Title

Arginine-induced insulin secretion in endoplasmic reticulum

Shorttitle

Arginine-induced insulin secretion

Authors

Makoto Umeda¹, Masaki Hiramoto^{1,2}, Atsushi Watanabe¹, Nobuyo Tsunoda¹, and Takeshi Imai^{1,*}

Affiliation

¹National Center for Geriatrics and Gerontology, Obu, Aichi 474-8511, Japan.

²Tokyo Medical University, Shinjyuku, Tokyo 160-8402, Japan

*Corresponding author: Takeshi Imai

E-mail address: dai.ncgg@gmail.com

Abstract

Arginine, a semi-essential amino acid, is known as one of the most strongest insulin secretagogues in a glucose-dependent manner, but major mechanism is unknown. Arginine induced insulin secretion in mice as well as β cell line, NIT-1, in which more than 90% of intracellular insulin is proinsulin without arginine cultivation. Arginine administration reduced proinsulin amount in 30 seconds, then insulin is secreted from NIT1 cells. These data indicated that the target factor(s) for arginine-induced insulin secretion located in endoplasmic reticulum (ER).

We established the screening system for identifying the arginine mimetics. Brazilian propolis, not Chinese propolis, induced insulin secretion.

To identify target factor(s) of arginine induced insulin secretion, our previous study was that nanobeads technology facilitated us to purify chemical-target factors. This time we chose the other way, proinsulin associating factor purification and arginine-immobilized agarose. Three proinsulin associating factors and 5 arginine interacting factors were identified. Among these factors, Calnexin (CNX) was the only one factor, which belonged to both groups, suggesting that CNX might play a key role in arginine-induced insulin secretion in ER.

Keywords

Arginine; insulin; Brazilian propolis; glucose; diabetes mellitus; endoplasmic reticulum

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Abbreviation

Arg, arginine

ATT, arginine tolerance test

CNX, calnexin

DM, diabetes mellitus

ER, endoplasmic reticulum

FK, forskolin

FCS, fetal calf serum

GTT, glucose tolerance test

GPRC6A, G Protein-Coupled Receptor, Class C, Group 6, Member A

Ins, insulin

IP, immunoprecipitation

NOS, NO synthase

OE, over expression

PKA, protein kinase A

PMSF, phenylmethylsulfonyl fluoride

T2DM, type 2 diabetes mellitus

WB, Western blot

1. Introduction

One of semi-essential amino acids, arginine, stimulates insulin secretion strongly in cells, mice and humans. Arginine is well known as substrates of 2 enzymes, Nitric oxide synthases (NOS) and arginase. NOS converts arginine into citrulline), and arginase changed to ornithine. But very small portion of arginine is only metabolized in pancreas[1]. Main action of arginine for insulin secretion might not be the substrates of these enzymes. The mechanism of arginine-induced insulin secretion is not known yet[2]. The minor mechanism is most probably arginine binds to G Protein-Coupled Receptor, Class C, Group 6, Member A (GPCR6A) and activates PKA signaling to granule of β cells. But there are several discrepancies [3, 4]. L-amino acids(not only cationic, but also non-cationic), calcium, osteocalcin, steroids, and so on, are the ligands of GPCR6A. Some L-amino acids which do not stimulate insulin secretion [1], for example Cystein binds to GPCR6A[5], but even Cysteininhibits insulin secretionin mice [6, 7]. Our purpose is to identify the molecular mechanism of arginine action on insulin secretion. It will be useful for developing new anti-DM drug with new mechanism.

2. Materials and methods

2.1. Materials

Arginine Sepharose 4B (Amersham Biosciences17-0524-01), insulin-agarose affinity support (ALP Alpha Diagnostic INSL19-AS), alanine-agarose (Sigma A-3435), ER Protein Folding Antibody Sampler Kit (Cell Signaling Technology 4759) were purchased.

2.2. Cell culture

The mouse pancreas-derived NIT1 cells (purchased from ATCC) were maintained in F12K supplemented with 10% fetal calf serum (FCS). Cells were plated at a density of $1.5-3 \times 10^6$ cells/60-mm dish, and after 48 hours, the culture medium was exchanged for α -MEM without phenol red supplemented with 0.5% charcoal-treated FCS.

