Chapter 2

Activating the NLRP3 Inflammasome Using the Amyloidogenic Peptide IAPP

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Abstract

In addition to several other extracellular substances, phagocytosis of amyloid-forming peptides can perturb cellular homeostasis, leading to activation of the cytoplasmic innate immune receptor NLRP3. Once triggered, NLRP3 forms an inflammasome complex that ultimately cleaves pro-IL-1β and pro-IL-18 into their mature, secreted forms. Here we describe a protocol by which one type of amyloidogenic peptide, islet amyloid polypeptide (IAPP, otherwise known as amylin) can be prepared and used to stimulate myeloid cells in vitro to engage the NLRP3 inflammasome. Methods for measuring the ensuing inflammasome activation are also described. Although initially soluble, IAPP monomers rapidly aggregate in solution to form oligomers and subsequently insoluble amyloid fibrils. More work is required to examine how this transition influences inflammasome activation for different types of amyloid. The course of amyloid formation and corresponding inflammatory capacity of these pre-fibrillar species following uptake also requires further examination, and we hope that our protocols are useful in these endeavors. While these protocols are restricted to examination of synthetic IAPP, isolation of IAPP aggregates from human and transgenic mouse pancreas will be required to definitively determine the proinflammatory effects of endogenous IAPP oligomers and fibrils.

Key words Inflammasome, Amyloid, Innate immunology, NLR, IAPP

1 Introduction

Activation of innate immune cells by several different types of amyloid-forming peptides has been reported for a number of years and may be mediated, in part, by cell surface Toll-like receptors [1, 2]. Recently, it has emerged that the cytoplasmic innate immune receptor, NLRP3, operates in myeloid cells to detect the danger presented by these uniquely folded proteins [3–7]. Although the exact mechanism by which amyloid triggers NLRP3 remains unknown, the downstream process of apoptosis-associated speck-like protein containing a CARD (ASC) oligomerization, autocatalytic activation of caspase-1, and subsequent cleavage of IL-1β and IL-18, in a complex called the inflammasome, has been well

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described [8]. Protocols to examine activation at several points in this pathway are described herein, namely, (a) measurement of caspase-1 activity using a fluorescent caspase-1 substrate and (b) Western blotting and ELISA measurement of mature, secreted IL-1β and IL-18, respectively. Furthermore, we describe preparation of the materials required (putative oligomers of IAPP capable of forming amyloid), and a protocol for detecting this amyloid when it is phagocytosed by macrophages.

IAPP is a 37 amino acid peptide that is co-secreted with insulin by beta cells [9, 10]. Insoluble amyloid deposits comprised primarily of IAPP fibrils are found in more than 90 % of patients with type 2 diabetes at autopsy [11, 12]. Amyloid fibrils are defined by a cross-beta sheet quaternary structure with a characteristic X-ray diffraction pattern. Several lines of evidence suggest that soluble oligomers of IAPP rather than mature fibrils may be the major cytotoxic species and also an important stimulus for islet inflammation, although both fibrils and oligomers likely contribute to beta cell dysfunction in vivo [13]. We have recently provided evidence to suggest that IAPP acts as a trigger for macrophage recruitment and secretion of proinflammatory cytokines that are toxic to beta cells, suggesting that islet amyloid may be a major stimulus for islet inflammation observed in patients with type 2 diabetes [6, 7]. Oligomers of human IAPP share a common structure with numerous other disease-causing amyloids [14]. The inflammatory capacity of these amyloids and other protein aggregates is currently an area of intensive research, with significant clinical implications for diseases such as Alzheimer's disease, age-related macular degeneration, Huntington's disease, Parkinson's disease, and others [8]. Despite recent advances in theoretical and empirical modeling of amyloid formation and toxicity, further work is required to determine the biochemical basis for the pro-inflammatory propensity of amyloidogenic peptides both in vitro and in vivo.

