillaries [1] and two predominant, distinct blood-flow patterns [2], as well as a very high blood-flow rate [3] that increases after stimulation with glucose [4] This may reflect the oxygen requirement of islets and their consequent sensitivity to hypoxia. Glycolysis and mitochondrial metabolism including oxidative phosphorylation might be changed or negatively affected by hypoxia with the outcome of reduction of glucose-stimulated insulin secretion, which is the net result of glucose metabolism. In this study, the glucose metabolism was followed in isolated rat islets cultured in different hypoxic conditions. This is especially pertinent for the field of islet transplantation. Islet isolation and purification are known to cause a rapid onset of hypoxia [5]. Recent studies have shown that pancreatic ischemia, including cold ischemia, can negatively influence both the quality and function of isolated islets [6]. In isolated islets, which depend only on diffusion for oxygen supply, consumption of oxygen results in external to internal oxygen concentration gradients that expose the islet to hypoxic or even anoxic conditions and causes

necrosis in the islet core, which consists mainly of insulin-secreting beta cells [7,8]. The expanded clinical application of islet transplantation is still limited by the large requirement for donor tissue. This need for large numbers of donor islets is directly related to the failure of transplanted insulin-secreting beta cells to survive in the recipients because of their sensitivity to hypoxia, and caused by the pro-apoptotic stresses present both during isolation and early post-transplantation period [9,10]. Regarding hypoxia-tolerance, we hypothesize that neonatal islets may be more advantageous than adult islets and therefore may prove to be more suitable source for islet transplantation. A higher resistance of fetal islets to ischemia has been early described [11]. Emamaullee *et al.* [10] reported a natural resistance of neonatal porcine islets to hypoxia-induced apoptosis. We also assume that the underlying reasons for their higher tolerance may be found in the glucose metabolic pathway.

In this study, in addition to insulin secretion under hypoxic conditions, we compared the islet cellular levels of pyruvate and lactate as glycolysis results, the oxidative phosphorylation product ATP and the signal transduction members cAMP and calcium between neonatal and adult rat islet cells cultured in different hypoxic conditions.

Material and methods

Islet isolation

Islets from adult Lewis rats (300–350 g) were isolated by pancreatic distension as described earlier [12]. Briefly, after opening the peritoneal cavity, the distal end of the bile duct was clamped near the duodenum with a metal clip. The proximal common bile duct was cannulated near the liver hilus with a 22-G catheter. The pancreas was distended by injection of 10 ml collagenase solution (Serva, Heidelberg, Germany, 0.78 PZ U/mg, 1 mg/ml), carefully removed, washed, freed from fat, and transferred into a digestion tube in which the volume was completed to 10 ml with collagenase solution. The digestion tube was incubated for 15 min in a shaking water bath at 37 °C. The digest was passed through a wire mesh filter with 350 μm pore size, washed several times with ice-cold Hanks' buffered salt solution (HBSS) containing 10% FCS. Islets were separated using Nycodenz (Nycomed, Oslo, Norway) with gradients of 1.095 and 1.08 g/ml, and RPMI as an uppermost layer.

For isolation of islets from neonatal rats, seven pancreases from 7- and 8-day-old Lewis rats were digested by shaking in 6 ml of collagenase warmed up to 37 °C (1 mg/ml) for 5 min. These seven pancreases were digested together as a single digestion unit. The digestion was stopped by filling the 15-ml tube with ice-cold Ca-Mg-free HBSS containing 10% fetal calf serum. The digest was washed several times with the same solution. Islets were separated with discontinuous density gradient using Nycodenz (Nycomed) with gradients of 1.09, 1.08, and RPMI 1640 as an uppermost layer.

Dissociation of islets to single cells

Pure islets from each isolation were further hand-picked, counted and incubated at 37 °C for 3 min in 2-ml Ca-Mg-free HBSS containing 0.2 mg/ml EDTA and 1 mg/ml glucose. No further enzymes were used so as to ensure avoidance of damage to the cells that may decrease their viability and count. Islets were then aspirated five to six times using a 19-G needle, washed with the culture medium and distributed to different groups. The applied EDTA/mechanical digestion was adequate to allow disruption of the intact islet structure. The small remaining cell clusters were left to dissociate within 1 day of preculture.

Islet cell culture

For insulin-secretion experiments, single cells belonging to 10 islets were cultured at 37 °C with 5% CO_2 with 0.5 ml/sample of RPMI-1640 medium (Invitrogen, Karlsruhe, Germany) at 5.6 mmol/l glucose supplemented with 10% fetal calf serum. Cells were cultured on cell culture membrane inserts made from polyethylene terephthalate (PET, Falcon, Becton Dickinson, USA). For the other experiments, cells from about 30 islets were used. The PET membranous inserts have pore sizes of 0.4 μm to allow gas exchange between the cultured cells and the surrounding environment. The inserts were installed in their 24-well culture plates (Falcon). Islet cells were cultured for 3 days at 37 °C. This preculture period was under conditions of normal oxygen availability. On the 3rd day of culture, the medium was changed with a fresh medium, in which the oxygen concentration was adjusted to reach a test concentration. To adjust this concentration, sterile nitrogen gas was bubbled at room temperature into the medium and the oxygen concentration was controlled using the oxygen meter (oxi 320; WTW, Weilheim, Germany). The applied test oxygen concentrations were: 1% (7.6 mmHg), 3% (22.8 mmHg), 5% (38 mmHg), 7% (53.2 mmHg), 10% (76 mmHg), and 21% (air). Islet cells were then cultured under one or the other of the different oxygen concentrations for 24 h in either basal medium containing 5.6 mmol/l glucose or stimulatory medium containing 16.7 mmol/l glucose. Oxygen concentration was also adjusted in the incubator to be the same as in the medium. After the 24-h culture, medium was kept in −20 °C until insulin was quantified, and cells were then disintegrated to determine the intracellular metabolites.