2.3. Insulin Production analysis

Insulin secretion was determined as previously described[2]. Briefly, after cultivation of NIT-1 cells, medium was replaced with F-12K medium without arginine for 10 min. The drugs (arginine, Baccharin) were added to the cells for 2 min and medium was collected. For mice experiments, mice plasma was collected at cognate time. Insulin concentration was measured by insulin ELISA kit according to the protocol of manufacture (Shibayagi, Gunma, Japan).

2.4. Construction of plasmids

The cDNA of mouse Ins2 was amplified by RT PCR from the total RNA prepared from NIT-1 cells and subcloned into the pcDNA6 vector (Invitrogen Life Technologies, Carlsbad, CA, USA) to allow the expression of Ins2 under the control of a CMV promoter. During PCR amplification, FLAG tag was fused at the C terminus of proinsulin. To introduce the point mutation, C96Y, into the Ins2 gene, site-directed mutagenesis was performed using a pair of primers of 5'-CATTGTAGATCAGTGCTACACCAGCATCTGCTCC-3' and 5'-GGAGCAGATGCTGGTGTAGCACTGATCTACAATG-3'. All the sequences were confirmed using an ABI PRISM 3700 sequence analyzer.

2.5. Immunoprecipitation [IP]

Over expression (OE) vectors of insulin were prepared by inserted cDNAs into pcDNA6 vectors described previously. The insulin expression vectors were transfected NIT1 cells using Lipofectamine 2000 (Invitrogen Life Technologies). After 48 hours the transfected NIT1 cell pellets were washed several times with PBS, and lysed in ice-cold lysis buffer (50 mM Tris-HCl (pH 8.0), 150mM NaCl, 1 mM EDTA, 1% Triton X-100). The soluble fractions from cell lysates were isolated by centrifugation. For IPs, anti-FLAG M2 affinity gel (Sigma Aldrich, St. Louis, MO, USA) were added to each lysate and incubated with rotation for 4 h at 4°C using a RT 50 rotator (Taitec Corp., Saitama, Japan). Immunoprecipitates were washed three times with lysis buffer, and then eluted with lysis buffer containing 3X FLAG peptide (100 µg/mL). Antibody raised against FLAG (sc28259, Santa Cruz for IP), was purchased. The dialyzed lysate was IPed with approximately 3 µg of the specified antibody and 30 µl of protein G-beads (GE Health care) and washed three times for 5 min each with the same buffer, followed by a brief centrifugation. Thirty microliters of the IP/bead suspension were mixed with binding buffer and boiled for 5 min. The input and IPs were analyzed by Western blot analysis [2, 8, 9].

2.6. Mass Spectrometry analysis

IPed purified proteins were separated by SDS-PAGE and gels subjected to silver staining as previously described [2]. IPed proteins were separated using 5%–20% SDS PAGE and subjected to silver staining. The specific protein bands were excised and mass spectrometry was then performed as previously described [9]. Band slices were reduced with 10 mM dithiothreitol, alkylated with 55 mM

iodoacetamide and digested with sequencing grade modified trypsin (12 µg/ml; Promega Corp., WI, USA) overnight. The extracted peptides were desalted with ZipTip C18 (Millipore, Billerica, MA, USA) and separated by nano-flow liquid chromatography (LC) (Paradigm MS4; Michrom BioResources, Inc., Auburn, CA, USA) using a reverse phase C18 column (Magic C18, 0.2 ×50mm; Michrom BioResources, Inc.). The LC eluent was coupled to a micro-ionspray source attached to an LCQ Advantage MAX mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). All MS/MS spectra were searched using the TurboSEQUEST algorithm within the BioWorks 3.2 software (Thermo Fisher Scientific Inc.).

2.7. Glucose/arginine tolerance test (GTT/ATT)

The WT B6 mice were maintained 12hr-light and 12hr-dark cycles. These mice were fasted for 4 hours, and glucose (1g/kg body weight) or arginine (1.5g/kg body weight) was administrated. Blood was collected at indicated time in chilled heparinized tubes, immediately centrifuged, and the plasma stored, and insulin levels were measured with kit described above.

