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Research Paper

Mild exposure of RIN-5F β-cells to human islet amyloid polypeptide aggregates upregulates antioxidant enzymes via NADPH oxidase-RAGE: An hormetic stimulus ☆

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ABSTRACT

The presence of amyloid aggregates of human islet amyloid polypeptide (hIAPP), a hallmark of type 2 diabetes, contributes to pancreatic β-cell impairment, where oxidative stress plays a key role. A contribution of NADPH oxidase to reactive oxygen species (ROS) generation after cell exposure to micromolar concentrations of hIAPP aggregates has been suggested. However, little is known about β-cells exposure to lower amounts of hIAPP aggregates, similar to those found in human pancreas. Thus, we aimed to investigate the events resulting from RIN-5F cells exposure to nanomolar concentrations of toxic hIAPP aggregates. We found an early and transient rise of NADPH oxidase activity resulting from increased Nox1 expression following the engagement of receptor for advanced glycation end-products (RAGE) by hIAPP aggregates. Unexpectedly, NADPH oxidase activation was not accompanied by a significant ROS increase and the lipoperoxidation level was significantly reduced. Indeed, cell exposure to hIAPP aggregates affected the antioxidant defences, inducing a significant increase of the expression and activity of catalase and glutathione peroxidase. We conclude that exposure of pancreatic β-cells to nanomolar concentrations of hIAPP aggregates for a short time induces an hormetic response via the RAGE-Nox1 axis; the latter stimulates the enzymatic antioxidant defences that preserve the cells against oxidative stress damage.

Introduction

hIAPP is a peptide hormone co-secreted with insulin by pancreatic β-cells with a glucomodulatory role in animal metabolism [1]. The presence of fibrillar deposits of hIAPP amyloid aggregates in the islets of Langerhans is a recognised hallmark of type 2 diabetes which is strongly related to pancreatic β-cell sufferance and death, a fundamental feature of the disease [2,3]. hIAPP fibrillar aggregates result from peptide misfolding, possibly favoured by the presence of lipid membranes or other biological surfaces, with structural reorganisation into early unstable oligomeric assemblies displaying a strong tendency to interact with the cell surface [4,5]. Amyloid oligomers further grow into increasingly ordered polymers eventually generating straight, unbranched, 6–10 nm wide, several μm long, β-structure enriched fibrils [6].

Growing evidence suggests that oxidative stress plays a key role in the onset of type 2 diabetes [7]. In addition, β-cells express low physiological levels of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) [8,9]. Many sources of ROS contributing to the development of type 2 diabetes have been described: a high level of mitochondrial oxidative phosphorylation, glucose auto-oxidation, production of Advanced Glycation End products (AGEs), overexposure to Angiotensin II (which increases NADPH oxidase activity) [10,11] and cell exposure to hIAPP aggregates which induce some alteration of membrane permeability [10,12]. The importance of ROS increase as a cause of cell sufferance during exposure to high concentrations of hIAPP aggregates has been indirectly confirmed by the

Abbreviations: hIAPP, human islet amyloid polypeptide; rIAPP, rat islet amyloid polypeptide; ROS, reactive oxygen species; RAGE, receptor for advanced glycation end-products; AGE, advanced glycation end products; DPI, diphenyleneiodonium; MDA, malonyldialdehyde; CAT, catalase; GPx, glutathione peroxidase; SOD, superoxide dismutase; ATZ, 3-amino-1,2,4-triazole; MS, mercaptosuccinic acid

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protection provided by antioxidants; the latter were found to improve the viability of either RINm5F β-cells and INS-1E β-cells exposed to 10 μM hIAPP aggregates [13] and to 10–30 μM hIAPP aggregates [14], respectively.

A problem with *in vitro* studies is that usually hIAPP doses are far from those normally found in the human pancreas; in fact, hIAPP can reach a millimolar concentration inside intracellular storage granules [15] but it is rapidly diluted outside the cell reaching picomolar concentrations in the plasma [16]. So, even though a local increase of hIAPP concentration in the islets can be postulated in the context of amyloid aggregation, it is hard to accept that it could reach micromolar levels. Then, we aimed to investigate the molecular and cellular events associated with the exposure of RIN-5F β-cells to nanomolar concentrations of hIAPP aggregates. In particular, we were interested in assessing the contribution of NADPH oxidase as a source of ROS at these experimental conditions, since its involvement in oxidative stress in cells exposed to 10–30 μM hIAPP had already been suggested [12].

NADPH oxidase is an ubiquitous multi-subunit enzyme which generates superoxide from molecular oxygen using NADPH as the electron donor. NADPH oxidase was originally identified in phagocytes, where it consists of two membrane-associated subunits, $p22^{phox}$ and $gp91^{phox}$ (renamed Nox2), and at least four cytosolic subunits ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and Rac1/2), which translocate to the membrane upon enzyme activation. Seven isoforms of the catalytic subunit Nox (Nox1-5, Duox 1 and 2), with different cellular localisation, tissue distribution and expression are known [17–19]. The RINm5F pancreatic β-cells (of which RIN-5F is a subclone) express only Nox1 and Nox4 [20]. Nox4 is constitutively expressed and active, whereas Nox1, like the Nox2 isoform, requires other components for activity. In fact, Nox1 is constitutively associated with NoxO1 and NoxA1, the $p47^{phox}$ and $p67^{phox}$ homologues, respectively [19].

