Published in final edited form as:

Biochim Biophys Acta Biomembr. 2018 September ; 1860(9): 1721–1733. doi:10.1016/j.bbamem.2018.03.024.

Apoptosis Signal Regulating Kinase-1 and NADPH Oxidase Mediate Human Amylin Evoked Redox Stress and Apoptosis in Pancreatic Beta-Cells

Sanghamitra Singh¹, Diti Chatterjee Bhowmick¹, Satyabrata Pany¹, Myungkuk Joe, Noor Zaghula, Aleksandar M. Jeremic*

Department of Biological Sciences, The George Washington University, Washington, DC 20052

Abstract

Misfolded toxic human islet amyloid polypeptide or amylin (hA) and plasma membrane-associated redox complex, NADPH oxidase (NOX), have been implicated in the islet β -cell demise associated with type-2 diabetes mellitus (T2DM). Studies show that hA accumulation is stressful to β -cells and that misfolding of human amylin evokes redox stress and activates mitogen activated protein (MAP) kinases, p38 MAPK and c-Jun N-terminal (JNK) kinase. However, the molecular link and causality between hA-evoked redox stress, NOX activity and MAP kinases signaling in pancreatic β -cells is incompletely understood. Here, we show that in the process of activating JNK, aggregation prone hA also activates an upstream apoptosis signal regulating kinase-1 (ASK1) with concomitant decrease in intracellular levels of reduced glutathione. Inhibition of ASK1 kinase activity, either by specific ASK1 inhibitor, NQDI1 or by thiol antioxidants reduces human amylin-evoked ASK1 and JNK activation and consequently human amylin toxicity in rat insulinoma Rin-m5F cells and human islets. β -cell specific overexpression of human amylin in mouse islets elicited ASK1 phosphorylation and activation in β -cells but not in other rodent's islet or exocrine cells. This ASK1 activation strongly correlated with islet amyloidosis and diabetes progression. Cytotoxic human amylin additionally stimulated pro-oxidative activity and expressions of plasma membrane bound NADPH oxidase (NOX) and its regulatory subunits. siRNA mediated NOX1 knockdown and selective NOX inhibitors, ML171 and apocynin, significantly reduced hA-induced mitochondrial stress in insulinoma beta-cells. However, NOX inhibitors were largely ineffective against hA-evoked redox stress and activation of cytotoxic ASK1/JNK signaling complex. Thus, our studies suggest that NOX1 and ASK1 autonomously mediate human amylin-evoked redox and mitochondrial stress in pancreatic β -cells.

*To whom correspondence should be addressed: Aleksandar M. Jeremic, Department of Biological Sciences, SEH Suite 6000, The George Washington University, 800 22nd Street NW, Washington, DC 20052, USA, Tel.: (202) 994-7899, Fax: (202) 994-6100; jerema@gwu.edu.

¹equal contribution

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Authors contributions

SS, DCB, SP and AJ designed the study and wrote the paper. SS, DCB, SP, MJ and NZ performed the experiments. All authors reviewed the results and approved the final version of the manuscript.

Antibodies validation, amylin aggregation, redox studies and phenotypic/histology analysis of hIAPP-transgenic mouse model are supplied as Supporting Information.

Keywords

human amylin; ASK-1 kinase; type-2 diabetes mellitus; oxidative stress; plasma membrane; NADPH oxidase; human islets

Introduction

Human islet amyloid polypeptide (hIAPP) or amylin (hA), a 4 kDa pancreatic hormone, is synthesized and secreted along with insulin from islet β -cells in response to elevated blood glucose levels [1, 2]. hA is produced as 6.2 kDa precursor that, following the proteolytic cleavage, is stored as a 37 amino acid long mature polypeptide in secretory granules of islet β -cells together with insulin [3]. The hormonal and metabolic functions of amylin are diverse, which includes modulation of insulin release, regulation of food intake, energy homeostasis and gastric emptying [3]. However, misfolded hA is aggregation prone similarly to other amyloidogenic proteins, and hence is a major component of islet amyloid, a hallmark of type-2 diabetes mellitus (T2DM) [1–3]. Both amylin oligomerization and aggregation (fibrilization), *in vitro* and *in vivo*, were linked to amylin's toxicity in the pancreas and progression of T2DM in hA-transgenic animals and humans [4–8]. Supporting this view, studies reveal that hA-treated or hA overexpressing rodent's β -cells show intracellular accumulation of reactive oxygen species (ROS) such as hydrogen-peroxide (H_2O_2). Increase in ROS, altered redox enzymes profile and mitochondrial dysfunction by hA suggests a role of oxidative stress in hA-mediated β -cell toxicity [9–11].

The ROS-producing enzymes recently gained much interest as possible contributors to oxidative stress resulting in β -cell dysfunction. Particularly, super oxide (SO) and ROS-producing membrane-bound redox enzyme NADP oxidase or NOX is recognized as an important contributor to the pathophysiology of islet beta-cells. Under physiological conditions NOX, together with mitochondria-derived ROS, regulates redox signaling and glucose-stimulated insulin secretion (GSIS) in β -cells [12–14]. However, diverse pathological conditions associated with T2DM stimulate NOX expression and activation in pancreatic β -cells leading to enhanced production and accumulation of ROS, which is detrimental to these cells [15]. Expressions of several NOX isoforms, namely NOX1–4, were reported for rodent and human islet beta-cells [16]. Likewise, studies show an upregulation of NOX isoforms in islet β -cells of diabetic animals [17]. Conversely, global inhibition of NOX activity/expression protected β -cells from cytotoxic insult evoked by free-fatty acids and pro-inflammatory cytokines [16, 18].

Chronic oxidative stress is a common pathological condition that has been implicated in the occurrence and progression of several amyloidogenic (protein misfolding) diseases such as type-2 diabetes mellitus (T2DM), Parkinson's and Alzheimer's disease [10, 19–23]. Prolonged oxidative stress is detrimental to many cells and tissues including pancreatic islets as it induces activation of various pro-apoptotic enzymes such as caspases and stress-activated kinases namely apoptosis signal regulating kinase (ASK1), c-JUN N-terminal kinase (JNK), and p-38 mitogen-activated protein kinase (p38MAPK), all of which were previously implicated in the etiology of T2DM [24–28]. For instance, all three major T2DM

risk factors, hyperlipidemia, hyperglycemia and amylin-derived islet amyloid, trigger β -cell apoptosis, at least in part, by activating p38MAP and/or JNK MAP kinase signaling cascades [26, 28]. The mammalian mitogen-activated protein kinase kinase kinase 5 (MAP3K5), also known as apoptosis signaling kinase 1 (ASK1), is readily activated in various cells by redox and ER stress inducing factors and enzymes such as NOX, leading to activation of its downstream target kinases, JNK and p38 [26, 29–33]. In agreement with these findings, studies show that ASK-1 deficiency protects pancreatic β -cells from glucolipotoxic insult as well as ER stress [34–36]. Collectively, recent data highlights the important contribution of this MAP kinase in β -cell failure induced by various stressors, including possibly hA.

Involvement of redox stress in hA-evoked β -cell toxicity is further suggested in studies showing that hA-mediated β -cell death is specifically blocked by thiol/disulfide reducing agents and antioxidants such as N-acetyl-L-cysteine (NAC) [20, 23]. Interestingly, in addition to suppressing redox stress, NAC also prevents reactive oxygen species (ROS)-induced activation of ASK1 and its downstream targets in cells [24]. While several studies have shown that hA stimulates MAPK signaling in β -cells, namely p38 and JNK [28, 37–40], the causality and molecular link between hA-evoked, thiol-sensitive oxidative stress and MAPK activation in β -cells and its pathological significance is unclear, and hence was investigated in this study. Here, we tested hypothesis that hA activates stress kinase JNK and consequently apoptosis of β -cells in ASK-1 and redox (NOX1) dependent manner, both *in situ* and *in vivo*. Our data suggest that ASK-1 but not NOX-1 links human amylin-induced redox stress and JNK activation in β -cells, which may be relevant for the onset and/or progression of T2DM in humans.

Materials and Methods

Materials

Antioxidant N-acetyl cysteine (NAC) (Sigma, MO) was used at a working concentration of 5 mM. Ask1 inhibitor NQDI1 (R&D Systems) and JNK inhibitor (JK1) (Abcam) were used at a working concentration of 10 μ M. Lactacystin (Lac) (Calbiochem, CA) was used at a concentration of 10 μ M. H₂O₂ (Sigma) and diamide (Sigma) were used at working concentrations of 500 μ M and 1mM respectively. Anti-PARP (rabbit, sc-7150), anti-actin (goat, sc-1616), anti-ASK1 (goat, sc-6368), anti-phospho-ASK1 (rabbit, sc-1099), anti-caspase-3 (goat, sc-1224), anti-JNK (rabbit, sc-572) and anti-phospho-JNK (goat, sc-12882) polyclonal primary antibodies were purchased from Santa Cruz Biotechnology. Insulin antibody (sc-9168) was purchased from Santa Cruz Biotech. For detection of overexpressed human amylin in transgenic mice islets, mouse monoclonal anti-human antibody (Santa Cruz Biotech., sc-377530) was used. For detection of internalized synthetic human amylin, rabbit polyclonal anti-amylin antibody (Santa Cruz Biotech., CA sc-20936) was used. While both anti-amylin antibodies show high binding affinity toward pro- and pre/pro-human amylin isoforms, only polyclonal anti-hA antibody detects synthetic human amylin (Figure S1A), which was used in amylin internalization studies (Fig. 1C) [41].

