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Hyperoxia inhibits glucose-induced insulin secretion and mitochondrial metabolism in rat pancreatic islets

Zuheng Ma [a], Noah Moruzzi [a], Sergiu-Bogdan Catrina [a], Valdemar Grill [a,b,c,1], Anneli Björklund [a,*,1]

[a] Dept. of Molecular Medicine and Surgery, Karolinska Institutet, 171 76 Stockholm, Sweden
[b] Institute of Cancer Research and Molecular Medicine, The Medical Faculty, Norwegian University of Science and Technology, 7006 Trondheim, Norway
[c] Dept. of Endocrinology, St. Olav University Hospital, 7006 Trondheim, Norway

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ABSTRACT

Isolated pancreatic islets containing the insulin-producing beta cells are devoid of circulation. They may therefore experience hypoxia with possible negative effects on beta cell function and survival. We investigated (1) whether hyperoxia in vitro would be beneficial by counteracting putative effects of lost circulation and, further, (2) whether previous hyperoxia would attenuate the impact of subsequently induced severe hypoxia. Islets from Sprague–Dawley rats were exposed to 95% O_2 for 18 h. This hyperoxic exposure diminished glucose-induced insulin secretion by 47% and inhibited oxygen consumption by 39–41%. Mitochondrial complexes I–III were decreased by 29–37%. Negative effects on insulin secretion and complexes III and IV waned after a 22 h period of normoxia following hyperoxia whereas complexes I and II were still diminished, ROS production was increased and rates of apoptosis tended to be increased ($P = 0.07$). The effects of previous hyperoxia on susceptibility to damage by subsequent hypoxia were tested after 5.5 h of 0.8% O_2. Previous hyperoxia did not affect hypoxia-induced enhancement of HIF-1 alpha but modestly and significantly attenuated hypoxia-induced decreases in insulin contents. We conclude that hyperoxia exerts largely negative effects on beta cells, effects which are functional and possibly also toxic. A paradoxical positive finding (attenuation of hypoxia-induced effects) could be secondary to a protective effect of the hyperoxia-induced reduction of oxidative metabolism.

1. Introduction

Islets of Langerhans, containing the insulin-producing beta cells are markedly sensitive to hypoxia [1]. Sensitivity is due, in part at least, to a high degree of metabolism. Indeed stimulation of metabolism by elevated glucose (to provide energy for insulin secretion and synthesis) is accompanied by modest increases in parameters of hypoxia [2]. Isolated islets lack functioning vasculature, something which may exacerbate intra-islet hypoxia in vitro as well as in vivo after transplantation. Hypoxic condtions are bound to be present with negative effects since an artificial increase in oxygenation improves transplantation results [3–5].

A hyperoxic environment could be thought to counter any negative effects of intra-islet hypoxia in vitro and in vivo. However, it is known from other tissues that hyperoxia can exert negative effects and that the balance between negative effects of hypoxia and hyperoxia can be precarious [6]. The influence of hyperoxia on isolated islets was investigated to some extent in a previous publication which demonstrated reversal of markers of intra-islet hypoxia but no positive or negative effects on insulin release by overnight exposure to hyperoxia [2]. Here we extend investigations into effects of hyperoxia with regard to effects of hyperoxia per se as well as a possibly modulating influence on damaging effects of severe hypoxia.

2. Materials and methods

2.1. Materials

All chemicals were from Sigma, St Louis MO.

2.2. Methods

2.2.1. Animals

Male Sprague–Dawley rats were from Scanbur (Sollentuna, Sweden). Animals were maintained in a 12-h (600–1800 h) light/dark cycle with free access to water and a standard diet. The rats weighed 250–350 g at the time of experiments. Ethical Guidelines of the Karolinska Institutet for the care and use of laboratory animals were followed.

* Corresponding author. Address: Dept. of Molecular Medicine and Surgery, Karolinska Institutet, Karolinska University Hospital M1:03, 171 76 Stockholm, Sweden. Fax: +46 8 5177 3658.
E-mail address: anneli.bjorklund@ki.se (A. Björklund).
[1] Contributed equally.

