Decreased β-Cell Proliferation and Vascular Density in a Subpopulation of Low-Oxygenated Male Rat Islets

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Low-oxygenated and dormant islets with a capacity to become activated when needed may play a crucial role in the complex machinery behind glucose homeostasis. We hypothesized that low-oxygenated islets, when not functionally challenged, do not rapidly cycle between activation and inactivation but are a stable population that remain low-oxygenated. As this was confirmed, we aimed to characterize these islets with regard to cell composition, vascular density, and endocrine cell proliferation. The 2-nitroimidazole low-oxygenation marker pimonidazole was administered as a single or repeated dose to Wistar Furth rats. The stability of oxygen status of islets was evaluated by immunohistochemistry as the number of islets with incorporated pimonidazole adducts after one or repeated pimonidazole injections. Adjacent sections were evaluated for islet cell composition, vascular density, and endocrine cell proliferation. Single and repeated pimonidazole injections over an 8-hour period yielded accumulation of pimonidazole adducts in the same islets. An average of 30% of all islets was in all cases positively stained for pimonidazole adducts. These islets showed a similar endocrine cell composition as other islets but had lower vascular density and β-cell proliferation. In conclusion, low-oxygenated islets were found to be a stable subpopulation of islets for at least 8 hours. Although they have previously been observed to be less functionally active, their islet cell composition was similar to that of other islets. Consistent with their lower oxygenation, they had fewer blood vessels than other islets. Notably, β-cell regeneration preferentially occurred in better-oxygenated islets.

Freeform/Key Words: islet vasculature, pancreatic islets, heterogeneity, β-cell proliferation

Islets receive ~10% of the pancreatic blood flow despite their small volume of 1% to 2% of the total pancreatic mass [1]. Islet blood flow is affected by many factors, including activity in the vagus nerve, sympathetic nerves, and endothelium-derived vasoactive substances, as well as metabolic factors released from islets [2]. The major factor changing blood flow is plasma glucose levels, which modulate blood flow according to the need of insulin release by stimulating vagal nerve activity and by glucose metabolism in the islets [2, 3]. β cells and vascular endothelial cells are closely connected, facilitating close intercellular communication wherein both cell types secrete various substances maintaining phenotype as well as stimulating the function of the other [4–6]. Importantly, β cells secrete vascular endothelial growth factor (VEGF) and insulin, which are important for the growth and maintenance of the endothelial cells, whereas endothelial cells secrete hepatocyte growth factor and laminins, which stimulate β-cell proliferation and function [7–10].

Abbreviations: BS-1, *Bandeiraea simplicifolia* agglutinin-1; HPI, pimonidazole hydrochloride; IGF1R, insulin-like growth factor-1 receptor; TBS, Tris-buffered saline; VEGF, vascular endothelial growth factor.

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There is increasing evidence of both β-cell and islet functional heterogeneity, with implied importance for the ability of the endocrine tissue to fine-tune hormone release and thereby obtain optimal glucose homeostasis [11]. We and others [12, 13] have identified high-functioning subpopulations of islets with intrinsically (or after a high-fat diet challenge) greater β-cell proliferation and insulin-release response to glucose. Interestingly, both the islet blood flow and the islet vasculature are widely heterogeneous and coupled to such differences in islet functionality [13, 14]. In addition to the high-functioning islets, low-functioning islets have also been identified, and recently, an insulin-like growth factor 1-receptor (IGF1R)–expressing islet subpopulation has been described as a potentially old and dysfunctional population of islets [15]. Moreover, dormant or resting islets, which may become activated when the metabolic demand is increased, have been described [16]. These islets constitute 25% to 30% of all pancreatic islets and can be identified by the low-oxygen-tension marker pimonidazole, which stains islet cells that have an oxygen tension <10 mm Hg [16]. Interestingly, when the metabolic demand per β cell is decreased by a whole pancreas transplant, the number of low-oxygenated islets is increased. On the other hand, when the metabolic demand per β cell instead is increased by a partial pancreatectomy, almost no pimonidazole-positive islets subsist. Pimonidazole-positive islets were also found to have a decreased blood flow and leucine-dependent protein biosynthesis, which includes (pro)insulin biosynthesis [16].

