Brief communication

MOLECULAR METABOLISM

Identification of fatty acid binding protein 4 as an adipokine that regulates insulin secretion during obesity

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ABSTRACT

A critical feature of obesity is enhanced insulin secretion from pancreatic β-cells, enabling the majority of individuals to maintain glycaemic control despite adiposity and insulin resistance. Surprisingly, the factors coordinating this adaptive β-cell response with adiposity have not been delineated. Here we show that fatty acid binding protein 4 (FABP4/aP2) is an adipokine released from adipocytes under obesogenic conditions, such as hypoxia, to augment insulin secretion. The insulinotropic action of FABP4 was identified using an in vitro system that recapitulates adipocyte to β-cell endocrine signalling, with glucose-stimulated insulin secretion (GSIS) as a functional readout, coupled with quantitative proteomics. Exogenous FABP4 potentiated GSIS in vitro and in vivo, and circulating FABP4 levels correlated with GSIS in humans. Insulin inhibited FABP4 release from adipocytes in vitro, in mice and in humans, consistent with feedback regulation. These data suggest that FABP4 and insulin form an endocrine loop coordinating the β-cell response to obesity.

Keywords Obesity; Adipokine; Adipocyte; Beta-cell; Insulin secretion; FABP4

1. INTRODUCTION

The current global obesity epidemic is associated with a cluster of diseases including type 2 diabetes (T2D) [1,2], cardiovascular disease [3,4] and cancer [5,6]. Obesity is often accompanied by insulin resistance and enhanced insulin secretion from pancreatic β-cells [7,8]. If GSIS remains tightly coupled to insulin resistance, normal glycaemic control is maintained. In contrast, failure of GSIS to compensate for insulin resistance leads to hyperglycaemia and in some cases T2D [9]. The molecular mechanisms that coordinate enhanced GSIS with increased adiposity to maintain glycaemic control in the pre-diabetic state are not understood.

Blood glucose, although a major determinant of insulin secretion, appears largely uncoupled from the pronounced enhancement of insulin secretion in obese mice [10] and humans [8] that display blood glucose levels within the normal range. Non-esterified fatty acids (NEFAs) potentiate GSIS [11], yet increases in adiposity often occur independently from changes in plasma NEFA [12] suggesting that NEFA are not a common driver of compensatory insulin secretion. Adipokines regulate β-cell function, however the two most well characterised, leptin and adiponectin, do not adequately explain enhanced insulin secretion during obesity. Leptin, which increases with adiposity [13], inhibits insulin release [14] while adiponectin, a potentiator of GSIS [15,16], decreases with obesity [17]. The adipose enriched, secreted enzyme eNampt/visfatin augments GSIS in mice [18], although only subtle alterations in serum concentrations accompany obesity and correlate negatively with insulin secretion in humans [19]. In the current study, we sought to address the hypothesis that another adipokine plays an integral role coupling β-cell function with adiposity, thereby facilitating enhanced insulin secretion during obesity.

2. MATERIALS AND METHODS

2.1. Cell culture and islet isolation

Cell culture reagents were from Life Technologies unless otherwise indicated. 3T3-L1 fibroblasts were differentiated into adipocytes as described [20] and used for experiments 8 days post-differentiation. For signalling experiments, 3T3-L1 adipocytes were stimulated with β-3 adrenergic agonists *CL*316243 and *BRL*37344 (Sigma) at 0.1 μg/ml in PBS, or 3-Isobutyl-1-methylxanthine (IBMX; Sigma) at 500 μmol/l in 0.1% DMSO. Human insulin (Actrapid; Novo Nordisk) was diluted in

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Abbreviations: T2D, type 2 diabetes; NEFA, non-esterified fatty acid; GSIS, glucose-stimulated insulin secretion; SILAC, stable-isotope labelling by amino acids in cell culture; ELISA, enzyme-linked immunosorbant assay; BMI, body mass index; cAMP, cyclic-AMP; IBMX, 3-Isobutyl-1-methylxanthine

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PBS. Islet isolation, culture and insulin secretion experiments were performed as described [21].

2.2. Conditioned media experiments

We conditioned serum-free islet media (RPMI1640 containing penicillin, streptomycin and L-glutamine) by culturing with 3T3-L1 adipocytes for 18 hours under normoxic (21% O_2, 5% CO_2, 74% N_2) or hypoxic (1% O_2, 5% CO_2, 94% N_2) atmospheres, followed by 0.45 μm filtration to remove detached cells. From this conditioned media, a >10 kDa protein fraction was dialysed against fresh islet media using centrifugal filtration (Amicon/Merck Millipore), then reconstituted at a 3× concentration (i.e. 33% of starting volume of conditioned media) in fresh islet media containing 10% foetal calf serum, before being used to culture isolated mouse islets for 24 h. Insulin secretion was subsequently assessed for 1 h. Replicate insulin secretion data were averaged to give a single data point (*n*) for each batch of conditioned media.

