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IAPP/amylin deposition, which is correlated with expressions of ASC and IL-1β in β-cells of Langerhans' islets, directly initiates NLRP3 inflammasome activation

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Abstract

Recent findings revealed that type 2 diabetes mellitus (T2D) is a chronic inflammatory disease and an islet amyloid polypeptide (IAPP)/amylin, is deposited within pancreatic islets. IAPP/amylin has been reported to activate NACHT, LRR, and PYD domains-containing protein 3 (NLRP3) inflammasome in infiltrated macrophages. NLRP3, an intracellular pattern recognition receptor, has been shown to recognize pathogens and/or metabolites and complexes with the adopter protein apoptosis-associated speck-like protein containing a caspase-recruitment domain ASC to form a huge complex, called an inflammasome, an interleukin (IL)-1β-processing platform. Although reactive oxygen species (ROS) were reported to be involved in activation of NLRP3 inflammasome, we were hypothesized that IAPP could directly activate NLRP3 inflammasome, leading to islets β-cell death. We analyzed expression of the inflammasome components ASC, NLRP3, caspase-1, IL-1β, IAPP/amylin, and insulin immunohistochemically in Langerhans' islets of autopsy cases. The initial event of NLRP3 inflammasome activation was assessed using a cell-free system consisting of NLRP3 and ASC with the amplified luminescent proximity homogeneous assay. IAPP/amylin deposition in Langerhans' islets was detected and significantly correlated with expressions of IL-1β and ASC. IAPP/amylin directly interacted with NLRP3 and initiated an interaction between NLRP3 and ASC in a cell-free system. The deposition of IAPP/amylin in β-cells of Langerhans' islets may act together with the expression level of an inflammasome component, ASC, to regulate IL-1β processing, and directly lead to the dysfunction of β-cells. The interaction between IAPP/amylin and NLRP3 could be an attractive drug target to avoid both inflammation and β-cell death for T2D therapy.

Keywords

ASC, cell-death, cell-free system, drug target, IAPP/amylin, IL-1β, inflammasome, NLRP3

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Introduction

Diabetes mellitus (DM) is a chronic disease. The number of patients with type 2 diabetes mellitus (T2D) has increased in the world. T2D is considered to be a chronic sterile inflammatory disease because of the elevation of serum inflammatory cytokines such as interleukin (IL)-1β and IL-6.[1] In addition, IL-1β has also been suggested to

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negatively affect the insulin-producing pancreatic β-cell function, and recent studies suggested that treatment with the IL-1 receptor antagonist anakinra cured T2D.[2]

NACHT, LRR and PYD domains-containing protein 3 (NLRP3), also known as cryopyrin, is an intracellular pattern recognition receptor that is activated by the recognition of pathogen-associated molecular pattern molecules and/or damage-associated molecular pattern molecules.[3] Activated NLRP3 interacts with the adaptor protein ASC to construct a huge signalosome called inflammasome, which activates pro-caspase-1, leading to IL-1β processing and activation in a complex.[4–6]

NLRP3 has also been reported to be involved in the pathogenesis of metabolic diseases including T2D, atherosclerosis, obesity, and gout.[7,8] Furthermore, the role of NLRP3 in diseases was associated with those in central nerve, lung disease, liver, kidney, and aging.[9–12] These diseases are thought to be caused by endogenous metabolites, which activate inflammasome.

Recently, it was reported that when a human islet amyloid polypeptide (IAPP)/amylin transgene is expressed in mice or rats, amyloid is deposited within pancreatic islets, leading to a reduced β-cell mass.[13,14] It was also reported that an endogenous metabolite, human amyloidogenic IAPP/amylin, can trigger activation of the NLRP3 inflammasome and IL-1β production in lipopolysaccharide (LPS)-primed infiltrated macrophages or dendritic cells, not in islet β-cells.[7] These findings prompted us to test the expression of inflammasome components in Langerhans' islets and investigate whether IAPP/amylin directly activates NLRP3 inflammasome.

In this study, we report an analysis of the expression of NLRP3, ASC, caspase-1, IL-1β, and IAPP/amylin in Langerhans' islets in the pancreas and the effect of IAPP/amylin on NLRP3 inflammasome using reconstituted NLRP3 inflammasome in a cell-free system.

Methods

Human pancreatic samples

Paraffin-embedded blocks of the human pancreas were selected from the autopsy files of the Department of Pathology of Ehime University. The study was approved by the Human Research Ethical Committee of Ehime University (reference number 1301001). Samples from diabetic patients should be included; however, because of the non-consolidated anonymous experiment, we do not know which samples were from diabetic patients.

Immunohistochemistry

Immunohistochemical analysis was carried out using the anti-ASC mouse monoclonal antibody developed in our previous study.[15] Anti-NLRP3 mouse monoclonal antibody [Nalpy3-b] was purchased from Alexis Biochemicals (San Diego, CA, USA). Anti-caspase-1 mouse monoclonal antibody (#2225) was purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-IL-1β rabbit polyclonal antibody (ab2105) and anti–IAPP/amylin mouse monoclonal antibody (ab115766) were purchased from Abcam (Cambridge, UK). Anti-insulin Guinea pig polyclonal antibody kit (A0654) was purchased from Dako (Carpinteria, CA, USA).

