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Imaging NF- κ B activity in a murine model of early stage diabetes

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Abstract

Early pro-inflammatory signaling in the endocrine pancreas involves activation of NF- κ B, which is believed to be important for determining the ultimate fate of β -cells and hence progression of Type 1 diabetes (T1D). Thus, early non-invasive detection of NF- κ B in pancreatic islets may serve as a potential strategy for monitoring early changes in pancreatic endocrine cells eventually leading to T1D. We investigated the feasibility of optical imaging of NF- κ B transcription factor activation induced by low-dose streptozocin (LD-STZ) treatment in the immunocompetent SKH1 mouse model of early stage diabetes. In this model we showed that the levels of NF- κ B may be visualized and measured by fluorescence intensity of specific near-infrared (NIR) fluorophore-labeled oligodeoxynucleotide duplex (ODND) probes. In addition, NF- κ B activation following LD-STZ treatment was validated by using immunofluorescence and transgenic animals expressing NF- κ B inducible imaging reporter. We showed that LD-STZ treated SKH1 mice had significantly higher (2–3 times, $p < 0.01$) specific NIR FI in the nuclei and cytoplasm of islets cells than in non-treated control mice and this finding was corroborated by immunoblotting and electrophoretic mobility shift assays (EMSA). Finally, by using semi-quantitative confocal analysis of non-fixed pancreatic islet microscopy we demonstrated that ODND probes may be used to distinguish between the islets with high levels of NF- κ B transcription factor and control islet cells.

Keywords

NF- κ B; oligodeoxynucleotide duplex; imaging probes; beta cells; type 1 diabetes

Introduction:

Type 1 Diabetes (T1D) is an inflammatory disease of the pancreas in which insulin-producing β -cells in the pancreatic islets of Langerhans are progressively destroyed months to years prior to the clinical onset of insulin insufficiency [1]. Beta cell death is preceded by the pancreatic invasion of macrophages, autoreactive T cells, and other immune cells [2] which secrete inflammatory cytokines (e.g., IL-1 β , TNF α), nitric oxide (NO), and oxygen free radicals [3, 4]. Exposure of β -cells to inflammatory cytokines results in activation of transcription factor NF- κ B which then modulates β -cell response to further cytokine

exposure [3, 5] and results in the activation of both pro- and anti-inflammatory genes. The fine balance between these gene expression products in the cells ultimately determines the fate of β -cells and the progression to full blown T1D [6]. In rodent and human β -cells, NF- κ B has been shown to be a critical effector of β -cell destruction induced by exposure to IL-1 β or IL-1 β /IFN- γ [7, 8]. *In vitro* exposure of human β -cells to IL-1 β + (IFN)- γ resulted in elevated proinsulin/insulin levels resembling changes of functional insulin status seen in early human T1D [9]. Prolonged exposure to IL-1 β + (IFN)- γ leads to activation of a pro-inflammatory gene network (e.g., iNOS, Fas, MIG, IP-10) resulting in β -cell death [3, 10, 11]. In contrast to its established pro-apoptotic role, NF- κ B activation was found to protect β -cells against TNF- α induced apoptosis through induction of anti-inflammatory NF- κ B target genes A20, XIAP and c-FLIP [12–14]. Histology of human islets has confirmed the presence of inflammation in recent-onset T1D patients, however, this approach is not feasible as a clinical diagnostic strategy due to the invasive nature of pancreatic biopsy [15]. Thus, non-invasive imaging of NF- κ B levels holds great promise as an alternative strategy for capturing the earliest inflammatory events in β -cells.

We previously designed and characterized near-infrared fluorescent ODND carrying NIR fluorophores positioned within the NF- κ B consensus binding site [16, 17]. Because of labeling with near-infrared fluorophores these ODND can be potentially detected during deep tissue imaging [18, 19]. The imaging of functionally active NF- κ B transcription factor in solution was previously demonstrated by analyzing fluorescence intensity (FI) and fluorescence lifetime (FLT) changes in a model system that included active purified NF- κ B p50 and p65 subunits [20, 21]. Here we report on the binding of NIR-ODND in tissues of SKH1 albino immunocompetent mice treated with several subsequently administered sub-diabetogenic doses of streptozocin (LD-STZ) resulting in pre-diabetes. The dependence of the induction of T1D on the activation of pro-inflammatory NF- κ B signaling in this model has been demonstrated in a study showing that the development of disease pathology following LD-STZ treatment could be prevented through expression of a β -cell-specific mutant degradation-resistant NF- κ B inhibitor ($\text{I}\kappa\text{B}\alpha$) [11]. The reduction of pancreatic NF- κ B activation through treatment with zinc sulfate-supplemented water also prevented LD-STZ induced diabetes in C57BL/6 mice, thus confirming the role of NF- κ B activation in the LD-STZ induction model of T1D [22]. In our experiments we determined that LD-STZ treatments were driving the expression of NF- κ B dependent firefly luciferase in tissues (including the pancreas) of transgenic reporter mice resulting in luminescence. This observation served as independent proof that LD-STZ dosing of mice can be used as a laboratory tool for creating non-acute pre-diabetic models with definite involvement of NF- κ B activation pathways. Using validated immunocompetent SKH1 mouse model we tested the feasibility of imaging NF- κ B expression at an early, pre-diabetic stage using fluorescent NIR-ODND. Our findings demonstrate that NIR ODND enables the detection and visualization of NF- κ B expression in pancreatic tissue, fresh isolated islets of Langerhans, and pancreatic cell extracts, and as such can be used as a tool for measuring quantitative differences in NF- κ B expression levels in a pre-diabetic versus control pancreas.

Materials and Methods:

Multiple low dose streptozocin (LD-STZ) model.