2.8. Animal study compliance

All experiments were performed in accordance with the National Center for Geriatrics and Gerontology (NCGG)'s ethical guidelines for animal care, and the experimental protocols were approved by the Animal Care Committee [10, 11].

2.9. Statistical analysis

The values are reported as the means $\pm$ SEMs. Statistical significance is indicated as follows: *, $p < 0.05$; **, $p < 0.005$; and ***, $p < 0.0001$. Non-significant differences ($p > 0.05$) are indicated as NS. Experimental reproducibility was measured and analyzed using at least 3 independent experiments [12, 13, 14, 15, 16, 17].

3. Results

3.1. Arginine induces conversion of proinsulin to insulin (Figure 1).

Arginine is one of the strongest insulin secretagogues with unknown mechanism. Some group studied that G Protein-Coupled Receptor, Class C, Group 6, Member A (GPRC6A) is the target protein for arginine (so called receptor), but with some discrepancies (see also above on introduction section).

There are two groups who established and analyzed GPRC6A KO mice [18, 19]. Pi *et al.* (2012) showed less (at least not major) effect on insulin secretion in GPRC6A KO mice, and concluded that GPRC6A PARTIALLY regulated insulin secretion [18]. The other paper Smajilovic *et al.* (2013) resulted that GPRC6A played no role for insulin secretion [19]. Taken together GPRC6A did not regulate arginine-induced insulin secretion dominantly with genetic approach in mice.

The second point is different affinity of the amino acids (arginine and its derivatives). For insulin secretion activity Arg > Orn = Lys >> Cit (data not shown, and [1]). And cationic amino acid induces insulin secretion, but GPRC6A is L-amino acid receptor (not only cationic amino acid, and [20]). Oya *et al.* (2013) reported that EC₅₀ to GPRC6A is Orn > Lys = Arg [4]. Final example is Cystein, it is one of the strongest affinity to GPRC6A [5], but inhibit insulin secretion in mice [6, 7]. Insulin secretagogues activity and affinity to GPRC6A of amino acid are different.

The third point is down stream of GPRC6A. The ligands (non-cationic L-amino acids, calcium etc) binds to GPRC6A, and major activation signal is protein kinase A (PKA, and [3, 4, 20]), and PKA activation stimulates insulin secretion for granule [1, 20-26]. Actually forskolin (FK) binds adenylyl cyclase, activates PKA, and induces insulin secretion (Figure 1C column 3 and 4, 5 and 6, and [1, 21-26]). With (columns 5 and 6) and without arginine (columns 3 and 4), FK induced insulin secretion. FK induced insulin secretion arginine-independent manner (and no synergy). These data indicated that arginine- and FK- signalings are independent.

Final (4th) point were in Figure 1A and B. Insulin form in NIT1 cells were analyzed without arginine (Figure 1A), surprisingly 92.7% insulin was proinsulin form (black arrow), 7.3% is matured (after cleavage) insulin in NIT1 cells. Insulin is translated as preproinsulin in cytoplasm and immediately transferred to endoplasmic reticulum (ER), Preproinsulin is converted to proinsulin SRP etc in ER immediately. Proinsulin moved in granule and cleaved to insulin +C-peptides [1]. Arginine was administrated to NIT1 cells, and proinsulin (in ER) and insulin (in granule) amount were analyzed after arginine administration (Figure 1B). Proinsulin amount was decreased to *ca* 60% after 30 to 60 seconds (P arrow in Figure 1B). Secreted insulin was increased (30-60-120 seconds) after proinsulin decrease (30-60 seconds). Insulin secretion is classical cascade ER to golgi to granule to secretion [1], and it's fitted.

Taken together major arginine signaling for insulin secretion is not via GPRC6A-PKA, but arginine-target for Insulin secretion (ATIS) complex. ATIS signaling stimulated the conversion of proinsulin (in ER) to insulin (in granule). It suggested ATIS complex/signals located in ER.

