Dual Radiotracer Analysis of Cholinergic Neuronal Changes in Prediabetic Mouse Pancreas

Paige B. Clark, M.D.,¹ Michael J. Plaza, M.D.,¹ Jonathan Kraas, M.D.,¹ Nicole Burbank, M.D.,¹ Allen W. Elster II, B.S.,¹ Pradeep Garg, Ph.D.,¹ Sudha Garg, Ph.D.,¹ H. Donald Gage, Ph.D.,¹ Jorge Calles-Escandon, M.D.,² Janice D. Wagner, D.V.M., Ph.D.,³ and Kathryn Morton, M.D.⁴

Abstract

Background: Pancreatic neuronal changes associated with beta cell loss in type 1 diabetes mellitus are complex, involving, in part, parasympathetic mechanisms to compensate for preclinical hyperglycemia. The parasympathetic neurotransmitter acetylcholine (ACh) mediates insulin release via M3 muscarinic receptors on islet beta cells. The vesicular ACh transporter (VAChT) receptor has been shown to be a useful marker of cholinergic activity in vivo. The positron emission tomography (PET) radiotracer (+)-4-[¹⁸F]fluorobenzyltrozamicol ([¹⁸F]FBT) binds to the VAChT receptor on presynaptic cholinergic neurons and can be quantified by PET. The compound 4-diphenylacetoxymethyl-N-methylpiperidine (4-DAMP), available in a tritiated form, binds to M3 muscarinic receptors on beta cells and is a potential target for assessing pancreatic beta cell mass. In this study, we investigate the feasibility of dual radiotracer analysis in identifying neurofunctional changes that may signify type 1 diabetes mellitus in its early preclinical state.

Methods: Ex vivo determinations of pancreatic uptake were performed in prediabetic nonobese diabetic mice and controls after intravenous injection of [¹⁸F]FBT or 4-[³H]DAMP. Beta cell loss in prediabetic mice was confirmed using immunohistochemical methods.

Results: [¹⁸F]FBT uptake was significantly higher in prediabetic pancreata than controls: 3.22 ± 0.81 and 2.51 ± 1.04 , respectively ($P < 0.03$). 4-[³H]DAMP uptake was significantly lower in prediabetic pancreata than controls: 0.612 ± 0.161 and 0.968 ± 0.364 , respectively ($P = 0.01$).

Conclusions: These data suggest that a combination of radiotracer imaging agents that bind to neuronal elements intimately involved in insulin production may be an effective method of evaluating changes associated with early beta cell loss using PET.

Introduction

INSULIN SECRETION by pancreatic beta cells is under the complex control of serum glucose, circulating neuropeptides, and the parasympathetic autonomic nervous system. As shown in several animal models, stimulation of parasympathetic cholinergic nerve fibers releases the neurotransmitter acetylcholine (ACh), which activates M3 muscarinic receptors on beta cells, leading to insulin release.¹ Markers of cholinergic activity are 10 times higher in the endocrine pancreas compared to the exocrine pancreas, confirming a major role for cholinergic control of endocrine pancreatic function.^{2,3}

Studies of cholinergic activity in the endocrine pancreas to date have been largely invasive or in vitro. For example, stud-

ies interrogating cholinergic influence on the endocrine pancreas include cross-perfused animal models, electrical stimulation of the vagus nerve, and administration of pharmacologic antagonists. In vitro studies to elucidate in vivo neuronal response on isolated islets are complicated by nonphysiologic laboratory conditions. In addition, the neuronal activity and innervation of the endocrine pancreas are highly adaptable to various conditions, e.g., islets transplanted in liver develop innervation similar to that in the endocrine pancreas.

Neuronal receptor-ligand positron emission tomography (PET) imaging of cholinergic activity has been studied extensively in the central nervous system with good success.⁴⁻⁹ However, few have applied neurofunctional radiotracers to the study of the peripheral nervous system, which includes

Departments of ¹Radiology, ²Internal Medicine, and ³Pathology/Comparative Medicine, Wake Forest University Health Sciences, Winston-Salem, North Carolina.