Male and female SKH1-mice aged 6–8 weeks (Charles River Laboratories, Wilmington, MA) were given intraperitoneal injections of STZ (Sigma Aldrich, St. Louis, MO) at a concentration of 50 mg/kg dissolved in sodium citrate buffer (0.1 M, pH 4.5) daily for 5 consecutive days. The same experimental setup was used for testing of the LD-STZ dosing regimen in transgenic NGL (NF- κ B-GFP-luciferase) mice (male and female, FVB.Cg-Tg(HIV-EGFP,luc)8Tsb/J, JAX Labs) with knocked-in NF- κ B-dependent enhanced green fluorescent protein-luciferase fusion protein expression cassette [23]. These mice served as positive imaging control. The first day of STZ administration was designated as day 1 of the study (Figure 1A). Non-fasting blood glucose readings were determined by using a glucose meter (Nova Diabetes Care, Billerica, MA). Imaging of luciferase expression was performed after an injection of 100 mg/kg D-luciferin in 0.2 ml of saline (I.P., right lower ventral quadrant of the abdomen). Animals were anesthetized with isoflurane (1.75% in 30% oxygen/nitrogen mixture) and images were acquired 5 min post injection every 5 min for 30 min onward to ensure the peak of luminescence was detected by using integrated optical/micro-computed tomography (CT) device (IVIS SpectrumCT, PerkinElmer, Waltham MA). The final image acquisition and image analyses were performed by using automated optical and CT image registration/integration to map the areas of luminescence and mouse anatomy (Living Image, PerkinElmer). Animal housing and all experiments involving animals were conducted in accordance with the guidelines of University of Massachusetts Medical Institutional Animal Care and Use Committee (IACUC).

Pancreas harvesting and fixation.

Pancreata were harvested and embedded in OCT medium (Electron Microscopy Sciences, Hatfield, PA) and snap frozen immediately in liquid nitrogen or fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) and embedded in paraffin for further sectioning and immunohistochemical processing.

Pancreatic cell harvesting.

Islets and acinar/exocrine cells were separated on day 8. Briefly, cold collagenase P (0.7 mg/mL, Roche, Basel, Switzerland) solution was injected into the pancreas via the common bile duct, followed by incubation at 37°C. After digestion, tissue was passed through a sterile strainer. Islets were separated from exocrine cells by a density gradient using Histopaque 1077 (Sigma Aldrich, St. Louis, MO) and the floating islets and the pellet containing exocrine cells were collected. Individual islets were double hand-picked under an inverted microscope. Collected islets and exocrine cells were suspended in RPMI 1640 medium (Sigma Aldrich, St. Louis, MO) supplemented with 5% horse serum (Gibco-Thermo Fisher Scientific, Waltham, MA).

Immunofluorescence (IF), immunohistochemistry (IHC) of pancreas slices and image analysis.

The frozen and paraffin embedded tissues were sectioned at a thickness of 5 μ m and mounted on microscope slides. Paraffin-embedded sections were deparaffinized and subjected to antigen retrieval (Tris/EDTA pH 9.0). Frozen sections were fixed with acetone. Sections were blocked for non-specific binding in the presence of 5% BSA, TBS, pH 7.5 and incubated with the primary antibody at 4°C, overnight, in a humidifier chamber. The following rabbit-anti-mouse polyclonal or monoclonal primary antibodies were used at optimized dilutions: anti-insulin, anti- NF- κ B p65 (phospho S536 or phospho S276) and anti-CD68 (Abcam, Cambridge, MA), anti-eGFP tag (Invitrogen, Rockford IL). For IHC staining, slides were incubated with secondary antibody conjugated with AP (dilution 1:1000, Sigma Aldrich, St. Louis, MO or Abcam). AP activity was detected using BCIP/NBT substrate mix (Roche) in the presence of 1 mM levamisole. Sections were counterstained with nuclear fast red (Vector laboratories, Burlingame, CA). For IF staining, slides were incubated with secondary antibody conjugated with Alexa Fluor 555 (Abcam, Cambridge, MA) for 1 hour at room temperature and were mounted with mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Microscopy images acquired in 16-bit tiff format were analyzed by using ImageJ 1.50b software (NIH, Maryland, USA). Fraction of insulin positive area (%) was determined by color thresholding and calculating the ratio of the insulin-positive area to the total tissue area on the entire section. Three nonadjacent sections from each harvested pancreas were analyzed (n=3). Fluorescence intensity of NF- κ B in cell compartments was quantified using nucleus and cytoplasm segmentation in at least 700 islet cells.

NIR-ODND probe.

The following sense and antisense complementary ODNs: 1) NF- κ B: 5'-AGC CTG GAA AGT CC--C ACA TCG-3' and 5'-CGA TGT GGG ACT TTC-- CAG GCT-3', 2) control: 5'-CGTATG--AGTGACTGCAGAGCT-3' and 5'-AGCTCTG--CAGTCACTCATACG-3' were synthesized, purified and labeled with NHS esters of IRDye 800 CW (Li-Cor Biosciences, Lincoln NE), sulfocyanines Cy3 or Cy5.5 (Lumiprobe Corp, Hunt Valley MD) essentially as described in [17, 24]. Aminodiethoxyethylene internucleoside linkers that were used for conjugating fluorophores [25] are marked with --, phosphorothioate-bond modified nucleosides are shown in bold. Both experimental and control ODN pairs were hybridized to form NIR ODNDs either resulting in a synthetic consensus sequence 5'-GGGACTTTCC/5'-GGAAAGTCCC of the immunoglobulin κ -light chain gene enhancer (Ig- κ B) [26], or in a control ODND lacking NF- κ B recognition motif. ODNs were mixed in a 1:1.1 molar ratio in buffer solution containing 25mM Hepes, 1mM MgCl₂ and 50mM NaCl, pH 7.4 followed by heating at 90–95°C for 5 min and then cooled at room temperature. Probe integrity was confirmed by gel electrophoresis of duplex and single strand on 4–20% polyacrylamide gels run in 0.5×TBE (40 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3). For some experiments following duplex formation, ODND were conjugated with digoxigenin. Digoxigenin-hydroxysuccinimide ester (Roche, Basel, Switzerland) in DMSO was added to ODND (5 μ M) at pH 8.0, final concentration of 100 μ M. The labeling efficiency was analyzed by using anti-digoxigenin-Fab' antibody