In total we outline four methods. The first uses fluorescence microscopy to detect IAPP amyloid when present in macrophages, and could be modified to detect different species of amyloid in other cell types. This could also be examined contemporaneously with measurement of inflammasome activation such as ASC "speck" formation. Secondly, measurement of caspase-1 activation by IAPP is described using the FAM-FLICA Caspase Detection Kit. This assay is based on the irreversible binding of a cell permeable fluorescein labeled inhibitor to the active enzyme, which can be detected by fluorescence microscopy; however, we describe its application in flow cytometry. Thirdly, we briefly describe Western blot analysis of cleaved IL-1β after IAPP activation. Finally, we outline a protocol to measure secreted IL-18 by ELISA that is more affordable than commercially available ready-made kits. It is important that for all experiments, cytotoxicity is measured by propidium

iodide, LDH or another method in parallel. This will ensure that the effect observed occurs at a time and concentration of IAPP that does not cause significant levels of cell death.

2 Materials

2.1 Preparation of IAPP Oligomers

1. Islet amyloid polypeptide (IAPP) (*see* **Note 1**).
2. Rodent IAPP (MW 3920).
3. 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Sigma).
4. 0.1 M acetic acid.
5. Sigmacote (Sigma).
6. Thioflavin T powder (Sigma): Dissolve in distilled water (dH_2O) to prepare a 100 μM (10×) stock solution prior to each assay. Pass the solution through a 0.2 μm filter to eliminate small aggregates (*see* **Note 2**). The stock solution can be stored in the dark at 4 °C for 1 month.
7. 0.2 μm filter.
8. Sterile 1.5 mL tubes.
9. Desiccator at −20 °C.
10. Nitrogen gas or lyophilizer.
11. 96-well plate for fluorescence measurement (clear bottom).
12. Fluorescence plate reader.

2.2 Detection of IAPP Amyloid in Macrophages by Fluorescent Microscopy

1. Phosphate buffered saline (PBS).
2. 4 % Paraformaldehyde.
3. 0.2 % Triton X-100.
4. 70 % Ethanol.
5. Distilled water (dH_2O).
6. Thioflavin S powder (Sigma) (*see* **Note 3**).
7. Bone marrow derived macrophages (BMDMs).
8. Microscope slides or glass-bottom plates for fluorescence microscopy.
9. Confocal or fluorescence microscope.

2.3 Analysis of Active Caspase-1 in Macrophages Using a Cleavable Fluorescent Substrate

1. BMDMs.
2. PBS, pH 7.4.
3. Dimethyl sulfoxide (DMSO).
4. 24-well plate.
5. Cell culture incubator at 37 °C under 5 % CO_2.
6. Cell scraper.

7. FACS tubes.
8. FAM-FLICA Caspase Detection Kit (ImmunoChemistry Technologies).
9. Flow cytometer with an argon ion laser.

2.4 Western Blotting for Cleaved, Secreted IL-1β

1. Ultrapure LPS from *E. coli* (Invivogen).
2. Methanol.
3. Chloroform.
4. 2× SDS loading buffer: 4 % SDS, 125 mM Tris–HCl, pH 6.8, 20 % glycerol, 0.02 % (w/v) bromophenol blue, 200 mM dithiothreitol.
5. 12 % or gradient SDS-polyacrylamide gel.
6. PVDF or nitrocellulose membrane.
7. Monoclonal rat anti-mouse IL-1β antibody (R&D Systems, Clone 166926).
8. Heat block for 50 and 95 °C incubations.

2.5 ELISA Analysis of Mouse IL-18

1. 96-well NUNC Maxisorp ELISA plate.
2. Wash buffer: PBS + 0.05 % Tween 20 (PBS-T).
3. Block: 1 % BSA + PBS-T.
4. Rat anti-mouse IL-18 capture antibody (clone 74).
5. Biotinylated rat anti-mouse IL-18 detection antibody (clone 93-10C).
6. Murine recombinant IL-18 cytokine standard (R&D systems, B004-5).
7. HRP-streptavidin.
8. TMB substrate solution.
9. Stop solution: 1 M H_2SO_4 or alternative.