In this study, we aimed to further characterize the subpopulation of low-oxygenated islets to investigate whether these islets are a rapidly changeable subpopulation or are more stable. As the latter was confirmed, we aimed to describe these islets with regard to islet cell composition, vascular density, and α- and β-cell proliferation.

1. Research Design and Methods

A. Experimental Animals

Adult male Wistar Furth rats (Harlan Laboratories, Indianapolis, IN) weighing 280 ± 2 g (mean ± SEM) were used. The regional ethics committee of Uppsala University approved all experiments.

B. Pimonidazole Administration

To identify low-oxygenated islets, pimonidazole hydrochloride (HPI; Hypoxyprobe, Burlington, MA), 60 mg/kg body weight, was injected intravenously into the tail vein of each animal. The animals had free access to standard chow and water throughout the study, and the first injection was administered at 8 AM in all groups to avoid differences in metabolic challenge. To evaluate whether islets cycle between a low-oxygenation state and conditions with better oxygenation, HPI was injected twice in some animals. The second injection was administered 2 or 6 hours after the first injection. Two hours after the last injection, the animals were anesthetized with thiobutabarbital (120 mg/kg body weight; Sigma-Aldrich, St. Louis, MO) and the pancreas was removed and sent for histological analysis. To certify that pimonidazole adducts were stable throughout the experiment, the pancreas was removed 4 or 8 hours after a single injection of HPI in separate control animals. In two animals, stability for 24 hours was also investigated.

C. Induction of Hypoxia In Vivo *in Pancreas*

Wistar Furth rats were anesthetized with thiobutabarbital, 120 mg/kg body weight and placed on a heating plate to maintain body core temperature at 38°C. A polyethylene catheter was inserted into the trachea to secure free airways, and another was inserted into the ascending aorta through the carotid artery to enable monitoring of blood pressure throughout the experiment. A graded aortic vascular clamp was positioned superior to the celiac artery and adjusted to allow an arterial blood pressure of 20 to 30 mm Hg, resulting in an intra-islet

oxygen tension of 5 to 10 mm Hg [16]. When the blood pressure was stabilized, HPI, 60 mg/kg body weight, was injected intravenously into the tail vein and the animal was monitored for 2 hours. Afterward, the animals were euthanized and each pancreas was removed for histological analysis. The tissue was used as positive control for low-oxygenated islets in immunohistochemistry.

D. Tissue Preparation for Histology

Directly after removal, the pancreases were fixed in 4% (v/v) buffered formalin (VWR BHD Prolabo, Fontenay-sous-Bois, France) and thereafter embedded in paraffin. The tissues were consecutively divided into 5-μm-thick sections to enable multiple stainings on adjacent sections.

E. Immunohistochemistry Staining for Pimonidazole

Pancreatic sections from animals treated with one or HPI injections, and positive control sections from aortic vascular clamp animals, were stained for pimonidazole adducts. After deparaffinization, sections were rehydrated and antigen retrieval was performed by incubation in 0.01% (wt/v) pronase (Roche Diagnostics, Mannheim, Germany) for 40 minutes at 40°C. The sections were thereafter incubated with blocking solution (protein block, serum-free; Dako, Glostrup, Denmark) for 30 minutes at room temperature. Primary antibody, antipimonidazole mouse IgG1 monoclonal antibody (MAb1, HPI) [17], diluted 1:60 in Tris-buffered saline (TBS) with 0.2% (wt/v) Brij-35 (ThermoFisher Scientific, Carlsbad, CA) was incubated overnight at 40°C. Washing steps were performed in TBS plus 0.02% Brij-35. The biotinylated secondary goat $F(ab)_2$ antimouse antibody [18] (Southern Biotech, Birmingham, AL), diluted 1:500 in TBS + 0.02% Brij 35, was incubated for 30 minutes at room temperature. Antibody detection was performed by incubation with Vectastain Elite ABC AP 500-Kit (Vector Laboratories, Inc., Burlingame, CA) for 30 minutes, followed by incubation with Vulcane Fast Red Chromogen kit 2 (Biocare Medical, Concord, CA) for 10 to 15 minutes. Before mounting, the sections were counterstained with hematoxylin for 30 seconds.