Biochemical measurements

Insulin-related data were determined by radioimmunoassay using kits from Biodata (Italy). Data were normalized by dividing by the corresponding DNA content value. For the determination of intracellular metabolites, islets were disintegrated by Triton X-100 and sonication [13]. ATP, lactate and pyruvate were measured directly after the cellular disintegration. For calcium and DNA determination, disintegrated cells were frozen at −20 °C and measured on the next day.

Determination of ATP

ATP concentrations were determined by bioluminescence as described by Malaisse *et al.* [14]. The principle of this method is the following equation:

$$\text{ATP + D-luciferin } + O_2 \xrightarrow{Mg^{2+},luciferase} \text{oxyluciferin + AMP + PPi} + CO_2 + 0.9\text{hv}$$

In the presence of luciferase from Photinus pyralis (Sigma), ATP reacts with Photinus pyralis luciferin (Sigma) to give pyrophosphate and adenylyl luciferin. The latter is then oxidized by molecular oxygen in the presence of the same enzyme, yielding CO_2, AMP, and oxyluciferin in the electronically excited singlet state. Upon relaxation, 0.9 photons of wavelength 562 nm are emitted per molecule of ATP. The intensity of the emitted light is proportional to the ATP concentration. Luminescence was measured using the luminometer (Biolumat LB-9505; Berthold, Wildbad, Germany). The equipment was programmed to measure the number of photons released from a sample every 1/10 min for 1 min at 30 °C.

Determination of calcium

Aequorin, a bioluminescent jellyfish protein, exhibits bioluminescence that results from calcium-activated photoproteins and is used to measure the concentration of free Ca^{2+} in a rapid, sensitive and specific manner [15]. Calcium-activated photoproteins emit blue light at a rate that is highly sensitive to the free calcium ion concentration, but independent of cofactors such as ATP, oxygen, or NADH [16]. The 1 mg Aequorin supplied by Sigma was dissolved in 10 ml water. A standard series was prepared with calcium concentrations ranging from 10 to 200 μmol/l in addition to blank (H_2O). Ten μl from the sample, blank or standard was used. Fifty μl of the Aequorin solution (that contained 5 μg Aequorin/sample) were added and the luminescence was measured and integrated for1 min.

Determination of cAMP

Cyclic adenosine monophosphate (cAMP) was determined quantitatively by the Enzyme Immunoassay (EIA) kit from DRG Instruments GmbH (Marburg, Germany). The plate reader Spectramax 340 (Molecular devices, Sunnyvale, CA, USA) was used for these measurements.

Determination of lactate and pyruvate

Lactate and pyruvate were measured fluorometrically by the micro-method described by Olsen [17] and Sener and Malaisse [18]. This method is based on the following lactate dehydrogenase (LDH)-catalysed, reversible reaction

$$\text{L-(+)-Lactate + NAD}^+ \xleftarrow{LDH} \rightarrow \text{Pyruvate + NADH + H}^+$$

This reaction proceeds rapidly to completion from right to left at pH 7 in the presence of NADH in excess. The reaction can be made to proceed in the opposite direction in the presence of excess of NAD if the pH is raised. The substrates on either side of the reaction can be estimated by measuring the change in the concentration of NADH, which can be determined by its native fluorescence. Lactate was measured directly after incubation with Tris buffer (pH 9) containing 2 mg/ml NAD. Pyruvate was measured indirectly as described by Lowry *et al.* [19]. After completion of the reaction, the excess NADH was destroyed by HCl and the produced NAD was then transformed in alkaline medium into NADH that was measured fluorometrically. Fluorescence was measured with the Perkin-Elmer (LS-3) fluorescence-spectrometer. Excitation wavelength was 340 and emission wavelength 460. All measures were performed at room temperature.

Determination of DNA

Determination of DNA was performed fluorometrically [20] by staining with the fluorescent agent propidium iodide in a hypotonic solution of citrate. Fluorescence was measured at excitation and emission wavelengths of 520 and 600 nm respectively.

Statistical analysis

The best curve fit for calculations of the standard curve of different parameters was performed using the software 'TableCurve 2D' from Jandel Scientific, CA, USA. A standard curve was achieved for every measurement from each isolation. The number of samples (n) refers to number of islet isolations and is mentioned in every figure in the results. From an isolation, six samples were

cultured under some definite condition and averaged to give $n = 1$; Samples were measured in duplicates. The stimulatory index was calculated as stimulated secretion/basal secretion, where stimulated and basal secretions were in response to 16.7 and 5.6 mmol/l glucose respectively. Values were expressed as mean ± SEM. Statistical analyses were performed by ANOVA followed by Mann–Whitney test for group comparisons. Unpaired Student's *t*-test was used when ANOVA was not necessary. A *P* value of <0.05 was considered statistically significant. All statistical analyses were formulated in line with the guidelines of and performed using the software SIGMASTAT (Jandel Scientific).