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2.2.2. Isolation, culture, and incubation of rat pancreatic islets

Islets of Langerhans were isolated by collagenase digestion (Roche Diagnostics) followed by sedimentation. Islets were selected under a stereomicroscope. Care was taken to select only medium-sized islets. Islets were transferred to 5 ml Petri dishes (Sterilin, Teddington, U.K.) containing RPMI-1640, 2 mmol/l glutamine, 10% (vol/vol) FCS, 100 units/ml benzylpenicillin, 0.1 mg/ml streptomycin, and 11 mmol/l glucose. Islets were cultured free-floating overnight either during continued normoxia in an atmosphere of 5% CO_2 in air or during hyperoxia (=hyperoxia period). Hyperoxia was achieved in a closed chamber (Billup-Rothenberg, Calif) that was equilibrated with a gas mixture of 95% oxygen and 5% CO_2. Hyperoxia-exposed and control islets were tested for various parameters either in sequence to the period of hyperoxia or after an overnight period of normoxia that succeded the hyperoxia period. A subset of experiments tested effects of hypoxia following hyperoxia (for study design see Fig. 1).

2.2.3. Immunoreactive insulin

Immunoreactive insulin (IRI) was measured by RIA [7]. Cross-reactivity with human proinsulin was about 80%. Isolated islets and transplants were extracted for IRI in acid–ethanol [8].

2.2.4. Western blotting

The procedure is described in [9]. For mitochondrial proteins we refrained from boiling the samples. Samples containing 30 (mitochondrial complexes I, II and IV) and 10 (complexes III and V, and beta actin) μg protein were prepared. Primary antibodies were used at the following dilutions: 1:2000 for HIF-1α (Novus Biologicals, Inc. USA), 1:20,000 for beta-actin (Sigma) and 1:500 for oxidative phosphorylation complexes 1–5 MS604 (MitoSciences, Eugene, OR, USA).

2.2.5. Oxygen consumption rate (OCR)

This was measured employing Sea-Horse technology basically as described [10]. Seventy equally sized islets were used for each well.

2.2.6. Total DNA and protein

Total DNA was measured by a fluorescent DNA Quantitation Kit (Bio-Rad) and protein content by the DC Protein Assay Kit (Bio-Rad).

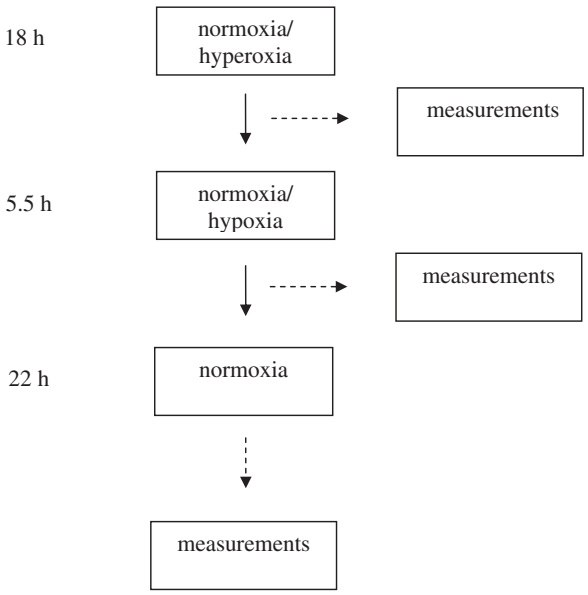

Fig. 1. Study design.

2.2.7. Necrosis and apoptosis

The Cell Death Detection ELISAPLUS kit (Roche Diagnostics) was used. Samples were placed into streptavidin-coated microplates and incubated with a mixture of anti-histone-biotin and anti-DNA-peroxidase. Islet culture media were analyzed for necrosis and islet lysates for apoptosis.