F. Immunohistochemistry Staining for Vascular Density

Sections, consecutively adjacent to HPI stained sections from animals given one injection of HPI, were analyzed for vascular density. The sections were treated with peroxidase blocking solution (Dako) for 5 minutes at room temperature. Thereafter, the sections were incubated with blocking solution (Background Sniper, Biocare Medical, Concord, CA) before being incubated with primary antibody anti-insulin [19] (1:400, polyclonal guinea pig anti-insulin; Fitzgerald, Acton, MA) and biotinylated lectin from *Bandeiraea simplicifolia* agglutinin-1 (BS-1, 1:100; Sigma-Aldrich) overnight at 4°C. BS-1 lectin was detected by incubation with 4plus Streptavidin AP Label (Biocare Medical) for 10 minutes and incubation with Vulcan Fast Red (Biocare Medical) for 10 minutes. Insulin was detected by secondary peroxidase-conjugated anti–guinea pig antibody [20] (1:1000; Jackson ImmunoResearch, West Grove, PA) incubation for 30 minutes at room temperature, followed by incubation with 3,3′-diaminobenzidine for 60 seconds. All sections were counterstained with hematoxylin for 30 seconds before mounting. The sections were scanned and analyzed in a laser microdissection microscope (Leica LMD6000; Leica Microsystems, Wetzlar, Germany) in a blinded manner. Islets with positive pimonidazole staining were size-matched with islets negative for pimonidazole staining, and vascular density was defined as the area positive for BS-1 per insulin-positive area.

G. Immunofluorescence Staining for Islet Cell Composition and Proliferation

Stainings of insulin, glucagon, and the proliferation marker Ki-67 were analyzed on sections also adjacent to HPI stained sections from animals given one HPI injection. Heat-induced

antigen retrieval was performed in Diva Decloaker solution (Biocare Medical) by a pressure cooker (2100 Retriever; Prestige Medical, Blackburn, United Kingdom). Thereafter, the sections were incubated with blocking solution (protein block, serum-free, Dako) for 15 minutes and incubated with primary goat anti-insulin antibodies [21] (Insulin A, dilution 1:100; Santa Cruz Biotechnology, Dallas, TX), mouse antiglucagon [22] (dilution 1:1000; Abcam, Cambridge, United Kingdom) and rabbit anti-Ki67 [23] (SP6, dilution 1:300; Abcam) at 4°C overnight. Secondary anti-goat antibodies [24] (AF 555, dilution 1:1000; ThermoFisher Scientific, anti-mouse antibodies [25] (AF 647, dilution 1:1000; ThermoFisher Scientific), and anti-rabbit antibodies [26] (AF 488, dilution 1:1000; ThermoFisher Scientific) at room temperature for 1 hour. Images were acquired by a confocal laser scanner microscope (LSM780; Zeiss, Oberkochen, Germany) and analyzed by Pancreas Image Detection software (Stardots, Uppsala,