In this study we report the effect of cell exposure to low concentrations of hIAPP aggregates for short time periods. Our results led us to uncover an unexpected hormetic effect of such treatment that involved NADPH oxidase activation via RAGE yet resulting in cell protection against oxidative stress through a significant increase of the enzymatic antioxidant defences.

Material and methods

Cell culture

Rat RIN-5F insulinoma cells, from American Type Culture Collection (ATCC), were cultured as described previously [21].

Production of hIAPP amyloid aggregates

hIAPP (Calbiochem, La Jolla, CA) was dissolved in 80% hexafluoroisopropanol (HFIP, Sigma-Aldrich, Steinheim, Germany) to a concentration of 512 μM and stored at −20 °C until use. hIAPP aggregates were prepared by diluting hIAPP to 6.5 μM in 10 mM phosphate buffer, pH 7.4, 1.0% HFIP, and by incubating the mixture at 25 °C for 30 min [21]. Aggregate morphology was determined by Transmission Electron Microscopy (TEM) as previously described [21]. rIAPP (Bachem, Bubendorf, Switzerland) was dissolved and incubated at the same conditions as hIAPP.

Cell treatments

Freshly prepared hIAPP aggregates were diluted in cell culture medium without phenol red, containing 0.5% FCS, and administered to RIN-5F cells. Cells were also treated with similarly diluted rIAPP. In some experiments, the cells were pre-treated for 1 h with (i) the NADPH oxidase inhibitors diphenyleneiodonium (DPI,10 μM) and apocynin (Apo,100 μM); (ii) a blocking anti-RAGE antibody (sc-5563, Santa Cruz Biotech), 20 μg/mL; (iii) the CAT and GPx inhibitors 3-amino-1,2,4-triazole (ATZ) and mercaptosuccinic acid (MS) (20 mM and 7.0 mM, respectively) [22]. All inhibitors were from Sigma. Experiments were carried out to optimise inhibitor concentrations.

Cell viability assessment

20,000 cells/well were seeded into 96-well plates in complete medium, cultured for 48 h and then treated for 3 h with hIAPP aggregates diluted to 60 nM (here and thereafter, aggregate concentration refers to the monomeric peptide concentration). Cell viability was determined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay as previously described [21]; cell mortality was assessed by determining the Lactate Dehydrogenase (LDH) release in the culture medium with the CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega, Italy) [23].

NADPH oxidase activity determination by lucigenin chemiluminescence

The cells were treated with 60 nM hIAPP aggregates for 0–4.5 h and with 60, 120 or 500 nM hIAPP, or with 120 nM rIAPP for 3.0 h. After washing twice with PBS, the cells were harvested, incubated for 60 min in 20 mM ice-cold Tris–HCl buffer, pH 7.4, containing 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1.0 mM PMSF and phosphatase inhibitors (Sigma, Italy) and sonicated to obtain total cell lysates. Protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce, Italy). Superoxide generation was assayed at room temperature using lucigenin (5.0 μM)-enhanced chemiluminescence, as previously described [24]. Each measurement was performed in 20 mM Tris HCl, pH 7.4 with 30–60 μg of total cell lysate proteins in the absence or in the presence of specific oxidase substrates (300 μM NADPH for NADPH oxidase, 5.0 mM succinate for mitochondrial oxidase complex I or 1.0 mM xanthine for xanthine oxidase). Further measurements were performed after the addition of the NADPH oxidase inhibitors DPI (20 μM) and Apo (100 μM). A buffer blank was subtracted from each reading. Superoxide production was detected using a Lumat LB 9507 luminometer (EG&G Berthold) and calculated as an arbitrary light unit (MLU) over 10 min.

Intracellular ROS content and lipid peroxidation assay

Intracellular ROS content was measured by monitoring the oxidation of 2',7'-dihydrodichlorofluorescein diacetate (H_2DCF-DA, Invitrogen, Italy) to the fluorescent dichlorofluorescein (DCF), as previously described [25]. The cells were treated with 10 μM H_2DCF-DA for 10 min before the end of a 3.0 h exposure to 60 nM hIAPP. After washing twice with PBS, the cells were lysed in RIPA buffer (50 mM Tris /HCl buffer, pH 7.5 containing 150 mM NaCl, 2.0 mM EGTA, 100 mM NaF, 2.0% Triton X-100, 1.0 mM vanadate, 0.1% SDS, 2.0 mM EDTA, 2.0 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). DCF fluorescence was measured on a Fluoroskan Ascent Fluorometer (Thermo Electron Corporation, Finland), and normalised to the total protein content. Lipid peroxidation was evaluated as malonyldialdehyde (MDA) content using a "Bioxytech LPO-586 Assay" kit (Oxis International Inc., Prodotti Gianni, Italy), according to the manufacturer instructions.