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Activation of Akt through 5-HT2A receptor ameliorates serotonin-induced degradation of insulin receptor substrate-1 in adipocytes

Qinkai Li [a,*], Toshio Hosaka [c], Nagakatsu Harada [b], Yutaka Nakaya [b], Makoto Funaki [a]

[a] Clinical Research Center for Diabetes, Tokushima University Hospital, Kuramoto-cho, Tokushima 770-8503, Japan
[b] Department of Nutrition and Metabolism, Institute of Health Biosciences, University of Tokushima Graduate School, Kuramoto-cho, Tokushima 770-8503, Japan
[c] Department of Endocrinology and Diabetes, Saitama Medical University, Saitama 350-1241, Japan

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ABSTRACT

Serotonin (5-hydroxytryptamine, 5-HT) was found to be elevated in the serum of diabetic patients. In this study, we investigate the mechanism of insulin desensitization caused by 5-HT. In 3T3-L1 adipocytes, 5-HT treatment induced the translocation of insulin receptor substrate-1 (IRS-1) from low density microsome (LDM), the important intracellular compartment for its functions, to cytosol, inducing IRS-1 ubiquitination and degradation. Moreover, inhibition of 5-HT-stimulated Akt activation by either ketanserin (a specific 5-HT2A receptor antagonist) or knocking-down the expression of 5-HT2A receptor promoted 5-HT-stimulated IRS-1 dissociation from 14-3-3β in LDM, leading to drastic ubiquitination. Interestingly, sarpogrelate, another antagonist of 5-HT2A receptor, protected IRS-1 from degradation through activation of Akt. This implicates the importance of Akt activation in extending IRS-1 life span through maintaining their optimal sub-location into adipocytes. Taken together, this study suggest that activation of Akt may be able to compensate the adverse effects of 5-HT by stabilizing IRS-1 in LDM.

1. Introduction

Insulin action on target tissues requires tyrosine autophosphorylation at multiple sites in insulin receptor (IR) and activation of the intrinsic tyrosine kinase to phosphorylated insulin receptor substrate-1 (IRS-1), in which, phosphatidylinositol-3 kinase (PI3K) was recruited for activation and stimulates Akt and glucose disposal into peripheral tissues, such as skeletal muscle and adipose tissue to maintain normal glucose homeostasis (White and Kahn, 1994). Impaired glucose disposal into these tissues in response to insulin stimulation, called insulin resistance, is observed in type 2 diabetes mellitus (T2DM) and obese subjects (DeFronzo and Ferrannini, 1991; Hoehn et al., 2008).

Serotonin (5-hydroxytryptamine, 5-HT) is a small molecular monoamine neurotransmitter. It is found extensively in the gastrointestinal tract. About 80–90% of 5-HT is located in the enterochromaffin cells in the gut. In humans, 5-HT is synthesized from the amino acid L-tryptophan by a short metabolic pathway. 5-HT is positively uptaken and stored in platelets in periphery (Rapport et al., 1948). In the blood, activated platelets aggregate and release 5-HT to exert various effects via 14 different receptors belonging to seven subfamilies (5-HT1–5-HT7) (Vanhoutte, 1991; Saxena, 1995). In healthy subjects, the serum level of 5-HT is approximately 146 μg/l (~1 μmol/l) (Sasa et al., 1978). However, due to the platelet hyper-reactivity, serum levels of 5-HT are elevated in diabetic subjects (Barradas et al., 1988; Malyszko et al., 1994). Moreover, it is well known that long-term use of selective serotonin reuptake inhibitors (SSRIs) significantly increases body weight gain and obesity (Schwartz et al., 2004; Raeder et al., 2006). These studies implicate that the glucose metabolism and obesity may be etiologically associated with 5-HT. In one study, acute treatment of either L6 myotubes or isolated rat skeletal muscle with 50 folds of physiological concentration of 5-HT (50 μmol/l) stimulates glucose uptake through activation of 5-HT2A receptor, implying an insulin mimetic effect of 5-HT in muscle cells (Hajduch et al., 1999). Our previous work also discovered that 5-HT induces insulin resistance by activating extracellular-regulated kinase (Erk) and mammalian target of rapamycin (mTOR) signals via the transactivation of epidermal growth factor (EGF) receptor (Li et al., 2012). In this study, we attempted to have a further assessment on how 5-HT modifies insulin signaling in adipocytes.