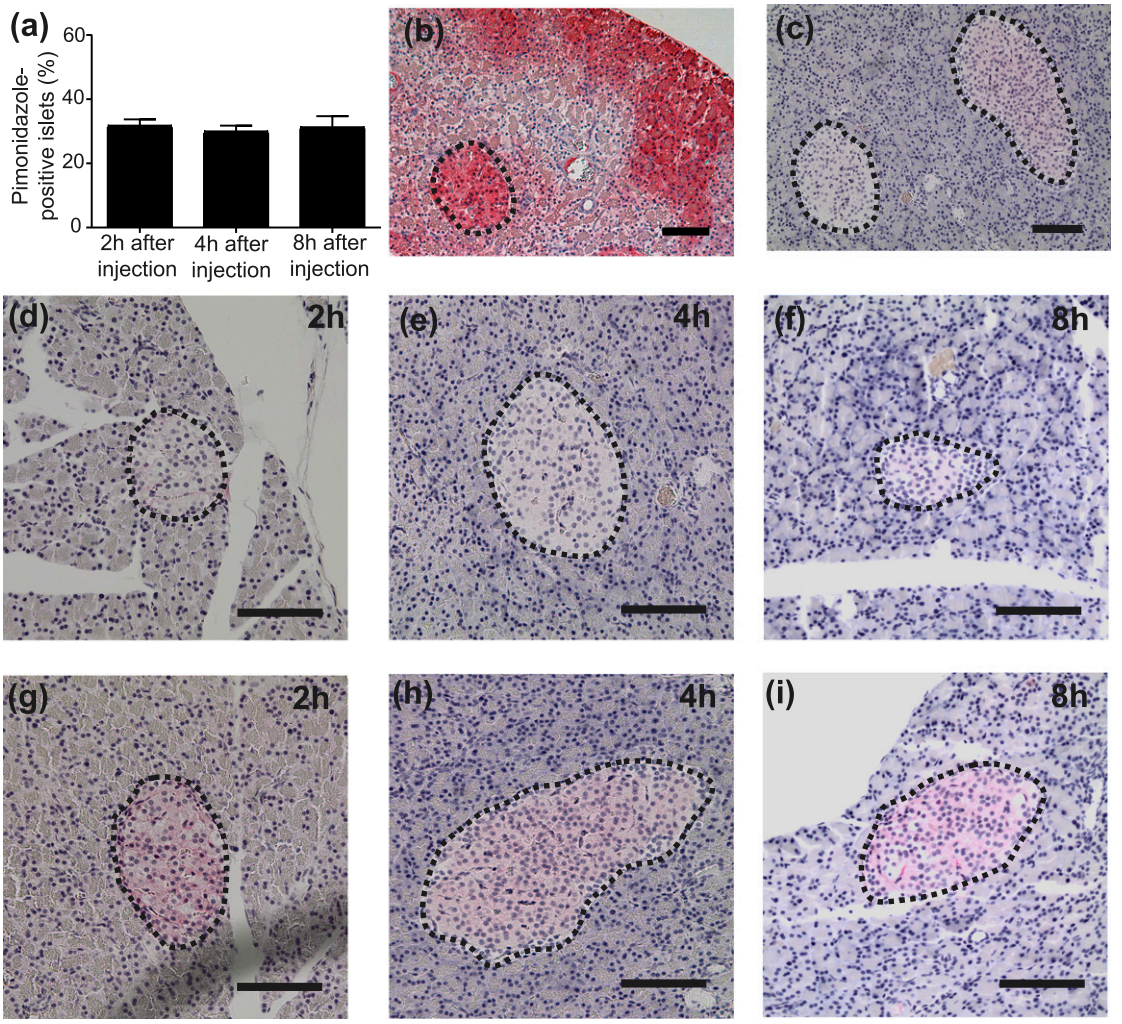

Figure 1. Pimonidazole adduct staining was stable at least up to 8 h after injection. (a) Pimonidazole adducts were found in ~30% of the pancreatic islets and identified in pancreases at least up to 8 h after administration. (b–i) Representative images of pimonidazole-stained (b) positive control pancreas; (c) pancreas with two adjacent islets with positive and negative staining, respectively; negative islets (d) 2 h, (e) 4 h, and (f) 8 h after pimonidazole administration; associated islets from the same pancreatic section positive for pimonidazole adducts (g) 2 h, (h) 4 h, and (i) 8 h after administration. Pimonidazole adducts in red; scale bar = 100 μm; dashed lines encircle islets. Values are given as mean ± SEM; n = 5 or 6 animals. Kruskal-Wallis test with Dunn multiple comparison *post hoc* test was used to calculate *P* values.

Sweden) in a blinded matter. Cell composition was evaluated as the fractions of α and β cells of all islet cells and the proliferation as the fraction of Ki-67–positive α and β cells.