fragment- alkaline phosphatase conjugate (Roche, Basel, Switzerland) after blotting the probe on Immobilon-NY+ transfer membranes (Millipore, Burlington, MA).

Binding assay 1 : pancreas slices.

Frozen pancreas slices were blocked in a solution containing 0.01 mg/ml fragmented salmon testes DNA (Sigma Aldrich, St. Louis, MO) in 1X Denhardt's solution for 30 minutes at room temperature. Anti-insulin staining (rabbit polyclonal antibody, Abcam, Cambridge, MA) was performed in a humidifier chamber overnight at 4°C. Sections were incubated with 40–250 nM Cy5.5-labeled NIR-ODND and control duplex probes in the presence of 10 mM Tris, 100 mM KCl, 2 mM MgCl₂, 10% v/v glycerol, 0.1 mM EDTA, 0.1 mg/mL tRNA, 0.25 mM DTT, pH 7.5 (ODND binding buffer) for 3 hours at room temperature. This step was followed by incubation with secondary antibody conjugated with AF555 for 1 hour at room temperature to complete insulin staining. Sections were mounted with mounting medium with and without DAPI (Vector Laboratories, Burlingame, CA). Images were captured using Nikon TE2000-U inverted microscope equipped with a standard Cy5.5 set (XF141–2, Omega optical). The images were acquired and processed by using IP Lab Spectrum (Beckton-Dickinson). Binding of digoxigenin-labeled to tissue sections was performed at 200 nM ODND incubated for 3 h, RT in the binding buffer described above followed by counterstaining with nuclear fast red. The competition binding assay was done by adding a 10 times excess of the unlabeled ODND. Bright field images were captured using Olympus AX70 inverted microscope and analyzed by using Image-Pro software.

Cell extract preparation.

Isolated islets and exocrine cells from each group of mice were fractionated into cytoplasmic/membrane and nuclear fractions. Briefly, cells were washed twice in ice-cold PBS and suspended in hypotonic buffer (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 1X protease inhibitors cocktail (Thermo-Fisher Scientific, Waltham, MA), incubated on ice for 15 min, passed through a 28 gauge needle and centrifuged at 4,000 rcf, 5 min at 4°C. The supernatant (the cytoplasmic fraction) was transferred to a pre-chilled tube. The nuclei pellet was suspended in high salt buffer (20 mM HEPES, 1.5 mM MgCl₂, 420 mM NaCl, 0.1 mM EDTA, 25% v/v glycerol, 0.25 mM DTT, 1X protease inhibitors, incubated on the ice for 30 min with agitation. Samples were centrifuged at 16,000 rcf for 10 min at 4°C, and the supernatant (nuclear fraction) was collected and stored at –80°C. To obtain control inactive islet lysates cell extracts were treated sequentially in the presence of 0.5 mM TCEP and an excess of iodoacetamide (1 mM). The protein concentration was determined by using Micro BCA Protein Assay (Pierce, Rockford, IL, USA).

Electrophoretic mobility shift assay (EMSA).

EMSA was performed using nuclear and cytoplasmic extracts of islets and endocrine cells and either NIR-ODND, or Cy3-labeled ODND. Pancreatic cell extracts (1–2 µg) or TNFα-treated HeLa cells nuclear extracts (1–2 µg, Santa Cruz Biotechnology, Dallas, Texas) as positive control were incubated with fluorescent ODND probe (0.22 pmol, final concentration 44 nM) for 30 min at room temperature in the presence of ODND-BB. In some control experiments 2 µl iodoacetamide-inactivated LD-STZ nuclear extracts were used in combination with 0.5 pmol of NIR-ODND or 2 µl active extracts were mixed together

with 0.5 pmol NIR-ODND and the excess of non-labeled ODND (5 pmol) was added. The complexes were analyzed by on 10% TBE minigels (Bio-Rad, Hercules, CA). The gels were imaged and digitized using an Odyssey Infrared Imaging system (Li-Cor Biosciences, Lincoln, NE) equipped with solid diode excitation sources. Supershift EMSA experiments were performed using similar conditions as above: samples containing 2.5 µg extracts and 0.4 pmol Cy3-labeled ODND were incubated for 1 h in ODND-BB (with no DTT) for 30 min and then 0.5 µl anti-NF-κB p105/p50 polyclonal antibody (ab7549, Abcam) or NF-κB p65 polyclonal antibody (ADI-KAS-TF110-D, Enzo Life Sciences, Farmingdale NY) for additional 30 min at RT and the bands were resolved using 7.5% TBE gels. Band intensity was measured using BioSpectrum UVP imaging systems and the obtained 16-bit images were quantified using ImageJ (NIH).

Western blot analysis.