Preparation of amylin peptides

Lyophilized C-terminal amidated synthetic human amylin (AS-60254) and rat amylin (AS-60253) (>95% purity) was purchased from Ana Spec, CA. Amylin stock solution (500 μ M) was prepared using hexafluoro-2-isopropanol (HFIP) as a solvent. The measured amounts of hA and rA were solubilized in HFIP overnight to completely dissolve the amylin and remove any small or large aggregates [42, 43]. Prior to an experiment, the HFIP solvent was evaporated with a gentle stream of nitrogen, and the peptide was then reconstituted in aqueous reaction buffer (PBS) to prepare a peptide's stock concentration of 500 μ M. Freshly prepared peptides were immediately diluted with cell culturing medium and added to cells. Except for dose dependent studies, all pancreatic cells irrespective of their origin were treated with human or rat amylin at a final concentration of 30 μ M for consistency.

Cell cultures and siRNA transfections

Rat insulinoma (RIN-m5F) β -cells (ATCC, Gaithersburg, MD, cat # CRL-11605) were cultured in RPMI 1640 medium (ATCC) supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin. Insulin secreting (INS) cells (parental line 832/13) were a generous gift from Christopher Newgard from Duke University. INS-832/13 cells were cultured in RPMI 1640 medium (ATCC) supplemented with 10% (v/v) fetal bovine serum, 1% penicillin/streptomycin, 10mM Hepes, 2mM L-glutamine, 1mM sodium pyruvate and 0.05 mM 2-mercaptoethanol. Both cell lines were cultured at 37 °C in a humidified incubator with 5% CO₂ and passaged bi-weekly. Passages 15–60 were used for all the experiments. β -cells were plated at density 50,000 cells/well and cultured for 12–16 hours prior to the experiments. Synthetic human or rat amylin was then added to cultures for additional 0–24 hours depending on experiments. Human islets from diabetic and non-diabetic cadavers with >90% purity and viability were obtained through the Integrated Islet Distribution Program (IIDP) for Human Islet Research. Upon arrival, islets were handpicked under a dissecting microscope to yield purity of 95% (based on dithiazone staining). Intact or partially dissociated islets were plated on 24-well tissue culture treated plastic plates at 20–30 islets per well and suspended in CMRL media (ATCC) for biochemical studies. For microscopy studies, islets were plated on a poly-D-Lysine coated 96-well glass bottom black microplates (Thermo-Fisher) and allowed to adhere for 24h prior to treatments. Islets were then exposed to amylin for additional 24 hour in euglycemic (5mM glucose) media. Under these conditions, human islets retain their functional and biochemical characteristics [41, 44, 45].

For small interfering RNA (siRNA) experiments, RIN-5mf cells (5×10^4 cells) were transfected with NOX1-siRNA or Silencer™ Negative siRNA control (ThermoScientific) at a concentration of 0–40 nM using Lipofectamine 3000 (ThermoScientific), in accordance with the manufacturer's protocol. The mRNA and protein intracellular levels of NOX1 were determined 48h following transfections using qPCR and western blot methods, respectively. Following siRNA transfections, RINm5F cells were treated with 30 μ M hA for additional 24 h and amylin toxicity in control and NOX1-silenced cells analyzed with MTT assay.

Animals

Hemizygous human amylin (hA^{+/-}) transgenic mice (FVB/N-Tg(Ins2-IAPP)RHFSol/J, stock no. 008232) and control non-carrier FVB/NJ breeder pair were purchased from Jackson Laboratories (Bar Harbor, ME). Transgenic mice express human amylin under the regulatory control of the rat insulin II promoter, and consequently human amylin protein is only expressed in islet β -cells in the pancreas, as described previously [46]. Hemizygous hA^{+/-} mice, which show no symptoms of spontaneous disease, were inbred to produce homozygous hA^{+/+} mice, which develops diabetes. Genotyping was performed according to supplier's protocol. Mice were maintained on a light/dark (12/12 hours) cycle with free access to water and standard mouse diet. All animal experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC)-approved guidelines at the George Washington University Medical Center (protocol #A300) and conform to NIH guidelines and federal law. Both female and male mice were used in our studies. Because female hA transgenic mouse exhibits similar diabetic phenotype as age-matched males, we report only data collected from male mice for consistency.

MTT reduction assay

RINm5F cells were plated onto 96 well plates for 24 hours followed by incubation with various reagents in the absence or presence of human or rat amylin for additional 24 hours. Treated cells were then incubated with 1mg/ml MTT (3-(4,5-dimethylthiazol-2-)-2,5-diphenyl tetrazolium bromide, Sigma) solution for the last 3 hours of incubations. Precipitated formazan crystals inside cells, product of mitochondrial metabolism, were dissolved in HCl-isopropanol solution and absorbance determined at 570 with subtraction at 690 nm with a Spectra Max M5 spectrofluorometer. A decrease in cell viability results in a decrease in the amount of MTT formazan formed and a decrease in absorbance. The data were expressed as fold change from control (taken as 100%).

Quantitative real time PCR (qRT-PCR) analysis—Cells (10^5 cells/well) were grown overnight in 24-well plate and then, treated as per the experimental requirement. Total RNA isolation from treated cells was carried using the RNeasy® Mini Kit (Qiagen) as per the manufacture's recommendations and with an additional step of on-column DNA digestion using RNase-Free DNase (Qiagen). cDNA synthesis was performed using 2 μ g of total RNA and High Capacity cDNA Reverse Transcription Kit (Thermo-Fisher) as per the manufacturer's instructions. Quantitative Real Time PCR (qRT-PCR) was performed on CFX Real-Time PCR Detection Systems (BioRad) using 20 ng of cDNA, iTaq™ Universal SYBR® Green Supermix (BioRad) and gene specific primers (Table 1). All primers were designed using NCBI-Primer-BLAST software [47]. Relative level of mRNA was calculated using C_q method as described previously[48] after value normalization with β -actin control.

Cellular reactive oxygen species and superoxide analysis—Intracellular accumulation of cellular reactive oxygen species (ROS) and superoxide (SO) production was measured using cellular ROS/Superoxide detection assay kit (Abcam), as per the manufacturer's instructions. Briefly, cells (0.2×10^5 cells/well) were grown overnight in basal media in 96-well black-clear bottom plates (Bio-One). Cells were treated with varying

concentration of amylin, apocynin, ML171, pyocyanin and NAC in basal media either for 1 or 24 h. Treated cells were labeled with green and orange fluorescence probe (Abcam) for ROS and SO detections, respectively for 1h. Fluorescence was measured using fluorescent microplate reader (Molecular Device). The relative changes in ROS and SO intracellular content was calculated from row fluorescence data (fluorescence intensity) after background subtraction, and expressed as % change from the control (taken as 100%).

ELISA (pASK1 Ser966 and pJNK) Assay

Changes in phosphorylation of Ser966 in ASK1 and JNK in pancreatic cells, reflecting the activation of the ASK1-JNK signaling cascade, were quantified by a colorimetric cell based pASK1 Ser966 ELISA kit (Cytoglow) and pJNK ELISA kit (Abcam) respectively, according to manufacturer's guidelines. The obtained absorbance values were either normalized to total protein content by BCA assay (pJNK) or to cell number by crystal violet cell staining method (pASK1). Data is expressed as % change from the control (pASK1 Ser 966) or arbitrary units (pJNK).

Reduced Glutathione Assay

Reduced glutathione (GSH) levels were measured using GSH assay kit as per the manufacturers protocol (Cayman Chemicals, catalog number 703002). This assay is based on the principle of enzymatic recycling of GSH to produce yellow colored 5-thio-2-nitrobenzoic acid (TNB) from 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB). Following treatments and prior to performing the glutathione assay, plated cells were homogenized using RIPA buffer and deproteinized using deproteinizing sample preparation kit (Abcam). GSH intracellular levels were estimated as a measure of absorbance of TNB at 405 nm in cell extracts and the absorbance values were used to calculate GSH concentrations from the standard curve. The obtained concentration values were normalized to total protein content by BCA assay (μM GSH/ μg protein).