H. Statistics

All values are given as means ± SEM. Differences between two groups of nonparametric data were analyzed by a paired Wilcoxon matched-pairs signed-rank test; differences between more than two groups of nonparametric data were analyzed by a Kruskal-Wallis test with a Dunn multiple comparison *post hoc* test. P values < 0.05 were considered to indicate statistically significant differences. Statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software, San Diego, CA).

2. Results

A. No Evidence of Variability in Low-Oxygenated Pimonidazole-Positive Islet Subpopulation

Pimonidazole adducts were identified in ~30% of the islets 2 hours after injection of HPI (Fig. 1). These adducts were also identified in islets 4 and 8 hours after injection (Fig. 1) but were faint or unidentifiable 24 hours after injection (data not shown). There was no difference in

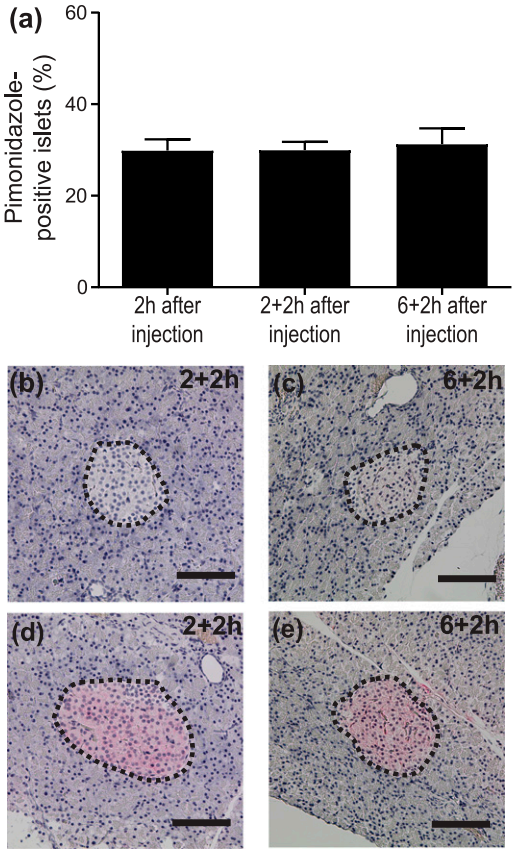

Figure 2. Similar percentage of pimonidazole-positive islets after single or repeated injections. (a) A similar percentage of pimonidazole-positive islets was detected after single or repeated (2 + 2 h, 6 + 2 h) injection of HPI. (b–e) Representative images of pimonidazole-stained pancreases with (b and c) negative islet at 2 + 2 and 2 + 6 h after injection, respectively. (d and e) Positive islet at 2 + 2 and 2 + 6 h after injection, respectively. Pimonidazole adducts in red; scale bar = 100 μm; dashed lines encircle islets. Values are given as mean ± SEM; n = 5 or 6 animals. Kruskal-Wallis test with Dunn multiple comparison *post hoc* test was used to calculate *P* values.

the percentage of islets that stained positive for pimonidazole adducts at 4 or 8 hours after injection when compared with 2 hours after injection (n = 5 or 6 animals) (Fig. 1a). In comparing the percentage of pimonidazole-positive islets after one and two injections of HPI, no difference could be detected between the groups (Fig. 2).

B. Vascular Density Decreased in Pimonidazole-Positive Islets

The mean vascular density was 6.5% ± 0.9% in the pancreatic islets of investigated animals (n = 6 animals). Islets that stained positive for pimonidazole had ~30% lower mean vascular density than size-matched islets negative for pimonidazole staining (n = 6 animals) (Fig. 3).

C. Similar Endocrine Cell Composition in Pimonidazole-Positive and -Negative Islets

The percentages of β cells and α cells were ~76% and ~17% of total islet cells, respectively, in islets that stained positive and negative for pimonidazole adducts (n = 6 animals) (Fig. 4a and 4b).

