Noninvasive Monitoring of Glycemia-Induced Regulation of GLP-1R Expression in Murine and Human Islets of Langerhans

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TECHNOLOGICAL ADVANCES

Glucagon-like peptide 1 receptor (GLP-1R) imaging with radiolabeled exendin has proven to be a powerful tool to quantify β-cell mass (BCM) in vivo. As GLP-1R expression is thought to be influenced by glycemic control, we examined the effect of blood glucose (BG) levels on GLP-1R–mediated exendin uptake in both murine and human islets and its implications for BCM quantification. Periods of hyperglycemia significantly reduced exendin uptake in murine and human islets, which was paralleled by a reduction in GLP-1R expression. Detailed mapping of the tracer uptake and insulin and GLP-1R expression conclusively demonstrated that the observed reduction in tracer uptake directly correlates to GLP-1R expression levels. Importantly, the linear correlation between tracer uptake and β-cell area was maintained in spite of the reduced GLP-1R expression levels. Subsequent normalization of BG levels restored absolute tracer uptake and GLP-1R expression in β-cells and the observed loss in islet volume was halted. This manuscript emphasizes the potency of nuclear imaging techniques to monitor receptor regulation noninvasively. Our findings have significant implications for clinical practice, indicating that BG levels should be near-normalized for at least 3 weeks prior to GLP-1R agonist treatment or quantitative radiolabeled exendin imaging for BCM analysis.

As a decline in functional β-cell mass (BCM) contributes to diabetes development, current drug development is geared toward compounds that can prevent β-cell death or increase BCM (1). Autopsy studies in patients with both type 1 (2,3) and type 2 (4) diabetes have demonstrated residual BCM, even decades after disease onset, supporting the notion that β-cell–enhancing strategies can be promising in humans suffering from diabetes. While numerous studies report on bioactive molecules that can induce β-cell proliferation or protection in animals (1,5), translation of these compounds to humans is hampered by the inability to measure BCM. As biopsying the human pancreas is associated with an unacceptable complication rate (6), a biomarker that reliably reflects BCM would be imperative to demonstrate the efficacy of candidate compounds in humans (7). Furthermore, such a biomarker would aid decision making early in the drug development process and would allow for patient stratification to identify patients with residual BCM who would be eligible for therapy.

We have previously developed a noninvasive imaging technology to quantify BCM based on the radiolabeled glucagon-like peptide 1 (GLP-1) analog exendin (8). Preclinical studies demonstrated that pancreatic uptake of exendin linearly correlates with BCM (9) and that both off-target specific and nonspecific binding are negligible in nonhuman primate pancreata (10). The first clinical study revealed marked differences in pancreatic uptake between patients with type 1 diabetes and controls (8). In line with the autopsy studies (2,3), residual exendin uptake was