Western blot analysis

The modulatory effects of human and rat amylin on the NOX subunits expression and activity of stress kinases and apoptotic proteins were analyzed by western blot. Following treatments, cells were solubilized with RIPA buffer and cell extract was loaded onto 4–15% gradient Tris-Glycine SDS-PAGE gels (BioRad). The protein bands were then electro-transferred to nitrocellulose membranes, and non-specific IgG binding sites were blocked by incubation with 5% nonfat dry milk. The intact membranes were probed with primary antibodies against human amylin, cleaved caspase-3 (Asp175), phospho-JNK (Tyr 183/ Tyr185), phospho-ASK1 (Thr845), ASK1, JNK, NOX1, p47^{phox} and actin. Except for ASK1 (1:1000, Abcam) and NOX1 (1:500, Novus Biologicals) all other primary antibodies were from Santa Cruz Co. and used at 1:200 dilutions. This was followed by addition of corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies at 1:2000 dilutions (Thermo Fisher). Blots were then developed using ECL substrate kit (Thermo Fisher) and documented using the Kodak 1500 gel imaging station equipped with Kodak digital camera. In these studies, the loading control and target proteins (bands) were consecutively developed on the same blot and then cropped and assembled side by side for the presentation.

Immunocytochemistry

Following treatments, cultured RIN-m5F or INS-832/13 pancreatic cells were washed in PBS (3×) and fixed with 4% paraformaldehyde for 20 minutes. ASK1 activation was detected by incubating the cells with rabbit anti-pASK1 Thr845 antibody (1:200) at 4°C overnight followed by goat anti-rabbit Alexa 647-conjugated secondary antibodies (1:300) for 60 minutes at room temperature. Similarly, p47^{phox} and p67^{phox} levels were detected by incubating the cells with mouse anti-p47^{phox} and anti-p67^{phox} antibodies (1:200; Santa Cruz Biotechnologies) at 4°C overnight followed by goat anti-mouse Alexa 488-conjugated secondary antibodies (1:500) for 60 minutes at room temperature. Nuclei were stained using DAPI. Intracellular amylin aggregates were detected by staining cells with 0.05% thioflavin-S (Th-S) solution for 10 minutes. Cells were then washed with 50% ethanol solution and PBS 3× prior to imaging. Three random fields in each well were imaged by confocal microscopy, with each treatment performed in duplicates. Single optical sections (1µm z axis) through the middle of the cells were acquired for each field using an LSCM-800 confocal microscope (Carl Zeiss, Thornwood, NY) equipped with EC Plan-Neofluar 10× (N.A. 0.3) and Plan-Apochromat 63× (N.A. 1.4) oil objectives. The pinhole was adjusted to keep the same size of z-optical sections for all channels and objectives used. Multitrack imaging was performed to ensure that there was no crosstalk between the channels. The intensity thresholds of the channels were set using the Zeiss cross-hair function to avoid an arbitrary background threshold being set. Images were assembled using Adobe Photoshop software (Adobe Systems Co.).

Immunohistochemistry

Following euthanasia, whole pancreases were isolated from 10–12-months old male homozygous *hA*^{+/+} transgenic mice and their non-transgenic littermates, immediately embedded in optimal cutting temperature compound and rapidly frozen using a dry ice/ethanol. 10 µm transverse cryosections taken from top, middle and bottom of the mice pancreas were then prepared using a cryotome. In addition to cryo-sections, paraffin-embedded 4 µm-thick pancreatic sections were also prepared after fixation in 10% neutral buffered formalin for 24 h. The cryosections were fixed in 10% neutral buffered formalin for 5 min and the paraffin-embedded sections were deparaffinized, followed by heat-mediated antigen-retrieval in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA Solution, 0.05% Tween 20, pH 9.0) before being processed for immunohistochemistry. The transverse sections were blocked in a blocking buffer (2% normal goat serum and 0.2% Triton X-100 in PBS) for 1 h and incubated with antibody against anti-amylin (1:100), pASK1 (1:100), or anti-insulin (1:100) in a blocking buffer for 16 h at 4°C. The sections were washed three times in PBS containing 0.01% Tween 20, followed by incubation with Alexa 555-conjugated anti-mouse secondary antibody and Alexa 488-conjugated anti-rabbit secondary antibody for 1 h. After staining with 4',6'-diamidino-2-phenylindole (DAPI) and washing three times for 5 min in PBS containing 0.01% Tween 20, the sections were mounted in Prolong gold mounting medium (Invitrogen), and samples examined at room temperature using a Zeiss LSM 800 confocal microscope (Zeiss, Jena, Germany), as described above. Islet amyloid in pancreatic sections was detected by staining cells with 0.05% thioflavin-S (Th-S) solution for 10 minutes. Slides were washed with 50% ethanol solution and PBS 3× prior to imaging. Pancreatic sections were examined and captured using multi-track imaging mode to reduce

possibility of crosstalk between the channels. A low-mag tile-stitching imaging approach for Hematoxylin and Eosin (H&E) stained sections were used to determine islets number in pancreatic sections (no. of islets per tissue surface area). In addition, the mean islet area (expressed as area per μm^2) was determined using the object selection and quantitative software (ZEN Blue, Zeiss). The histological data was collected from four random fields per each section per experimental group.

Non-fasting blood glucose and glycosylated hemoglobin measurements

Long term changes (7–54 weeks) in non-fasting blood glucose and glycosylated hemoglobin levels were assessed in wild type, hemizygous (IAPP^{+/-}) and homozygous (IAPP^{+/+}) male and female mice. Blood was collected from mice tail vein, and blood glucose levels were determined using glucometer (ONE TOUCH Ultra, LIFESCAN). Glycated hemoglobin (HbA1c) levels were measured using Mouse Hemoglobin A1C kit (Crystal Chem., IL, cat. No. 80310) following manufacturer's instruction. Briefly, equal volumes (5 μl) of total blood from each group were collected and subjected to extensive proteases digestion, following which glycated hemoglobin levels were determined using horseradish peroxidase based colorimetric assay. The raw data (absorbance values at 700nm) were converted to % HbA1c using manufacturer protocol.

Statistical Analysis

The Graph Pad Prism 5 Program was used for plotting of data and statistical analysis. The unpaired Student's t test, One-way or Two-way analysis of variance (ANOVA) followed by the *Tukey* post-hoc test were used for pair wise comparisons among groups when appropriate with significance established at $p < 0.05$.

Results

Human amylin triggers ASK-1 kinase activation in pancreatic β -cell lines and human islets

Studies show that prolonged extracellular and intracellular accumulation and signaling of hA triggers redox stress in pancreatic β -cells, which induces apoptosis [20, 21, 37, 49]. Stress-activated terminal kinase, JNK, plays a decisive role in this process [20, 26, 28, 37, 39, 50]. However, it is unclear which cellular factors and intermediate signaling pathways couple hA-evoked redox stress and JNK activation in islet β -cells. Here, using hA- and ASK-1-specific antibodies (Fig. S1A, B) and combination of microscopy and biochemical approaches we tested the role of stress-activated kinase ASK1 in amylin-induced redox stress and toxicity in β -cells. First, we use mitochondrial stress assay MTT and western blot to verify the toxic potential of hA and rat amylin (rA) in our model systems, rat insulinoma RIN-m5F and cultured human islet cells (Fig. S2). Pro-oxidants, H_2O_2 and diamide, were used as positive controls to amylin in these experiments. hA triggered mitochondrial stress in RIN-m5F cells in a dose-dependent manner (Fig. S2A) as expected. Within the same time frame (24 hours) and the same concentration range, hA induced cleavage of chief executors of apoptosis, caspase-3 and PARP (Fig. S2B–D). As a control, non-amyloidogenic rA was significantly less stressful to cells as compared to hA (Fig. S2A) and consequently slightly activated caspase-3 (Fig. S2B) and PARP (Fig. S2D). In agreement with these findings, using amyloid-specific fluorescent probe thioflavin-S (Th-S) we detected intracellular

amylin aggregates in hA-treated but not in control or rat amylin (rA) treated cells (Fig. S1C). These results suggest that hA oligomerization and/or aggregation also play important role in hA toxicity in our cells, as previously suggested [37, 41, 44]. Our results additionally revealed that hA induces apoptotic (also evoked by H₂O₂) but not necrotic (evoked by diamide) cell death [22], reflected by disparity in caspase-3 activation in these cells by diamide and pro-apoptotic agents hA and H₂O₂ (Fig. S2B).

Next, we sought to determine if hA in the course of its toxicity (Fig. S2) also activates redox-sensitive pro-apoptotic MAP kinase, ASK1. ELISA revealed that in response to hA, RINm5F cells exhibited a marked (2 fold) and sustained (24h) decline in phosphorylated Ser966 (p)ASK1 levels as compared to controls (Fig. 1A). This long-lasting stimulatory effect of hA was mimicked by the other redox stressors, H₂O₂ and diamide, suggesting similar, redox-sensitive mechanism of ASK1 activation. Next, we tested whether this modulatory effect of hA on ASK1 activity is cell-specific by analyzing the regulatory effect of hA on ASK1 phosphorylation state in another insulin-secreting pancreatic β -cell line, INS 832/13 [51] (Fig. 1B). A similar potent (2.5 fold) and long lasting pattern (24h) in ASK1 activation by hA and other pro-oxidants, reflected by decrease in pASK Ser966 levels, have been observed in INS cells (Fig. 1B). These results suggest that ASK1 is common target of hA and other redox stressors in pancreatic cells. As seen in RIN-m5F cells (Fig. 1A), addition of rA to INS cells triggered similar decrease in pSer966 levels as compared to other stressors, indicating that, at least in insulinoma cells aggregation state of amylin is not a sole determinant of its pro-stress activity. This conclusion is in agreement with findings showing ability of rA to induce moderate metabolic and cellular stress in these cells (Fig. S2 A, B, [52]).