Three-micrometer-thick sections were obtained from formalin-fixed paraffin-embedded tissues. The sections were deparaffinized in xylene and rehydrated in an ethanol series with a decreasing concentration. Endogenous peroxidase activity was blocked by the addition of 1% H_2O_2 methanol for 30 min. The primary antibody was diluted in blocking buffer and exposed to the tissues overnight at 4°C. Their binding was detected using EnVision+ Mouse/Rabbit/HRP (Dako, Carpinteria, CA, USA), and positive signals were revealed by the addition of diaminobenzidine tetrahydrochloride (DAB). Tissue sections were counterstained with hematoxylin. A control experiment was performed by omitting the primary antibody from the staining procedure, and no specific staining was found.

Evaluation of expressions of ASC, NLRP3, capsase-1, IL-1β, and IAPP/amylin

For the evaluation of protein expressions of Langerhans' islets in immunochemistry, we defined the protein-positive score as follows: 3 (plus) is strong, complete submembranous and uniform, intense intracellular staining; 2 (plus) is weak complete submembranous staining or uniform, intense intracellular staining; 1 (plus) is weak, incomplete submembranous or partial intracellular staining in any proportion of cells; and 0 is no specific staining. Ten Langerhans' islets were evaluated using the categories above from each specimen under

Table 1. Clinicopathological data and expression of protein-positive score of inflammasome components and amylin in islets of Langerhans in autopsy cases.

Case	Age (years)	Sex	HbA1c (NGST%)	NLRP3	ASC	Caspase-1	IL-1β	Amylin
1	66	F	6.3	1.7 ± 0.48	1.7 ± 0.67	0.4 ± 0.52	2.1 ± 0.57	1.6 ± 0.52
2	75	M	8.3	1.2 ± 0.42	1.8 ± 0.32	0.3 ± 0.48	1.8 ± 0.42	1.1 ± 0.32
3	93	M	5.8	1.4 ± 0.52	1.3 ± 0.48	1.4 ± 0.84	1.4 ± 0.52	0.6 ± 0.52
4	69	M	6.1	0.4 ± 0.52	1.1 ± 0.32	1.6 ± 0.51	1.4 ± 0.70	0.9 ± 0.32
5	62	M	6.7	1.1 ± 0.32	2.1 ± 0.57	0.6 ± 0.84	2.1 ± 0.88	1.7 ± 0.67
6	65	M	8.5	1.5 ± 0.53	1.2 ± 0.42	0.1 ± 0.32	1.4 ± 0.52	1.7 ± 0.67
7	81	M	7.2	0.6 ± 0.52	0.7 ± 0.48	0.1 ± 0.32	1.3 ± 0.52	0.6 ± 0.52
8	57	M	5.3	0.8 ± 0.42	0.4 ± 0.71	0.9 ± 0.74	1.1 ± 0.32	0.8 ± 0.42
9	59	M	11.2	1.3 ± 0.48	2.2 ± 0.63	0.4 ± 0.52	2.4 ± 0.70	2.7 ± 0.48

NLRP3: NACHT, LRR, and PYD domains-containing protein 3; IL: interleukin.
Age: age at autopsy. Sex: female (F) and male (M); Values are mean ± SD of protein positive-scores from 10 individually evaluated islets of Langerhans in each case.

200× magnification in the best-stained area of each section. Data are presented in Table 1.

Immunofluorescence microscopy

Three-micrometer-thick sections were obtained from formalin-fixed paraffin-embedded tissues. The sections were deparaffinized in xylene and rehydrated in an ethanol series with a decreasing concentration. The tissue was exposed to anti-IL-1β rabbit polyclonal antibody (ab2105) for 1 h at room temperature followed by tetramethylrhodamine (TRITC)-conjugated swine anti-rabbit immunoglobulins (R156) (Dako, Carpinteria, CA, USA). The tissue was then exposed to anti-insulin Guinea pig polyclonal antibody (A0654) (Dako, Carpinteria, CA, USA) for 1 h at room temperature followed by fluorescein (FITC)-conjugated affinity pure goat anti-Guinea pig IgG F(ab′)2 fragment specific antibody (106-095-006) (Jackson ImmunoResearch, West Grove, PA, USA).

Recombinant protein synthesis using a wheat germ cell-free system

The NLRP3 entry clones, pDONR221-NLRP3-FL, DONR221-NLRP3-(Exons 1-2), pDONR221-NLRP3-(Exons 3-9), pDONR221-NLRP3-(Exons 4-9), and pDONR221-ASC, were constructed as previously reported.[16] The constructed plasmids were used to synthesize specific proteins with the WEPRO1240 Expression Kit (Cell-free, Inc., Matsuyama, Japan) followed by Western blotting.

Amplified luminescent proximity homogeneous assay

Synthesized protein–protein interactions were assessed using the amplified luminescent proximity homogeneous assay (Alpha). A total of 100 ng of each protein was applied to Alpha buffer (100-mM Tris-HCl (pH 8.0), 0.01% (v/v) Tween20), 1 mg/mL BSA, 17 μg/mL streptavidin-conjugated Alpha donor beads (PerkinElmer, Waltham, MA, USA), 17 μg/mL protein-A-conjugated Alpha acceptor beads, and 5 μg/mL anti-FLAG mAb M2, and incubated in an AlphaPlate-384 shallow well (PerkinElmer, Waltham, MA, USA) at 25°C for 24 h. The fluorescence emission signals of each well were measured using an EnSpire™ Multimode Plate Reader (PerkinElmer, Waltham, MA, USA).

