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Development of an ultrasensitive immunoassay using affinity maturated antibodies for the measurement of rodent insulin

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ABSTRACT

The measurement of plasma insulin is important for clinical diagnosis of diabetes and for preclinical research of metabolic diseases, especially in rodent models used in drug discovery research for type 2 diabetes. Fasting immunoreactive insulin (F-IRI) concentrations are used to calculate the homeostasis model assessment ratio (HOMA-R), an index of insulin sensitivity. However, even the most sensitive commercially available enzyme-linked immunosorbent assay (ELISA) kits cannot measure the very low F-IRI concentrations in normal rats and mice. Therefore, we sought to develop a new rodent insulin ELISA with greater sensitivity for low F-IRI concentrations. Despite repeated efforts, high-affinity antibodies could not be generated by immunizing mice with mouse insulin (self-antigen). Therefore, we generated two weak monoclonal antibodies (13G4 and 26B2) that were affinity maturated and used to develop a highly sensitive ELISA. The measurement range of the sandwich ELISA with the affinity maturated antibodies (13G4m1 and 26B2m1) was 1.5 to 30,000 pg/ml, and its detection limit was at least 10 times lower than those of commercially available kits. In conclusion, we describe the development of a new ultrasensitive ELISA suitable for measuring very low plasma insulin concentrations in rodents. This ELISA might be very useful in drug discovery research in diabetes.

Type 2 diabetes is a metabolic disorder characterized by high blood glucose in the context of insulin resistance and relative insulin deficiency. The metabolic manifestations of type 2 diabetes include hyperglycemia, which results from insulin resistance in peripheral tissues and inadequate secretion of insulin [1], and impaired suppression of glucagon secretion in response to glucose ingestion. Thus, type 2 diabetes involves at least two primary pathogenic mechanisms: (i) a progressive decline in pancreatic islet function, resulting in a reduction in insulin secretion and inadequate suppression of glucagon secretion [2,3], and (ii) peripheral insulin resistance, which decreases the metabolic responses to insulin [4].

Insulin resistance in type 2 diabetes is characterized by hyperglycemia and hyperinsulinemia, and the homeostasis model assessment ratio (HOMA-R),[2] the product of fasting plasma glucose and fasting immunoreactive insulin (F-IRI), is often used as an index of insulin sensitivity [5]. As peripheral insulin resistance increases, the demand for insulin to suppress blood glucose also increases, resulting in elevated F-IRI and HOMA-R. Thus, high HOMA-R values are indicative of insulin resistance. HOMA-R is widely used in clinical settings [6] and in preclinical research [7] because of the convenient, noninvasive sample collection and testing methods. Rodent models are commonly used in preclinical research of type 2 diabetes, but the F-IRI concentration is very low in normal rodents and is often undetectable using existing enzyme-linked immunosorbent assay

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[2] *Abbreviations used:* HOMA-R, homeostasis model assessment ratio; F-IRI, fasting immunoreactive insulin; ELISA, enzyme-linked immunosorbent assay; KLH, keyhole limpet hemocyanin; PEG, polyethylene glycol; IgG, immunoglobulin G; HRP, horseradish peroxidase; PBST, phosphate-buffered saline supplemented with Tween 20; TMB, tetramethyl benzidine; cDNA, complementary DNA; VL, variable light; VH, variable heavy; PCR, polymerase chain reaction; Igκ, immunoglobulin kappa; HCDR3, heavy chain complementarity determining region 3; IPTG, isopropyl β-D-1-thiogalactopyranoside; ALP, alkaline phosphatase; HPLC, high-performance liquid chromatography; CV, coefficient of variation.

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(ELISA) kits. Indeed, in the Results section of this article, we report that two very sensitive assay kits with nominal detectable limits of less than 100 pg/ml were unable to measure the plasma insulin concentration in normal lean mice after an overnight fast (~50 pg/ml; see Fig. 4 in Results), consistent with previous reports [8–11]. Therefore, it is not possible to calculate HOMA-R in such cases when using the currently available assays. To aid the discovery of antidiabetic drugs, it is important to determine HOMA-R to estimate the severity of insulin resistance in diabetic rodent models compared with normal rodents and to evaluate the efficacy of novel drugs in terms of improvements in insulin sensitivity. Thus, there is a need to measure low plasma insulin concentrations in normal rodents, and the development of more sensitive insulin ELISAs will facilitate drug discovery research in diabetes.

Insulin is the principal hormone responsible for the control of glucose metabolism. Its precursor, proinsulin, is synthesized in the beta cells of the islets of Langerhans in the pancreas and is processed to form C peptide and insulin. Both are secreted in equimolar amounts into the portal circulation. The mature insulin molecule comprises two polypeptide chains, A and B (21 and 30 amino acids, respectively), which are linked by two disulfide bridges. In addition, a third disulfide bridge joins two amino acids in the A chain. In most species the insulin gene exists in a single copy, whereas rats and mice have two closely related genes that produce two non-allelic insulins: insulin I and insulin II [12]. In rats insulin I is more abundant than insulin II, whereas in mice the opposite is true [13]. To establish an ELISA that is able to measure insulin concentrations in the mouse and rat models frequently used in drug discovery research in diabetes, it is necessary to develop antibodies that target insulin I and insulin II equally.

In this study, we sought to develop an ELISA with a detection limit of less than 10 pg/ml in order to measure the low plasma insulin concentrations in healthy rodents and in rodent models of diabetes.