The stimulatory effect of hA on ASK1 activity was retested in cultured human islets by western blot analysis and using another marker of ASK1 activity, Thr845 (Fig. 1C). Islets exposed to hA show a progressive and concurrent increase in phosphorylation levels of ASK1 (Thr845) and JNK over 24h (Fig. 1C). Time-lapse western blot analysis also revealed that internalized hA dwarfs endogenous amylin and progressively accumulates in these cells in response to chronic exposure to the peptide (Fig. 1C, [41]). Ensuing intracellular and PM accumulation of hA (Fig. 1C, [44]) corresponded with gradual activation of both kinases suggesting that excessive intracellular and/or extracellular hA buildup, at least in part, stimulates cytotoxic ASK1/JNK stress signaling cascade in pancreatic human islets.

Redox but not protein stress mediates amylin-induced ASK1 activation in pancreatic cells

In order to infer more about the potential mechanism of ASK1 activation in pancreatic cells we treated RIN-m5F cells with oligomer/fibril-forming hA (Fig. S3) or nonamyloidogenic rA [41, 44] for 24h. These studies were performed in the presence or absence of ASK-1 blocking peptide or antioxidant NAC broadly used to reduce amylin toxicity. ASK1 activation was monitored by confocal microscopy using specific Thr845 pASK1 antibody (Fig. 1D, Fig. S1C). hA triggered the phosphorylation of ASK1 in the absence but not in the presence of the blocking peptide, which confirmed the specificity of the pASK1 signal (Fig. S1B). Because accumulation of hA induces redox and ER/protein stress in β -cells [53, 54], here we examined contribution of these two stress pathways in hA-induced ASK1 activation

and signaling. As positive controls for oxidative and protein-stress-induced ASK1 signaling in these cells we used hydrogen peroxide (H_2O_2) and proteasome inhibitor lactacystin (Lac), respectively. Both stressors (Lac and H_2O_2) as well as hA potently activated ASK-1 in majority of cells as demonstrated by potent 7-fold increase in number of ASK1 pThr845-positive cells (94 ± 3 %) as compared to controls (12 ± 4 %) (Fig. 1D). Likewise, there was a visible increase in ASK1 fluorescence signal (puncta) in hA-treated cells as compared to respective controls (Fig. 1D). If oligomerization and/or aggregation are essential for amylin-induced ASK1 activation in these cells then rA should be less potent in activating this kinase than its human isoform. However, confocal microscopy revealed comparable levels in overall phospho-ASK1 signal (fluorescence puncta and pASK-positive cells) in cells treated with equimolar hA and rA (Fig. 1D), in agreement with biochemical studies (Fig. 1A, B). Collectively, our findings indicate that loss in mitochondrial function along with redox and protein stress are major factors causing ASK1 activation in pancreatic β -cells, the former of which is readily evoked by hA and to lesser extent rA accumulation (Fig. 1, Fig. S2, Fig. 4A, B).

Previous studies have shown that an optimal cellular redox state, maintained by thiol redox proteins and molecules such as glutathione (GSH), is indispensable for anti-oxidative stress response in many cells including β -cells [19, 22, 23, 55]. Thiol reducing agents such as N-acetyl-L-cysteine (NAC), glutathione and dithiothreitol, which modulate cellular redox state by increasing intracellular levels of GSH and by reducing disulfide bonds in oxidizing proteins, reversed hA-evoked apoptosis in RIN-m5F cells [20]. Since phosphorylation and activation of ASK1 is often induced in cells undergoing oxidative stress, we assessed whether changes in cell's redox (thiol) state affects basal and stress-induced phosphorylated levels (Thr845) of ASK1 in pancreatic cells by confocal microscopy (Fig. 1D). We used NAC to test the redox mechanism of ASK1 activation in these cells because unlike some antioxidants, NAC does not interfere with hA aggregation (Fig. S3, [41]). Microscopy and biochemical studies revealed that NAC not only reversed the ASK1 activation by hA (Fig. 1D, Fig. 2B) but also completely restored GSH levels in RIN m5F cells, which were significantly depleted by additions of hA and H_2O_2 (Fig. 2A). Moreover, NAC abolished hA-induced PARP cleavage (Fig. S2D). These results postulate a link between hA-induced redox stress and hA-evoked ASK1 activation and toxicity in these cells. NAC addition did not prevent or reduce Lac- (protein stress) induced ASK1 activation (Fig. 1D), suggesting that redox but not protein stress mediates hA-evoked ASK1 activation in β -cells. In addition to suppressing hA-evoked ASK1 activation, NAC supplementation effectively increased intracellular levels of reduced GSH by $\sim 30\%$ compared to controls (Fig. 2A). Conversely, NAC supplementation to non-treated cells (control) further reduced cell number with detectable pASK1 signal by ~ 2 fold (from 12 ± 4 % to 7 ± 2 %, Fig. 1D). These results suggest that ASK1 activation in β -cells under normal and stress conditions is interrelated and dependent on β -cell's GSH content. Importantly, redox assay revealed that even modest depletion of cellular GSH levels by hA (14 ± 1 %) and H_2O_2 (24 ± 2 %) respective to controls (Figure 2A) is sufficient to co-activate pro-apoptotic ASK-1 and JNK (Fig. 1, Fig. 2, Fig. S2). Similarly to their action in insulinoma cells (Fig. 1D), NAC and another disulfide reducing agent, β -mercapthoethanol (BME) suppressed hA-evoked ASK1 and JNK

activation in human islets (Fig. 2B), thus postulating a molecular link between these two redox stress-activated MAP kinases and hA toxicity in pancreatic cells.

ASK1 mediates human amylin toxicity in pancreatic cells

To further confirm the causal role of ASK1 in hA toxicity, RINm5F cells were treated with cytotoxic concentrations of hA, rA, H₂O₂ or Lac in the presence or absence of a specific ASK1 inhibitor NQDI1 and its effect on ASK1 activation (Fig. 3A) and cell viability (Fig. 3B, C) was assessed. Confocal microscopy revealed that in the presence of NQDI1, hA, H₂O₂ and Lac-evoked ASK1 phosphorylation returned to control levels, thus confirming ASK1 activation by hA and other redox and protein stress inducers (Fig. 3A). The causal role and effect of ASK1 inhibition on amylin's toxic potential was then determined in RIN m5F cells and human islets by MTT assay (Fig. 3B) and PARP cleavage assay (Fig. 3C), respectively. While PARP cleavage indicates cellular stress and activation of upstream executor caspases, namely pro-apoptotic caspase-3 [56], the prolonged decrease in mitochondrial enzymatic activity is indicative of mitochondrial stress and compromised viability of cells including pancreatic cells (Fig. S2). Toxicity assays revealed that NQDI1 significantly reversed hA and H₂O₂ induced PARP cleavage and loss of mitochondrial function suggesting that ASK1 signaling plays a key role in cellular stress induced by these pro-oxidants (Fig. 3B, C). NQDI1 also effectively reduced rA-induced ASK-1 activation and PARP cleavage in pancreatic cells (Fig. 3A, C).

ASK1 is required for human amylin-evoked JNK activation and apoptosis

JNK has widely been reported as one of the downstream targets of ASK1 and has particularly been implicated in ER and redox stress induced β -cell death [57]. Because JNK also mediates hA toxicity in β -cells [28, 37] we explored here a causal link between hA-toxicity and ASK1 / JNK signaling and activation. Cultured human islets were treated with hA in the presence or absence of ASK1 inhibitor (NQDI1) or JNK inhibitor (JNKI) and the extent of ASK1 and JNK phosphorylation, reflecting their activation, were determined by western blot analysis (Fig. 4A, C) and ELISA (Fig. 4B). In contrast to insulinoma cells (Figs. 1 and 2), western blot assay and ELISA revealed that in human islets hA was more potent in inducing ASK1/JNK signaling cascade as compared to non-amyloidogenic rA, which slightly activated these two MAP kinases (Fig. 4A, B). This result implies that in primary human islet cells oligomerization and aggregation of hA play a significant role in hA-evoked stress kinase activation. Analogous to RIN-m5F cells (Fig. 3), in human islets NQDI1 effectively inhibited hA-induced ASK1 and JNK activation revealed by western blot (Fig. 4A) and ELISA (Fig. 4B). The reversal of JNK activation by ASK1 inhibitor NQDI1 suggests a causal link between these two kinases in hA toxicity, in analogy with other amyloid proteins [58]. Next, specific ASK1 and JNK inhibitors were concurrently used to determine the sequence of MAPK activation. ASK1 inhibitor NQDI1 completely inhibited hA-evoked ASK1 phosphorylation and significantly reduced JNK activation, which in turn abolished hA toxicity, reflected by low levels of cleaved PARP (Fig. 4C). In contrast, JNK inhibitor blocked hA-evoked JNK activation and toxicity (PARP cleavage) but not ASK1 activation by hA (Fig. 4C). Taken together, these results place ASK1 upstream of JNK in hA-evoked stress and apoptosis in pancreatic β -cells.