Immunoprecipitation

Three micrograms of biotinylated IAPP/amylin and 1 μg of FLAG-NLRP3-FL, FLAG-NLRP3-(Exons 1-2), FLAG-NLRP3-(Exons 3-9), and FLAG-NLRP3-(Exons 4-9) lysed in 300 μL of NP-40 buffer (1% Nonidet P-40, 142.5 mmol/L KCl, 5 mmol/L $MgCl_2$, 10 mmol/L HEPES (pH 7.6), 0.2 mmol/L phenylmethylsulfonylfluoride (PMSF), and 1 mmol/L ethylenediaminetetraacetic acid (EDTA)) were precipitated with 20 μL of streptavidin-conjugated agarose beads (Invitrogen, Carlsbad, CA, USA) and incubated for 3 h at 4°C. The precipitations were subjected to SDS-PAGE and immunoblotting. Detection on the blotting membranes was performed using anti-FLAG mAb M2 (Sigma-Aldrich, St. Louis, MO, USA).

Chemicals

Human synthesized α−IAPP/amylin (4219-v) was purchased from PEPTIDE INSTITUTE, INC. (Osaka, Japan). Biotinylated IAPP/amylin (AS-64451-05) was purchased from AnaSpec (Fremont, CA, USA).

Statistics

Results are presented as the mean and standard deviation of data from three independent experiments, and the significance of differences was evaluated using Mann–Whitney U test. A $P<0.05$ was considered significant. Correlations were examined using Pearson's product–moment correlation coefficient.

Results

Expression of inflammasome components in Langerhans' islets of human pancreatic tissues

Immunohistochemical staining of Langerhans' islets. We examined the expression of inflammasome components such as ASC, NLRP3, caspase-1, and the substrate IL-1β in paraffin-embedded pancreatic tissue sections from autopsy cases by immunohistochemistry (Figure 1: ASC, NLRP3, caspase-1, and IL-1β, and Table 1). We also examined the expression of IAPP/amylin and a β-cell marker, insulin, in the same specimens to evaluate the deposition of IAPP/amylin and amounts of β-cells in Langerhans' islets by immunohistochemistry (Figure 1). In general, varied positive staining signals of ASC, NLRP3, caspase-1, IL-1β, IAPP/amylin, and insulin were observed (Figure 1).

Positive staining signals of ASC were located in subpopulations of cells of Langerhans' islets. Cytosolic signals were also found in cells of Langerhans' islets (Figure 1, ASC). A weak staining signal of NLRP3 was noted in Langerhans' islets and even diffusely in acinar cells of exocrine cells (Figure 1, NLRP3). A weak staining signal of NLRP3 was found in Langerhans' islets (Figure 1, Caspase-1). An intense staining signal of IL-1β was noted in subpopulations of cells in the cellular periphery of Langerhans' islets (Figure 1, IL-1β). A positive staining signal of IAPP/amylin was detected in cells and interstitial tissue in Langerhans' islets of pancreatic tissues, especially in hyaline-degenerated islets of Case 9 (Figure 1, Amylin).

Immunofluorescence staining of IL-1β and insulin in Langerhans' islets of pancreatic tissues. We investigated whether IL-1β-positive cells were β-cells that release insulin in Langerhans' islets using immunofluorescence microscopy. The data revealed that the green insulin-positive cells were almost identical to the red IL-1β-positive cells on dual immunofluorescence microscopy (Figure 2).

ASC-positive score correlated with IL-1β-positive score

Since ASC was reported to be indispensable for IL-1β processing by inflammasome, we examined whether the ASC-positive score was correlated with the IL-1β-positive score in the same patient samples as presented in Table 1. We found that the ASC-positive score was significantly correlated with the IL-1β-positive score based on Pearson's product–moment correlation coefficient $R=0.937$ with $P=0.000191$ (Figure 3(a), Table 1).

IAPP/amylin-positive score correlated with ASC and IL-1β-positive score

Since IAPP/amylin was reported to activate inflammasome, we examined whether an IAPP/amylin-positive score was correlated with NLRP3, ASC, caspase-1, and IL-1β−positive scores in the same patient samples as presented in Table 1. We found that an IAPP/amylin-positive score was significantly correlated with ASC and IL-1β-positive scores with based on Pearson's product–moment correlation coefficient $R=0.742$ with $P=0.0220$ (Figure 3(b)) and $R=0.823$ with $P=0.0640$, respectively (Figure 3(c)). However, there was no significant correlation between an IAPP/amylin-positive score and NLRP3 or caspase-1 (Figure 3(d) and (f)).

IAPP/amylin-positive score correlated with most recent HbA1c value of patients

We examined whether an IAPP/amylin-positive score was correlated with the most recent HbA1c value of patients presented in Table 1. We found