2.2.8. ROS-production

Batches of 30 islets were washed in cold PBS and then incubated in 200 μl culture medium containing 10 μM 2′7′-dichlorofluorescin diacetate (DCFH-DA, Sigma). Upon crossing the cell membrane this compound undergoes de-acetylation by intracellular esterases, producing the non-fluorescent compound, DCFH, which is trapped inside the cells. Oxidation by ROS produces the fluorescent compound 2′7′-dichlorofluorescein (DCF).Islets were cultured with DCFH at 37 °C for 40 min. Fluorescence was detected at 485/535 nm excitation/emission using a microplate analyzer (Victor2 1420, Perkin-Elmer). Values were calculated after subtracting background fluorescence.

2.2.9. Statistical analysis

Results are expressed as mean ± SEM. Significant differences were tested using Student's paired *t* test (two-sided) or, for multiple comparisons, one way ANOVA. The Student–Newman–Keuls method was used for post hoc analysis. A P value <0.05 was considered significant.

3. Results

3.1. Hyperoxia acutely inhibits insulin secretion

Overnight exposure to hyperoxia inhibited glucose-induced insulin secretion by 47 ± 4% when measured in sequence to the period of hyperoxia (Fig. 2A). Insulin contents of islets were not significantly affected (Fig. 2B). Hyperoxia when present reduced the accumulation of insulin into culture media (Fig. 2C). The negative effect on glucose-induced secretion waned with time of continued culture at normoxia and was not apparent after 22 h of normoxia, separate experiments, $n = 12$. Glucose-induced insulin release was 90 ± 6% of that of islets which had experienced continuous normoxia, Fig. 4A).

3.2. Hyperoxia does not acutely affect islet DNA

Islet contents of DNA were not affected when measured in sequence to the hyperoxic event (10.0 ± 0.7 for hyperoxia vs. 10.3 ± 0.5 ng/islet for normoxia, $n = 5$).

3.3. Hyperoxia inhibits oxidative phosphorylation

Oxygen consumption during continuously normoxic conditions was enhanced by elevated glucose (16.7 mmol/L), inhibited by oligomycin and further enhanced by the addition of FCCP to test media (Fig. 2D). Hyperoxia decreased oxygen consumption when measured in sequence to the hyperoxic period (Fig. 2D and E). Consumption was decreased both during conditions of "basal" (3.3 mmol/L glucose) as well as during stimulation with elevated glucose. Oxygen consumption in hyperoxia-treated islets was lower than in controls also after the addition of oligomycin (which blocks ATP synthase activity). Differential effects of oligomycin and/or FCCP were otherwise difficult to discern and interpret due to the absence of a "plateau" of oxygen consumption after the administration of oligomycin.

The molecular basis for the inhibitory effects of hyperoxia was further explored by Western blotting of mitochondrial complexes