Phospho-ASK1 levels are elevated in the hypotrophic islets of hIAPP-transgenic mice

To confirm our *in vitro* and *ex vivo* data concerning ASK-1 activation by hA, we used an established hIAPP transgenic mice model. The selective expression of human amylin in transgenic mice pancreatic β -cells is achieved via rat insulin II promoter [46]. β -cell specific overexpression of human but not rat amylin is cytotoxic to this islet cells, ultimately triggering type-2 diabetic phenotype in homozygous transgenic mice [46]. In order to determine detrimental effect of hA expression in these islets, if any, we performed morphometric analysis of the pancreatic sections of homozygous transgenic males and their non-transgenic male littermates (Fig. S4, S5). Homozygous transgenic mice were further divided into two aged groups, weeks 40 and 54, that exhibited moderate and severe diabetic symptoms manifested by persistent elevated glucose and glycosylated hemoglobin levels in the blood (Fig. 5A, B). Hematoxylin and eosin (H&E) staining showed that, compared to the islet structure of non-transgenic wild type mice, islet architecture was disrupted and the round islet boundary was obliterated in aged transgenic mice (hA^{+/+}, 54 wks, Fig. S5). The pancreatic islets number (Fig. S4) and the area (Fig. S5) were significantly reduced by respectively ~45% and ~55% in transgenic mice with severe diabetes (hA^{+/+}, 54 wks) as compared to sex and age-matched wild type or younger mice with pre-diabetic syndrome (hA^{+/+}, 40 wks). Similarly, there was a marked (~2 fold) decrease in number of insulin-positive cells in islets of aged (hA^{+/+}, 54 wks) as compared to sex and age-matched wild type mice (Fig. 5C). These results point to hA-induced β -cell hypotrophy and/or hypoplasia in conjunction with aging as a main cause for the disease. Conversely, there were no significant difference in the number or the size of islets between nontransgenic (wild type) and transgenic mice with mild diabetic syndrome (Fig. S4, Fig. S5). Although pancreatic islets from moderately diabetic hA transgenic mice (hA^{+/+}, 40 wks) and its age- and sex-matched controls (wild type mice) show comparable morphometric values, altered islet architecture in 40 weeks-old hA mice indicates that early pathology was already in progress (Fig. S5). Indeed, mild hyperglycemia was apparent from 38 weeks of age (Fig. 5A).

Next, we examined the pancreatic sections of transgenic mice and their non-transgenic littermates after staining with anti-pASK1 (Thr845) and hIAPP antibodies to see whether ASK1 is activated in hA transgenic mice (Fig. 5D). Notably, pASK1 immunoreactivity was markedly elevated (< 2 folds) in the hA-expressing β -cells compared to other islet or exocrine cells of transgenic or control littermates (Fig. 5D). Importantly, we detected phospho-ASK1 signal in both moderate and severe diabetic groups of hA transgenic (homozygous) mice and the pattern of pASK1 signal was not notably different between both groups (Figure 5D). Similarly, in the islets from homozygous but not wild type mice we detected a significant and progressive accumulation of Th-S-positive amyloid-laden plaques (Fig. 5E). These results suggest that hIAPP expression and accumulation inside and outside of β -cells induce islet amyloidosis and ASK1 activation in islet β -cells *in vivo* and before the onset of the disease (Fig. 5A–E).

Human amylin regulates expression of NOX subunits in pancreatic insulinoma cells—Here, we examined a possible link between NADPH oxidase (NOX) expression/activation and hA-mediated MAP kinase signaling in rat insulinoma cells. To this end, we used qPCR and NOX isoform-specific primers (Table 1) to determine which of the four

major NOX isoforms (NOX1–4) are expressed in INS-832/13 and RIN-m5F cells. We observed high transcript levels of NOX1 both in INS-832/13 and RIN-m5F cells as compared to other NOX isotypes (Fig. 6A, B), which appears to be the main PM-associated NOX isoform constitutively expressed both by rodent islets and insulinoma cells [59]. Next, we analyzed if and how toxic hA affects NOX1 expression in these cells. Hence, we treated INS-832/13 and RIN-m5F cells with hA at a concentration of 30 μ M (which induces JNK activation and apoptosis) over 24 h and quantified dynamical changes in mRNA levels of NOX1 by qRT-PCR. In INS-832/13 cells, we observed a marked (~5-fold) and prompt (~1h) increase in NOX1 mRNA levels respective to control cells (Fig. 6A, inset). The hA-induced mRNA levels of NOX1 remained elevated throughout the treatment despite the decrease after reaching its peak (~20-fold) at 6 h (Fig. 6A, inset). We also observed a similar potent (10-fold) stimulatory effect of hA on NOX1 expression in RIN-m5F cells, peaking at 12 h (Fig. 6B, inset). For fully functional/activated NOX signaling complex, presence of regulatory subunits, p67^{phox} and p47^{phox} or their isoforms, are critically required [16]. In lieu of this, we used confocal microscopy to evaluate the modulatory effect of hA on p67^{phox} and p47^{phox} expressions in RIN-m5F cells. Our time-lapse microscopy approach revealed that hA induced robust and progressive expression of p67^{phox} and p47^{phox} in majority of cells (<70%) over 24h (Fig. 6C), indicating their possible involvement in hA-induced β -cell stress.

NOX silencing and inhibition of its activity attenuate human amylin toxicity—

An up-regulation of NOX catalytic and regulatory subunits in cells is indicative of complex activation [16]. Hence, we evaluated the functional consequence of amylin's stimulatory effect on NOX subunits expression in β -cells. Specifically, we examined the effect of ML171, a NOX1 selective inhibitor [60], on hA-evoked mitochondrial dysfunction (Fig. 7), redox stress (Fig. 9A) and pro-apoptotic JNK signaling (Fig. 9B). INS-832/13 and RIN-5mF cells were either treated or co-treated with amylin (30 μ M) and ML171 (0.1–1 μ M) for 24 h following which amylin toxic/stress potential was assessed. hA significantly reduced (by ~90 %) mitochondrial activity in INS-832/13 and by ~70% in RIN-5mF cell as compared to the untreated cells (Fig. 7). To avoid nonselective action of ML171 on different NOX isoforms and to limit its possible side effects, we used low non-toxic concentrations (0.1–1 μ M) of ML171. At these sub-micromolar concentrations, ML171 showed high selectivity towards NOX1 over other NOX isoforms [16] [60]. Under these conditions, we observed a small (~25 %) but significant improvement in mitochondrial activity/function in hA/ML171 co-treated cells as compared to hA-treated cells (Fig. 7). To reconfirm the NOX involvement in hA-induced cellular stress, we measured the effect of apocynin (1 μ M), a pan-NOX inhibitor, on hA-evoked mitochondrial dysfunction (Fig. S6). Analogous to ML171, apocynin treatment slightly (~20 %) but significantly improved mitochondrial function in hA/apocynin co-treated RIN-m5F cells as compared to hA-treated cells (Fig. S6). Unlike NAC (Fig. S2D, [37]), neither of the potent NOX inhibitors could achieve full protection of amylin-induced mitochondrial stress (Fig. 7, Fig. S6).

We retested NOX1 involvement in hA-induced mitochondrial test by silencing NOX1 using siRNA approach. Transfection of RINm5F cells with NOX1 specific siRNA (0–40 nM) effectively silenced NOX1 transcription in these cells (Fig. S7). We then used the lowest

effective dose (20 nM) to probe NOX1 involvement in amylin toxicity in order to avoid possible off-target effects of siRNA (Fig. 8). Following NOX1-siRNA transfection for 48 h, the NOX1 protein levels were reduced by ~70%, compared to non-transfected cells and cells transfected with scrambled siRNA (Fig. 8, top). Human amylin induced a significant mitochondrial stress in non-transfected (NT) and negative control (Ctr-siRNA) transfected cells reflected by ~50% and 40% decrease in mitochondrial activity, respectively (Fig. 8, bottom). In cells transfected with NOX1 siRNA, we observed a significant increase (~30%) in mitochondrial activity in amylin-treated cells as compared to control cells (Fig. 8, bottom). Collectively, these results demonstrate reduced amylin cytotoxicity in NOX1-silenced cells.