Nuclear and cytoplasmic extracts of islets and exocrine (acinar) cells (2 µg total protein/sample), and 2 µg HeLa nuclear extract (positive control) were separated on a 4–15% TGX precast protein gels (Bio-Rad) and transferred onto a PVDF membranes (Bio-Rad). Membranes were blocked with TBS-B overnight at 4°C. Primary anti-p65 NF-κB antibody (rabbit monoclonal D14E12, Cell Signaling Technology, Danvers MA) and anti-GAPDH antibody (mAb 6C5, Santa Cruz Biotechnology, Dallas TX) for signal normalization were diluted at 1:1000 in 0.1x TBS-B and incubated overnight at 4°C. Membranes were incubated with secondary anti-idiotypic alkaline phosphatase antibody conjugates (Abcam Cambridge, MA) diluted at 1:1500 in 0.1xTBS-B. Membranes were developed using NBT-BCIP solution (Sigma-Aldrich) diluted in 2 mM MgCl₂ in TBS, pH 9.5. Signals were analyzed as integrated intensities of regions defined around the bands of interest using ImageJ software (NIH).

Binding assay 2: freshly isolated islets- confocal microscopy.

Isolated islets were incubated with 0.3 µM NIR-ODND in CMRL medium 1066 (Gibco-Thermo Fisher Scientific, Waltham, MA) supplemented with 10%FBS (Gibco-Thermo Fisher Scientific, Waltham, MA), 1% pen-strep-Glutamine(Gibco-Thermo Fisher Scientific, Waltham, MA), 1% sodium pyruvate (Gibco-Thermo Fisher Scientific, Waltham, MA) for 4 h at 37°C. Following incubation, islets were washed with TBS, fixed with 3.7% paraformaldehyde, blocked with 10% FBS in TBS and permeabilized with 0.1% Tween-20 in TBS. Islets were stained with primary anti- insulin antibody (Abcam, Cambridge, MA) in 1%FBS in TBS followed by incubation with secondary antibody conjugated with AF568. Cell nuclei were stained with DAPI. Islets and exocrine cells were mounted using mounting medium (Vector Laboratories). Confocal microscopy was performed by using Leica TCS SP8 confocal scope and images were captured using LAS AF software. Successive optical sections were acquired at 0.5 µm intervals in the z-dimension.

Statistical analyses.

Statistical analyses were performed by using one-way ANOVA, Graphpad Prism 7.0 (GraphPad Software Inc., San Diego, CA). Difference between groups were considered to be significant at if P<0.05.

Results:

LD-STZ effects on blood glucose concentration, islet morphology of SKH1 mice and macrophage infiltration.

Our initial hypothesis was in that LD-STZ regimen of STZ administration (Figure 1A) will result in a pre-diabetic condition involving continuous NF- κ B activation via production of excessive levels of NO resulting in β -cell stress and macrophage-mediated cytokine release (Figure 1B). We monitored mice during the first 5 days of the study and observed small increase of blood glucose levels of treated male SKH1 mice compared to control ones. However, this increase did not achieve statistical significance and LD-STZ treated mice remained normoglycemic throughout the study ($p>0.05$, Figure 2A). To determine the changes in the size and number of islets following LD-STZ administration we performed a morphometric analysis of pancreata of control and LD-STZ treated mice 3 days after final injection (day 8). LD-STZ treatment significantly decreased insulin positive fraction of pancreas (by 74%) compared to control mice at the end of scheduled treatments ($p<0.05$; Figure 2B). Furthermore, the number of islets was reduced by 57% in pancreata of the treated mice ($P<0.03$; Figure 2C). Representative islets from each group are shown in Figure 3-D&E. Morphological assessments by H&E and insulin labeling of pancreatic sections showed that the pancreatic islets of the control group displayed normal histological features (Figure 2D,F). However, the islets of LD-STZ treated mice were smaller in size with irregular outlines (Figure 2E,G). Infiltration of CD68-positive macrophages into the islets, following LD-STZ treatment was observed on day 4 and day 7 (>10 cells/section) while control islets in non-treated animals contained no more than 3–4 positive cells/ section (Supplementary Figure 1).

LD-STZ treatment activation of NF- κ B mediated expression of imaging marker in transgenic animals.

To obtain direct in vivo evidence of the induction of NF- κ B mediated expression of genes during the LD-STZ administration and to confirm the endpoints suggested by ex vivo tissue analysis we used a transgenic FVB.Cg-Tg(HIV-EGFP,luc)8Tsb/J murine model in which fLuc fusion with EGFP is expressed under the control of NF- κ B dependent HSV minimal thymidine kinase promoter cassette containing four tandem copies of an enhancer from the 5'-HIV-long terminal repeat [23]. The animals rapidly developed strong luminescence signal in the area of IP injections in the lower right quadrant of peritoneum suggesting local activation of NF- κ B (Figure 3B). After day 5 injection on tomographic reconstruction of luminescence images we observed the appearance of localized and discrete areas of luminescence in the upper abdomen of mice (Figure 3C). Luminescence signal in these areas peaked between day 3 and day 8 of LD-STZ treatment and was sustained until the study endpoint, i.e. day 8 (Figure 3C). The experiments in transgenic animals showed the evidence of sexual dimorphism when animals of both sexes were compared side-by-side with male developing similar by magnitude but delayed and sustained imaging signal in the upper abdominal area in response to the injections of STZ (Figure 3D). Immunohistochemistry of frozen sections using a polyclonal antibody raised against EGFP (a component of fLuc-EGFP fusion reporter protein) confirmed an overall higher level of signal in the tissues of LD-STZ treated male pancreas as opposed to female mice (Figures 3 E and F). The majority

of islet cells (pointed to by arrows, Figure 3E) showed positive reaction with the antibody. Multiple cells in the acini also showed high levels of fusion reporter protein expression (visible as blue spots outside the margins of the islets) and their occurrence was noted in male pancreas after the last day of STZ injection.