⁴Department of Radiology, University of Utah Hospitals and Clinics, Salt Lake City, Utah.

autonomic control of the richly innervated pancreas.^{10–13} The goal of this project is to adapt the use of tracers used primarily in the central nervous system to develop a strategy that will ultimately allow an *in vivo*, radiotracer-based method of imaging cholinergic function by PET to identify type 1 diabetes mellitus in its earliest stages, when therapeutic interventions may be most effective.

There is evidence that cholinergic innervation is reduced as a late event in type 1 diabetes, but that enhanced cholinergic activity in the pancreas may actually precede other biochemical or clinical signs of the disease.^{14,15} This may occur as an early compensatory mechanism to maintain insulin production in the face of dwindling numbers of functional beta cells early in the disease.

The PET radiotracer (+)-4-[¹⁸F]fluorobenzyltrozamicol ([¹⁸F]FBT) binds to active vesicular ACh transporter (VACHT) receptors on presynaptic cholinergic neurons. This tracer has been most extensively studied in the brain, where increased uptake of [¹⁸F]FBT reflects increased activation of VACHT receptors.^{5,8,9} The VACHT is responsible for loading presynaptic vesicles with the neurotransmitter ACh and has been shown to be a reliable marker of acetylcholine activity *in vivo*.¹⁶ We have previously reported that [¹⁸F]FBT localizes in the pancreas.¹⁰ Therefore, [¹⁸F]FBT may be a suitable tracer to detect pancreatic neurofunctional changes in preclinical diabetes with PET.

ACh-mediated release of insulin occurs through activation of M3 muscarinic receptors located on beta cells.^{17,18} Diminished insulin release and impaired glucose tolerance have been demonstrated in knockout mice lacking the pancreatic M3 receptor.¹ Immunohistochemical staining of rat pancreas with antibodies to the M3 muscarinic receptor shows preferential accumulation in the islets compared to the exocrine pancreas.¹⁷ The compound 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP) binds to the M3 muscarinic receptor *in vivo*.¹⁹ While M3 muscarinic PET ligands are not commercially available, the use of 4-DAMP (4-[³H]DAMP) allows quantification of M3 muscarinic binding in organs and may indicate a direction for the subsequent development M3 radiotracers for noninvasive PET imaging. As the M3 muscarinic receptor is present on beta cells themselves, 4-[³H]DAMP activity in the pancreas may correlate with beta cell mass.

An *in vivo* imaging method that could detect ACh activity and M3 muscarinic binding in the pancreas may allow detection of type 1 diabetes in its preclinical stages. The development of such an imaging strategy would provide a valuable research tool in defining the natural course of the disease and in the development of novel therapies, which are likely to be most effective early in the course of the disease. The hypothesis of this project is that increased pancreatic binding of [¹⁸F]FBT and decreased binding of 4-[³H]DAMP will identify type 1 diabetes in its earliest preclinical stages. To test this hypothesis, binding of the two radiotracers was compared in type 1 prediabetic nonobese diabetic (NOD) mice and in control (NOD/SCID) mice.

Materials and Methods

Ethics

Institutional Animal Care and Use Committee approval was obtained.

Mouse model of type 1 diabetes mellitus

NOD mice undergo a progressive, autoimmune-mediated loss of insulin-producing beta cells (insulinitis) similar to type 1 diabetes in humans. By 18 weeks of age, approximately 80% of female NOD mice are diabetic. The NOD/SCID mouse is the control strain. It is genetically similar to the NOD mouse, but has no functional T-cells, which mediate autoimmune insulinitis.²⁰

Blood glucose determinations

Blood glucose was measured by a glucometer. Normal glucose levels for mice are 75–143 mg/dL (fasting). NOD mice with normal fasting blood glucose levels were considered euglycemic.