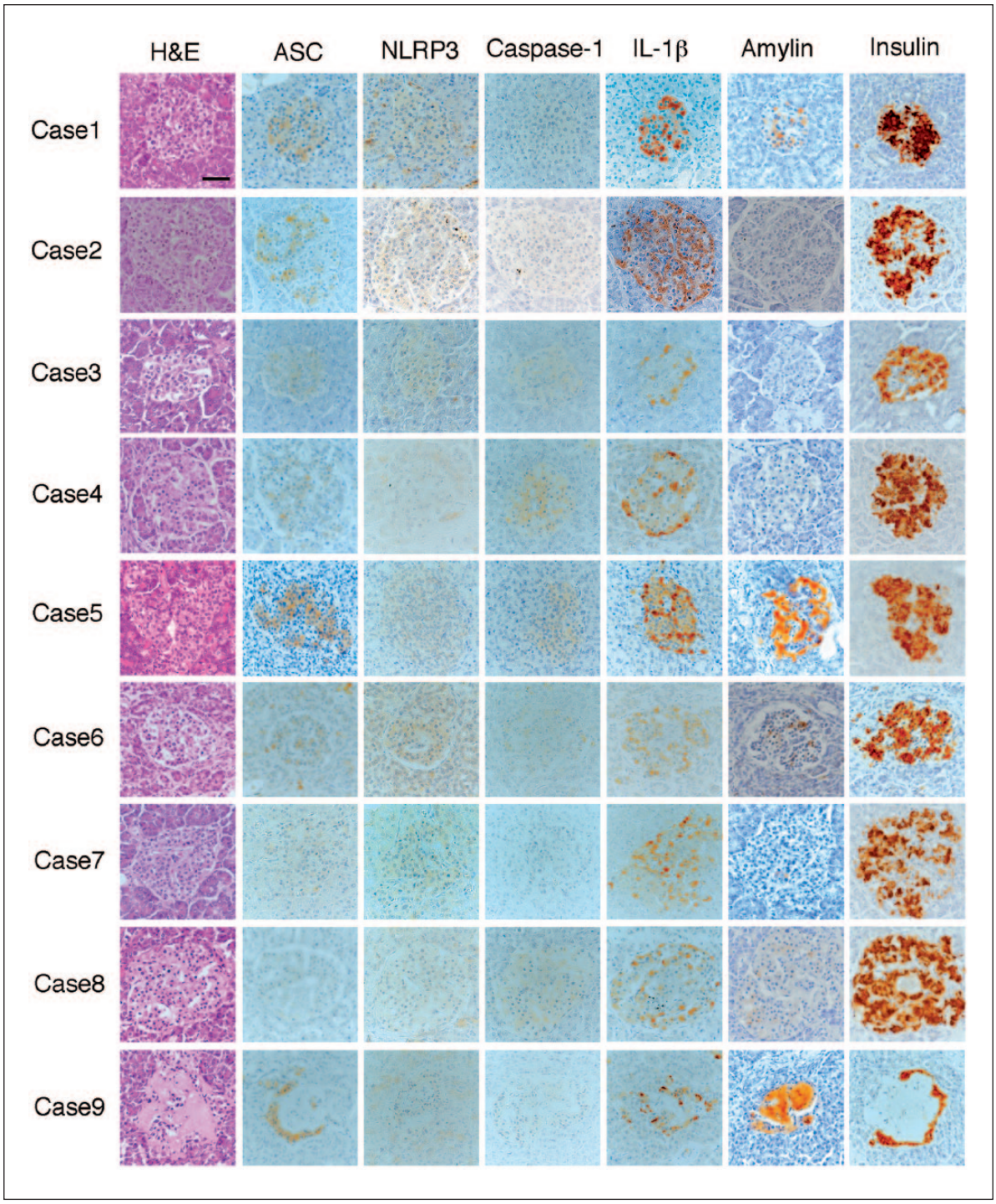

Figure 1. Expression of NLRP3, ASC, caspase-1, IL-1β, and IAPP/amylin in Langerhans' islets of pancreas. Formalin-fixed and paraffin-embedded specimens of nine autopsy cases were immunostained with specific antibodies to ASC, NLRP3, caspase-1, IL-1β, amylin, and insulin as indicated. Representative Langerhans' islets in each case are presented. Parallel hematoxylin and eosin staining is presented on the left. The bar is 100 μm. The cases are summarized in Table 1.

that an IAPP/amylin-positive score was significantly correlated with the HbA1c value based on Pearson's product–moment correlation coefficient $R = 0.774$ with $P = 0.0143$ (Figure 3(f)).

IAPP/amylin-induced interaction between NLRP3 and ASC in a cell-free system

C-terminal biotinylated full-length NLRP3 (NLRP3-FL-Btn) and N-terminal FLAG-tagged

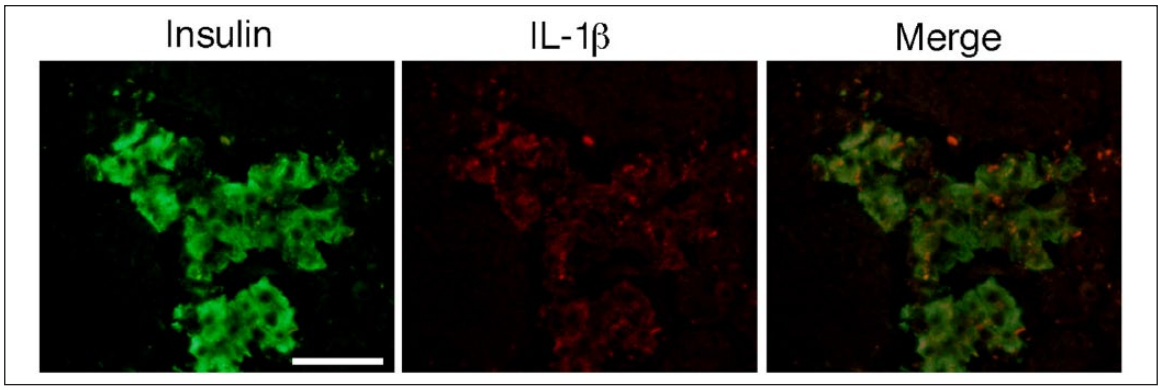

Figure 2. Dual immunofluorescence microscopy of islet of Langerhans in pancreas. Formalin-fixed and paraffin-embedded specimens were stained with anti-insulin Guinea pig polyclonal antibody with FITC-conjugated secondary antibody (left, insulin, green) and anti-IL-1β rabbit polyclonal antibody with TRITC-conjugated secondary antibody (middle, IL-1β, red) and were observed using a fluorescence microscope. A merged image is shown (right, merge, yellow). The bar is 10 μm.