Amylin evokes NOX1 independent oxidative stress and MAP kinase activation

—It was reported that NOXs play significant roles in oxidative stress-induced apoptosis of β —cells [16]. Hence, we tested effects of hA and NOX activators and inhibitors on ROS and superoxide (SO) production in insulinoma cells. Amylin treatment (hA, 30 μ M over 24h) significantly increased (<1 fold) intracellular ROS levels in INS-832/13 and RIN-m5F cells as compared to untreated controls (Fig. 9A). Similarly, hA increased SO production by ~2 folds and ~0.7 fold in INS-832/13 and RIN-m5F cells, respectively (Fig. 9A). These results are in agreement with previous reports [61]. However, pan-NOX inhibitor, apocynin or selective NOX1 inhibitor, ML171, did not show any modulatory (inhibitory) effect on hA-induced ROS and SO production in these cells (Fig. 9A). By contrast, ML171 and apocynin significantly reduced ROS and SO production evoked by NOX activator, pyocinin (Fig. S8). The NOX inhibitors were equally effective as potent ROS inhibitor NAC in reducing NOX-mediated redox stress in these cells (Fig. S8). These results imply that hA evokes NOX-independent oxidative stress in pancreatic β -cells. In agreement with this notion ML171, even at its highest effective concentration (0.1 μ M, Fig. 7), failed to block or significantly reduce hA-evoked activation of ASK1 (data not shown) and JNK (Fig. 9B), suggesting NOX1-independent redox-sensitive mechanism of MAP kinase activation by hA. As demonstrated by confocal microscopy (Fig. 6C), hA also increased expression of an important NOX regulatory subunit, p47^{phox} (Fig. 9B). Consistent with above findings, hA-induced expression of p47^{phox} was not blocked or reduced by ML171 (Fig. 9B), suggesting that activation of NOX1 complex is not requirement for stimulatory effect of hA on NOX subunits expression in these β -cells.

Discussion

The activation of ASK1-mediated apoptotic pathway in response to ER, protein and redox stress has been previously explored in several model systems including human islets [24, 31, 62]. However, the possible role of ASK1 in hA toxicity in pancreatic β -cells is unclear and hence was investigated here. The present study demonstrates that hA, in the course of its toxicity, activates ASK1-mediated apoptotic pathway in several pancreatic β -cell lines and human and mouse islets. Our *in vivo* and *ex vivo* data are consistent with the mechanism in which both extracellular and intracellular amylin accumulation stimulate beta-cell stress via ASK1-activation. *In vivo* studies revealed a marked and sustained increase in islet amyloid and phosphorylated ASK1 levels, reflecting enhanced MAP kinases signaling, in response to

accumulation of amyloidogenic hA. In contrast, islet amyloid and ASK1 activation was almost undetectable in islets of wild type mice. Similarly, aggregation incapable rodent amylin was less potent than hA in activating these two MAP kinases (though only in primary human and mouse islet cells), indicating that aggregation is important but not sole requirement for hA-induced ASK1 activation and stress signaling in pancreatic islet cells.

Likewise, inhibitory studies suggest that an alternative ASK1-independent, JNK-mediated signaling pathway operates together with ASK1 in hA-evoked β -cell death. In line with this idea, although the ASK1 inhibitor NQDI1 completely reversed the stimulatory effects of hA on ASK1, in some experiments, it only partially protected human islet cells against hA induced JNK activation and toxicity. However, the ability of thiol antioxidant NAC to completely reverse the stimulatory effect of hA on ASK1 and JNK activation and hA-evoked β -cell death further reaffirms the idea that an alternative redox-sensitive mechanism or pathway, in conjunction with ASK1, mediates hA-induced JNK signaling and toxicity. A study by Konarkowska and colleagues showed that hA triggers β -cell death through signaling pathways that are sensitive to the action of thiol (disulfide bond) reducing agents [20]. Our previous study reiterated this finding and showed that hA-induced oxidative stress in RIN-m5F cells could be completely reversed in the presence of thiol antioxidant NAC [37]. In agreement with these reports, our current confocal and biochemical studies revealed that thiol antioxidants NAC and BME completely reversed hA-induced GSH oxidation and markedly attenuated ASK1 activation by human amylin and other pro-oxidants. These findings suggest that hA induces ASK1 activation in pancreatic cells in a redox and thiol sensitive manner. It is interesting to note that while ASK1 activation by the oxidative stress inducers H_2O_2 and hA was successfully reversed by NAC, it failed to inhibit ASK1 activation by proteasome inhibitor Lac, reflecting a divergent pathways and mechanism of ASK1 activation by redox and protein stress in human islet and insulinoma cells. This conclusion is in agreement with our previous study showing that extracellular hA readily evokes redox but not protein stress in these cells [41]. Taken together, our data suggests that (i) ASK1 serves as a broad molecular sensor for various stressors in pancreatic β -cells including pro-oxidant hA ([37]; and (ii) hA-evoked ASK1-mediated β -cell death can be partially or completely reversed by specific ASK-1 inhibitor owing to whether additional pro-apoptotic factors or signaling pathways have been co-activated with ASK1.

Because human amylin exerts β -cell cytotoxicity via oxidative stress induced apoptosis [20, 23] (Fig. 2, Fig. S2), we evaluated contribution of cell's main ROS/SO-producing enzyme, NOX, to this pathological process. Previously, NOXs were linked to mitochondrial dysfunction and β -cell apoptosis [63, 64]. Specifically, NOX1 has been implicated in β -cell oxidative stress response [65], which is major hA-responsive NOX isoform in rodent β -cells (Fig. 6, [59]. Contrary to our initial hypothesis, NOX activity and function (SO/ROS production) were dispensable for hA-induced redox stress and MAP kinase activation. Notably, while NOX1 inhibitors partially restored mitochondrial function in insulinoma cells compromised by hA accumulation, they were ineffective against hA-induced redox stress. On other hand, they efficiently blocked ROS and SO accumulation evoked by NOX activator, pyocinin. Taken together, these results suggest that hA induces redox stress and pro-apoptotic MAP kinase signaling in β -cells via NOX-independent mechanism, likely by compromising mitochondrial function. Previous studies provided strong evidence for both

direct and indirect pathways of mitochondrial dysfunction by hA [41, 66, 67]. Thus, our studies suggest that hA-induces mitochondrial stress and beta-cell death via complex NOX1-dependent and -independent mechanisms. The exact mechanism by which mitochondrial-produced ROS mediates hA-evoked MAP kinase activation and pro-apoptotic signaling in β -cells remains to be determined in the future studies.

In summary, our studies suggest that overexpression or exogenous addition of human amylin depletes the cellular GSH levels, which in turn activate redox sensitive ASK1/JNK pathway leading to apoptosis of islet β -cells. Our studies identified ASK-1 as a novel molecular sensor of human amylin-induced redox stress that together with NOX1 mediates mitochondrial dysfunction in pancreatic β -cells. Interventions to prevent or reverse hA-induced ASK1- and NOX1-mediated stress signaling pathways in islet β -cells might be beneficial against T2DM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Prof. R Donaldson for valuable discussions on the subject. We also thank Dr. Chris Newgard lab for making INS 832/13 cells available for this study. This work was supported by the NIH grant RO1DK091845 and the ICR Basic Science Islet Distribution Program (to A.J.).

Abbreviations

ASK1	apoptosis signal regulating kinase-1
BME	β -mercapthoethanol
GSH	glutathione
hA	human amylin
hIAPP	human islet amyloid polypeptide
HFIP	hexafluoride isopropanol
JNK	c-Jun N-terminal kinase
Lac	lactacystin
MAP	mitogen activated protein kinases
NAC	N-acetyl-L-cysteine
NOX	NADPH oxidase
PM	plasma membrane
rA	rat amylin
ROS	reactive oxygen species