2.3. Proteomics

Stable-isotope labelling by amino acids in cell culture (SILAC) [22] labelling of 3T3-L1 fibroblasts was performed as described [23], using "heavy" amino acid isotopes arginine U-$^{13}C_6$ U-$^{15}N_4$ (CNLM-539) and lysine U-$^{13}C_6$ U-$^{15}N_2$ (CNLM-291) from Cambridge Isotope Laboratories, and non-labelled "light" equivalents from Sigma. To reduce amino acid conversion to proline [24], arginine was used at 0.021 g/L, and lysine at 0.0365 g/L. Differentiated labelled 3T3-L1 adipocytes were used to condition media, with "light" cells cultured under normoxia and "heavy" cells under hypoxia. Conditioned media were collected and mixed in a 1:1 ratio to reduce subsequent inter-sample handling variability. Secreted proteins were enriched, precipitated, resolved by SDS-PAGE and subjected to in-gel trypsin digestion, as described [25]. Resulting peptides were subjected to liquid chromatography and tandem mass spectrometry using a Waters Ultima instrument at the Bioanalytical Mass Spectrometry Facility at the University of New South Wales. Data were analysed using Mascot Distiller v2.3.1.0. The SwissProt reference database was queried using Mascot Server 2.2 to identify peptide spectra. Only proteins represented by >5 unique peptide spectra were retained for further analysis. Potential for secretion was predicted using SecretomeP2.0 [26].

2.4. Recombinant FABP4 production

Recombinant FABP4 (rFABP4) used in Figure 2A and B was from Cayman Chemicals. rFABP4 used in all other figures was produced as follows. Mouse *Fabp4* (GenBank BC054426.1) was cloned into His-tag expression vector pDEST17, transformed into BL21 *Escherichia coli*, and 0.5 ocular density cultures induced to produce rFABP4 by 1 mmol/l IPTG treatment. After 6 h, cultures were DNAseI and lysozyme treated, before addition of 1% Triton X-100. Clarified lysates were applied to Co^{2+} coated agarose bead columns and washed. For in vivo rFABP4—linoleate experiments, 0.4 mmol/l linoleate was passed over the column, followed by washing, to enable coupling with rFABP4. rFABP4 was eluted with 300 mmol/l imidazole and dialysed against PBS. Size, identity and purity of rFABP4 were confirmed by SDS-PAGE with Western blotting and Coomassie blue (Sigma) protein staining (Figure S1A and B). Our rFABP4 preparations contain very low endotoxin levels <0.02 EU/μg protein (Lonza LAL assay).

2.5. Preparation of fatty acids

Linoleate (Nu-Chek prep) was stored at −80 °C under nitrogen. Immediately before use, 0.4 mmol/l linoleate or sodium-palmitate (Sigma) were solubilised by coupling to 0.92% BSA (Sigma).

2.6. Mouse studies

2.6.1. Welfare

12-week old C57Bl/6J mice were purchased from Australian BioResources and housed in groups of 2—5, within a barrier facility with 12 h light/dark cycles. Environmental enrichment was provided and mice had free access to a standard lab diet [27] and water. Approval for mouse studies was issued to J.Cantley by the Garvan Institute/St.Vincent's Hospital Animal Ethics Committee.

2.6.2. Diet-induced obesity

10-week old male mice were fed either a standard lab diet (lean) or high-fat diet (obese) for 40 weeks. Diet composition was described previously [27].

2.6.3. rFABP4 treatment

Mice were treated with rFABP4—linoleate via i.p. injection (80 μg) or by subcutaneous implantation of osmotic mini-pumps (Alzet) providing a 0.5 μl/h flow rate and mean rFABP4 dose of 1.22 μg/h. Mini-pumps were loaded, primed and implanted (mid-scapular) into mice under gaseous general anaesthesia (1—4% isoflurane in oxygen), with ketoprofen (5 mg/kg) and marcaine (8 mg/kg) used as local analgesics.

2.6.4. Metabolic testing

Glucose tolerance testing (GTT) was performed after a 6 h fast (with free access to water), by intraperitoneal (i.p.) injection of sterile 20% glucose (2 g/kg). Insulin action was assessed by i.p. injection of insulin (0.75 U/kg) following a 6 h fast. Blood was obtained from the tail tip for glucose monitoring (Roche Accu-Chek Performa glucometer) and insulin assay (Crystal Chem ELISA). Circulating FABP4 was assayed by ELISA (Circulex/MBL).

2.7. Human studies

2.7.1. Participants

Sedentary, non-diabetic Caucasian participants were recruited ($n = 17$, 4 males). Mean age and body mass index (BMI) ± standard error (SEM) were 56 ± 2 years and 26 ± 1 kg/m^2, respectively. Exclusion criteria were: weight instability (>2 kg change in the preceding 6 months), exercising more than 60 min per week, taking medications known to affect glucose homeostasis, or a personal history of type 2 diabetes or cardiovascular disease. The study protocol was approved by the Human Research and Ethics Committee at St Vincent's Hospital, Sydney. Participants provided informed written consent before commencement.

2.7.2. Metabolic testing

Participants attended the Clinical Research Facility at 8 am after a 12-h fast. Weight, height and blood pressure were measured and fasting blood samples drawn. Glucose tolerance testing (0.3 g/kg intravenous glucose dose; max 25 g) was performed, followed by a 2-h hyperinsulinaemic (60 mU/m^2/min)—euglycemic (5.0 mmol/L) clamp. Human serum FABP4 levels were determined by ELISA (Biovendor) and insulin levels determined by RIA (Merck Millipore).

2.7.3. Body composition

Fat mass and fat-free mass were assessed by dual energy X-ray absorptiometry (DXA; Lunar DPX-Lunar Radiation, GE Healthcare).