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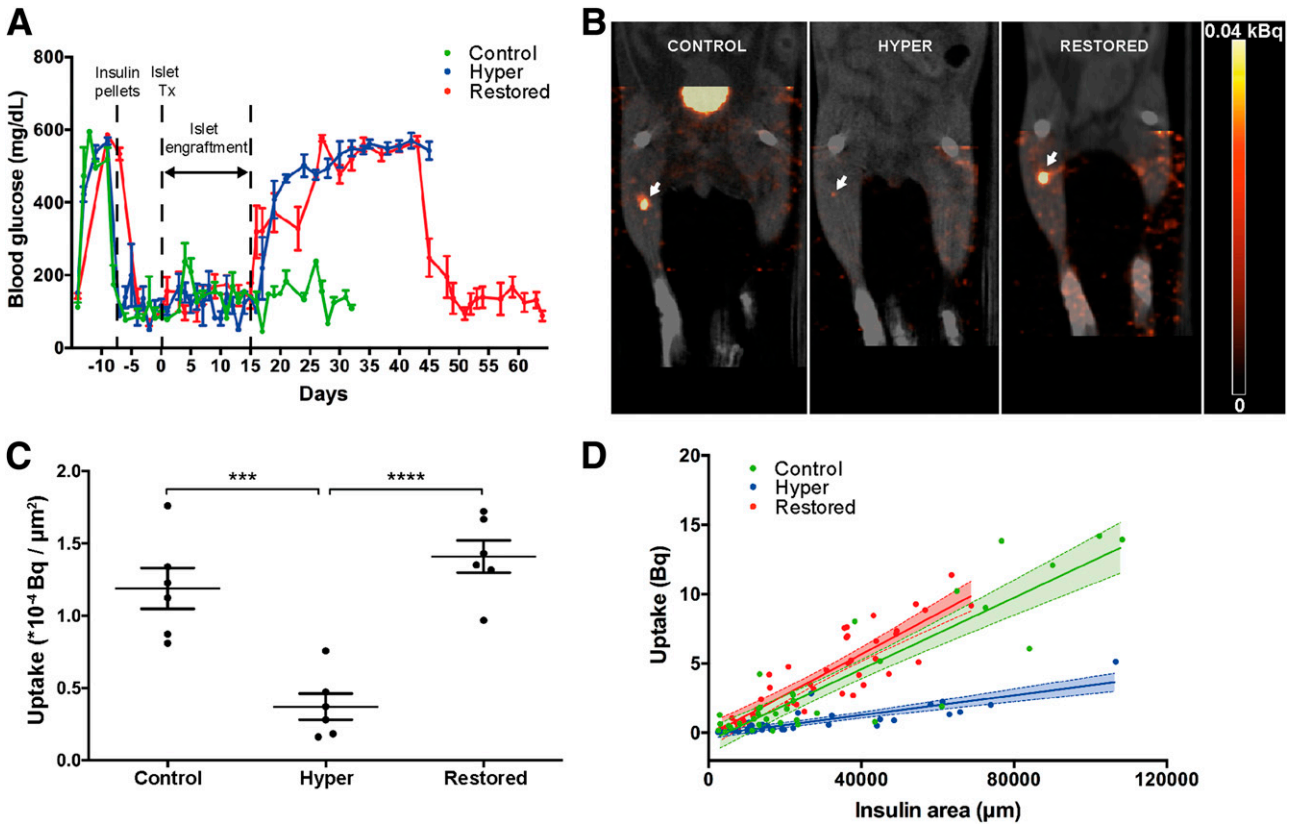

Figure 1—GLP-1R–mediated exendin uptake is significantly reduced in murine islets after sustained hyperglycemia, which is restored upon normalization of glycemic levels. *A*: BG levels of murine syngeneic islet transplants. After hyperglycemia was confirmed, mice received subcutaneous insulin implants to normalize BG levels prior to islet transplantation. Two weeks after transplantation, mice were randomly assigned to one out of three experimental groups (control, hyper, and restored). The BG levels of the control group were maintained within physiological range with insulin pellets (BG <200 mg/dL). Insulin pellets were removed from mice assigned to the hyper or restored group to induce severe hyperglycemia (BG >350 mg/dL). After 4 weeks of hyperglycemia, mice assigned to the restored group received insulin pellets again for a period of 3 weeks. *B*: ^{111}In-labeled exendin-3 SPECT/CT scans of control, hyper, and restored mice. *C*: GLP-1R–mediated exendin-3 uptake in islets in the control, hyper, and restored groups. Data are presented as mean ± SEM. ***P <0.001, ****P <0.0001, evaluated by ANOVA with Tuckey post hoc test with n = 6 per group. *D*: Correlation between exendin-3 uptake and β-cell area in the control, hyper, and restored groups. The slope of the correlation curve was significantly decreased in the hyper group compared with the control group (P = 0.013). Normalization of BG levels completely restored the slope of the correlation curve (P = 0.51).

found in two out of five patients, illustrating the potential of this imaging strategy, which is currently considered the most advanced approach to noninvasively quantify BCM (7).

Nevertheless, there are indications that hyperglycemia downregulates GLP-1 receptor (GLP-1R) expression (11,12) and signaling (12) in β-cells in vitro, possibly affecting GLP-1R–based BCM quantification in individuals with diabetes. Here, we examined the effect of chronic hyperglycemia and subsequent normalization of blood glucose (BG) levels on GLP-1R–mediated exendin uptake in both murine and human islets and its implications for BCM quantification. For this, we used an islet transplantation setup in a chemically induced diabetic mouse model as this allows control over islet-cell mass and glycemic stress exposure.