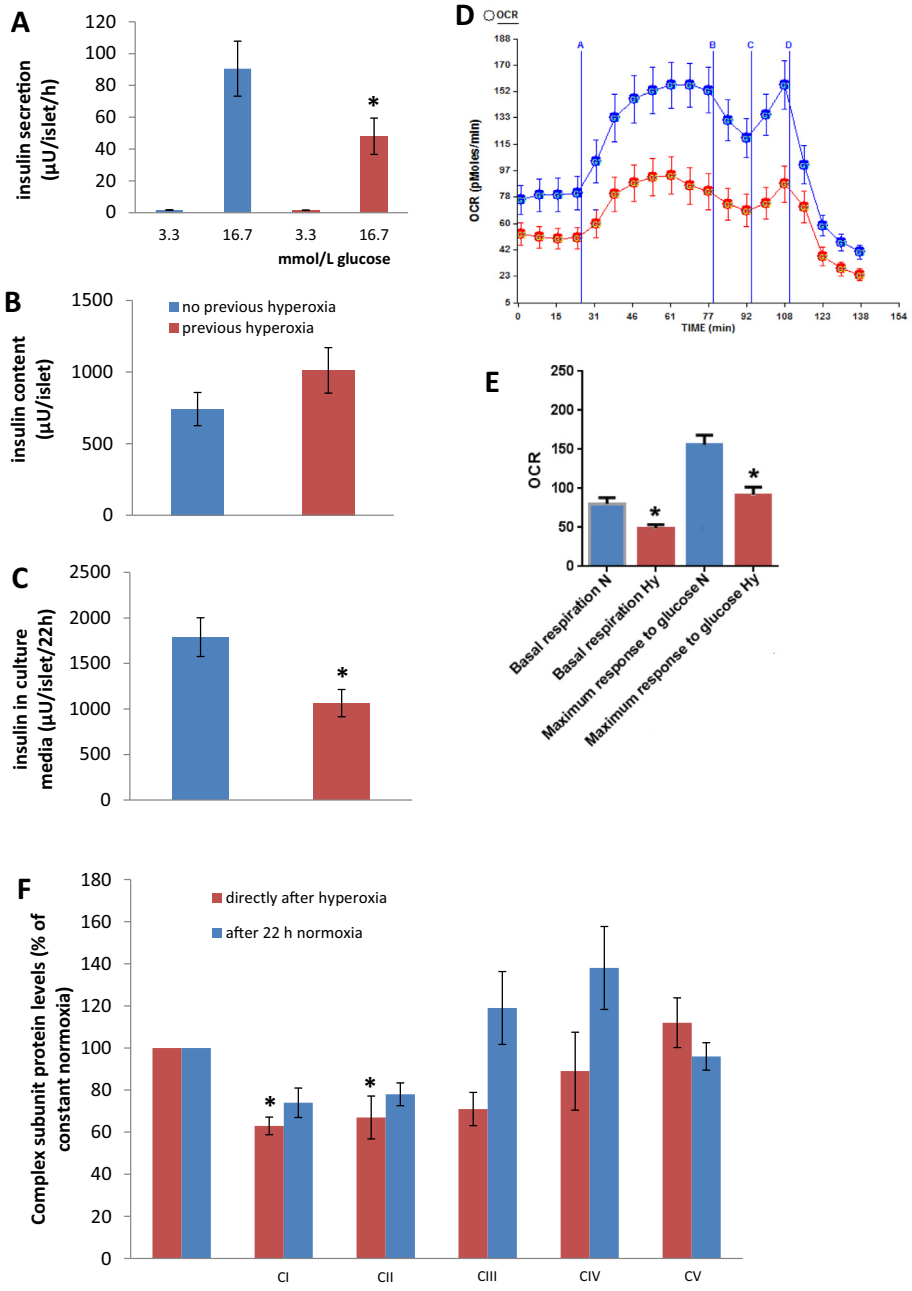

Fig. 2. Effects of hyperoxia on insulin secretion (A), islet insulin contents (B), culture medium (C) and oxygen consumption rate (OCR) (D and E) measured directly after hyperoxia. Islet insulin secretion was measured after 0.5 h preincubation at 3.3 mmol/L glucose followed by final incubations for 1 h at 3.3 and 16.7 mmol/L glucose, $n = 4$, $^{*}P < 0.02$ vs. no previous hyperoxia. Oxygen consumption was measured by a "Sea Horse" instrument. In (D), mean and SEM are shown from islets of four rats which were – separately for each rat – placed in parallel wells and processed in the same experiment. A in figure (D) indicates change from 3.3 to 16.7 mmol/L glucose, B addition of oligomycin, C addition of FCCP and finally D addition of antimycin. (E) Presents a summary of basal and glucose-stimulated oxygen consumption from ten experiments, measured at basal (3.3 mmol/l) and glucose stimulated (16.7 mmol/l) respiration for hyperoxia and normoxia-treated islets, $^{*}P < 0.05$ vs. no previous hyperoxia. (F) Shows Western blotting of mitochondrial subunit complexes I–V measured immediately after hyperoxia and after 22 h continued culture in normoxia, $n = 5$. $^{*}P < 0.05$ for effect of hyperoxia.

I–V. Hyperoxia significantly reduced complexes I and II, tended to reduce complex III but did not affect complex IV and V (Fig. 2F). Restitution of complex III and enhancement of complex IV was seen 22 h after return to normoxia; however complexes I and II were not completely normalized.