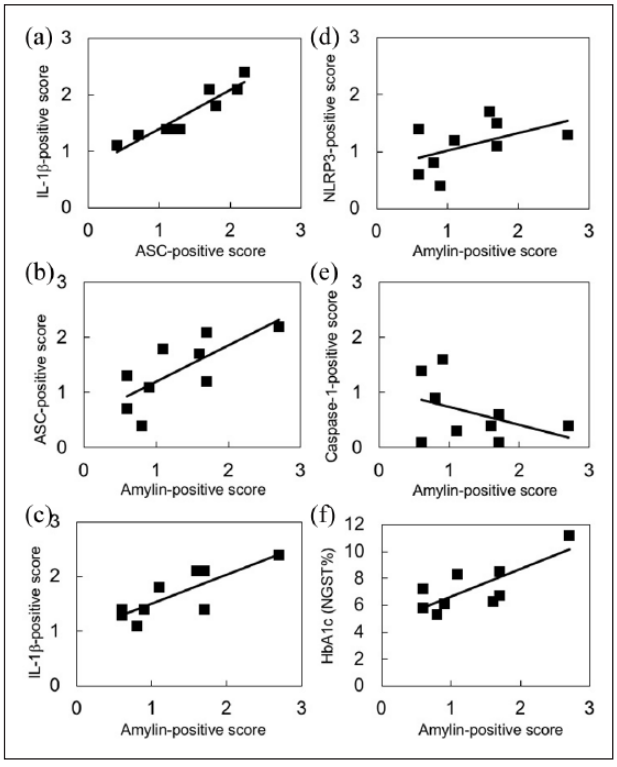

Figure 3. Correlation diagram comparing IAPP/amylin-positive scores and each value. (a) Correlation analysis between ASC-positive score and IL-1β-positive score. (b) Correlation analysis between IAPP/amylin-positive score and ASC-positive score. (c) Correlation analysis between IAPP/amylin-positive score and IL-1β-positive score. (d) Correlation analysis between IAPP/amylin-positive score and NLRP3-positive score. (e) Correlation analysis between IAPP/amylin-positive score and caspase-1-positive score. (f) Correlation analysis between IAPP/amylin-positive score and serum HbA1c (%).

full-length ASC (FLAG-ASC-FL) are schematically indicated in Figure 4(a).

Using wheat germ cell-free system–specific expression plasmids, NLRP3-FL-Btn and FLAG-ASC-FL proteins were also synthesized as described previously.[16,17]

Since we previously reported the construction of NLRP3 inflammasome in a cell-free system that is able to detect NLRP3 inflammasome–activating ligands, we investigated whether IAPP/amylin was able to induce interaction between NLRP3 and ASC using the reconstituted NLRP3 inflammasome. Expectedly, IAPP/amylin induced the interaction between FLAG-ASC-FL and NLRP3-FL-Btn in the cell-free system (Figure 4(b), Amylin). A positive control poly(I:C) also induced the interaction between FLAG-ASC-FL and NLRP3-FL-Btn (Figure 4(b), poly(I:C)), whereas neither MDP, LPS nor poly(C) induced this interaction (Figure 4(b), muramyl dipeptide (MDP), LPS, and poly(C), respectively).

To confirm the direct interaction between NLRP3 and IAPP/amylin, we examined the interaction between N-terminal FLAG-tagged full-length NLRP3 (FLAG-NLRP3-FL) and biotinylated IAPP/amylin. The Alpha signals of these interactions gradually increased with IAPP/amylin in dose-dependent manner (Figure 4(c)).

IAPP/amylin directly interacted with NLRP3

We assessed the specific binding site of NLRP3 with biotinylated IAPP/amylin by Alpha and immunoprecipitation. FLAG-NLRP3-FL and its truncated proteins, such as FLAG-NLRP3-(Exons 1-2), FLAG-NLRP3-(Exons 3-9), and FLAG-NLRP3-(Exons 4-9), were prepared (Figure 5(a)). Stronger Alpha signals were detected between