Analysis of LD-STZ induced activation of NF- κ B.

We followed the changes in total levels of NF- κ B p65 expression and levels of NF- κ B activation by performing: a) microscopy image analysis of pancreas sections and b) Western blotting of cell lysates obtained from LD-STZ treated and control mice. Figure 4 shows immunohistochemistry (A-D) and immunofluorescence (E-H) of pancreas sections reflecting NF- κ B phospho-p65 expression in the islets of control (A, B, E and F) and LD-STZ treated (C, D, G and H) mice. Image analysis of anti- NF- κ B phospho-p65 labeled pancreatic sections indicated that LD-STZ treatment induced a significantly higher level of NF- κ B phospho-p65 in the islets of treated mice compared to the control ($P<0.004$; Figure 4I). In agreement with this result, both nuclear and cytoplasmic levels of NF- κ B phospho-p65 in individual cells of islets were approximately 2 times higher in LD-STZ treated mice compared to control (nuclear: $P<0.003$; cytoplasmic: $P<0.001$; Figure 4J). To further investigate whether LD-STZ treatment resulted in higher NF- κ B content we performed Western blot on nuclear and cytoplasmic extract of islets of pancreas using an antibody to total NF- κ B p65 (phosphorylated and non-phosphorylated isoforms, Figure 5 A) and compared the observed results to the NF- κ B p65-specific signals detected on the exocrine cell blots. The NF- κ B p65-specific band intensities were normalized by GAPDH-specific band intensities that served as loading controls (alongside with HeLa-TNF α treated positive control cell extracts). The normalized intensity analysis of bands of nuclear and cytoplasmic lysates isolated from the islets indicated that LD-STZ treatment induced accumulation of p65 at higher levels (approximately 5.7 times, $p<0.05$) in the nuclei of islet cells if compared to control islet cells (Figure 5B). In addition, Western blot performed on nuclear and cytoplasmic extract of exocrine cells confirmed the expression of NF- κ B in exocrine cells at high levels. The results obtained using exocrine cell lysates consistently showed a decline in NF- κ B p65 after LD-STZ treatment, which was statistically significant ($p<0.05$) in the case of cytoplasmic fraction of NF- κ B p65 (Figure 5B).

ODND probe- duplex formation and electrophoretic mobility shift assay (EMSA).

The formation of an oligonucleotide duplex probe with a NIR fluorophore covalently linked to it (Figure 6A,B) was initially confirmed by electrophoresis which indicated the formation of fluorescent products with mobility different from that of ODN. Mobility shift assay served as test of affinity and specificity of NF- κ B components binding to ODND in the pancreatic cell extracts (Figure 6 C,D). The binding of NF- κ B to NIR-ODND was confirmed by presence of shifted bands shown by arrowhead in Figure 6C. EMSA confirmed the presence and binding in both nuclear (wells 3–4) and cytoplasmic extracts (wells 5–6) of islet cells. Moreover, higher band intensities (i.e. NIR-ODND probe binding) were observed in nuclear and cytoplasmic extracts of islet cells of LD-STZ treated mice at study endpoint if compared to control extracts (Figure 6D). We also observed a band shift resulting from adding either nuclear, or cytoplasmic extract of exocrine (acinar) cells confirming the detection of NF- κ B activation by NIR-ODND in these cells (Supplementary Figure 4C,D).

Figure 6E summarizes the results obtained in competition tests using the added excess of non-labeled ODND or inactivated extracts (Figure 6E, lanes 2–7) which showed similar decrease of shifted band intensity in the case of the islets isolated from LD-STZ treated animals and TNF α -treated HeLa cells, i.e. a positive control containing activated NF- κ B. The presence of active, ODND binding NF- κ B p65 in the lysates isolated from the treated islets was further verified by using polyclonal anti-p65 antibody that resulted in a supershift migration of fluorescent shifted ODND complex with NF- κ B (Figure 6E, lanes 8–10).

NIR ODND detects NF- κ B in the pancreas.

After we established that NIR-ODND specifically binds to NF- κ B in islet cell extracts we examined whether NIR-ODND is capable of NF- κ B in pancreatic tissue sections. Tissue sections obtained from LD-STZ and control animals were incubated with various concentration of NIR-ODND (40–250 nM) under conditions designed maximizing transcription factor activity and minimizing non-specific interactions. As illustrated in Figure 7 higher fluorescence intensity of LD-STZ treated pancreas captured in 700 nm channel (specific for NIR Cy5.5 dye) indicated the higher level of binding of NIR ODND to LD-STZ treated pancreas compared to control ($p < 0.01$). Binding was present in cytoplasm as well as in cell nuclei and NIR-ODND showed concentration-dependent binding. To investigate, whether insulin-positive cells exhibit NIR-ODND binding, pancreas tissue sections were labeled with anti-insulin antibody. As shown in Figure 7 (panels E-H) NIR signal in the cytoplasm of islet cells was co-localized with insulin-specific fluorescence in both control and LD-STZ treated pancreas. However, LD-STZ treated pancreas exhibited higher level of NIR signal and lower insulin signal compared to control. In agreement with results obtained using NIR-ODND, digoxigenin-labeled ODND showed an overall higher level of binding to the cells of LD-STZ treated pancreas compared to control (Supplementary Figure 5 A,B). The specificity of ODND binding was additionally tested by using comparative microscopy of pancreas sections after adding an excess of the unlabeled probe (see Supplementary Figure 5C), by comparing EMSA shifts of ODND signal in the presence of the iodoacetamide-inactivated LD-STZ islet nuclear lysate or in the presence of an excess of unlabeled ODND (Supplementary Figure 5C). The microscopy showed a strong decrease of ODND signal in the islets isolated from LD-STZ treated animals (Supplementary Figures 5B vs. 5C).