Animal preparation

Food and water were withheld 4 h prior to experiments. Induction of anesthesia was achieved using intramuscular ketamine (90–150 mg/kg).⁷

Quantification of [¹⁸F]FBT uptake

[¹⁸F]FBT was synthesized following a previously published procedure.²¹ In brief, [¹⁸F]fluoride was reacted with the quaternary salt of dimethylamino benzaldehyde. The resulting 4-[¹⁸F]fluorobenzaldehyde was reacted with lithium borohydride, followed by its conversion to 4-[¹⁸F]fluorobenzyl iodide using hydrogen iodide. The precursor *des*-fluorobenzyl-FBT (trozamicol; 1 mg) in 250 μ L of dimethylformamide was added to a solution of 4-[¹⁸F]fluorobenzyl iodide in 5 mL of pentane, which was subsequently evaporated. The reaction mixture was injected onto a reverse-phase high-performance liquid chromatography column, and [¹⁸F]FBT was isolated using 0.02 M ammonium acetate/methanol (30:70 vol/vol). [¹⁸F]FBT was produced with >95% radiochemical purity as determined by high-performance liquid chromatography.

Female NOD ($n = 21$) and NOD/SCID ($n = 22$) mice were used in these experiments. Each mouse (average weight 20 g) was injected in the tail vein with 50 μ Ci of [¹⁸F]FBT. Preliminary studies by our lab have shown that [¹⁸F]FBT activity within the pancreas reaches a peak and is stable between 45 and 75 min. At 60 min postinjection, mice were euthanized by decapitation, and organs were harvested, weighed, and counted for radioactivity in a gamma well counter (Packard Minaxi γ Auto-Gamma 5000 series, Packard Instruments Co., Downers Grove, IL). Data were reported as counts per minute (cpm) and corrected for radioactive decay. Data are presented as mean $\pm$ standard deviation values. Data were subjected to statistical analysis using a Wilcoxon two sample test. Values of $P < 0.05$ were considered significant. Percentage of injected dose (ID) per gram of organ was calculated using the following formula:

$$\frac{\text{Tissue uptake } (\mu\text{Ci})/\text{g of tissue}}{\text{Total ID } (\mu\text{Ci})} \times 100 = \% \text{ID/g}$$

Differential uptake ratio (DUR) values were calculated using the following formula:

$$\frac{\% \text{ID/g} \times \text{body weight (g)}}{100} = \text{DUR}$$

TABLE 1. CHARACTERISTICS OF MICE EVALUATED WITH [^{18}F]FBT, WHICH BINDS TO THE VACHT RECEPTOR

	Control (NOD/SCID)	Prediabetic NOD
Pancreatic [^{18}F]FBT uptake (DUR)	2.51 ± 1.04^a	3.22 ± 0.81^a
Fasting serum glucose (g/dL)	118 (92–132)	120 (98–142)
Beta cells (% of control)	100	75–85
Body weight (g)	23.3 ± 2.56	24.4 ± 1.75
Number of mice	22	21

^a $P < 0.03$.

Quantification of 4-[^3H]DAMP uptake

4-[^3H]DAMP was obtained commercially from Perkin-Elmer Life and Analytical Sciences (Shelton, CT). Female NOD ($n = 13$) and NOD/SCID ($n = 5$) mice were used in these experiments. Euthanasia and necropsy were performed on mice 60 min after tail vein injection of 50 μCi of 4-[^3H]DAMP. Pancreata were harvested, weighed, and individually solubilized using Soluene[®]-350 (Packard) and added to a liquid scintillation cocktail. Radioactivity in the specimens was quantified by liquid scintillation counting in a Beckman Coulter (Foster City, CA) LS 6500 multipurpose scintillation counter. Quench correction was applied as well as correction for sample size and background counts. Tissue uptake was expressed as a DUR: (cpm recovered/g of tissue)/(cpm injected/g of body mass).¹⁹ An unpaired t test analysis was used for comparison of biodistribution data between normal and prediabetic mice. Values of $P < 0.05$ were considered significant.