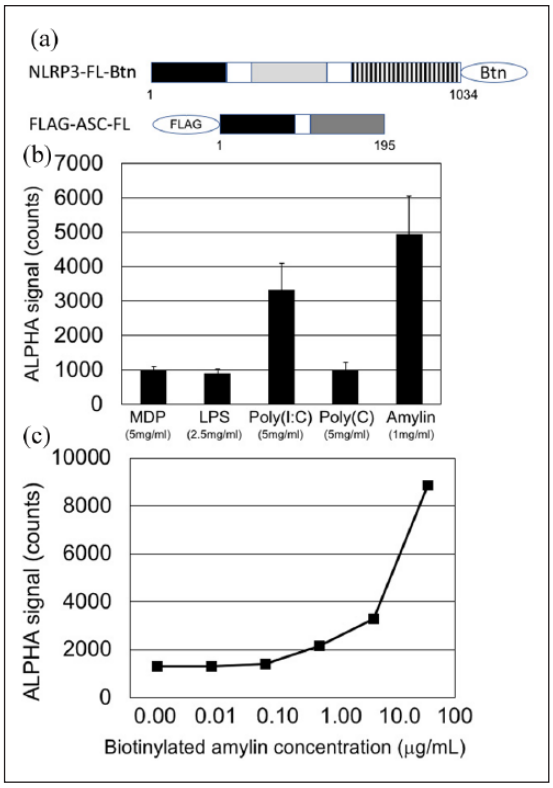

Figure 4. IAPP/amylin-induced interaction between NLRP3 and ASC in a cell-free system. (a) Schematic representations of NLRP3 and ASC. Synthetic C-terminal biotinylated full-length NLRP3 (NLRP3-FL-Btn) and N-terminal FLAG-tagged ASC (FLAG-ASC-FL) are indicated. The pyrin domain (PYD) is indicated by black boxes. The caspase recruitment domain (CARD) is indicated by a dark gray box. The nucleotide-binding oligomerization domain (NOD) is indicated by a light gray box. Leucine-rich repeats are indicated by a striped box. Amino acid sequence numbers are indicated under each schema. (b) Interaction between NLRP3-FL-Btn and FLAG-ASC-FL was detected by an amplified luminescent proximity homogeneous assay (Alpha). Responses (counts) were measured using an EnSpire™ Multimode Plate Reader. The results are given as means ± standard deviation from triplicate wells. *P-value < 0.05 was considered statistically significant using Mann–Whitney U test. (c) IAPP/amylin directly interact with NLRP3 in a dose-dependent manner. Representative results are shown from three independent experiments.

FLAG-NLRP3-FL, FLAG-NLRP3-(Exon 3-9), or FLAG-NLRP3-(Exon 4-9) and biotinylated IAPP/amylin (Figure 5(b)).

These interactions between IAPP/amylin and NLRP3 or its truncated forms were confirmed by immunoprecipitation. FLAG-NLRP3-FL or FLAG-NLRP3-(Exons 4-9) was co-precipitated with 10 μg/mL IAPP/amylin (Figure 5(c)).

Discussion

Over the past decade, accumulating data have indicated that the pattern recognition receptors in innate immunity can recognize excessive nutrient intake associated with a stress-related biological event. Especially, the NLRP3 inflammasome was reported to mediate inflammatory and stress responses in various tissues that contribute to the development of metabolic disorders.[18]

T2D was reported to be an inflammatory disease linked to the pathogenesis of hypersecretion of IL-1β and activation.[1,2,19,20] IAPP/amylin is co-secreted with insulin from islet β-cells into the extracellular spaces in islets by the exocytosis of secretory granules.[21] Oxidative stress was reported to induce pancreatic β-cell death.[22] The pancreatic β-cell death was considered to be caused by IL-1β released by infiltrated macrophages.[23] The sources of IL-1β were reported to be primary macrophages that had phagocytosed IAPP/amylin through frustrated phagocytosis, resulting in reactive oxygen species (ROS) formation.[24] The IAPP/amylin or generated ROS are thought to be involved in the inflammasome complex nucleated by NLRP3.[25]

Consistent with this, a recent paper reported that amyloidogenic human IAPP/amylin can trigger the processing of IL-1β from bone marrow–derived macrophages in a mouse model.[7] The inflammasome activation and pancreatic β-cell death mediated by IAPP/amylin were suggested to be involved in a ROS-sensitive manner.[26]

In this study, we have found additional evidence that both ASC and IL-1β are highly expressed in β-cells in Langerhans' islets of human pancreatic tissues, which correlates with the deposition of IAPP/amylin. Also, IAPP/amylin directly initiates an interaction between NLRP3 and ASC, which may lead to NLRP3 inflammasome activation in a cell-free system.

The distributions of inflammasome components in Langerhans' islets are interesting (Figure 1). Both NLRP3 and caspase-1 expressions were diffusely distributed in the cells of Langerhans' islets (Figure 1, NLRP3 and Caspase-1). However, ASC and IL-1β were likely to be restricted in specific cells (Figure 1, ASC and IL-1β). Dual immunofluorescence microscopy revealed that red-fluorescence-positive IL-1β-expressing cells are almost identical to green-fluorescence-positive insulin-expressing cells suggested to be β-cells in Langerhans' islets (Figure 2). Thus, we suggested that expressions of both ASC and IL-1β are involved in β-cell functions.

In support of the description above, there is a significant correlation between ASC-positive and IL-1β-positive scores (Figure 3(a)), and both