NIR ODND detects NF- κ B in freshly isolated islets.

To investigate the binding of NIR ODND and its intracellular localization in freshly isolated islets and examine differential binding to LD-STZ treated and control cells, confocal optical sectioning of cells was performed. Following isolation, islets of LD-STZ treated and control mice were incubated with NIR-ODND (0.3 μ M). To determine the binding of NIR-ODND to insulin positive cells, islets were fixed and labeled with anti-insulin antibody. As shown in Figure 8 A-D, NIR-ODND bound to islet cells in both LD-STZ and control mice. However, LD-STZ treated islets contained higher number of NIR-positive cells compared to control islets. In agreement with our previous results, LD-STZ treated islets contained fewer insulin positive cells compared to control islets. Quantified NIR fluorescence signal of NIR-ODND indicated significantly higher binding of NIR ODND to LD-STZ treated islet compared to control as shown in Figure 8E ($p < 0.01$). This signal is higher in cytoplasm as well as nuclei

of LD-STZ treated islet cells compared to control (Figure 8F). Successive optical sections of islet cells acquired with 1 μ m intervals shown in Supplementary Figures 2 and 3 and Supplementary videos illustrating the localization of NIR-ODN in the cell compartments. The arrows in the enlarged images point to binding of NIR-ODND to cytoplasm and nuclei of islet cells at each optical plane. LD-STZ treated islet cells exhibit an overall higher binding of NIR-ODND in the nuclei and cytoplasm compared to control. Both control exocrine cells and cells isolated from LD-STZ treated mice showed NIR-ODND binding that was less dependent of LD-STZ treatment than in the islet cells (Supplementary Figure 4-A-B).

Discussion

There has been a concerted effort in the last decade to develop a non-invasive imaging technique for functional assessment of endocrine pancreas. Development of β -cell specific imaging probes and techniques is based on the premise that the measurable reduction of β -cell mass (BCM) known to occur over the course of the disease is concomitant with T1D progression. One promising target for potential image-guided assessment of BCM are glucagon-like peptide-1 receptor (GLP-1R) which is specifically expressed on β -cell surface. Its agonist, exendin-4 [27] has been modified to accommodate various fluorescent [28] and superparamagnetic pendant groups and as such has been used successfully for measuring β -cell loss [29]. Although no doubt useful for assessing BCM, these techniques may be limited as a stand-alone universal predictive tool for diagnosing T1D due to the inherent variability of BCM found within diseased and even healthy human pancreas [30]. It is more likely that BCM monitoring will be further developed as a personalized medicine approach enabling reliable longitudinal monitoring of BCM number in a given patient in response to therapy.

One of the earliest observable changes in humans and in rodent models of T1D is increased pancreatic vascular permeability [31, 32]. Because these changes occur prior to diabetes onset, imaging pancreatic vascular permeability has been recognized as a potential strategy for early T1D prognosis. Using fluorescent and MR-detectable imaging probes [33] in mouse and rat models [34, 35], as well as human studies [36] it has been shown that vascular leakage and probe accumulation correlate with the volume of insulinitis-induced lesions and thus have predictive and translational value in identifying subjects developing full blown T1D [36–38].

In this study, we investigated a low molecular weight near-infrared imaging probe (NIR-ODND, Figure 6) containing an NF- κ B transcription factor binding site as a potential indicator for NF- κ B activation. Our approach was supported by a previous observation showing that LD-STZ -induced diabetes could be completely abrogated following the inducible expression of a dominant-negative form of I κ B α in β -cells [11], which points to the key role of NF- κ B in mediating β -cell death and progression to diabetes in this model. It has also been shown that M1 macrophages infiltrate into the pancreas following LD-STZ treatment [39]. Macrophages exert their effects via release of soluble mediators such as oxygen free radicals, nitric oxide and other cytokines including IL-1 β and IFN- γ [40–42]. IL-1 β -mediated NF- κ B activation leads to activation of pro-inflammation genes such as iNOS (and subsequent nitric oxide formation) in addition to other pro-inflammatory genes

that mediate β -cell death (Figure 1B). Nitric oxide contributes to apoptosis of β -cells via potentiating the activity of JNK and inhibition of Akt [43]. Our own data obtained in using imaging of transgenic mice (Figure 3) showed that LD-STZ regimen did result in expression of a NF- κ B-mediated fusion reporter protein and detectable longitudinal evolvement of bioluminescent signal *in vivo*. After collecting necessary evidence *in situ* and *in vivo* that unambiguously identified the role of NF- κ B in LD-STZ-mediated type 1 diabetes model (Figures 3–5), we tested the feasibility of using an NIR-ODND for imaging changes in NF- κ B activation at the pre-diabetes stage in this model, prior to the occurrence of hyperglycemia. We observed that NIR-ODND exhibited sufficient sensitivity for detecting NF- κ B at the level of whole tissue sections (Figure 7), isolated islets (Figure 8) as well as cell fractions in both cytoplasmic and nuclear compartments of pancreatic cells (Figure 6). Furthermore, higher signal resulted from NIR-ODND binding to nuclei and nuclear fraction of pancreatic islet cells correlated with increased NF- κ B expression after LD-STZ treatment of the pancreas as assessed by IHC and Western blot analysis (Figure 5). We anticipate that NIR-ODND will be useful in differentiating between control islets and islets subjected to pro-inflammatory cytokine stimulation such as during the pre-diabetic normoglycemic stage. Considering the role of NF- κ B in mediating islet insulinitis [11], imaging of NF- κ B transcription factor activation in the pancreas and assessment of its levels may serve as a tool for detection of inflammatory processes in β -cells prior to occurrence of sever insulinitis and BCM decline in individuals at high risk of developing diabetes. Moreover, monitoring therapeutic response to anti-inflammatory interventions may be critical for ensuring that pancreatic inflammation and ensuing β -cells damage is controlled. Whereas current clinical practice relies on the evaluation of glucose tolerance and serum A1C levels for the detection of pre-diabetes, imaging of NF- κ B activity may enable a more direct assessment of early islet inflammation, thus allowing disease prevention (diet, exercise, therapeutics) to begin earlier interventions when success is more likely. Although this study was focused on diabetes, the NIR-ODND is not species specific and should be potentially useful in detection of NF- κ B transcription factor activation in other animal models of inflammatory mediated disease.