Quantification of beta cells

Pancreatic tissue was fixed in 4% paraformaldehyde and paraffin-embedded. Immunohistochemistry was performed on 4- μm sections using a rabbit polyclonal cocktail containing antibodies to glucagon (diluted 1:500, DAKO Corp., Carpinteria, CA), pancreatic polypeptide (diluted 1:500, DAKO Corp.), and somatostatin (diluted 1:1,000, ImmunoStar, Inc., Hudson, WI). Five to 10 islets per mouse were evaluated. Beta and non-beta islet cells were counted. The number of beta cells was divided by the total number of islet cells. These values were averaged and recorded. The number of beta cells in prediabetic NOD mice was expressed as a percentage of the number of beta cells in normal controls.²²

Results

[^{18}F]FBT pancreatic uptake in prediabetic mice

Table 1 shows [^{18}F]FBT data for both control and prediabetic mice (mean body weight, blood glucose level, percentage of islet beta cells present, and pancreatic [^{18}F]FBT uptake). Note that prediabetic NOD mice are normoglycemic, yet they have lost 15–25% of pancreatic beta cells compared with controls. The mean pancreatic [^{18}F]FBT uptake in prediabetic NOD mice (3.22 ± 0.81) was statistically higher than in nondiabetic (control) NOD/SCID mice (2.51 ± 1.04) ($P < 0.03$). Both prediabetic NOD and control mice were normoglycemic.

4-[^3H]DAMP pancreatic uptake in prediabetic mice

Table 2 shows 4-[^3H]DAMP data associated with control and prediabetic NOD mice (mean body weight, blood glucose level, percentage of islet beta cells present, and pancreatic 4-[^3H]DAMP uptake). Note that prediabetic NOD mice are normoglycemic, yet they have lost 15–25% of pancreatic beta cells compared with controls. The average pancreatic 4-[^3H]DAMP uptake in prediabetic NOD mice was significantly lower (0.612 ± 0.161) than in nondiabetic (control) NOD/SCID mice (0.968 ± 0.364) ($P = 0.01$). Both prediabetic NOD and control mice were normoglycemic.

Discussion

The results of this study support that noninvasive imaging of pancreatic cholinergic activity and M3 receptor density may be a viable strategy for evaluating pancreatic beta cell function and mass, respectively. Total pancreatic [^{18}F]FBT binding is increased and 4-[^3H]DAMP binding is decreased in prediabetic NOD mice when compared to controls. The prediabetic state was confirmed in the NOD/SCID

TABLE 2. CHARACTERISTICS OF MICE EVALUATED WITH 4-[^3H]DAMP, WHICH BINDS TO THE M3 MUSCARINIC RECEPTOR

	Control (NOD/SCID)	Prediabetic NOD
Pancreatic 4-[^3H]DAMP uptake (DUR)	0.968 ± 0.364^a	0.612 ± 0.161^a
Fasting serum glucose (g/dL)	114 (99–124)	108 (93–126)
Beta cells (% of control)	100	75–85
Body weight (g)	21.3 ± 1.86	21.5 ± 2.41
Number of mice	5	13

^a $P = 0.01$.

mouse by demonstration of a 15–25% loss in pancreatic beta cells compared with controls. All mice were normoglycemic.

An inverse relationship between pancreatic [^{18}F]FBT and 4- ^{3}H]DAMP uptake is logical in light of several physiological considerations. The presynaptic cholinergic vesicles, to which FBT binds, are located in intrapancreatic neural elements that are not part of the beta cells themselves. Increased FBT binding likely reflects a compensatory up-regulation of cholinergic activity due to early loss of beta cell mass or diminished insulin production. Whether this enhanced presynaptic cholinergic activity is due to an increased number of presynaptic vesicles or an increased affinity for binding of the substrate is unknown. The binding of 4- ^{3}H]DAMP occurs on M3 receptors located on the beta cells themselves. Decreased 4- ^{3}H]DAMP binding in preclinical type 1 diabetes likely reflects a loss of beta cell mass and receptor number through an autoimmune process. However, whether the density of M3 receptors on each remaining beta cell remains constant during the development of type 1 diabetes remains to be determined.