Results

Effect of hypoxia on insulin secretion

In adult rat islet cells (Fig. 1a) that were cultured for 24 h in different hypoxic levels [(21%, control), 10%, 7%, 5%, 3% and 1% oxygen], insulin secretion was dependent on oxygen concentration and decreased when the latter was reduced. Basal insulin secretion was not largely affected and reduced from 0.137 ± 0.013 (control) to 0.099 ± 0.006 μU/μg DNA at 1%O_2. Only this value was significantly lower than that of the control, while other basal values were not different from the control one. On the other hand, glucose-stimulated insulin secretion was significantly ($P < 0.05$, ANOVA) decreased at 5% oxygen (0.144 ± 0.008 μU/μg DNA) in comparison with the normoxic value (0.273 ± 0.028 μU/μg DNA), and further at 3 and 1% O_2 (0.129 ± 0.007 and 0.099 ± 0.004 μU/μg DNA respectively). Glucose was able to stimulate insulin secretion over the basal value except for that at 3% and 1% O_2, where no difference between basal and stimulated secretions was observed ($P > 0.05$, *t*-test).

A lesser effect of hypoxia on insulin secretion was observed in neonatal islet cells (Fig. 1b). No statistically significant difference between control basal and glucose-stimulated insulin secretions was noticed under different hypoxic levels until reaching 1% O_2, where both basal (0.094 ± 0.007 μU/μg DNA) and stimulated (0.13 ± 0.01 μU/μg DNA) secretions were significantly lower than the corresponding normoxic values (0.138 ± 0.009 and 0.338 ± 0.056, respectively). In addition, even at 1% O_2, the glucose-stimulated secretion was significantly higher than the basal one. However, the stimulatory index (Fig. 1c) was changed earlier at 3% and 1% O_2 in neonatal rat islet cells. These data suggest a better function of neonatal islet cells than that of the adult cells, whose stimulatory index significantly decreased at 7% O_2 and downwards, when compared with the control value.

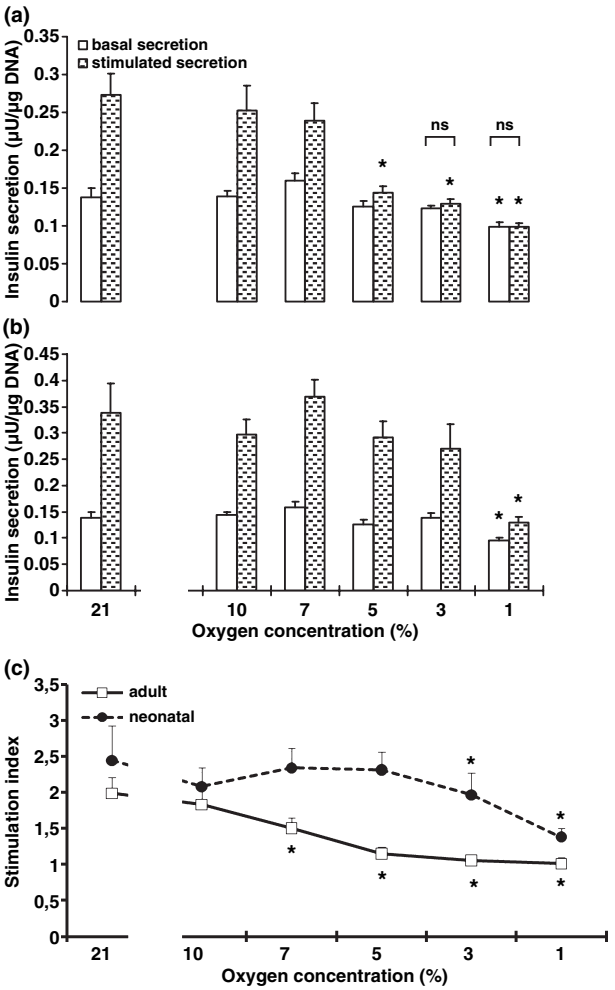

Figure 1 Basal and glucose-stimulated insulin secretions from pancreatic islet cells isolated from adult (a) and neonatal (b) rats and cultured in different hypoxic conditions. (c) Effect of hypoxia on insulin stimulation index of neonatal and adult rat islets. All insulin-secretion values were correlated to the DNA content of the same samples. Data are means ± SE for a number of isolations (n) = 4 in all cases. Statistical analyses: *significantly lower than the corresponding control value at normal air; NS, no significant difference between basal and stimulated values.

Another sign of neonatal islet cells having better function under hypoxia than the adult cells is shown in Fig. 2, which shows the daily insulin release (at basal glucose concentration) of neonatal and adult islet cells that were statically cultured for 4 days at different hypoxic levels. After the first hypoxic day, neonatal islet cells cultured at 10%, 7%, 5%, 3% and 1% O_2 restored their insulin release to the normoxic value. All values of insulin release under hypoxia were similar to the normoxic value after 4 days, except for that at 1% O_2. On the other hand, adult rat islet cells could not show this adaptation to hypoxia.