3 Methods

3.1 Preparation of IAPP Oligomers (See Notes 1 and 4)

1. Dissolve 0.5 mg of lyophilized human IAPP (MW 3,903 Da) at a concentration of 500 μM by adding 256 μL of HFIP. Use rodent IAPP as a non-amyloidogenic control. Allow the peptide to dissolve in HFIP for several hours or overnight, and then aliquot as desired (e.g., 50 μL per tube in 1.5 mL tubes).
2. Remove the HFIP using nitrogen gas. To do this, gently blow the inert gas into the tube (*see* **Note 5**).
3. Resuspend an aliquot in 5 μL of 0.1 M acetic acid (*see* **Note 6**).

4. This can then be diluted in H_2O, PBS, or media to the same volume as before the HFIP was removed, to return a 500 μM concentration, which we typically use as a 50× stock (*see* **Note 7**).
5. Measure IAPP aggregation using Thioflavin T fluorescence. Dissolve IAPP at the desired concentration in media under conditions used in parallel tissue culture experiments. Add Thioflavin T at a final concentration of 10 μM for a final volume of 50–100 μL/well in a 96-well plate (black with clear bottom) (*see* **Note 8**).
6. Incubate the plate at 37 °C and monitor amyloid formation by fluorescence for up to 24 h at 450/482 nm (Thioflavin T excitation/emission peaks) (*see* **Notes 9** and **10**). Ensure the plate is well sealed to prevent evaporation.

3.2 Detection of IAPP Amyloid in Macrophages by Fluorescence Microscopy

1. Seed cells of interest such as bone marrow derived macrophages (BMDMs) on multi-well glass microscope chamber slides or a 96-well plate for fluorescence microscopy (10^5 cells/well).
2. Allow cells to adhere overnight at 37 °C.
3. Treat cells with human IAPP (use rat IAPP as a non-amyloidogenic control) from 0 to 10 μM over 0–24 h to assess peptide uptake and aggregation (*see* **Note 11**).
4. Wash cells with PBS and fix with 4 % paraformaldehyde for 15 min at room temperature (RT). Use sufficient paraformaldehyde to cover the cells completely (e.g., 50 μL/well for a 96-well plate or 500 μL for an 8-well chamber slide).
5. Wash cells with PBS and then store at 4 °C until ready for staining.
6. Permeabilize cells by adding sufficient 0.2 % Triton X-100 to immerse them entirely for 5 min at RT.
7. Wash 3× in PBS for 5 min each wash.
8. Incubate cells with 0.01 % Thioflavin S for 10 min at RT. Use enough Thioflavin S to fully immerse fixed cells.
9. Wash stained cells 3× for 5 min each with 70 % ethanol followed by dH_2O (*see* **Note 12**).
10. Mount cells as desired and examine by confocal or fluorescence microscopy (*see* **Note 13**).

3.3 Analysis of Active Caspase-1 in Macrophages Using a Cleavable Fluorescent Substrate

1. Seed BMDMs in a 24-well plate (500 μL/well) at a density of 1×10^6 per mL, the day before carrying out this assay.
2. Treat cells with 10 μL/well of a 500 μM IAPP stock in order to give a final concentration of 10 μM and incubate for 1 h at 37 °C (*see* **Note 14**).
3. Detach cells from the base of the wells using a cell scraper and transfer to FACS tubes.