It should be noted that both the endocrine and exocrine portions of the pancreas are modulated by the autonomic nervous system. Approximately 2% of pancreatic mass is devoted to endocrine function. However, ACh-related neuronal activity is 10 times more abundant in these regions when compared to the exocrine pancreas.^{22,23} The majority of cholinergic activity appears focused on modulating beta cell insulin secretion, glucose tolerance, and beta cell proliferation since beta cells are the predominant cell type in the islets.²⁴ Enhanced cholinergic activity in the face of falling insulin production may actually precede other biochemical or clinical signs of type 1 diabetes. Later in the course of the disease, cholinergic innervation of the pancreas is reduced.^{14,15} Therefore, an analysis of the ratio of [^{18}F]FBT to 4- ^{3}H]DAMP binding in the pancreas may actually unveil the compensatory reserve of the pancreas in preclinical type 1 diabetes as well as providing a more sensitive way to diagnose the disease in its earliest stages. Such an approach will also facilitate the development and monitor the efficacy of novel therapies to treat patients with early type 1 diabetes prior to the onset of clinically apparent signs of irreversible disease. The results of this ex vivo study encourage a future commitment to the development of radiopharmaceuticals such as [^{18}F]FBT and positron-labeled 4-DAMP to allow PET imaging of the pancreas.

Acknowledgments

This study was supported by grant DK67247 from the National Institutes of Health. The authors thank Kineka Hull, Kimberly Black, Holly Smith, Stephanie Rideout, and Hermina Borgerink for their contribution to this work.

Author Disclosure Statement

No competing financial interests exist.

References

- Gautam D, Han S-J, Hamdan FF, Jeon J, Li B, Li JH, Cui Y, Mears D, Lu H, Deng C, Heard T, Wess J: A critical role for beta cell M3 muscarinic acetylcholine receptors in regulating insulin release and blood glucose homeostasis in vivo. *Cell Metab* 2006;3:449–461.
- Kiba T: Relationships between the autonomic nervous system and the pancreas including regulation of regeneration and apoptosis. *Pancreas* 2004;29:e51–e58.
- Brunicaudi FC, Shavelle DM, Andersen DK: Neural regulation of the endocrine pancreas. *Int J Pancreatol* 1995;18:177–195.
- Efange SMN, Garland EM, Staley JK, Khare AB, Mash DC: Vesicular acetylcholine transporter density and Alzheimer's disease. *Neurobiol Aging* 1997;18:407–413.
- Efange SMN: In vivo imaging of the vesicular acetylcholine transporter and the vesicular monoamine transporter. *FASEB J* 2000;14:2401–2413.
- Gage HD, Gage JC, Chiari A, Xu Z-M, Mach RH, Efange SMN, Ehrenkauf RLE, Eisenach JC: In vivo imaging of the spinal cord cholinergic system with PET. *J Comput Assist Tomogr* 1999;23:25–33.
- Gage HD, Voytko ML, Ehrenkauf RLE, Tobin JR, Efange SMN, Mach RH: Reproducibility of repeated measures of cholinergic terminal density using [^{18}F](+)-4-fluorobenzyltrozamicol and PET in the rhesus monkey brain. *J Nucl Med* 2000;41:2069–2076.
- Mach RH, Voytko ML, Ehrenkauf RLE, Nader MA, Tobin JR, Efange SMN, Parsons SM, Gage HD, Smith CR, Morton TE: Imaging of cholinergic terminals using the radiotracer [^{18}F](+)-4-fluorobenzyltrozamicol: in vitro binding studies and positron emission tomography studies in nonhuman primates. *Synapse* 1997;25:368–380.
- Voytko ML, Mach RH, Gage HD, Ehrenkauf RLE, Efange SMN, Tobin JR: Cholinergic activity of aged rhesus monkeys revealed by positron emission tomography. *Synapse* 2001;39:95–100.
- Clark PB, Gage HD, Brown-Proctor C, Buchheimer N, Calles-Escandon J, Mach RH, Morton KA: Neurofunctional imaging of the pancreas utilizing the cholinergic PET radioligand [^{18}F]4-fluorobenzyltrozamicol. *Eur J Nucl Med Mol Imaging* 2004;31:258–260.
- Ribeiro MJ, Boddaert N, Delzescaux T, Valayannopoulos V, Bellanné-Chantelot C, Jaubert F, Verkarre V, Nihoul-Fékété C, Brunelle F, De Lonlay P: Functional imaging of the pancreas: the role of [^{18}F]fluoro-L-DOPA PET in the diagnosis of hyperinsulinism of infancy. *Endocr Dev* 2007;12:55–66.
- Kauhanen S, Seppänen M, Minn H, Gullichsen R, Salonen A, Alanen K, Parkkola R, Solin O, Bergman J, Sane T, Salmi J, Välimäki M, Nuutila P: Fluorine-18-L-dihydroxyphenylalanine (^{18}F -DOPA) positron emission tomography as a tool to localize an insulinoma or beta-cell hyperplasia in adult patients. *J Clin Endocrinol Metab* 2007;92:1237–1244.
- Simpson NR, Souza F, Witkowski P, Maffei A, Raffo A, Heron A, Kilbourn M, Jurewicz A, Herold K, Liu E, Hardy MA, Van Heertum R, Harris PE: Visualizing pancreatic beta-cell mass with [^{11}C]DTBZ. *Nucl Med Biol* 2006;33:855–864.
- Ostenson C-G, Grill V: Evidence that hyperglycemia increases muscarinic binding in pancreatic islets of the rat. *Endocrinology* 1987;121:1705–1710.
- Komabayashi T, Sawada H, Izawa T, Kogo H: Altered intracellular Ca^{2+} regulation in pancreatic acinar cells from acute streptozotocin-induced diabetic rats. *Eur J Pharmacol* 1996;298:299–306.
- Van der Kloot W: Loading and recycling of synaptic vesicles in the *Torpedo* electric organ and the vertebrate neuromuscular junction. *Prog Neurobiol* 2003;71:269–303.
- Iismaa TP, Kerr EA, Wilson JR, Carpenter L, Sims N, Biden TJ: Quantitative and functional characterization of muscarinic receptor subtypes in insulin-secreting cell lines and rat pancreatic islets. *Diabetes* 2000;49:392–398.