3.4. Previous hyperoxia affects ROS production

Islet ROS production as estimated by the accumulation of DCF was measured directly after hyperoxia as well as 22 h after the return to normoxia. Accumulation of DCF was decreased directly after hyperoxia by 42 ± 3% ($P < 0.001$). In contrast the accumulation was increased by 160 ± 23% ($P < 0.03$) when measured 22 h after the return of normoxia, $n = 8$.

3.5. Previous hyperoxia tends to increase rate of apoptosis

Measurements of apoptosis and necrosis sequential to hyperoxia did not reveal any perturbations (results not shown). Following 22 h of culture at normoxia the rate of apoptosis tended to be increased (by 48 ± 24%, $P = 0.07$, $n = 4$). No effect was apparent on the parameter of necrosis (results not shown).

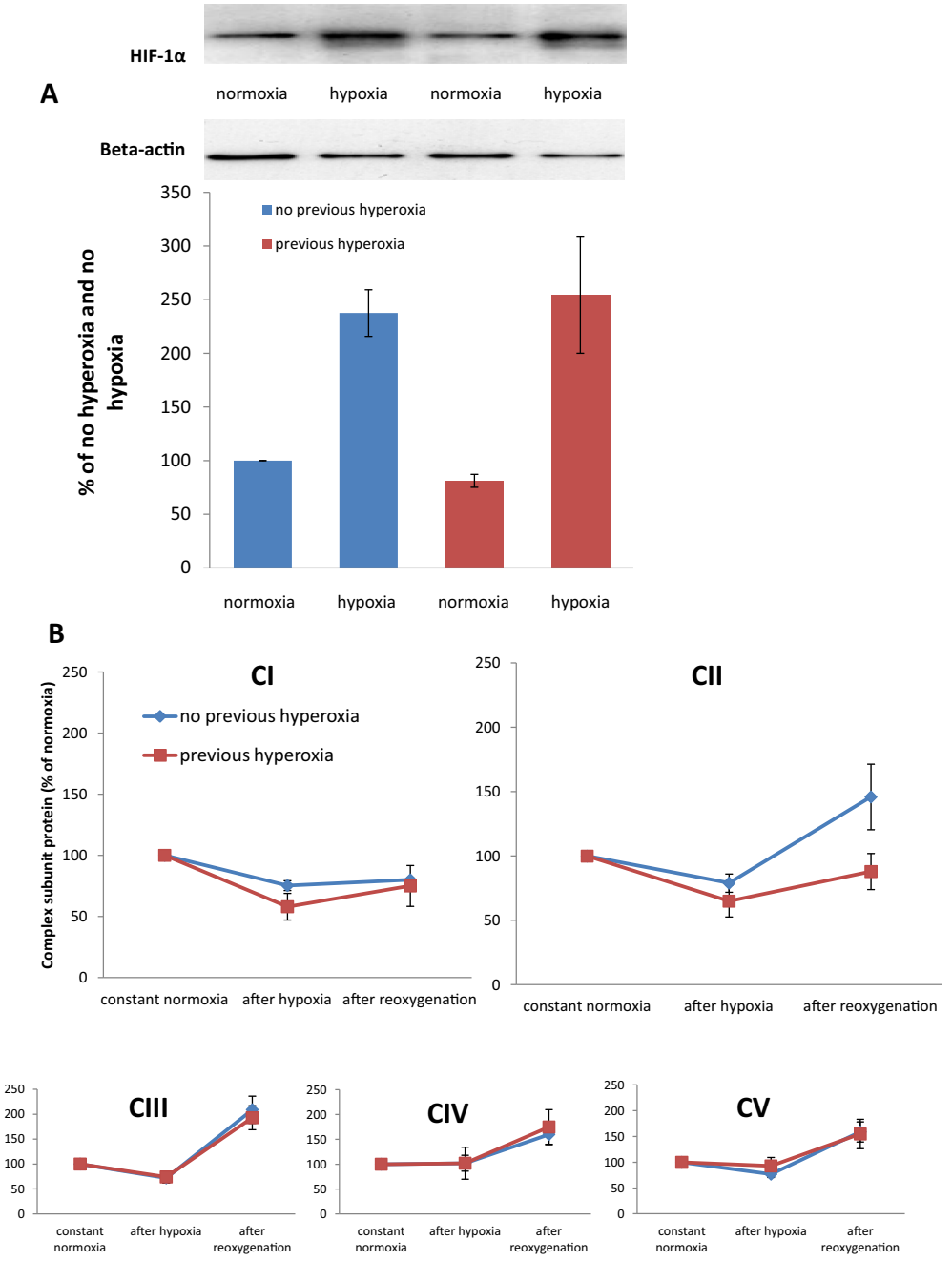

Fig. 3. Effects of hypoxia with or without previous hyperoxia on HIF-1-alpha (A) and mitochondrial subunit complexes I–V (B). In (A) measurements were performed directly after 5.5 h of hypoxia, $n = 4$. In (B) measurements were performed directly after hypoxia and after 22 h further culture at normoxia (re-oxygenation), $n = 5$.

3.6. Previous hyperoxia does not modify a hypoxia-induced increase in HIF-1-alpha or decreases of mitochondrial complexes I–V but attenuates effects on cellular insulin contents

A previous oxygen environment could be thought to influence susceptibility to damage by hypoxia. It was therefore of interest to test for such influences of hyperoxia in our experimental system.

The transcription factor HIF-1 alpha is known to play a key role in activating cellular defense mechanisms against hypoxia [11]. Levels of HIF-1 alpha tended to be reduced by previous hyperoxia when measured after normoxia ($P = 0.076$, paired t-test). Following hypoxia the levels of HIF-1 alpha were – predictably – markedly elevated. However, this augmenting effect of hypoxia was similar in islets previously exposed to hyperoxia or not (Fig. 3A).

Hypoxia per se leads to decline of mitochondrial complexes I–V [12]. Previous hyperoxia did not affect this decline (Fig. 3B). The partial normalization that takes place following 22 h of re-oxygenation [12] was not significantly affected by hyperoxia preceeding the hypoxic event (Fig. 3B).

In agreement with reported findings [12], previous hypoxia reduced glucose-induced insulin secretion and cellular contents considerably. Previous hyperoxia failed to restitute insulin secretion but attenuated the reduction in islet insulin contents (Fig. 4).

4. Discussion

Our study demonstrates that 18 h of hyperoxia inhibits glucose-induced insulin secretion when measured subsequent to the period