4. Reconstitute the FLICA reagent with 50 μL of DMSO to generate a 150× stock solution (*see* **Note 15**). Dilute the required amount of 150× stock solution 1:5 in PBS immediately prior to use; this will form a 30× stock solution.
5. Add 16 μL/well of 30× FLICA reagent to IAPP treated and untreated cells and incubate for 1 h at 37 °C under 5 % CO_2 (Unlabeled IAPP treated and untreated cells should be included as additional controls).
6. Centrifuge the plate at <400 × *g* for 5 min at RT.
7. Prepare the required amount of wash buffer by diluting the 10× wash buffer stock solution from the FLICA kit 1:10 with dH_2O.
8. Wash cells with 2 mL of 1× wash buffer and centrifuge at <400 × *g* for 5 min at RT. Repeat this step twice more.
9. Discard the supernatant and resuspend cells in 300 μL of 1× wash buffer. Run cells on a flow cytometer with an argon ion laser at 488 nm and detect in the FL1 channel (515–535 nm) (*see* **Note 16**).

3.4 Western Blotting for Cleaved, Secreted IL-1β

1. Seed BMDMs in a 24-well plate (500 μL/well) at a density of 1×10^6 per mL, in a low concentration of FCS, the day before carrying out this assay (*see* **Note 17**).
2. Prime cells with 100 ng/mL ultrapure LPS for 3 h at 37 °C.
3. Stimulate cells with 10 μM IAPP overnight.
4. Collect the supernatant and mix with 500 μL methanol and 125 μL chloroform to precipitate all proteinaceous material.
5. Centrifuge the sample at 16,000 × *g* for 5 min and then remove the top layer, leaving the precipitate at the interface and a lower liquid layer.
6. Add 500 μL of methanol and resuspend.
7. Centrifuge the sample for 5 min at 16,000 × *g* and then aspirate the supernatant and dry the pellet at 50 °C for 5 min.
8. Resuspend the pellet in 50–100 μL 2× SDS loading buffer, and mix and heat at 95 °C until the pellet is dissolved.
9. Run the sample on a low percentage or gradient SDS-polyacrylamide gel, then transfer to PVDF or nitrocellulose, and blot using an antibody such as rat anti-mouse IL-1β.

3.5 ELISA Analysis of Mouse IL-18

1. Coat each well of a 96-well NUNC Maxisorp ELISA plate with 100 μL IL-18 capture antibody diluted 1/1,000 in PBS (final concentration 1 μg/mL), overnight at 4 °C (*see* **Note 18**).
2. Wash the plate 2× with PBS-T (approximately 200 μL, can be filled by immersion).

3. Block the plate with 200 μL of 1 % BSA + PBS-T per well, for 2 h at RT.
4. Wash the plate as in **step 2** of Subheading 3.5.
5. Add samples and recombinant mouse IL-18 standard made up in 1 % BSA + PBS-T, then incubate the plate for 2 h at RT or overnight at 4 °C (*see* **Note 19**).
6. Wash the plate 5× with PBS-T then add 100 μL IL-18 detection antibody diluted 1/10,000 in 1 % BSA + PBS-T and incubate for 1 h at RT (*see* **Note 18**).
7. Wash the plate 5× with PBS-T, then add HRP-streptavidin and incubate for 30 min at RT.
8. Wash the plate 5× with PBS-T then add 100 μL TMB substrate solution per well and incubate for 5–10 min.
9. Add 50 μL stop solution per well and read absorbance at 450 nm, subtracting background reading at 620 nm.

4 Notes

1. Milligram quantities of IAPP peptide can be synthesized by traditional chemistries. Alternatively, these can be purchased from commercial sources, such as Bachem or Merck. When working with IAPP, and other amyloid species, it may be important to use glassware, tips, and other equipment that are treated to minimize adherence. In this protocol, when resuspending IAPP, using tips that are treated with Sigmacote promotes recovery.
2. Thioflavin T is a benzothiazole salt that undergoes a red shift of its emission spectrum upon binding to beta sheet-rich structures. The increase in emission at 482 nm can be used to monitor amyloid formation [15]. Fluorescence correlates with fibril formation as determined by electron microscopy, although Thioflavin T may also bind some pre-fibrillar IAPP aggregates [16].
3. Thioflavin S, similar to Thioflavin T, binds to beta sheet-rich structures and is particularly well suited for detecting intracellular IAPP amyloid by fluorescence microscopy. Thioflavin S powder can be purchased from Sigma and stock solutions prepared at a concentration of 0.5 % in dH_2O followed by passage through a 0.2 μm filter.
4. Appropriate preparation of IAPP is critical to obtain species that are capable of activating the inflammasome. In our hands, freshly prepared soluble IAPP must be quickly added to macrophages to have this effect, while the insoluble fibrils that form over time do not. However, this may not necessarily be the case for other types of amyloid, and further work is required

to characterize the nature of the IAPP aggregate responsible for NLRP3 activation.