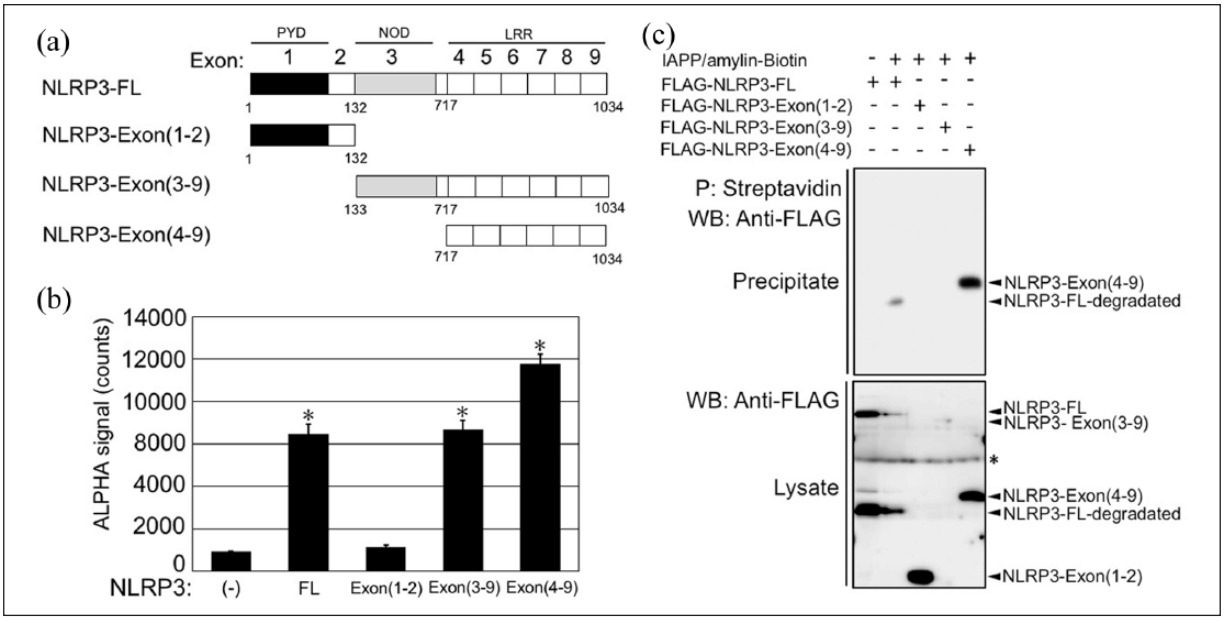

Figure 5. IAPP/amylin directly interacted with LRR domain of NLRP3. (a) A schematic representation of the NLRP3 structure depicts Exon components and truncated proteins. (b) Synthetic protein-protein interactions between biotinylated IAPP/amylin and FLAG-NLRP3-FL, FLAG-NLRP3-(Exons 1-2), FLAG-NLRP3-(Exons 3-9), or FLAG-NLRP3-(Exons 4-9) were assessed by amplified luminescent proximity homogeneous assay (Alpha). The results are given as means ± standard deviation from triplicate wells. *P-value < 0.05 was considered statistically significant using Mann–Whitney U test. (c) Interactions between biotinylated IAPP/amylin and FLAG-NLRP3-FL, FLAG-NLRP3-(Exons 1-2), FLAG-NLRP3-(Exons 3-9), or FLAG-NLRP3-(Exons 4-9) were assessed by immunoprecipitation. The asterisk indicates a non-specific band.
WT: wild-type; P: precipitation; WB: western blot; Lysate: lysate after immunoprecipitation.

scores were significantly correlated with an IAPP/amylin-positive score (Figure 3(b) and (c)). However, there were no significant correlations between an NLRP3-positive score or capase-1-positive score and IAPP/amylin-positive score (Figure 3(d) and (e)). IAPP/amylin deposition in Langerhans' islets is associated with T2D progression.[27] Consistent with this, an IAPP/amylin-positive score was significantly correlated with the most recently obtained HbA1c value (Figure 3(f)).

Does IAPP/amylin induces an interaction between NLRP3 and ASC without ROS machinery? To answer this, we performed recently developed cell-free experiments.[17] Cell-free experiments, with no ROS, revealed that IAPP/amylin directly induces the interaction between NLRP3 and ASC confirmed by Alpha and immunoprecipitation, which could be an initial event of NLRP3 inflammasome activation. Consistent with the known function of NLRP3, IAPP/amylin directly interacted with LRR domain (NLRP3-Exon (4-9)) of NLRP3 (Figures 4 and 5). Inflammasome activation reportedly leads to IL-1β secretion accompanied by pyroptotic cell death.[6] In addition, pancreatic β-cells and endothelial cells were reported to express IL-1R1, which could mediate the function of increased IL-1β.[28,29] Thus, direct activation of the NLRP3 inflammasome in β-cells in Langerhans' islets by IAPP/amylin may contribute inflammation and β-cell death in T2D in addition to the ROS-mediated mechanism in living cells. Although we cannot deny the accumulated evidence that ROS are crucial intrinsic triggers of NLRP3 inflammasome activation in infiltrating macrophages,[7] we provide evidence that IAPP/amylin could directly trigger NLRP3 inflammasome in β-cells of Langerhans' islets in human tissues.

In conclusion, IAPP/amylin directly interacts with NLRP3 to activate NLRP3 inflammasome, and this interaction could be an attractive drug target to avoid inflammation and β-cell death during therapy for diabetes, although there are mechanisms in NLRP3 inflammasome and diabetes pathology in human tissues that still require elucidation. Our data may help to advance understanding of these mechanisms.