In conclusion, we demonstrate the use of an NIR-ODND which has sufficient sensitivity for detecting the increased expression of NF- κ B in a normoglycemic pancreas prior to the onset of diabetes. Because of the dual protective/destructive role played by NF- κ B in the viability of β -cells we anticipate that non-invasive imaging of NF- κ B may lead to greater understanding of the complex pathways that underlie the development of T1D. This information will be essential for in-vivo translational studies aimed at early assessment of pre-diabetic changes in the pancreas using non-invasive fluorescence imaging techniques.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AP	alkaline phosphatase
BCM	beta cell mass
ODND	oligodeoxyribonucleotide duplex
NIR-ODND	near-infrared dye labeled ODND
EMSA	electrophoretic mobility shift assay
T1D	type 1 diabetes
LD-STZ	low dose streptozocin treatment
TBS	Tris-buffered saline, 20 mM Tris, 137 mM NaCl pH 7.4
TBS-B	5% BSA, 0.1% Tween 20 in TBS
NHS	N-hydroxysuccinimide
TCEP	tris(2-carboxyethyl)phosphine
ODND-BB	10 mM Tris, 100 mM KCl, 2 mM MgCl ₂ , 10% v/v glycerol, 0.1 mM EDTA, 0.1 mg/mL tRNA, 0.25 mM DTT, pH 7.5

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Figure 1.

A. Flow chart showing the optimized scheduling of low dose streptozocin (LD-STZ) treatment of SKH1 mice for inducing activation of NF- κ B in the pancreatic islets. The IP injections of STZ were followed by islets isolation as described in Materials and Methods.

B. A schematic showing LD-STZ hypothetical treatment effect on NF- κ B-activation via excess levels of NO release and β -cell stress.

Figure 2. Blood glucose levels in mice during LD-STZ treatment of SKH1 mice. The treatment (50 mg STZ /kg/d) was performed for 5 days.

Histological and morphometric analyses of pancreatic islets of treated and control SKH1 mice are shown. **A.** Non-fasting blood glucose levels were monitored on days 1–5 and 8. No significant differences were observed between LD-STZ treated and control mice groups ($p > 0.05$). Glucose levels are presented as mean $\pm$ SD ($n=6$). **B.** IHC analysis of insulin-positive area in the pancreas. Insulin-positive area was significantly reduced 3 days after LD-STZ treatment compared to control ($n=3$; $p < 0.05$). **C.** Region-of-interest analysis of islet number per area in pancreatic sections. Islet number was significantly reduced in LD-STZ mice in comparison to controls ($n=3$; $p < 0.03$). **D-E.** Representative hematoxylin and eosin (H&E)-stained sections showing islet morphology of control (D) and LD-STZ treated (E) mice on day 8 (Scale bar 100 μ m, 20X magnification). **F-G.** Representative IHC of insulin in pancreatic sections of control (F) and LD-STZ treated (G) mice on day 8 (Scale bar 50 μ m; 40X magnification; blue- anti-insulin; red- nuclear fast red).

Figure 3. Longitudinal in vivo bioluminescence imaging of transgenic mice.

Mice carried a knock-in expression cassette with a marker fusion protein (firefly luciferase-EGFP) under the control of NF- κ B dependent enhancer/minimal HSV promoter. Representative 3D (tomographic) reconstructions of luminescent signal (coronal projections) measured in a male mouse on days 0 (before LD-STZ), day 3 (after 2 injections of STZ) and day 8 (after 5 injections of STZ) are shown in panels A, B and C, respectively. On day 8 images (panel C) the presence of several discrete areas of luminescence was noted in the upper abdomen (arrow). Panel D shows the difference in bioluminescence signal evolvement over time in the upper abdominal area in female mice (red trace) and male mice (blue trace). Luminescence photon flux was measured omni-directionally and expressed in photons/s. Panels E and F show representative immunohistochemistry images of the islet and acinar cells in the male (E) and female (F) mouse frozen pancreas sections stained with primary anti-EGFP antibody (1:500) followed by anti-rabbit -alkaline phosphatase conjugate (1:1000, blue) and counterstained with nuclear fast red (pink). The islets are shown by black arrows. Bar=100 μ m.

Figure 4. Analysis of total and phosphorylated NF-κB p65 following LD-STZ treatment endpoint in tissue sections.

A-D Representative immunohistochemistry of anti-NF-κB p65 (phospho S536) in the pancreas of control (A-B) and LD-STZ treated (C-D) mice. Enlarged views of cells marked with yellow squares are shown in (B) and (D), bar=50 μm; magnification – 40x. Blue- anti-NF-κB p65; red- nuclear fast red counterstain. **E-H**. Representative immunofluorescent images of anti-NF-κB staining of control (E-F) and LD-STZ treated (G-H) pancreas sections using primary anti-NF-κ p65-phospho S276 antibody and secondary antibody conjugated with Alexa Fluor 555. Enlarged views of islet cells are shown in (F) and (H). Scale bar=25 μm; magnification- 40x; red - anti-NF-κB; blue- DAPI. Arrows point to NF-κB-specific fluorescence in the nuclei of LD-STZ treated pancreas sections. **I-J**. Fluorescence signal quantification in the pancreas labeled with anti- NF-κB p65-phospho S276 antibody. LD-STZ treated pancreas exhibited significantly higher level of NF-κB expression in the islets (I). This overall increase in islets could be accounted for by increases in both nuclei and cytoplasm (J) of the individual islet cells compared to control. At least 700 islet cells were analyzed in each group.