RESEARCH DESIGN AND METHODS

Animal Models and Human Islets

Animal experiments were approved by the Animal Welfare Committee of the Radboud University (the Netherlands) or the German Center for Diabetes Research (Germany) and carried out in accordance with the local and national guidelines. Female C3H/HeNCrl (Charles River, Sant'Angelo Lodigiano, Italy) and male NOD.CB17-*Prkdc*^{scid}/J (NOD-Scid) mice (The Jackson Laboratory) were used for syngeneic and allogeneic islet transplantations, respectively. Human islets were obtained from a 37-year-old female organ donor without diabetes (BMI 19.57 kg/m^2, HbA_{1c} 4.6%, 90% viability and purity; tebu-bio, Le Perray-en-Yvelines, France).

Radiolabeling

Mice were injected with 0.1 µg of ^{111}In-labeled [Lys^{40}(DTPA)] exendin-3 (Peptide Specialty Laboratories, Heidelberg, Germany) (±15 MBq), as previously described (13).

Syngeneic Islet Transplantation and Imaging

For the induction of hyperglycemia, C3H/HeNCrl mice were injected i.v. with 100 mg/kg Alloxan (Sigma). BG levels were measured three times a week. After hyperglycemia was confirmed, mice received subcutaneous insulin implants

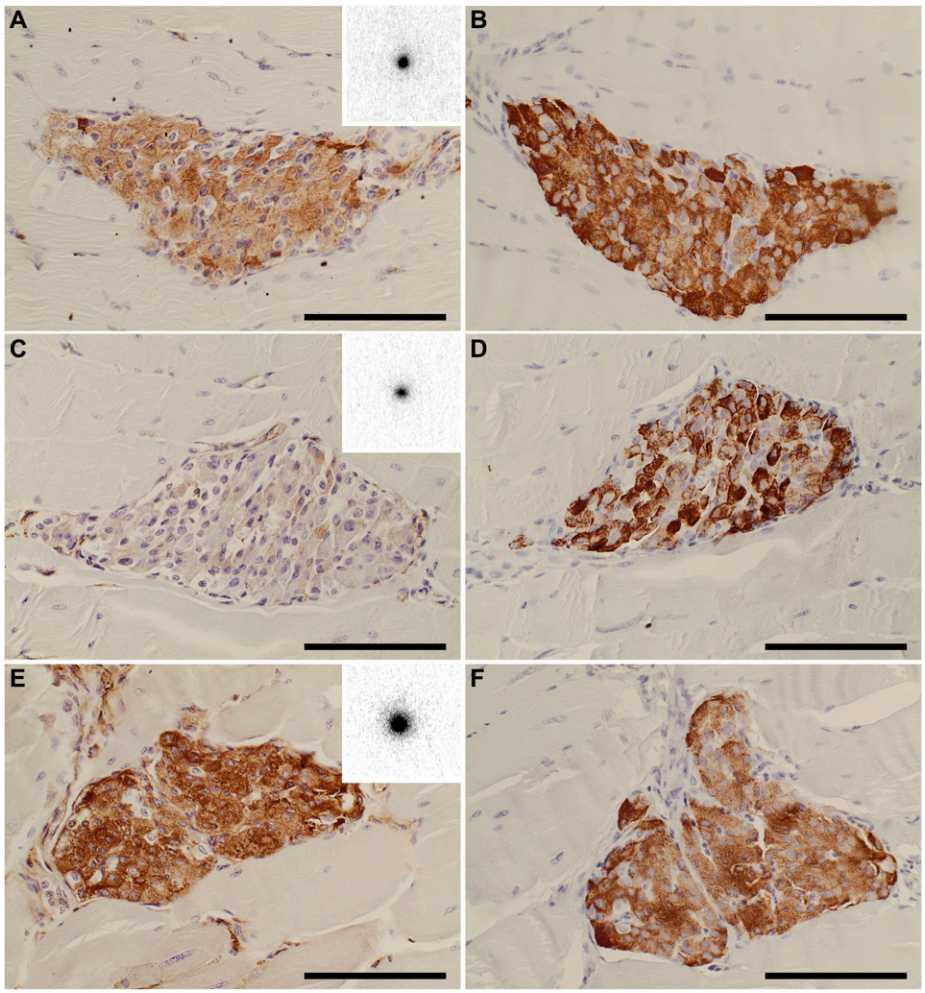

Figure 2—Anti-insulin and GLP-1R staining of islet grafts in control, hyper, and restored mice. *A–F*: Immunohistochemistry images of islet grafts stained for GLP-1R of control (*A*), hyper (*C*), and restored (*E*) mice and for insulin of control (*B*), hyper (*D*), and restored (*F*) mice. *A*, *C*, and *E*: Inserts depict autoradiography images of grafts with comparable insulin areas. Scale bars = 100 μm.