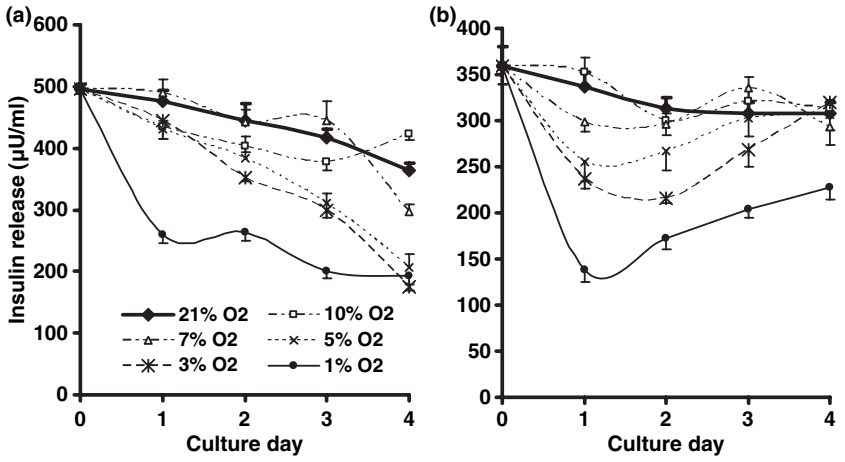

Figure 2 Basal insulin release from adult (a) and neonatal (b) islet cells after static culture in different hypoxic levels for 4 days. Data are summary of four experiments and presented as mean ± SE.

Effect of hypoxia on intracellular pyruvate and lactate levels

Data of neonatal and adult rat islets were compared. Values were computed as a percentage of the normoxic values and presented in Fig. 3. At basal glucose concentration, neither hypoxic neonatal nor adult rat islet cells were largely reduced by reduction in oxygen concentration. Pyruvate level of neonatal cells was reduced at 1% O_2 to 97.8 ± 12.0% of the control normoxic value, while basal pyruvate level of the adult cells was reduced at 1% O_2 to 81.4 ± 4.1% of the normoxic value. However, the reduction was more remarkable in adult cells and significantly lower than that of neonatal cells at 3% and 1% O_2. At stimulatory glucose (Fig. 3b), pyruvate level in the adult hypoxic cells was significantly reduced in response to the reduction of O_2, as compared with that at normoxic conditions (ANOVA), while limited nonsignificant effect was observed in neonatal cells. Consequently, under hypoxic conditions (5%, 3% and 1% O_2), the pyruvate levels

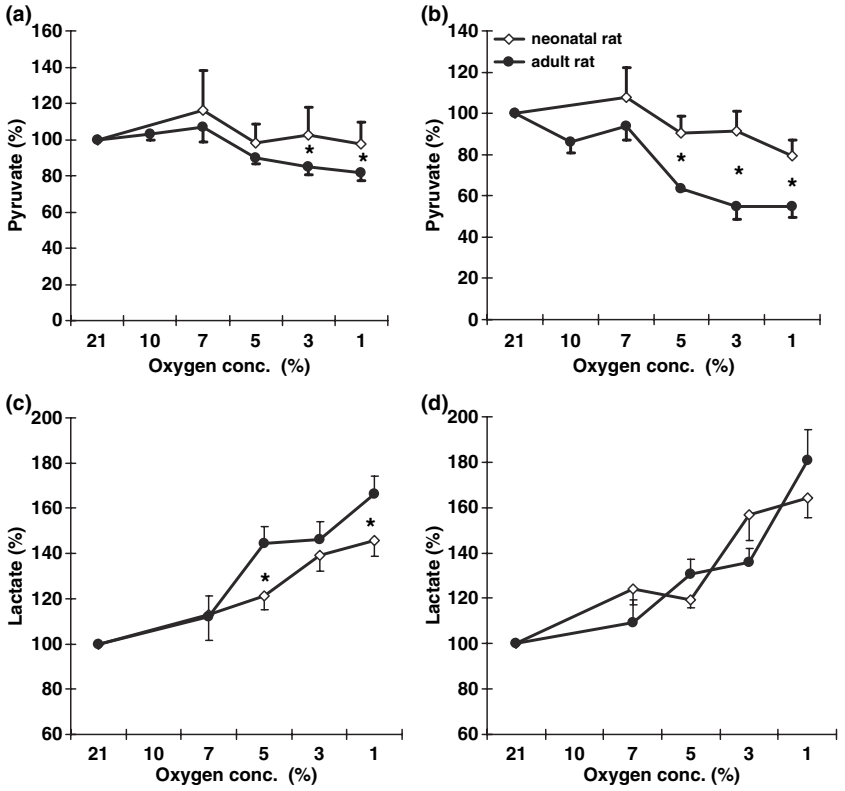

Figure 3 Basal (a and c) and glucose-stimulated (b and d) pyruvate and lactate levels, respectively, of neonatal and adult rat islet cells cultured in different hypoxic levels. All values were equalized by dividing by the DNA content of the same samples. To facilitate comparisons, data were related to the control value at normal air, which is considered as 100%. Data are presented as mean ± SE of at least $n = 3$ islet isolations. Statistical analyses: *the difference between neonatal and adult islet cell values is significant.

in neonatal cells were significantly higher than that of the adult cells.

Hypoxia caused a significant increase of lactate level in neonatal and adult islet cells at both basal and stimulatory glucose concentrations. Lactate was higher in hypoxic adult islet cells at basal glucose level (Fig. 3c) but no differences were observed between both at stimulatory glucose concentration (Fig. 3d).