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S.M., N.K., C.O., H.T., M.K., T.Y., H.O., A.N., T.Z., and J.M. were responsible for the acquisition of data. S.M., N.K., and J.M. analyzed the data. All authors contributed to the interpretation of the data. J.M. drafted the article. All authors critically reviewed and edited the manuscript. J.M. is the guarantor of this work. S.M. and N.K. contributed equally to this work.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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References

1. Spranger J, Kroke A, Möhlig M, et al. (2003) Inflammatory cytokines and the risk to develop type 2 diabetes: Results of the prospective population-based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. *Diabetes* 52: 812–817.
2. Larsen CM, Faulenbach M, Vaag A, et al. (2007) Interleukin-1-receptor antagonist in type 2 diabetes mellitus. *New England Journal of Medicine* 356: 1517–1526.
3. Franchi L, Muñoz-Planillo R and Núñez G (2012) Sensing and reacting to microbes through the inflammasomes. *Nature Immunology* 13: 325–332.
4. Manji GA, Wang L, Geddes BJ, et al. (2002) PYPAF1, a PYRIN-containing Apaf1-like protein that assembles with ASC and regulates activation of NF-kappaB. *Journal of Biological Chemistry* 277: 11570–11575.
5. Srinivasula SM, Poyet JL, Razmara M, et al. (2002) The PYRIN-CARD protein ASC is an activating adaptor for caspase-1. *Journal of Biological Chemistry* 277: 21119–21122.
6. Martinon F, Burns K and Tschopp J (2002) The inflammasome: A molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Molecular Cell* 10: 417–426.
7. Masters SL, Dunne A, Subramanian SL, et al. (2010) Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1β in type 2 diabetes. *Nature Immunology* 11: 897–904.
8. Wen H, Ting JP and O'Neill LA (2012) A role for the NLRP3 inflammasome in metabolic diseases—Did Warburg miss inflammation? *Nature Immunology* 13: 352–357.
9. Anders HJ and Muruve DA (2011) The inflammasomes in kidney disease. *Journal of the American Society of Nephrology* 22: 1007–1018.
10. Szabo G and Csak T (2012) Inflammasomes in liver diseases. *Journal of Hepatology* 57: 642–654.
11. Youm YH, Grant RW, McCabe LR, et al. (2013) Canonical NLRP3 inflammasome links systemic low-grade inflammation to functional decline in aging. *Cell Metabolism* 18: 519–532.
12. De Nardo D, De Nardo CM and Latz E (2014) New insights into mechanisms controlling the NLRP3 inflammasome and its role in lung disease. *American Journal of Pathology* 184: 42–54.
13. Verchere CB, D'Alessio DA, Palmiter RD, et al. (1996) Islet amyloid formation associated with hyperglycemia in transgenic mice with pancreatic beta cell expression of human islet amyloid polypeptide. *Proceedings of the National Academy of Sciences of the United States of America* 93: 3492–3496.
14. Janson J, Soeller WC, Roche PC, et al. (1996) Spontaneous diabetes mellitus in transgenic mice expressing human islet amyloid polypeptide. *Proceedings of the National Academy of Sciences of the United States of America* 93: 7283–7288.
15. Masumoto J, Taniguchi S, Ayukawa K, et al. (1999) ASC, a novel 22-kDa protein, aggregates during apoptosis of human promyelocytic leukemia HL-60 cells. *Journal of Biological Chemistry* 274: 33835–33838.
16. Kaneko N, Ito Y, Iwasaki T, et al. (2017) Poly (I:C) and hyaluronic acid directly interact with NLRP3, resulting in the assembly of NLRP3 and ASC in a cell-free system. *European Journal of Inflammation* 15: 85–97.
17. Kaneko N, Iwasaki T, Ito Y, et al. (2017) Applications of reconstituted inflammasomes in a cell-free system to drug discovery and elucidation of the pathogenesis of autoinflammatory diseases. *Inflammation and Regeneration* 37: 9.
18. Li HB, Jin C, Chen Y, et al. (2014) Inflammasome activation and metabolic disease progression. *Cytokine & Growth Factor Reviews* 25: 699–706.
19. Lamkanfi M, Mueller JL, Vitari AC, et al. (2009) Glyburide inhibits the Cryopyrin/NALP3 inflammasome. *Journal of Cell Biology* 187: 61–70.
20. Donath MY and Shoelson SE (2011) Type 2 diabetes as an inflammatory disease. *Nature Reviews Immunology* 11: 98–107.
21. Rorsman P and Renström E (2003) Insulin granule dynamics in pancreatic beta cells. *Diabetologia* 46: 1045–1029.

22. Sakuraba H, Mizukami H, Yagihashi N, et al. (2002) Reduced beta-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese type II diabetic patients. *Diabetologia* 45: 85–96.
23. Dinarello CA, Donath MY and Mandrup-Poulsen T (2010) Role of IL-1beta in type 2 diabetes. *Current Opinion in Endocrinology, Diabetes and Obesity* 17: 314–321.
24. Hornung V, Bauernfeind F, Halle A, et al. (2008) Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nature Immunology* 9: 847–856.
25. Zraika S, Hull RL, Udayasankar J, et al. (2009) Oxidative stress is induced by islet amyloid formation and time-dependently mediates amyloid-induced beta cell apoptosis. *Diabetologia* 52: 626–635.
26. Zhou R, Tardivel A, Thorens B, et al. (2010) Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nature Immunology* 11: 136–140.
27. Ohsawa H, Kanatsuka A, Mizuno Y, et al. (1992) Islet amyloid polypeptide-derived amyloid deposition increases along with the duration of type 2 diabetes mellitus. *Diabetes Research and Clinical Practice* 15: 17–21.
28. Böni-Schnetzler M, Boller S, Debray S, et al. (2009) Free fatty acids induce a proinflammatory response in islets via the abundantly expressed interleukin-1 receptor I. *Endocrinology* 150: 5218–5229.
29. Song A, Zhu L, Gorantla G, et al. (2018) Salient type 1 interleukin 1 receptor expression in peripheral non-immune cells. *Scientific Reports* 8: 723.