(LinShin, Scarborough, Canada) to normalize BG levels (14). Syngeneic islets were isolated from 8- to 10-week-old mice by collagenase digestion and 200 islets were transplanted in the calf muscle, as previously described (15). Two weeks after transplantation, mice were randomly assigned to one of three experimental groups. The BG levels of the control group were maintained at <200 mg/dL. Insulin pellets were removed from mice assigned to the hyper or restored groups to reinduce hyperglycemia (>350 mg/dL). After 4 weeks of hyperglycemia, the restored group received insulin pellets again. At the end of the experiment, mice were injected with radiolabeled exendin-3 and scanned 1 h after injection on a small animal U-SPECT-II/CT system (MILabs, Utrecht, the Netherlands) with a 1-mm multipinhole ultra-high sensitivity mouse collimator for 50 min. Two hours after injection, mice were sacrificed, and grafts were embedded in paraffin for histology and autoradiography.

Autoradiography

The 4-μm-thick sections from different levels of the islet grafts were exposed to an imaging plate (Fuji Film BAS-SE 2025; Raytest GmbH, Straubenhardt, Germany) for 1 week. Images were visualized with a Typhoon FLA 7000 laser scanner (GE Healthcare Life Sciences). Tracer uptake quantification was done with AIDA Image Analyzer software (Raytest GmbH). To normalize the uptake to the insulin area, sections were stained for insulin (see the IMMUNOHISTOCHEMISTRY AND MORPHOMETRIC ANALYSIS section).

Human Islet Transplantation Into the Eye, STZ Treatment, and Longitudinal In Vivo Imaging

Eight-week-old male NOD.CB17-*Prkdc*^{scid}/J (NOD-Scid) mice were used as transplant recipients. Upon arrival, human islets were cultured overnight in CMRL-1066 (Corning Cellgro, Manassas, VA), supplemented with 2 g/L human serum albumin, 100 units/mL penicillin/streptomycin. Mice were transplanted with 15 islets into the anterior chamber of the eye as previously described (16). Islets were allowed to engraft for 8 weeks as human islets require a prolonged time for proper revascularization and engraftment.