Abbreviations: T2DM, type 2 diabetes mellitus; 5-HT, 5-hydroxytryptamine (Serotonin); IRS-1, insulin receptor substrate-1; LDM, low density microsome; PI3 K, phosphatidylinositol-3 kinase; CHX, cycloheximide; Erk, extracellular-regulated kinase; mTOR, mammalian target of rapamycin; GLUT1, glucose transporter type 1; GLUT4, glucose transporter type 4.

* Corresponding author. Tel./fax: +81 88 633 9679.
E-mail address: lqk@clin.med.tokushima-u.ac.jp (Q. Li).

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2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM, 4.5 g/l glucose), 5-hydroxytryptamine, 3-isobutyl-1-methylxanthine (IBMX), α-Me-5-HT, SB-206553, DOI, DOB and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Calf serum (CS) and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA). Rapamycin was from Calbiochem (La Jolla, CA). Sarpogrelate hydrochloride was from Mitsubishi Pharma Co. (Tokyo, Japan). Anti-phosphotyrosine monoclonal antibody (PY20), anti-5-HT2A receptor, anti-14-3-3β and anti-IRS-1 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p85 PI 3-kinase antibody was from Upstate Biotechnology (Lake Placid, NY). Anti-phospho-Akt (Ser473), anti-phospho-Akt (Thr308), anti-Akt, anti-phospho-IRS-1 (Ser636/639, mouse: Ser632/635), anti-phospho-IRS-1 (Ser307) and anti-phospho Akt substrate antibody were from Cell Signaling Technology (Danvers, MA). Anti-poly-Ub antibody (FK1) was from Affiniti Research Products (Exeter, UK). The anti-GLUT4 antibody was from Biogenesis (Brentwood, NH). The anti-GLUT1 antibody was from FabGennix Inc (Frisco, TX). Protein G Sepharose 4 Fast Flow and ECL Plus Western Blotting Detection Reagent were from GE Healthcare (Piscataway, NJ). 2-deoxy-D-[1,2-^{3}H]glucose (50 Ci/mmol) was from American Radiolabeled Chemicals. Inc. (St. Louis, MO). Ketanserin and MG132 were purchased from Alexis Biochemicals (San Diego, CA). PD98059 and all the other reagents were from Wako Pure Chemicals (Osaka, Japan).

2.2. Cell culture and treatment

3T3-L1 fibroblasts were cultured and differentiated into adipocytes as described previously (Hosaka et al., 2005). Briefly, 3T3-L1 fibroblasts maintained in DMEM supplemented with 10% CS (vol/vol) were differentiated by incubating the cells for 48 h in DMEM containing 10% FBS (vol/vol), 0.5 mmol/l IBMX, 1 μmol/l dexamethasone and 1.7 μmol/l insulin. Thereafter, the cells were maintained in DMEM supplemented with 10% FBS (vol/vol). The experiments were conducted 7–9 days after inducing differentiation, when > 90% of cells expressed the adipocyte phenotype. Cells were serum starved overnight before each experiment. In this study, 10 μmol/l of 5-HT (10 folds physiological concentration) was selected as the treatment of adipocytes. 5-HT was dissolved in phosphate-buffered saline (PBS), insulin was dissolved in 10 mmol/l hydrogen chloride, ketanserin was dissolved in dimethyl sulfoxide (DMSO) and applied under the concentration of 10 nmol/l for specific antagonizing 5-HT2A receptor (Glennon et al., 2002). All the other reagents were dissolved in DMSO before each experiment.