D. Lower β-Cell Proliferation in Pimonidazole-Positive Islets

Analysis of nuclear Ki-67 positivity in islets revealed that ~3.2% of the β cells and ~1.6% of the α cells in pimonidazole-negative islets stained for the proliferation marker. In islets that stained positive for pimonidazole adducts, there were ~44% fewer Ki-67–positive β cells (n = 6 animals) (Fig. 4c). However, no difference in the frequency of Ki-67–stained nuclei in α cells between islets that stained positive or negative for pimonidazole adducts was observed (Fig. 4d). Representative images of stainings are shown in Fig. 4e and 4f.

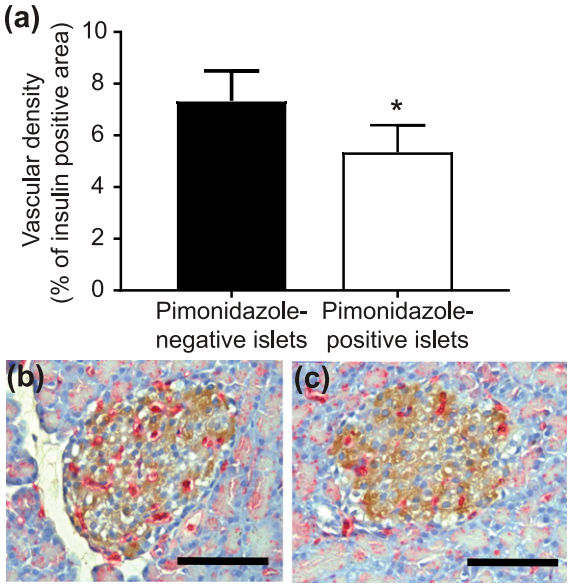

Figure 3. Vascular density in islets positive and negative for pimonidazole adducts. (a) The vascular density was decreased by ~30% in pimonidazole-positive islets. (b and c) Representative images of the vasculature in (b) pimonidazole-negative and (c) pimonidazole-positive islets. Insulin in brown and endothelial cells (BS-1) in red; scale bar = 50 μm. Values are given as mean ± SEM; n = 6 animals. *$P < 0.05$. Paired Wilcoxon matched-pairs signed-rank test was used to calculate P values.

3. Discussion

Previously, a population of low-oxygenated islets had been identified as a reserve pool of low-blood-perfused islets that can be functionally activated when needed [16]. However, on the basis of this study it was not clear whether the islets at rest are the same or whether all islets cycle through activation and inactivation. Because the half-life of pimonidazole in plasma is only 30 minutes [27], we designed an experiment in which the low-oxygenation marker pimonidazole was injected singly or repeatedly during an 8-hour period. Control experiments showed that pimonidazole adducts that accumulated in low-oxygenated cells were stable for at least 8 hours.

Two different outcomes were possible: (i) that the fraction of islets that accumulated pimonidazole adducts increased after a second pimonidazole injection, which would indicate cyclicity of islets being low oxygenated or (ii) that the number of low-oxygenated islets did not increase after repeated pimonidazole injection. The latter was the result in the current study. This finding supports the argument that the low-oxygenated resting islets normally are a