SO	superoxide
TTDM	Type-2 Diabetes Mellitus

References

- Clark A, Nilsson MR. Islet amyloid: a complication of islet dysfunction or an aetiological factor in Type 2 diabetes? *Diabetologia*. 472004; :157–169. [PubMed: 14722650]
- Hoppener JW, Lips CJ. Role of islet amyloid in type 2 diabetes mellitus. *Int J Biochem Cell Biol*. 382006; :726–736. [PubMed: 16459127]
- Westermarck P, Andersson A, Westermarck GT. Islet amyloid polypeptide, islet amyloid, and diabetes mellitus. *Physiol Rev*. 912011; :795–826. [PubMed: 21742788]
- Cao P, Abedini A, Wang H, Tu LH, Zhang X, Schmidt AM, Raleigh DP. Islet amyloid polypeptide toxicity and membrane interactions. *Proc Natl Acad Sci U S A*. 1102013; :19279–19284. [PubMed: 24218607]
- Engel MF, Khemtemourian L, Kleijer CC, Meeldijk HJ, Jacobs J, Verkleij AJ, de Kruijff B, Killian JA, Hoppener JW. Membrane damage by human islet amyloid polypeptide through fibril growth at the membrane. *Proc Natl Acad Sci U S A*. 1052008; :6033–6038. [PubMed: 18408164]
- Lorenzo A, Razzaboni B, Weir GC, Yankner BA. Pancreatic islet cell toxicity of amylin associated with type-2 diabetes mellitus. *Nature*. 3681994; :756–760. [PubMed: 8152488]
- Pappalardo G, Milardi D, Magri A, Attanasio F, Impellizzeri G, La Rosa C, Grasso D, Rizzarelli E. Environmental factors differently affect human and rat IAPP: conformational preferences and membrane interactions of IAPP17-29 peptide derivatives. *Chemistry*. 132007; :10204–10215. [PubMed: 17902185]
- Milardi D, Sciacca MF, Pappalardo M, Grasso DM, La Rosa C. The role of aromatic side-chains in amyloid growth and membrane interaction of the islet amyloid polypeptide fragment LANFLVH. *Eur Biophys J*. 402011; :1–12. [PubMed: 20809197]
- Mattson MP, Goodman Y. Different amyloidogenic peptides share a similar mechanism of neurotoxicity involving reactive oxygen species and calcium. *Brain Res*. 6761995; :219–224. [PubMed: 7796173]
- Lim YA, Rhein V, Baysang G, Meier F, Poljak A, Raftery MJ, Guilhaus M, Ittner LM, Eckert A, Gotz J. Abeta and human amylin share a common toxicity pathway via mitochondrial dysfunction. *Proteomics*. 102010; :1621–1633. [PubMed: 20186753]
- Milardi D, Sciacca MF, Randazzo L, Raudino A, La Rosa C. The role of calcium, lipid membranes and islet amyloid polypeptide in the onset of type 2 diabetes: innocent bystanders or partners in a crime? *Front Endocrinol (Lausanne)*. 52014; :216. [PubMed: 25566188]
- Imoto H, Sasaki N, Iwase M, Nakamura U, Oku M, Sonoki K, Uchizono Y, Iida M. Impaired insulin secretion by diphenyleneiodium associated with perturbation of cytosolic Ca²⁺ dynamics in pancreatic beta-cells. *Endocrinology*. 1492008; :5391–5400. [PubMed: 18617620]
- Leloup C, Tourrel-Cuzin C, Magnan C, Karaca M, Castel J, Carneiro L, Colombani AL, Ktorza A, Casteilla L, Penicaud L. Mitochondrial reactive oxygen species are obligatory signals for glucose-induced insulin secretion. *Diabetes*. 582009; :673–681. [PubMed: 19073765]
- Newsholme P, Morgan D, Rebelato E, Oliveira-Emilio HC, Procopio J, Curi R, Carpinelli A. Insights into the critical role of NADPH oxidase(s) in the normal and dysregulated pancreatic beta cell. *Diabetologia*. 522009; :2489–2498. [PubMed: 19809798]
- Morgan D, Oliveira-Emilio HR, Keane D, Hirata AE, Santos da Rocha M, Bordin S, Curi R, Newsholme P, Carpinelli AR. Glucose, palmitate and pro-inflammatory cytokines modulate production and activity of a phagocyte-like NADPH oxidase in rat pancreatic islets and a clonal beta cell line. *Diabetologia*. 502007; :359–369. [PubMed: 17151863]
- Taylor-Fishwick DA. NOX, NOX Who is There? The Contribution of NADPH Oxidase One to Beta Cell Dysfunction. *Front Endocrinol (Lausanne)*. 42013; :40. [PubMed: 23565109]
- Nakayama M, Inoguchi T, Sonta T, Maeda Y, Sasaki S, Sawada F, Tsubouchi H, Sonoda N, Kobayashi K, Sumimoto H, Nawata H. Increased expression of NAD(P)H oxidase in islets of

- animal models of Type 2 diabetes and its improvement by an AT1 receptor antagonist. *Biochem Biophys Res Commun.* 3322005; :927–933. [PubMed: 15922295]
18. Michalska M, Wolf G, Walther R, Newsholme P. Effects of pharmacological inhibition of NADPH oxidase or iNOS on pro-inflammatory cytokine, palmitic acid or H₂O₂-induced mouse islet or clonal pancreatic beta-cell dysfunction. *Biosci Rep.* 302010; :445–453. [PubMed: 20178457]
  19. Curtin JF, Donovan M, Cotter TG. Regulation and measurement of oxidative stress in apoptosis. *J Immunol Methods.* 2652002; :49–72. [PubMed: 12072178]
  20. Konarkowska B, Aitken JF, Kistler J, Zhang S, Cooper GJ. Thiol reducing compounds prevent human amylin-evoked cytotoxicity. *FEBS J.* 2722005; :4949–4959. [PubMed: 16176268]
  21. Newsholme P, Haber EP, Hirabara SM, Rebelato EL, Procopio J, Morgan D, Oliveira-Emilio HC, Carpinelli AR, Curi R. Diabetes associated cell stress and dysfunction: role of mitochondrial and non-mitochondrial ROS production and activity. *J Physiol.* 5832007; :9–24. [PubMed: 17584843]
  22. Ueda S, Masutani H, Nakamura H, Tanaka T, Ueno M, Yodoi J. Redox control of cell death. *Antioxid Redox Signal.* 42002; :405–414. [PubMed: 12215208]
  23. Zraika S, Hull RL, Udayasankar J, Aston-Mourney K, Subramanian SL, Kisilevsky R, Szarek WA, Kahn SE. Oxidative stress is induced by islet amyloid formation and time-dependently mediates amyloid-induced beta cell apoptosis. *Diabetologia.* 522009; :626–635. [PubMed: 19148619]
  24. Hsieh CC, Papaconstantinou J. Thioredoxin-ASK1 complex levels regulate ROS-mediated p38 MAPK pathway activity in livers of aged and long-lived Snell dwarf mice. *FASEB J.* 202006; :259–268. [PubMed: 16449798]
  25. Liu Y, Min W. Thioredoxin promotes ASK1 ubiquitination and degradation to inhibit ASK1-mediated apoptosis in a redox activity-independent manner. *Circ Res.* 902002; :1259–1266. [PubMed: 12089063]
  26. Matsukawa J, Matsuzawa A, Takeda K, Ichijo H. The ASK1-MAP kinase cascades in mammalian stress response. *J Biochem.* 1362004; :261–265. [PubMed: 15598880]
  27. Shen HM, Liu ZG. JNK signaling pathway is a key modulator in cell death mediated by reactive oxygen and nitrogen species. *Free Radic Biol Med.* 402006; :928–939. [PubMed: 16540388]
  28. Subramanian SL, Hull RL, Zraika S, Aston-Mourney K, Udayasankar J, Kahn SE. cJUN N-terminal kinase (JNK) activation mediates islet amyloid-induced beta cell apoptosis in cultured human islet amyloid polypeptide transgenic mouse islets. *Diabetologia.* 552012; :166–174. [PubMed: 22038516]
  29. Nadeau PJ, Charette SJ, Landry J. REDOX reaction at ASK1-Cys250 is essential for activation of JNK and induction of apoptosis. *Mol Biol Cell.* 202009; :3628–3637. [PubMed: 19570911]
  30. Nishitoh H, Matsuzawa A, Tobiume K, Saegusa K, Takeda K, Inoue K, Hori S, Kakizuka A, Ichijo H. ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. *Genes & development.* 162002; :1345–1355. [PubMed: 12050113]
  31. Nishitoh H, Saitoh M, Mochida Y, Takeda K, Nakano H, Rothe M, Miyazono K, Ichijo H. ASK1 is essential for JNK/SAPK activation by TRAF2. *Mol Cell.* 21998; :389–395. [PubMed: 9774977]
  32. Watanabe T, Sekine S, Naguro I, Sekine Y, Ichijo H. Apoptosis Signal-regulating Kinase-1 (ASK1)-p38 Pathway-dependent Cytoplasmic Translocation of the Orphan Nuclear Receptor NR4A2 Is Required for Oxidative Stress-induced Necrosis. *The Journal of biological chemistry.* 2902015; :10791–10803. [PubMed: 25752609]
  33. Liu H, Zhang H, Iles KE, Rinna A, Merrill G, Yodoi J, Torres M, Forman HJ. The ADP-stimulated NADPH oxidase activates the ASK-1/MKK4/JNK pathway in alveolar macrophages. *Free Radic Res.* 402006; :865–874. [PubMed: 17015265]
  34. Pepin E, Higa A, Schuster-Klein C, Bernard C, Sulpice T, Guardiola B, Chevet E, Alquier T. Deletion of apoptosis signal-regulating kinase 1 (ASK1) protects pancreatic beta-cells from stress-induced death but not from glucose homeostasis alterations under pro-inflammatory conditions. *PLoS One.* 92014; :e112714. [PubMed: 25383781]
  35. Widenmaier SB, Ao Z, Kim SJ, Warnock G, McIntosh CH. Suppression of p38 MAPK and JNK via Akt-mediated inhibition of apoptosis signal-regulating kinase 1 constitutes a core component of the beta-cell pro-survival effects of glucose-dependent insulinotropic polypeptide. *J Biol Chem.* 2842009; :30372–30382. [PubMed: 19748889]