Effect of hypoxia on ATP

Adenosine triphosphate (ATP) decreased significantly in both neonatal and adult cells at both basal and stimulatory glucose concentrations (ANOVA, Fig. 4) in relation to the corresponding normoxic value. Considering ATP control level as 100%, basal neonatal and adult islet cell ATPs decreased at 1% O_2, for example, to 65.19 ± 10.67% and 44.90 ± 7.17% of the normoxic values respectively, while the ATP level at stimulatory glucose concentration decreased to 48.88 ± 8.15% and only 21.77 ± 2.81% of the normoxic value in neonatal and adult cells, respectively, at 1%O_2. No difference was observed between neonatal and adult islet ATP levels at basal conditions. However, adult cells were more affected by hypoxia at stimulatory glucose concentration, especially at 3% and 1% O_2, where the neonatal ATP level was significantly higher than the corresponding values for adult cells.

Effect of hypoxia on calcium and cAMP

The change in calcium was not as obvious as that observed for ATP (Fig. 5). Hypoxia reduced the intracellular calcium. However, the reduction was not significant when compared with the normoxic value for either neonatal or adult cells at both basal and stimulatory glucose concentrations (ANOVA). Also, no difference was observed in calcium levels between neonatal and adult cells in response to hypoxia, when compared with each other.

Cyclic AMP levels, on the other hand, were more affected by hypoxia. However, this reduction was limited and insignificant at basal glucose concentration and was more obvious at stimulatory concentration, where neonatal and adult cells' cAMP decreased at 1% O_2, for example, to 55.24 ± 10.66% and 23.11 ± 11.40% of the normoxic value respectively. In comparison to each other, the cAMP level of neonatal cells was significantly higher than that of the adult cells at 7%, 3%, and 1% O_2.

Discussion

Oxygen supply is important for the function and survival of isolated and particularly encapsulated tissue [7,8]. In fact, hypoxia represents a most crucial factor limiting the function and survival of islet tissue, especially after immunoisolation and transplantation. It was the assumption that there might be differences in hypoxia tolerance between islets of different age categories. In this study, a direct comparison of the islet beta cell functions under different oxygen tensions was performed between neonatal and adult islet cells. Moreover, studying the metabolic pathways may have a biologic importance by elucidating the biologic strategies of hypoxia adaptation in different age categories. In this context, it should be mentioned that the metabolism in beta cells is characterized by some particularities with the aim of insulin secretion as an end result. Islet cells have a unique feature that they possess a transduction system for calorigenic nutrient signals, which is entirely different from that of other neuromodulators and peptide hormones. Fuel stimuli must be metabolized in the beta cell to cause secretion [21–24]. Therefore, regarding hypoxia, not only its effect under the basal conditions should be studied, but also in combination with fuel (glucose) stimulation to examine both the basal function of any cell (to live and survive) and the ability to perform the main function of a beta cell (insulin secretion in response to glucose).

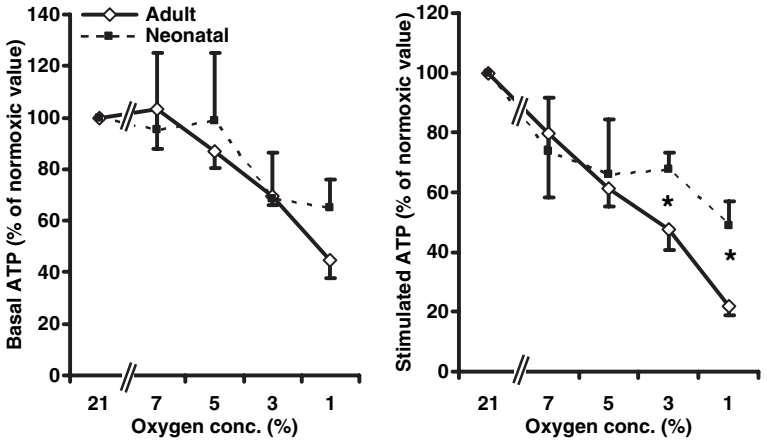

Figure 4 Basal and glucose-stimulated ATP level in neonatal and adult rat islet cells cultured for 24 h in different hypoxic levels. All values were equalized by dividing by the DNA content of the same samples. To facilitate comparisons, data are related to the control value at normal air, which is considered as 100%. Data are presented as mean ± SE of $n = 4$ islet isolations. Statistical analyses: *means that the difference between neonatal and adult islet cell values is significant.