3.2. Brazilian propolis as arginine mimetics (Figure 2).

We screened the extracts with arginine-induced insulin secretion activity in NIT1 cells, and Brazilian propolis was identified (column 5 of Figure 2B). Surprisingly no significant activity was observed from Chinese propolis extracts (column 6), indicated that Brazilian propolis specific-compounds/contents stimulated insulin secretion. Artepillin C and Drupanin are candidates, because these compounds are from Brazilian *Baccharis Dracunculifolia* (Figure 2A, and [24]). Actually Drupanin (column 3), Artepillin C (column 4), and arginine (column 2) induced insulin secretion similarly. Brazilian propolis stimulated insulin secretion more strongly, indicated that other unknown compound(s) in Brazilian propolis had much more higher activity than arginine, Drupanin and Artepillin C.

Did Brazilian propolis induce insulin secretion in arginine-dependent manner? We examined Brazilian propolis-induced insulin secretion with various concentrations of arginine and glucose (Figure 2C). In the absence of arginine Brazilian propolis stimulated insulin secretion in a glucose dependent manner. With normal concentration of arginine no strong insulin induction was observed in a glucose dependent manner. Brazilian propolis administration induced plasma insulin concentration, and decreased glucose concentration of mice in dose-dependent manner (Figure 2D). Taken together, Brazilian propolis behaved arginine mimetics, which induced insulin secretion in arginine- and glucose-dependent manner.

3.3. Identification of proinsulin associated factors (Figure 3)

How arginine regulates insulin secretion? Our previous paper [2, 16] reported that arginine-immobilized magnetic nanobeads facilitated to purify arginine target factors for insulin secretion. In this paper proinsulin (insulin form in ER) associated factors were purified with immunoprecipitated (IPed)

methods,

Preproinsulin expression vectors were introduced into NIT1 cells, and the cell extracts were IPed with antibody. Protein bands, whose intensities were stronger than control (empty) vector transfected cell extracts, were excised and identified using LC/MS analysis. These were BiP and PDIA6, and both are the proteins in the Endoplasmic Reticulum (ER) with KDEL sequences (ER retention signals,[28, 29]). As expected 2 proinsulin associated factors were purified and identified, and they located in ER.

3.4. Identification of arginine associated factors (Figure 4)

Arginine-associated factors were identified using commercial available arginine-immobilized agarose. Both insulin-associated factors are located in ER, indicated that intracellular localization of arginine target factors for insulin secretion (designated ATIS in [2]) were in the ER. Using commercial arginine-immobilized agarose, some proteins in the ER and interacted with arginine were screened, and 5 factors, ERp42 [30], ERp72[31, 32], PDI, gp96/GRP94[32], and Calnexin (CNX, and [33]), were identified as ATIS complex (Figure 4D – H). Comparing with other factors, CNX bound to Insulin-immobilized agarose (Figure 4E), suggested that CNX interacted with arginine and insulin.

3.5. Possible model of arginine-induced insulin secretion (Graphic abstract, Figure 5)

Several factors located in the endoplasmic reticulum were identified for arginine-induced insulin secretion. Total complex for insulin secretion were named ATIS (arginine target for insulin secretion), and ATIS complex was composed of 2 small complexes (arginine and proinsulin interacted factors). Calnexin (CNX) might behave as bridging factor of 2 small complexes.

4. Discussion

Partially identified the molecular mechanism of arginine action for insulin secretion [1]. First we demonstrated that proinsulin form in ER is major target for arginine-induced insulin secretion, and showed that all the arginine/insulin-associated factors displayed KDEL sequences located in the ER. Arginine target factors for insulin secretion might locate in ER (Graphic abstract). Using IP and arginine-immobilized agarose, several factors which interacted with arginine/proinsulin were identified, but these bindings to proinsulin/arginine were not cleared directly or indirectly. Recombinant proteins produced by E coli will be useful for identify to direct target proteins (so called receptors).

We screened extracts to find arginine mimetics for insulin secretion (Figure 2), fortunately Brazilian propolis stimulated insulin secretion, but Chinese propolis not. At least Brazilian propolis specific 2 chemicals, cinnamic acid derivatives, were examined for insulin secretion. They have weak activities than original Brazillan propolis extract, indicated that other chemical(s) in Brazilian propolis had strong insulin secretagogues activities with similar to arginine, indicated that the target factor(s) of arginine and Brazilian propolis are similar.