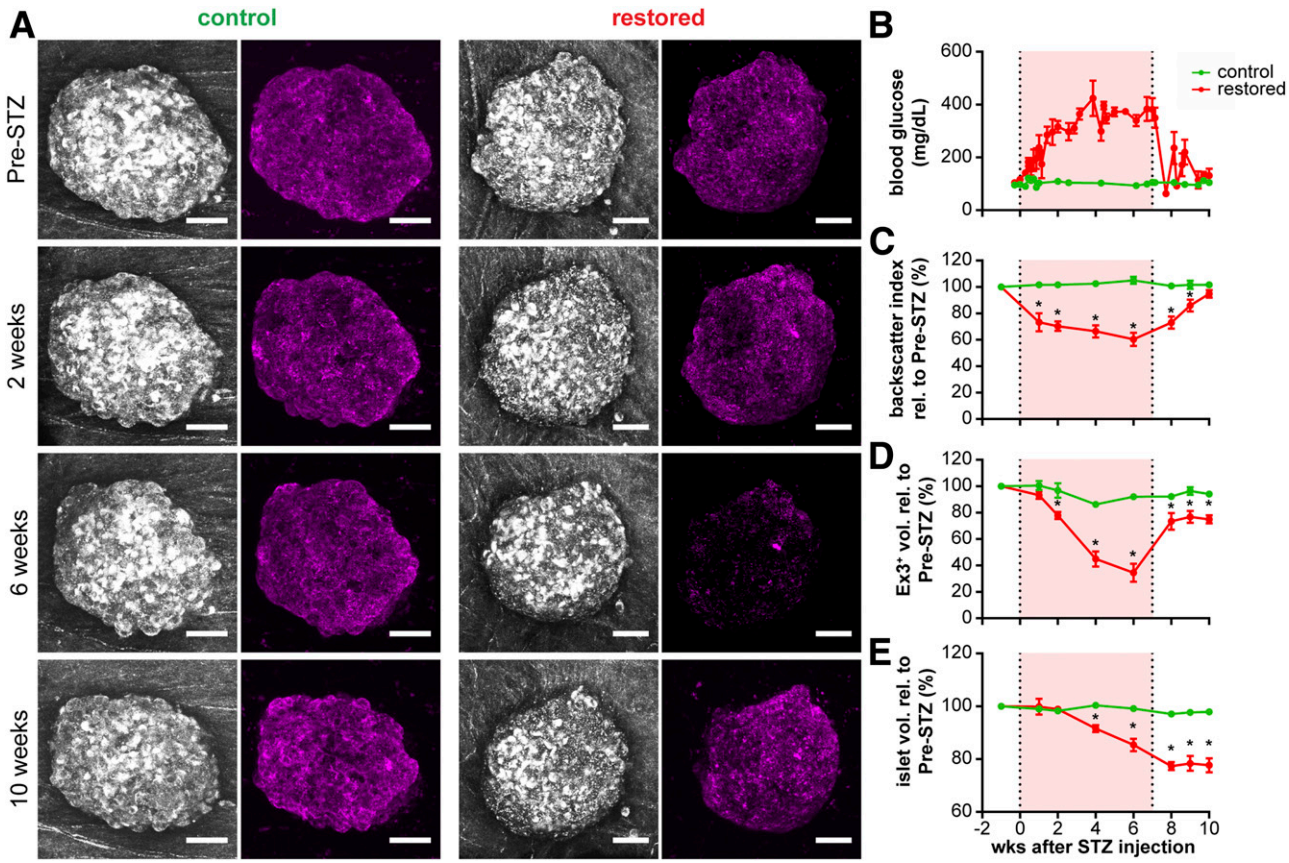

Figure 3—Exendin uptake in human islets depends on the glycemic status of the recipient. *A*: Maximum intensity projections of the same human islets in the control and restored group with islet backscatter (gray) and alexa647-labeled exendin-3 (Ex3) (magenta) at indicated time points. *B*: BG profile of the control and restored groups throughout the experiment. *C–E*: Quantification of backscatter signal in transplanted human islets (*C*), $Ex3^+$ islet volume (*D*), and islet volume relative to the initial imaging time point (*E*). *B–E*: The first dotted line indicates STZ injection, and second dotted line indicates insulin pellet implantation; red background indicates the period of hyperglycemia in the restored group. $n = 3$ mice (control), 4 mice (restored) with 16 and 22 individual islets, respectively. Data are presented as mean ± SEM, $^*P < 0.05$ evaluated by mixed model analysis. Scale bars = 50 μm. wks, weeks.

Hyperglycemia was induced by injecting 150 mg/kg streptozotocin (STZ) (Sigma-Aldrich, Darmstadt, Germany), and BG levels were monitored. To restore normoglycemia, insulin pellets were implanted after 7 weeks of hyperglycemia.