Materials and methods

Materials

N-terminal peptide (7mer: FVKQHLC) and C-terminal peptide (12mer: CGERGFFYTPKS) of mouse insulin B chain were synthesized by Sigma–Aldrich Japan (Tokyo, Japan) and conjugated to maleimide-activated keyhole limpet hemocyanin (KLH; Thermo Scientific, Waltham, MA, USA) as the immunogen. Two highly sensitive mouse insulin ELISA kits (Mouse Insulin ELISA Kit [U-type, ultrasensitive] and Ultra Sensitive PLUS Mouse Insulin ELISA Kit) were purchased from Shibayagi (Shibukawa, Japan) and Morinaga (Yokohama, Japan) and are referred to as Kits A and B, respectively, in this study (Table 4). Mouse insulin I recombinant protein was internally prepared according to the method reported by Goeddel and coworkers [14] and biotinylated using Sulfo-NHS-LC-Biotin (Thermo Scientific). Mouse insulin II and rat insulin (whose type was not disclosed) were purchased from Morinaga as the standard proteins in the respective ELISA kits and were used to evaluate cross-reactivity.

Establishment of anti-mouse insulin monoclonal antibodies

Monoclonal antibodies were established according to the method reported by Kohler and Milstein [15]. Female 4- to 6-week-old Balb/c mice were intraperitoneally administered with 0.1 mg of the KLH-conjugated peptide emulsified in complete Freund's adjuvant (Difco/Becton Dickinson, Franklin Lakes, NJ, USA). The mice were immunized four times at 3-week intervals with an equal amount of the KLH-conjugated peptide emulsified in incomplete Freund's adjuvant. Eight days after the last immunization, the mice were boosted with 100 μg of the adjuvant-free antigen. Three days after immunization, splenocytes were aseptically collected and fused with P3U1 murine myeloma cells using 50% polyethylene glycol (PEG) 4000. Hybridomas were selected in hypoxanthine/aminopterin/thymidine medium. Ten days later, the culture supernatants of hybridoma cells were screened as follows. First, 384-well MaxiSorp plates (Nunc/Thermo Scientific) were coated with goat anti-mouse immunoglobulin G (IgG; Shibayagi) and blocked with 1% BlockAce (Dainippon Pharmaceuticals, Osaka, Japan). The culture supernatants were added to individual wells and incubated with the biotinylated insulin and streptavidin-conjugated horseradish peroxidase (streptavidin–HRP; Thermo Scientific) overnight at 4 °C. The wells were washed with phosphate-buffered saline supplemented with Tween 20 (PBST). Then, tetramethyl benzidine (TMB) substrate solution (DAKO, Tokyo, Japan) was added to each well for the colorimetric assay. Cells in positive wells were cloned twice by limiting dilution, and the immunoglobulin class and subclass were determined using a Mouse Immunoglobulin Isotyping ELISA Kit (Becton Dickinson) according to the manufacturer's instructions. The established hybridomas were expanded in the ascites of Balb/c mice, and monoclonal antibodies were purified from the ascites using Protein G HP (GE Healthcare, Piscataway, NJ, USA).

Surface plasmon resonance

The binding affinities of the anti-insulin monoclonal antibodies to recombinant mouse insulin I were analyzed using a BIAcore T200 (GE Healthcare). Goat anti-mouse IgG antibody was immobilized on a CM5 chip using an amine coupling kit (GE Healthcare). The anti-mouse insulin antibodies were captured by the immobilized anti-mouse IgG on the chip. Five graded concentrations of mouse insulin (160, 80, 40, 20, and 10 nM) were injected into running buffer (10 mM HBS–EP: 0.01 M Hepes, 0.15 M NaCl, 3 mM ethylenediaminetetraacetic acid [EDTA], and 0.005% [v/v] surfactant P20, pH 7.4) (GE Healthcare) at a flow rate of 30 μl/min at 25 °C for more than 3 min (association phase). The chips were then washed for more than 1.5 h (dissociation phase). For serial measurements, the sensor chip was regenerated by quickly injecting Gly–HCl (pH 1.7) after each measurement. Double-referencing was applied to eliminate responses from the reference surface and the buffer-only control. The affinity was determined as the equilibrium dissociation constant (K_d) by simultaneously fitting the association and dissociation phases of the sensorgram from the analyte concentration series using the 1:1 Langmuir model on BIAevaluate software version 1.0 (GE Healthcare).

Determination of cDNA sequences of antibodies and production of recombinant antibodies

Total RNA was extracted from the established hybridoma cells producing the 13G4 and 26B2 antibodies. After synthesizing complementary DNA (cDNA) from total RNA using a 5′ rapid amplification of the cDNA end system (Life Technologies, Carlsbad, CA, USA), the variable light (VL)/variable heavy (VH) regions were amplified by polymerase chain reaction (PCR). The primers MG1-C (5′-CAGG GTCACCATGGAGTTAGTTTG-3′), MG2b-C (5′-TCCAGAGTTCCAAGTC ACAGTCAC-3′), Mk-C (5′-GACTGAGGCACCTCCAGATGTTAA-3′), MG1-P (5′-AGGGGCCAGTGGATAGACAGATGGGGGTGT-3′), MG2b-P (5′-AGGGGCCAGTGGATAGACTGATGGGGGTGT-3′), and Mk-P (5′-GGATGGTGGGAAGATGGATACAGTTGGTGCAGC-3′) and the nucleotide sequences were determined as described by Debat and coworkers [16]. The VH and VL genes of the antibodies were cloned into mouse IgG1 and immunoglobulin kappa (Igκ) expression vectors, respectively, and were cotransfected into FreeStyle