Taken together, our next purpose is to find the receptor for arginine and strong insulin secretagogues in Brazilian propolis.

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Figure legends

Figure 1. Arginine induces conversion of proinsulin to insulin.

(A) Pancreatic cell line NIT1 cells were cultivated in F-12k without arginine for 2 hours, whole cell lysate was prepared, and WB using anti-insulin antibody was analyzed (lane 1 Sigma's reagent; lane 2 NIT1 lysate). Black arrow indicates proinsulin (92.7%, 86 AA, and 9.0 kDa), and gray arrow indicates insulin (7.3%, 51 AA, and 5.8 kDa). (B) NIT1 cells were treated with 2.4mM arginine for indicate time (0, 0.1, 0.25, 0.5, 1, 2, 30 min), intracellular proinsulin (open circle with dot lines) and secreted insulin in medium (filled rectangle) were plotted. Descending arrow P indicates decreased proinsulin, and ascending dot arrow S indicates increased secreted insulin. (C) Forskolin (FK) induced insulin secretion arginine-independent manner. NIT1 was treated with/without arginine and FK, and secreted insulin was analyzed. *, $p < 0.05$. (D) Possible model of arginine induced insulin secretion mainly via ER.

Figure 2. Brazilian propolis induces insulin secretion.

(A) Brazilian propolis contains Drupanin, Artepillin C, and other (unknown) compounds. Chemical structures of Drupanin and Artepillin C are shown. (B, C and D) Brazilian propolis stimulated insulin secretion in cells (B and C) and in mice (D). NIT-1 cells were treated with arginine free-medium and 0.1mM arginine (column 2), 10uM Drupanine (column 3), 10uM Artepillin C (column 4), 0.01% Brazilian (column 5), and Chinese (columns 6) propolis were administrated (B). Brazilian propolis induced insulin secretion in arginine and glucose-dependent manner (C). The cells in the mediums of 0mM arginine (open circles, open rectangles, and open triangles), 2.4mM arginine (filled circles, filled rectangles, and filled triangles), 0mM glucose (open circles and filled circle), 7mM glucose (open rectangles, and filled rectangles), and 20mM glucose (open triangles, and filled triangles) were administrated with 0, 0.001, 0.03, 0.01 % Brazilian propolis for 2 minutes. (D) Brazilian propolis stimulated circulating insulin concentration, and reduced plasma glucose concentration in mice. The values are expressed as the means $\pm$ SEMs (n=5). *p<0.05, **p<0.005, and NS, p>0.05.

Figure 3. Identification of insulin complex in endoplasmic reticulum.

(A, B, and C) Purification and identification of BiP and PDIA6 as insulin associated proteins. Expression vectors of empty (co, lane 1), WT (Ins, lane 2), and mutated (Akt, lane 3) insulin were transfected to NIT1, and the cell extracts were prepared. These proteins were immuno-precipitated, and were visualized Ag stained gel (A). Peptides from SDS-PAGE were shown in B (BiP) and C (PDIA6).

Figure 4. Identification of arginine complex in endoplasmic reticulum

Identification of ERp44 (BA), ERp72 (B), PDI (C), gp96/GRP94 (D), Calnexin (E), and ERp57 (F) as arginine complex, NIT1 lysate (lanes 1, 5, 9, 13, 17, and 21) were mixed with control (Co; lanes 2, 6, 10, 14, 18, and 22), insulin- (Ins; lanes 3, 7, 11, 15, 19, and 23), and arginine- (Arg; lanes 4, 8, 12, 16, 20, and 24) immobilized agarose. Binding fractions were PAGEed (polyacrylamide gel electrophoresis), and visualized with antibodies against ERp44 (A), ERp72 (B), PDI (C), gp96/GRP94 (D), Calnexine (E), and ERp57 (F).

Figure 5. Possible model of arginine-induced insulin secretion

Several factors located in the endoplasmic reticulum were identified for arginine-induced insulin secretion. Total complex for insulin secretion were named ATIS (arginine target for insulin secretion), and ATIS complex was composed of 2 small complexes (arginine and insulin-interacting factors). Calnexin (CNX) might behave as bridging and common factor.

Figure 1

Figure 2

Figure 3

Figure 4