18. Duttaroy A, Zimlikli CL, Gautam D, Cui Y, Mears D, Wess J: Muscarinic stimulation of pancreatic insulin and glucagon release is abolished in m3 muscarinic acetylcholine receptor-deficient mice. *Diabetes* 2004;53:1714–1720.
19. Van Waarde A, Visser GM, Visser TJ, Bouwer J, Paans AMJ, Vaalburg W: Rodent biodistribution and metabolism of tritiated 4-DAMP, a M3 subtype-selective cholinergic ligand. *Nucl Med Biol* 1994;21:41–47.
20. Eisenbarth GS: Type I diabetes mellitus. A chronic autoimmune disease. *N Engl J Med* 1986;314:1360–1368.
21. Efange SM, Mach RH, Khare A, Michelson RH, Nowak PA, Evora PH: [¹⁸F]Fluorobenzyltrozamicol ([¹⁸F]FBT): molecular decomposition-reconstitution approach to vesamicol receptor radioligands for positron emission tomography. *Appl Radiat Isot* 1994;45:465–472.
22. Gilon P, Henquin J-C: Mechanisms and physiological significance of the cholinergic control of pancreatic beta cell function. *Endocr Rev* 2001;22:565–604.
23. Ahren B: Autonomic regulation of islet hormone secretion: implications for health and disease. *Diabetologia* 2000; 43:393–410.
24. Godfrey DA, Matschinsky FM: Enzymes of the cholinergic system in islets of Langerhans. *J Histochem Cytochem* 1975;23:645–651.

Address correspondence to:

Paige B. Clark, M.D.

Assistant Professor

Department of Radiology/Nuclear Medicine

Wake Forest University Health Sciences

Medical Center Boulevard

Winston-Salem, NC 27157

E-mail: pbclark@wfubmc.edu