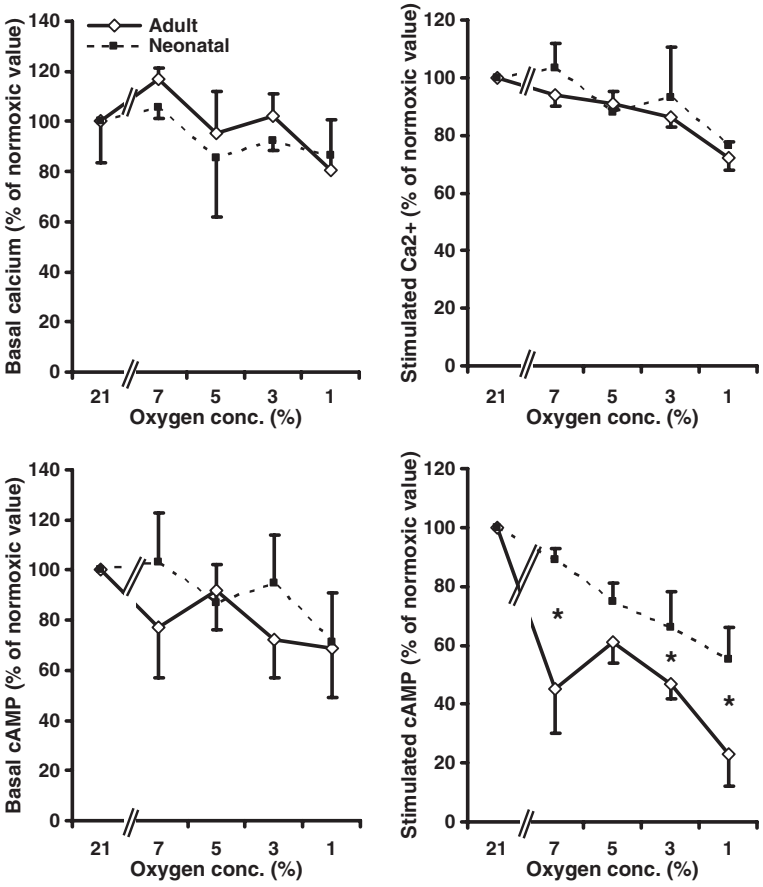

Figure 5 Second messengers: effect of culture for 24 h in different hypoxic levels on the basal and glucose-stimulated neonatal and adult rat islet cellular content of calcium and cAMP. All values were equalized by dividing by the DNA content of the same samples. To facilitate comparisons, data are related to the control value at normal air, which is considered as 100%. Data are presented as mean ± SE of $n = 4$ islet isolations in all cases. Statistical analyses: *the difference between neonatal and adult islet cell values is significant.

In this study, the islet cells were cultured in different tensions of oxygen. The process of oxidative phosphorylation is completely oxygen-dependent. Alteration of oxygen level distorts this process and consequently the cellular energy-state level and the production of ATP [25]. The action of ATP is exerted at several levels, leading to control of the activity of ATP-sensitive K^+-channel and thereby the electrical and secretory activities.

In fact, the results show that the effect of hypoxia on insulin secretion (Fig. 1) was more obvious than on the other parameters. Insulin secretion was strongly dependent on oxygen supply and showed a continuous reduction on lowering of the latter. Our results, which related to the reduction of insulin secretion under hypoxia, are consistent with that of several previous studies. Hypoxia is known to reduce insulin secretion and produce a central necrosis in islet tissue [25–28]. Hypoxia decreased gradually the magnitude of both basal and stimulated secretion. The gradual fashion of changes in the different metabolites that were studied was a characteristic feature in response to the gradual lowering of oxygen. The alterations in oxygen tensions are expected to manipulate the energy-state level of the cell [25], which control the whole cell functions, including insulin secretion.

The reduction in glucose-stimulated insulin secretion magnitude was more remarkable under any hypoxic level than the reduction in basal secretion. This difference may indicate that oxygen supply plays its major role during stimulation with glucose, when the metabolism in islet cells are more active, and that the effect of hypoxia was more obvious, when higher levels of oxygen supply was needed. This may indicate also a gradual dependency of glucose metabolic activity on oxygen. Dionne *et al.* [27] reported that basal secretion rate of perifused rat islets was not different at pO_2 of 25 mmHg (3.3% O_2) from that at normoxic pO_2, and that there was a slight reduction at 10 mmHg (1.2% O_2), while the maximum effect was on glucose-stimulated secretion. In this study, the effect of hypoxia was clearer on the basal secretion, notwithstanding the fact that the stimulated secretion was higher in all cases and under all hypoxic levels than the basal level. This may be attributable to the long-term culture under hypoxic conditions, which represents another design of this study in contrast to the short perifusion system.

Islet cells of the two different ages behaved variably in response to hypoxia. Neonatal rat islet cells could perform their function under hypoxic conditions even at 1%

O_2, while adult rat cells stopped their function at 3% O_2, where glucose could not stimulate insulin secretion any more over the basal value. This study shows that adult cells are more sensitive to hypoxia than the neonatal form. Numerous metabolic, ionic and synthetic changes accompany glucose-induced insulin secretion and may be part of the stimulus-secretion coupling mechanism or modulate the same. One or more factors may behave differently in islets of different ages, leading to the variable responses. However, such expected factor(s) should be related to insulin secretion, oxygen supply and glucose metabolism. The results of this study show that at low glucose, most studied metabolites were essentially independent of oxygen tension. In consistence with our results, Ohta *et al.* [25] reported no change in adenine nucleotides in nonstimulated islets under hypoxia. The response to hypoxia of the other metabolites mentioned above has not been measured in islet cells in earlier studies.

The functional reduction in response to hypoxia was metabolically expected, as insulin secretion is a result of glucose metabolism that depends in several of its steps on oxygen supply. In addition, a logical increase in intracellular lactate level during hypoxia was also expected, and could already be determined in this study. The lactate elevation may expose the islet cells to acidic conditions. The relationship between hypoxia and acidity in the different tissues is well known [12]. Acidity was reported to markedly reduce glucose-stimulated insulin secretion. Thus, hypoxia may exert its effect on insulin secretion through not only its negative effects on the glucose metabolism but also through other indirect effects like the acidic toxicity.