Four hours prior to imaging, mice were injected i.v. with 4 nmol of exendin3-Alexa647 in PBS. Mice were intubated and anesthetized by 2% isoflurane in 100% oxygen. To limit pupil dilation and iris movement, 0.4% pilocarpine (Pilomann; Bausch & Lomb) was placed on the cornea. Repetitive in vivo imaging was performed at indicated time points on an upright laser scanning microscope (LSM780 NLO; Carl Zeiss, Ulm, Germany) with a water-dipping objective (W Plan-Apochromat 203/1.0 DIC M27 75 mm; Carl Zeiss) using vidisic eye gel (Bausch & Lomb) as immersion. The total volume of transplanted islets was assessed by detecting 633-nm laser backscatter. Exendin3-Alexa647 uptake was assessed by spectral analysis on an internal array gallium arsenide phosphide (GaAsP) detector at a resolution of 8.7 nm using the lambda unmixing algorithm of ZEN2009 (Zeiss), using combined, 488-, 561-, and 633-nm laser excitation. Reference spectra were acquired for exendin3-Alexa647 after in vivo labeling and for human islet autofluorescence from transplanted human islets without labeling. Islet volume was determined by semiautomatic volume creation, and exendin3-Alexa647 was determined by automatic surface rendering from median filtered Z-stacks with Imaris 8.1 (Bitplane AG, Zurich, Switzerland).

Immunohistochemistry and Morphometric Analysis

Paraffin sections of murine grafts were immunostained for insulin (17), or GLP1R. For the latter, pronase (1 mg/mL, 10 min, 37°C) was used as antigen retrieval. After blocking with 3% H_2O_2 (10 min), sections were immunostained with mouse anti–GLP-1R (Novo Nordisk, Copenhagen, Denmark) (1:50, o/n, 4°C) followed by horseradish peroxidase–conjugated secondary antibody (DAKO, Copenhagen, Denmark) (1:100, 30 min). The staining was developed with DAB (Immunologic BV, Duiven, the Netherlands), and sections were counterstained with hematoxylin. Morphometric analysis was performed with the trainable Weka segmentation plugin in ImageJ/Fiji (https://rsb.info.nih.gov/ij/).

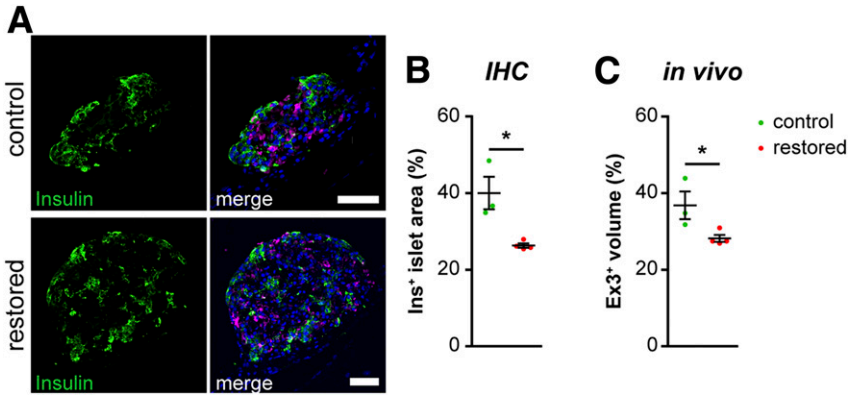

Figure 4— Insulin area correlates with exendin uptake in human islets. *A*: Images of explanted islets after termination of the experiment stained for insulin (green, left panel) as well as glucagon (magenta) and DAPI (blue) (merged image, right panel). *B* and *C*: Quantification of the insulin (Ins)-positive islet area in tissue sections (*B*) and the % exendin-3 (Ex3)-positive islet volume in vivo at the last imaging time point (*C*). Data are presented as mean ± SEM, *P <0.05 evaluated by Mann-Whitney rank sum test with n = 3 mice (control) and 4 mice (restored). Scale bars = 50 μm.

Islet-containing eyes were fixed in polyformaldehyde and mounted in TissueTek; 10-μm-thick cryosections were prepared and immunostaining was performed with guinea pig anti-insulin (1:200; DAKO) and mouse antiglucagon (1:2,000; Sigma-Aldrich). Secondary antibodies goat anti-guinea pig Alexa Fluor-488 and goat anti-mouse Alexa Fluor-633 (both 1:200; Thermo Fisher, Bremen, Germany) were used, and sections were counterstained with DAPI.