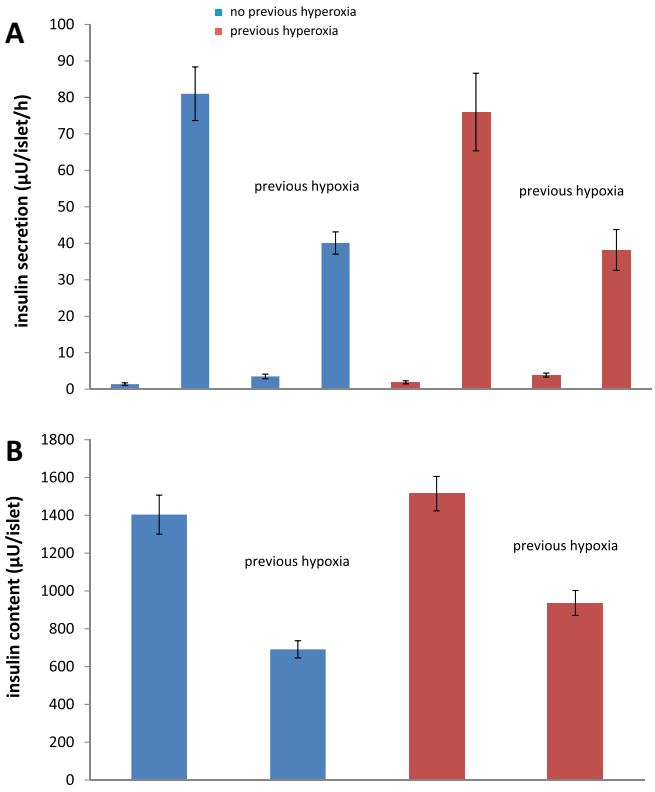

Fig. 4. Effects of previous hyperoxia on hypoxia-induced reduction of insulin secretion (A) and islet insulin contents (B). Measurements were done after 5.5 h of hypoxia followed by 22 h at normoxia (re-oxygenation), $n = 12$, $^{*}P < 0.01$ vs. previous hypoxia only (i.e. no previous hyperoxia).

of hyperoxia. We also find that accumulation of insulin was reduced in the medium in which islets were cultured during hyperoxia. The latter finding makes it likely (1) that the reduction of insulin secretion is caused by hyperoxia (rather than being an after-effect due to the subsequent switch to normoxia), (2) that shorter times of exposure to hyperoxia than 18 h would also exert inhibitory effects.

A previous study demonstrated toxic effects after long term culture in serum free media [2]. Our data are not incompatible with long term negative effects by hyperoxia on viability. Importantly our results indicate that shorter exposures to hyperoxia exert largely functional and reversible effects.

Glucose-induced insulin secretion is dependent on oxidative metabolism [13]. It is thus likely that the hyperoxia-induced reduction of insulin secretion is linked to the inhibition of oxidative phosphorylation that we document here by oximetry and by measurement of mitochondrial complexes. The question remains which molecular events that are responsible for the inhibition. Such events should be linked to cellular sensing of oxygen pressure for which more is known on the sensing of hypoxia than of hyperoxia.

Our results indicate that he inhibitory effects on insulin secretion and metabolism measured sequentially to hyperoxia are largely functional. Islet contents of DNA and insulin contents were thus not affected at the time of oximetry. Also the negative effect on insulin secretion was reversible as were some of the negative effects on mitochondrial complexes. However, after continued culture at normoxia mitochondrial complexes I and II were not normalized and there was a tendency for increased apoptosis all in all implying a toxic effect of hyperoxia.

Hyperoxia can enhance the formation of ROS from mitochondrial metabolism [14] hence inhibition of metabolism could possibly be a beneficial adaptation. Such inhibition could possibly explain the finding that DCF, a probe for ROS production, was actually reduced after hyperoxia. On the other hand we find enhanced ROS production 22 h after return to normoxia. The molecular mechanisms behind this late onset enhancement of ROS production has not been elucidated. It may indicate a pathological situation which could endanger viability.

Hypoxia after transplantation is a main hindrance to the success of islet or isolated beta cell transplantations to cure patients with type 1 diabetes. To find ways to increase cellular defense against hypoxia already before transplantation is thus clinically relevant. A previous period with hypoxia (ischemic preconditioning) has been shown to increase the resilience of tissues such as heart and nervous tissue against new exposures to hypoxia; however such an approach in islets gave negative results [15], implying that correction of mild hypoxia would be a better way to prepare islets for transplantation. This proposition was tested here. The results indicate a modest beneficial effect on islet insulin contents and no effect on insulin secretion. At first glance the possibly protective effect on insulin contents seems paradoxical, given the evidence for the functional deficits induced by hyperoxia that have been discussed above. However, evidence indicates that reduced oxidative metabolism per se attenuates hypoxia-induced cellular damage [12]. Obviously, one must seek other ways than by hyperoxia to exploit reduced metabolism for protection against hypoxia-induced damage.

Are our results clinically relevant in other aspects? The situation in premature newborns comes to mind. A recent study demonstrated optimal survival using 95% oxygen delivery in markedly premature babies – but at the expense of oxygen-induced microangiopathy [16]. In light of our findings one cannot exclude a negative impact by hyperoxia also on beta cells during prematurity. Obviously this notion can only be tested later in life.

Acknowledgment

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