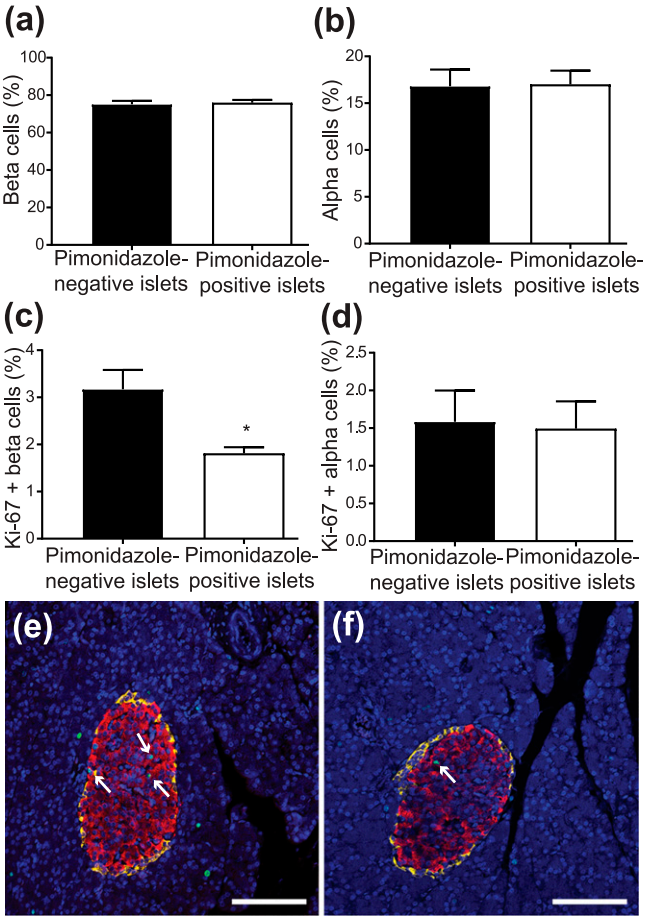

Figure 4. Islet cell composition and α-cell and β-cell proliferation in islets positive and negative for pimonidazole adducts. (a) Pimonidazole-positive and -negative islets were composed of similar amounts of β cells and (b) α cells. (c) The proliferation of βcells was decreased by 40% in pimonidazole-positive islets whereas no difference in proliferation was detected in (d) α cells. (e and f) Representative images of islet cell composition and proliferation in (e) pimonidazole-positive and (f) pimonidazole-negative islets. Insulin in red, glucagon in yellow, Ki-67 in green, and nuclei in blue. Arrows indicate proliferative islet cells; scale bar = 100 μm. Values are given as mean ± SEM; n = 6 animals. *$P < 0.05$. Paired Wilcoxon matched-pairs signed-rank test was used to calculate P values.

stable population, although the stability of pimonidazole adducts in islet cells restricted the study to an 8-hour period.

This finding of stability of the low-oxygenated pool of islets opened the possibility to further characterize these islets. The islets had similar fractions of α and β cells as other islets, but a lower vascular density of the insulin-producing endocrine tissue was identified. This shows that their low blood perfusion was reflecting not merely different regulation of blood perfusion at the arteriolar level (islet blood flow may be increased 10-fold [2]) but also fewer vascular structures. No technique is available to isolate tissue from the pimonidazole-positive islets, which limits the possibilities to study the cause of their reduced vascular density. However, we have previously observed that the most functional of islets in the pancreas are highly blood perfused and also have a higher vascular density and *VEGF* gene expression than other islets [13]. Because the vascularization of islets has also previously been shown to critically depend on VEGF secretion of β cells [9, 28], decreased *VEGF* expression in pimonidazole-positive islets could possible explain their decreased vascular density.

Islet endothelial cells support the function, differentiation, and proliferation of pancreatic β cells by paracrine factors, such as laminins and hepatocyte growth factor [7, 8, 10, 13]. The lower β-cell proliferation in the low-vascularized, low-oxygenated islets may reflect a decrease in such signals or merely reflect less metabolic activity. In fact, because the population of low-oxygenated islets has been observed to have 50% lower leucin-dependent protein biosynthesis [16], it can be expected that their glucose metabolism is also lowered (*i.e.,* the main positive regulator of basal β-cell proliferation) [29]. Islet hypoxia induced by VEGF ablation resulting in hypovascularization, on the other hand, does not affect the proliferation of β cells during mouse pregnancy [30]. No difference in α-cell proliferation could be detected in this study; however, it is difficult to determine whether this results from similar rates of proliferation between the groups of islets or whether a decrease could not be detected because of the low baseline Ki-67 expression in this cell type.

In summary, our data show that the pool of low-oxygenated islets is not interchangeable but consists of the same islets over at least 8 hours. This is also supported by the fact that they are distinguished by anatomical differences to other islets with lower vascular density. An interesting observation was their lower β-cell proliferation, which would over time favor the expansion of better-oxygenated functional β cells.

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Disclosure Summary: The authors have nothing to disclose.

Data Availability: The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author by reasonable request.

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