Statistical Analysis

Statistical analysis was performed using SPSS version 22 (SPSS). $P < 0.05$ was considered statistically significant. Results were presented as mean ± SEM. Group comparisons were performed using ANOVA with Tukey post hoc test or Mann-Whitney rank sum test, as indicated. Results obtained from longitudinal in vivo imaging studies were analyzed by linear mixed models (18).

Data and Resource Availability

The data sets generated and/or analyzed during the current study are available from the corresponding authors on reasonable request.

RESULTS AND DISCUSSION

Severe Hyperglycemia Reduces GLP-1R–Mediated Exendin Uptake in Murine Islets

To analyze the effect of hyperglycemia on GLP-1R–mediated exendin uptake, chemically induced diabetic mice were transplanted with a submarginal islet mass, and BG levels were regulated with insulin implants (Fig. 1*A*). Hyperglycemia had a substantial effect on exendin uptake, which was evidenced from ^{111}In-exendin SPECT scans (Fig. 1*B*) and autoradiography images (inserts in Fig. 2*A* and *B*). ^{111}In-exendin accumulation within islet grafts significantly decreased when islets were exposed to hyperglycemia (control $1.19 \times 10^{-4} \pm 0.34 \times 10^{-4}$ Bq/m^2 vs. hyper $0.37 \times 10^{-4} \pm 0.22 \times 10^{-4}$ Bq/μm^2, $P < 0.001$) (Fig. 1*C*). This was accompanied by a marked reduction in GLP-1R staining intensity compared with the control (Fig. 2*A* and *C*), while the insulin staining intensity was maintained (Fig. 2*B* and *D*). The observation that hyperglycemia reduced GLP-1R staining intensity implies that the reduced tracer uptake was caused by a reduction in GLP-1R expression, which is in line with previous in vitro reports (11,12).

Normalization of BG Levels Restores GLP-1R–Mediated Exendin Uptake in Murine Islets

Since near-normalization of BG levels improves β-cell responsiveness to GLP-1 in patients with type 2 diabetes (19,20), we hypothesized that strict regulation of BG may reinstate GLP-1R expression. Normalization of BG levels restored the observed decrease in tracer uptake (control $1.19 \times 10^{-4} \pm 0.34 \times 10^{-4}$ Bq/μm^2 vs. restored $1.41 \times 10^{-4} \pm 0.27 \times 10^{-4}$ Bq/μm^2, $P = 0.40$) (Fig. 1*B* and *C*), which was paralleled by normalization of GLP-1R staining intensity (Fig. 2*E*).

Linear Correlation Between Exendin Uptake and β-Cell Area Is Preserved After Severe Hyperglycemia

We have previously shown that exendin uptake linearly correlates with absolute BCM (8,9), indicating the potential of exendin as an imaging biomarker for BCM (7). To examine whether hyperglycemia affects this linear relationship, we correlated the uptake of exendin with the β-cell area. Exendin uptake linearly correlated with the insulin-positive β-cell area (control group, Pearson $r = 0.89$, $P < 0.0001$) (Fig. 1*D*). This linear correlation was maintained after a period of hyperglycemia (hyper group, Pearson $r = 0.84$, $P < 0.0001$) (Fig. 1*D*), though the slope of the correlation curve was significantly decreased compared with the control group (hyper group 0.36×10^{-4} vs. control group 1.29×10^{-4}, $P = 0.013$), reflecting the decrease in absolute uptake. Normalization of BG levels completely reinstated the slope of the correlation curve (restored group 1.47×10^{-4} vs. control group 1.29×10^{-4}, $P = 0.51$) (Fig. 1*D*).