Glucose-stimulated calcium level was found in our study to decrease in neonatal cells similarly or more than that in adult cells, although ATP production was higher than the same in adult cells. A decrease of $[Ca^{2+}]_i$ and impairment of its inflow in neonatal islets were reported before [29,30]. The normal ATP level in neonatal cells suggests that the lower levels of calcium are not attributable to an impairment of glucose metabolism as hypothesized previously [31–33], but caused by insensitivity of KATP channels in neonatal phase as reported by Rorsman *et al.* [34], or insensitivity of Ca^{2+} channels as suggested by Boschero *et al.* [29], or a combination of both. We have, however, used islet cells of relatively old neonatal rats (7- and 8-day-old neonatal rats). In such cells, glucose metabolism and insulin secretion are nearly as in adults. Fortunately, Some experiments on adult islets [35–37] suggest that a rise in $[Ca^{2+}]_i$ does not necessarily trigger secretion, also that secretion can occur independently of cytosolic free calcium level, and that there is a calcium-independent mechanism of exocytosis. Although high Ca^{2+} level is essential for insulin secretion, oscillations in Ca^{2+} are not necessarily needed for oscillations in insulin secretion [38]. This may clarify the normal insulin secretion from neonatal cells in our study.

The effect of hypoxia on increasing lactate level was similar in neonatal islet cells to that of adult islet cells. But pyruvate was higher in neonatal than in adult cells. These results may indicate that LDH is synthesized and active in neonatal islet cells, and that anaerobic glycolysis is similar in neonatal and adult islet cells and increase in response to hypoxia, consistent with a previous study [33] that reported a higher total and anaerobic rates of glycolysis in neonatal than in adult islets at normal conditions. On the other hand, pyruvate was higher in neonatal cells than in adults in response to hypoxia. This indicates that in adult cells, the anaerobic glycolysis substitutes gradually the aerobic one. In other words, hypoxia induces an increase in lactate production, which is accompanied by a decrease in oxidative metabolism. In neonatal cells, interestingly, elevated lactate level was accompanied by a parallel elevation of pyruvate. This may mean that both aerobic and anaerobic glycolysis are active at the same time in neonatal cells, on the one hand to produce a required maximal level of energy for synthetic and proliferative functions, and on the other hand to overcome the low availability of oxygen.

This may represent an adaptation of neonatal islet cells to tolerate hypoxia, which is also reflected by the observed insulin secretion ability of neonatal islet cells at more hypoxic levels than the adult cells. Several studies were aimed at investigating the adaptation of neonatal tissues to hypoxia. For example, Plunkett *et al.* [39] reported some metabolic adaptations to chronic hypoxia in neonatal myocardium and Kolesnik *et al.* [40] reported that intermittent exposure to hypoxia stimulates both insulin synthesis and secretion by beta cells and activates *de novo* formation of beta cells in the acinar tissue. In this study, adaptation of neonatal cells to hypoxia was clear in Fig. 2, in which basal insulin release was decreased within the first day of exposure to hypoxia, and restored to normoxic values after chronic hypoxia for 4 days.

In conclusion, the present results indicate that neonatal rat islet cells are more hypoxia-tolerant and adaptable than adult islet cells, and that an underlying reason of that may be found in the active glycolysis that was detected in neonatal cells.

Authorship

AH: performed research, analysed data, and wrote paper. CL: contributed reagents. JS: designed research and performed proof reading.

Funding

This work was supported by the foundation 'Stiftung Inseltransplantation'.