2.3. Construction of shRNA and electroporation of 3T3-L1 adipocytes

The multi-shRNA expression vector was purchased from Addgene (#12391) (Xia et al., 2006). The Mir-30a precursor containing the sequence targeting the mouse 5-HT2A receptor (5′-GCA GAA TGC CAC CAA CTA TTT-3′) was synthesized and inserted into KpnI/EcoRI sites of the vector. The plasmid (500 μg) was electroporated into adipocytes as described previously (Okada et al., 2003). Briefly, differentiated 3T3-L1 adipocytes were washed with PBS, dislodged by trypsin-EDTA, and centrifuged followed by PBS wash. Cells were suspended again in PBS at a concentration of 1.0×10^7 cells/500 μl and pipetted into a cuvette containing plasmid. This mixture was charged with 975 μF capacitance at 0.16 kV in a Gene Pulser II apparatus (Bio-Rad Laboratories, Hercules, CA). Cells were then transferred to DMEM supplemented with 10% FBS in a six-well plate. Expression of the vector was induced by continuous supplementation of doxycycline (1 μg/ml) in the medium. Approximately 24 h after electroporation, cells were serum starved for 8 h before each experiment.

2.4. Animal experiments

Animals were handled in accordance with the principles and guidelines established by the University of Tokushima. Briefly, overnight fasted male C57BL/6 mice and ob/ob mice (SLC, Shizuoka, Japan) at 8 weeks of age were orally administered single dose of sarpogrelate (1 g/kg BW) or PBS, followed by the *ad libitum* access to food and tap water, after 8 h, the animals were anesthetized and killed. Epididymal fat tissues were dissected and sampled from the midsection and then frozen in liquid nitrogen for a further analysis of IRS-1 expression by western blot.

2.5. Immunoprecipitation

After rinsing with ice-cold PBS (NaCl, 137 mmol/l; KCl, 2.7 mmol/l; Na_2HPO_4, 10 mmol/l; KH_2PO_4, 2 mmol/l, pH 7.4), cells were lysed in RIPA buffer (50 mmol/l Tris, 150 mmol/l NaCl, 1 mmol/l EDTA, 0.5% sodium deoxycholate (wt/vol), 1% Nonidet P-40 (vol/vol), 0.1% SDS (wt/vol), 1 μmol/l aprotinin, 10 μmol/l leupeptin, 0.1 μmol/l phenylmethylsulfonyl fluoride (PMSF), 20 mmol/l sodium fluoride (NaF), 20 mmol/l β-glycerolphosphate and 1 mmol/l sodium orthovanadate, pH 7.4). The lysates were centrifuged for 20 min at 15,000*g* at 4 °C to remove insoluble materials. The supernatants were incubated with the indicated antibodies, after which the immune complexes were precipitated with protein G sepharose. The immunoprecipitates were subject to SDS-PAGE and analyzed by western blot analyses.

2.6. Western blot

After the treatment as described in figure legends, cells or tissues were rinsed three times with ice-cold PBS and lysed in RIPA buffer. Cell lysates were obtained by centrifugation at 15,000*g* and 4 °C for 20 min. Equal amounts of proteins were subject to SDS-PAGE (Laemmli, 1970). Proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, MA). Immunoblotting was performed with an ECL PLUS system. Densitometric analysis was performed using Scion Image (Scion Corporation, Frederick, MD).

2.7. Protein degradation assay

Protein degradation was assessed by cycloheximide chase assay as previously described (Sehat et al., 2007; Xu et al., 2008). The effect of insulin (100 nmol/l) or 5-HT (10 μmol/l, medium refreshed every 12 h) on the stability of the entire IRS-1 pool was examined by western blot analysis at a variety of time points after treatment with cycloheximide (CHX, 25 μg/ml). Cells were grown in complete culture medium in order to follow receptor downregulation under physiological conditions. The protein synthesis of the cells was subsequently inhibited with cycloheximide (CHX, 25 μg/ml) that was maintained during the whole experiment.

2.8. Glucose uptake assay

Glucose uptake was measured in triplicate as described previously (Pekala et al., 1983). Briefly, cells treated with 5-HT or vehicle control were washed three times with 37 °C Krebs–Ringer phosphate (KRP) buffer (128 mmol/l NaCl, 6 mmol/l KCl, 1 mmol/l $CaCl_2$, 1.2 mmol/l $MgSO_4$, 4 mmol/l NaH_2PO_4, 6 mmol/l Na_2HPO_4, pH 7.4) containing HEPES (25 mmol/l). Then, in the presence or absence of treatment, cells were either treated with vehicle or treated with insulin for 15 min. Glucose uptake was initiated by adding