Normalization of BG Levels Restores Exendin Uptake in Human Islets

To assess whether these observations are representative for the human situation, we transplanted human islets into the anterior chamber of the eye of NOD-Scid mice. The advantage of this model is the ability to repetitively image an individual islet at a cellular resolution (21) using fluorescently labeled exendin. After engraftment, mice were imaged and distributed into two groups: one control group and one group receiving a single dose of STZ to induce endogenous β-cell destruction (22) (Fig. 3*B*). One week after STZ treatment, a significant reduction in islet backscatter signal intensity was observed (Fig. 3*A* and *C*), indicative for β-cell exhaustion leading to degranulation (18). In this group, the exendin uptake progressively decreased to 34.45 ± 0.07% of the pretreatment value (Fig. 3*D*), which was accompanied by a reduction in islet volume (Fig. 3*E*). Normalization of BG levels (Fig. 3*B*) resulted in cellular regranulation within a 3-week period and stabilization of the islet volume (Fig. 3*E*), which is in agreement with previously reported data on human islet recovery in response to transient hyperglycemia (18). Tracer uptake was found to immediately improve after glycemic levels were restored. After 3 weeks of near-normalized BG levels, tracer uptake was 76.81 ± 0.07% of the initial time point (Fig. 3*D*). This reduction in tracer uptake (23.19%) corresponded to the observed reduction in islet volume (21.64 ± 0.03%) (Fig. 3*E*), which suggests that the reduction in tracer uptake may be attributed to hyperglycemia-induced loss in BCM. To test this hypothesis, sections of explanted eyes were immunostained for insulin (Fig. 4*A*), revealing a significantly reduced insulin-positive area in the restored group compared with controls (restored 26.30 ± 0.55% vs. control 40.00 ± 4.26%, $P < 0.05$) (Fig. 4*B*). When we compared the tracer uptake in these islets at the last in vivo imaging time-point, a similar reduction in tracer uptake was observed (restored 28.19 ± 0.92% vs. control 36.81 ± 3.63%, $P < 0.04$) (Fig. 4*C*). These results indicate that a loss in BCM was responsible for the reduction in tracer uptake.

Taken together, our results highlight a critical role for hyperglycemia in GLP-1R regulation. Although hyperglycemia results in a substantial decrease in exendin uptake in murine and human islets, we have demonstrated that the uptake linearly correlates with BCM and that normalization of BG levels restores exendin uptake. The observed threefold reduction in tracer uptake in murine and human islets under hyperglycemic conditions may be an underestimation, as there are indications that hyperglycemia can increase capillary blood perfusion in islets (23). An important caveat of this study is that we used animal models with prolonged levels of hyperglycemia, whereas patients with diabetes experience fluctuating BG levels, with 2 h-postprandial BG levels of at least 200 mg/dL (24–26). Therefore, the reduction in tracer uptake may not be as dramatic in patients as described here. Nevertheless, this outcome emphasizes the potency and clinical relevance of nuclear imaging techniques to monitor receptor regulation noninvasively. Our results indicate that BG levels should be near-normalized for at least 3 weeks prior to GLP-1R agonist treatment or radiolabeled exendin imaging for BCM quantification.

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Duality of Interest. M.G. declares that he is an inventor and holder of the patent "Invention affecting GLP-1 and exendin" (Philipps-Universität Marburg, June 17, 2009). No other potential conflicts of interest relevant to this article were reported.

Author Contributions. M.Bu., C.M.C., and W.A.E. contributed to the design and conduct of the study and the analysis and interpretation of the data. M.Bu. and C.M.C. wrote and edited the manuscript. L.C.-J., C.F., D.B., and G.S. provided technical assistance in radiolabeling, tissue sectioning, immunostaining, and imaging. M.Br., S.S., and M.G. contributed to the experimental design, interpretation of the results, and conceptualization of the manuscript. S.S. and M.G. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. Parts of this work were given as an oral presentation at the European Association of Nuclear Medicine, Barcelona, Spain, 15–19 October 2016; as a poster presentation at the European Association for the Study of Diabetes, Munich, Germany, 12–16 September 2016; and as a poster presentation at the 2nd Joint Meeting of the European Association for the Study of Diabetes Islet Study Group and Beta Cell Workshop, Dresden, Germany, 7–10 May 2017.

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