References

1. Bonner-Weir S. Morphological evidence for pancreatic polarity of beta-cell within islets of Langerhans. *Diabetes* 1988; **37**: 616.
2. Nyman LR, Wells KS, Head WS, *et al.* Real-time, multidimensional in vivo imaging used to investigate blood flow in mouse pancreatic islets. *J Clin Invest* 2008; **118**: 3790.
3. Jansson L, Hellerström C. Stimulation by glucose of the blood flow to the pancreatic islets of the rat. *Diabetologia* 1983; **25**: 45.
4. Jansson L. Glucose stimulation of pancreatic islet blood flow by redistribution of the blood flow within the whole pancreatic gland. *Pancreas* 1988; **3**: 409.
5. Pileggi A, Ricordi C, Alessiani M, Inverardi L. Factors influencing islet of Langerhans graft function and monitoring. *Clin Chim Acta* 2001; **310**: 3.
6. Pileggi A, Ribeiro MM, Hogan AR, *et al.* Impact of pancreatic cold preservation on rat islet recovery and function. *Transplantation* 2009; **87**: 1442.
7. Schrezenmeir J, Gerö L, Laue C, *et al.* The role of oxygen supply in islet transplantation. *Transplant Proc* 1992; **24**: 2925.
8. Schrezenmeir J, Kirchgessner J, Gerö L, *et al.* Effect of microencapsulation on oxygen distribution in islets organs. *Transplantation* 1994; **57**: 1308.
9. Hyder A, Laue C, Schrezenmeir J. Variable responses of islet cells of different ages and species to hypoxia. *Transplant Proc* 1998; **30**: 578.
10. Emamaullee JA, Shapiro AM, Rajotte RV, *et al.* Neonatal porcine islets exhibit natural resistance to hypoxia-induced apoptosis. *Transplantation* 2006; **82**: 945.
11. Mandel T, Koulmanda M. Effect of culture conditions on fetal mouse pancreas in vitro and after transplantation in syngeneic and allogeneic recipients. *Diabetes* 1985; **34**: 1082.
12. Hyder A, Laue C, Schrezenmeir J. Effect of extracellular pH on insulin secretion and glucose metabolism in neonatal and adult rat pancreatic islets. *Acta Diabetol* 2001; **38**: 171.
13. Collins MP, Salton MR. Solubility characteristics of Micrococcus lysodeikticus membrane components in detergents and chaotropic salts analysed by immunoelectrophoresis. *Biochim Biophys Acta* 1979; **553**: 40.
14. Malaisse W, Hutton J, Kawazu S, Sener A. The stimulus-secretion coupling of glucose-induced insulin release. Metabolic effects of menadione in isolated islets. *Eur J Biochem* 1978; **87**: 121.
15. Azzi A, Chance B. The ''energized state'' of mitochondria: lifetime and ATP equevalence. *Biochem Biophys Acta* 1969; **189**: 141.
16. Blinks J, Wier W, Hess P, Prendergast F. Measurement of Ca2+ concentration in living cells. *Prog Biophys Mol Biol* 1982; **40**: 1.
17. Olsen C. An enzymatic fluorometric micromethod for the determination of acetoacetate, b-hydroxybutyrate, pyruvate and lactate. *Clin Chim Acta* 1971; **33**: 293.
18. Sener A, Malaisse W. Measurements of lactic acid in nanomolar amounts: reliability of such a method as an index of glycolysis in pancreatic islets. *Biochem Med.* 1976; **15**: 34.
19. Lowry O, Passonneau J, Sculz D, Rock M. The measurement of pyridine nucleotides by enzymatic cycling. *J Biol Chem* 1961; **236**: 2746.
20. Krishan A. Rapid DNA content analysis by the propidium iodide-hypoxic citrate method. *Methods Cell Biol* 1990; **33**: 121.
21. Newgard C, McGarry J. Metabolic coupling factors in pancreatic beta-cells signal transduction. *Annu Rev Biochem* 1995; **64**: 689.
22. Matschinsky FM. Banting lecture 1995: a lesson in metabolic regulation by the glucokinase glucose sensor paradigm. *Diabetes* 1996; **45**: 223.
23. Prentki M. New insights into pancreatic beta-cell metabolic signaling in insulin secretion. *Eur J Endocrinol* 1996; **134**: 272.
24. Prentki M, Tornheim K, Korkey B. Signal transduction mechanisms in nutrient-induced insulin secretion. *Diabetologia* 1997; **40**: S32.
25. Ohta M, Nelson D, Nelson J, *et al.* Oxygen and temperature dependence of stimulated insulin secretion in isolated rat islets of Langerhans. *J Biol Chem* 1990; **265**: 17525.
26. Dionne K, Colton C, Yarmush L. A microperifusion system with environmental control for studying insulin secretion by pancreatic tissue. *Biotechnol Prog* 1991; **7**: 359.
27. Dionne K, Colton C, Yarmush L. Effect of hypoxia on insulin secretion by isolated rat and canine islets of Langerhans. *Diabetes* 1993; **42**: 12.
28. Kühtreiber W, Lanza R, Beyer A, *et al.* Relationship between insulin secretion and oxygen tension in hybrid diffusion chambers. *ASAIO J* 1993; **39**: M247.
29. Boschero A, Bordin S, Plasman P, Herchuelz A. Effects of glucose and K+ depolarisation on cytosolic free Ca2+ in cultured neonatal islets. *Horm Metab Res* 1992; **24**: 51.
30. Weinhaus A, poronnik P, Cook D, Tuch B. Insulin secretogogues, but not glucose, stimulate an increase in [Ca2+]i in the fetal rat b-cell. *Diabetes* 1995; **44**: 118.
31. Freinkel N, Lewis N, Johnson R, *et al.* Differential effects of age versus glycemic stimulation of the maturation of insulin stimulus-secretion coupling during culture of fetal rat islets. *Diabetes* 1984; **33**: 1028. 38.

32. Hole R, Pian-smith N, Sharp G. Development of the biphasic response to glucose in fetal and neonatal rat pancreas. *Am J Physiol* 1988; **254**: E167.
33. Boschero A, Bordin S, Sener A, Malaisse W. D-glucose and L-leucine metabolism in neonatal and adult cultured rat pancreatic islets. *Mol Cell Endocrinol* 1990; **73**: 63.
34. Rorsman P, Arkhammar P, Bokvist K, *et al.* Failure of glucose to elicit a normal secretory response in fetal pancreatic beta cells results from glucose insensitivity of the ATP-regulated K+ channels. *PNAS* 1989; **86**: 4505.
35. Knight D, Von Grafenstein H, Athayde C. Calcium-dependent and calcium-independent exocytosis. *Trends Neurosci* 1989; **12**: 451.
36. Gembal M, Detimary P, Gilon P, *et al.* Mechanism by which glucose can control insulin release independently from its action on adenosine triphosphate-sensitive K^+ channels in mouse b -cells. *J Clin Invest* 1993; **91**: 871.
37. Aizawa T, Sato Y, Ishihara F, *et al.* ATP-sensitive K^+ channel-independent glucose action in rat pancreatic b-cell. *Am J Physiol* 1994; **266**: C622.
38. Cunningham B, Deeney J, Bliss C, *et al.* Glucose-induced oscillatory insulin secretion in perifused rat pancreatic islets and clonal b-cells (HIT). *Am J Physiol* 1996; **271**: E702.
39. Plunkett M, Hendry P, Anstadt M, *et al.* Chronic hypoxia induces adaptive metabolic changes in neonatal myocardium. *J Thorac Cardiovasc Surg* 1996; **112**: 8.
40. Kolesnik YM, Seredenko MM, Abramov AV. Adaptation to hypoxia stimulates the pancreatic. Islet apparatus in intact and diabetic rats. *Bull Exp Biol Med* 1995; **120**: 1183.