5. When removing the HFIP using the nitrogen gas, take care not to use excessive force, as this would cause the liquid to spill, or evaporate over a large surface area. Alternatively, the sample can be lyophilized until the peptide becomes a dry, fluffy white powder. When completely dry, the peptide can be stored at −20 °C in a desiccator for extended periods of time.
6. Resuspending the aliquot in 0.1 M acetic acid improves the solubility of IAPP.
7. Resuspending this aliquot in H_2O, the IAPP will persist as oligomers longer than if resuspended in a buffer such as PBS or media, which favors fibril formation [6]. We see maximum NLRP3 inflammasome activation when this material is quickly added to macrophages or dendritic cells after being reconstituted in H_2O.
8. Thioflavin T can be used at a final concentration of 10 μM in media, PBS, H_2O, or Tris–HCl, pH 7.4. Parameters such as IAPP concentration, temperature, agitation, dissolved solutes, pH, and the presence of cell membranes all affect aggregation kinetics.
9. The kinetics of aggregation should be determined in parallel with each culture experiment to ensure consistency among experiments in relation to time points for cell treatment and lysis. Amyloid formation typically occurs within several hours at 37 °C.
10. Cells may be treated with IAPP at different times following peptide dissolution to evaluate the effects of early and late aggregates on inflammasome activation. However, more precise characterization of aggregation state may be achieved using methods such as atomic force microscopy, SDS-PAGE, and binding to monoclonal anti-monomer/oligomer antibodies. Despite recent experimental and theoretical models of IAPP aggregation, there remains considerable uncertainty regarding the details of the steps of IAPP fibril formation.
11. We typically grow BMDMs for 7 days in DMEM + 10 % FCS + 10–20 % L-cell supernatant, on non-tissue-culture-treated 10 cm dishes. The cells can then be recovered by removing the medium, adding 10 mL cold PBS and incubating for 10 min at 4 °C, followed by scraping. These cells can then be seeded on tissue-culture-treated plates for use the next day.
12. If other stains will be used (e.g., lysosomal markers), these should be carried out prior to the Thioflavin S staining.
13. While uptake of IAPP fibrils is thought to occur by receptor-mediated phagocytosis, macropinocytosis is responsible for

clearance of soluble amyloid-β. It is unclear whether this is also the case for oligomeric forms of IAPP. Detection of prefibrillar IAPP in cultured cells may be achieved using commercially available antibodies that bind to IAPP monomers and/or oligomers.

14. Priming these cells with LPS does not seem to increase caspase-1 activity in this assay.
15. The 150× stock FLICA solution can be stored in the dark at –20 °C for up to 6 months. During this time it can be thawed and used twice.
16. If cells will not to be analyzed immediately: Add 30 μL of fixative (2 % paraformaldehyde in PBS, pH 7.4), incubate in the dark for 15 min at room temperature and store fixed cells at 2–8 °C for up to 24 h.
17. For this protocol, cells are grown in a low concentration of FCS as excess protein can overload an SDS-polyacrylamide gel. As we typically use BMDMs that are supplemented in 10–20 % L-cell supernatant containing FCS, this represents a final concentration of 1–2 % FCS which is suitable. A minimum of 500 μL supernatant is desirable.
18. The capture and detection antibodies should both be titrated for robust and sensitive detection of IL-18 in each laboratory.
19. We normally use a concentration of 2 ng/mL IL-18 for the top standard and dilute this twofold sequentially across 8–12 wells.

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