Figure 5. NF-κB p65 expression in the islets and acinar cells before and after LD-STZ treatment. Cytoplasmic and nuclear extracts of analyzed by Western blotting.

A – western blot with anti- NF-κB p65 antibody and nuclear fraction of islet and acinar cells. The arrowhead points to NF-κB p65 band. Nuclear extracts: lane 1- TNFα-treated HeLa cell extract (positive control); lane 2 and 4 – islets, control group, lanes 3 and 5 – islets, LD-STZ treated group; lanes 6 and 8- acinar cells, control group; lane 7 and 9- acinar cells, LD-STZ treated group. Below – Western blot obtained by using anti-GAPDH for signal normalization. **B** – bar graphs showing the results of quantification and normalization of band intensities (n=3, a representative blot is shown in panel A). NF-κB p65 band intensities were determined for nuclear and cytoplasmic extracts and normalized by GAPDH signals in the same cell isolates. Three sets of blotting quantification data obtained in two independent LD-STZ treatment experiments are summarized in panel B.

Figure 6. ODN probe properties and binding to the components of LD-STZ treated vs. control cell extracts.

A. Molecular model of the near-infrared ODN duplex probe (NIR-ODND) with a single NF- κ B consensus binding site carrying a Cy5.5 fluorophore on one of the ODN strands. **B-** electrophoretic mobility (4–20% polyacrylamide gradient /TBE) of the NIR-ODND and single ODNs. ODND was labeled with either Cy5.5 (lane 3, red) or 800CW (lane 4, green). The migration of single strand NIR-fluorophore labeled ODNs serve as controls and are shown in lanes 1 and 2. **C-** electrophoretic mobility shift assay (EMSA, 10% PAGE):

quantitative analysis of shifts using Cy5.5-labeled ODND (fluorescence emission – 700 nm) as a probe for activated NF- κ B in pancreatic extracts collected on day 8. NIR-ODND was incubated in the presence of: lane 1- none; lane 2- HeLa nuclear extract (positive control); lane 3- islet nuclear extract-control; lane 4 - islet nuclear extract-LD-STZ; lane 5- islet cytoplasmic extract-control; lane 6- islet cytoplasmic extract-LD-STZ. The arrowhead points to the shifted ODN bands. **D**-quantification of fluorescence intensity of shifted bands due to NIR-ODND banding to the components of islet extracts. **E**-electrophoretic mobility shift assay for testing the specificity of NIR-ODND binding: lane 1- Cy5.5-labeled ODND, lane 2- HeLa nuclear extract; lane 3 – HeLa and excess of non-labeled ODND; lane 4: inactive HeLa extract; lane 5- LD-STZ treated islet extract; lane 6- LD-STZ treated islet extract and excess of non-labeled ODND; lane 7- inactive LD-STZ treated islet extract. For testing band supershift in the presence of anti- NF- κ B p65 antibody Cy3-labeled ODND was analyzed on 7.5% PAGE gels: lane 8- ODND only, lane 9 - LD-STZ treated islet extract; lane 10 - LD-STZ treated islet extract and anti- NF- κ B p65 antibody.

Figure 7. Microscopy of nuclear and cytoplasmic NF- κ B in pancreatic sections by using NIR-fluorophore labeled ODND.

A-D. Control (A-B) and LD-STZ treated (C-D) pancreas. Enlarged view is shown in (B) and (D). Arrows point to nuclear binding of NIR probe. Nuclear translocation of NF- κ B results in magenta after merging red and blue pseudo color channels shown in LD-STZ treated pancreas (C-D) (NIR ODND: 40nM; scale bar 25 μ m; 40X magnification; red- NIR-ODND; blue -DAPI). **E-H.** Co-localization of NIR-ODND with insulin in tissue of control (E-F) and LD-STZ treated (G-H) pancreata on day 8. Enlarged view is shown in (F) and (H). LD-STZ treatment results in NF- κ B expression and a stronger NIR signal (**I**; $P < 0.01$) as well as in a decrease in insulin content compared to control. (NIR-ODND: 250nM; scale bar 25 μ m; 40X magnification; red - NIR-ODND; green- anti-insulin antibody; blue - DAPI).

Figure 8. Confocal microscopy of islets isolated from LD-STZ treated and control pancreas. Freshly isolated islets were incubated with NIR-ODND (0.3 μ M), fixed and labeled with anti-insulin antibody. **A-B.** Control islet. **C-D.** LD-STZ treated islet (scale bar 20 μ m; red - NIR ODND; green- anti-insulin antibody; blue - DAPI). Enlarged views of islet cells are shown in (B) and (D). LD-STZ treated islets contain insulin-positive cells with lower insulin content and a higher number of NIR positive cells compared to the control. Arrows point to the binding of NIR-ODND to islet cells with various insulin content. **E-F.** Quantification of NIR-ODND fluorescence calculated using two confocal planes positioned 5 μ m apart. LD-STZ treated islets show a higher signal compared to control islets (E). Fluorescence signal was higher ($P<0.01$) in the nuclei and cytoplasm of the LD-STZ treated islet compared to control islets (F).