36. Yamaguchi K, Takeda K, Kadowaki H, Ueda I, Namba Y, Ouchi Y, Nishitoh H, Ichijo H. Involvement of ASK1-p38 pathway in the pathogenesis of diabetes triggered by pancreatic ss cell exhaustion. *Biochim Biophys Acta*. 18302013; :3656–3663. [PubMed: 23416061]
37. Lee EC, Ha E, Singh S, Legesse L, Ahmad S, Karnaukhova E, Donaldson RP, Jeremic AM. Copper(II)-human amylin complex protects pancreatic cells from amylin toxicity. *Phys Chem Chem Phys*. 152013; :12558–12571. [PubMed: 23793354]
38. Rumora L, Hadzija M, Barisic K, Maysinger D, Grubiic TZ. Amylin-induced cytotoxicity is associated with activation of caspase-3 and MAP kinases. *Biol Chem*. 3832002; :1751–1758. [PubMed: 12530540]
39. Zhang S, Liu J, MacGibbon G, Dragunow M, Cooper GJ. Increased expression and activation of c-Jun contributes to human amylin-induced apoptosis in pancreatic islet beta-cells. *J Mol Biol*. 3242002; :271–285. [PubMed: 12441106]
40. Sinopoli A, Magri A, Milardi D, Pappalardo M, Pucci P, Flagiello A, Titman JJ, Nicoletti VG, Caruso G, Pappalardo G, Grasso G. The role of copper(II) in the aggregation of human amylin. *Metallomics*. 62014; :1841–1852. [PubMed: 25080969]
41. Singh S, Trikha S, Sarkar A, Jeremic AM. Proteasome regulates turnover of toxic human amylin in pancreatic cells. *Biochem J*. 4732016; :2655–2670. [PubMed: 27340132]
42. Cho WJ, Trikha S, Jeremic AM. Cholesterol regulates assembly of human islet amyloid polypeptide on model membranes. *J Mol Biol*. 3932009; :765–775. [PubMed: 19720065]
43. Cho WJ, Jena BP, Jeremic AM. Nano-scale imaging and dynamics of amylin-membrane interactions and its implication in type II diabetes mellitus. *Methods Cell Biol*. 902008; :267–286. [PubMed: 19195555]
44. Trikha S, Jeremic AM. Clustering and internalization of toxic amylin oligomers in pancreatic cells require plasma membrane cholesterol. *J Biol Chem*. 2862011; :36086–36097. [PubMed: 21865171]
45. Trikha S, Jeremic AM. Distinct internalization pathways of human amylin monomers and its cytotoxic oligomers in pancreatic cells. *PLoS One*. 82013; :e73080. [PubMed: 24019897]
46. Janson J, Soeller WC, Roche PC, Nelson RT, Torchia AJ, Kreutter DK, Butler PC. Spontaneous diabetes mellitus in transgenic mice expressing human islet amyloid polypeptide. *Proc Natl Acad Sci U S A*. 931996; :7283–7288. [PubMed: 8692984]
47. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*. 132012; :134. [PubMed: 22708584]
48. Huggett J, Dheda K, Bustin S, Zumla A. Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun*. 62005; :279–284. [PubMed: 15815687]
49. Bai JZ, Saafi EL, Zhang S, Cooper GJ. Role of Ca²⁺ in apoptosis evoked by human amylin in pancreatic islet beta-cells. *The Biochemical journal*. 343(Pt 1)1999; :53–61. [PubMed: 10493911]
50. Zhang S, Liu J, Dragunow M, Cooper GJ. Fibrillogenic amylin evokes islet beta-cell apoptosis through linked activation of a caspase cascade and JNK1. *J Biol Chem*. 2782003; :52810–52819. [PubMed: 14532296]
51. Hohmeier HE, Mulder H, Chen G, Henkel-Rieger R, Prentki M, Newgard CB. Isolation of INS-1-derived cell lines with robust ATP-sensitive K⁺ channel-dependent and -independent glucose-stimulated insulin secretion. *Diabetes*. 492000; :424–430. [PubMed: 10868964]
52. Milton NG, Harris JR. Fibril formation and toxicity of the non-amyloidogenic rat amylin peptide. *Micron*. 442013; :246–253. [PubMed: 22854213]
53. Casas S, Gomis R, Gribble FM, Altirriba J, Knuutila S, Novials A. Impairment of the ubiquitin-proteasome pathway is a downstream endoplasmic reticulum stress response induced by extracellular human islet amyloid polypeptide and contributes to pancreatic beta-cell apoptosis. *Diabetes*. 562007; :2284–2294. [PubMed: 17563070]
54. Huang CJ, Lin CY, Haataja L, Gurlo T, Butler AE, Rizza RA, Butler PC. High expression rates of human islet amyloid polypeptide induce endoplasmic reticulum stress mediated beta-cell apoptosis, a characteristic of humans with type 2 but not type 1 diabetes. *Diabetes*. 562007; :2016–2027. [PubMed: 17475933]

55. Trotter EW, Grant CM. Non-reciprocal regulation of the redox state of the glutathione-glutaredoxin and thioredoxin systems. *EMBO Rep.* 42003; :184–188. [PubMed: 12612609]
56. Soldani C, Lazze MC, Bottone MG, Tognon G, Biggiogera M, Pellicciari CE, Scovassi AI. Poly(ADP-ribose) polymerase cleavage during apoptosis: when and where? *Exp Cell Res.* 2692001; :193–201. [PubMed: 11570811]
57. Sano R, Reed JC. ER stress-induced cell death mechanisms. *Biochim Biophys Acta.* 18332013; :3460–3470. [PubMed: 23850759]
58. Kadowaki H, Nishitoh H, Urano F, Sadamitsu C, Matsuzawa A, Takeda K, Masutani H, Yodoi J, Urano Y, Nagano T, Ichijo H. Amyloid beta induces neuronal cell death through ROS-mediated ASK1 activation. *Cell Death Differ.* 122005; :19–24. [PubMed: 15592360]
59. Uchizon Y, Takeya R, Iwase M, Sasaki N, Oku M, Imoto H, Iida M, Sumimoto H. Expression of isoforms of NADPH oxidase components in rat pancreatic islets. *Life Sci.* 802006; :133–139. [PubMed: 16979190]
60. Gianni D, Taulet N, Zhang H, DerMardirossian C, Kister J, Martinez L, Roush WR, Brown SJ, Bokoch GM, Rosen H. A novel and specific NADPH oxidase-1 (Nox1) small-molecule inhibitor blocks the formation of functional invadopodia in human colon cancer cells. *ACS Chem Biol.* 52010; :981–993. [PubMed: 20715845]
61. Dubey R, Minj P, Malik N, Sardesai DM, Kulkarni SH, Acharya JD, Bhavesh NS, Sharma S, Kumar A. Recombinant human islet amyloid polypeptide forms shorter fibrils and mediates beta-cell apoptosis via generation of oxidative stress. *Biochem J.* 4742017; :3915–3934. [PubMed: 29046394]
62. Cai DT, Jin H, Xiong QX, Liu WG, Gao ZG, Gu GX, Qiu YH. ER stress and ASK1-JNK activation contribute to oridonin-induced apoptosis and growth inhibition in cultured human hepatoblastoma HuH-6 cells. *Mol Cell Biochem.* 3792013; :161–169. [PubMed: 23580093]
63. Matti A, Kyathanahalli C, Kowluru A. Protein farnesylation is requisite for mitochondrial fuel-induced insulin release: further evidence to link reactive oxygen species generation to insulin secretion in pancreatic beta-cells. *Islets.* 42012; :74–77. [PubMed: 22504835]
64. Syed I, Kyathanahalli CN, Jayaram B, Govind S, Rhodes CJ, Kowluru RA, Kowluru A. Increased phagocyte-like NADPH oxidase and ROS generation in type 2 diabetic ZDF rat and human islets: role of Rac1-JNK1/2 signaling pathway in mitochondrial dysregulation in the diabetic islet. *Diabetes.* 602011; :2843–2852. [PubMed: 21911753]
65. Weaver JR, Grzesik W, Taylor-Fishwick DA. Inhibition of NADPH oxidase-1 preserves beta cell function. *Diabetologia.* 582015; :113–121. [PubMed: 25277953]
66. Li XL, Chen T, Wong YS, Xu G, Fan RR, Zhao HL, Chan JC. Involvement of mitochondrial dysfunction in human islet amyloid polypeptide-induced apoptosis in INS-1E pancreatic beta cells: An effect attenuated by phycocyanin. *Int J Biochem Cell Biol.* 432011; :525–534. [PubMed: 21163363]
67. Magzoub M, Miranker AD. Concentration-dependent transitions govern the subcellular localization of islet amyloid polypeptide. *FASEB J.* 262012; :1228–1238. [PubMed: 22183778]

Highlights

- Toxic human amylin aggregates accumulates on PM and inside beta-cells which induces mitochondrial stress
- Amylin induces mitochondrial stress in part via activation/expression of NAPH oxidase complex
- NOX pro-oxidative activity was dispensable for hA-induced redox stress and MAP kinase activation
- ASK1 serves as redox sensor in pancreatic β -cells and mediates amylin-induced MAP kinase signaling

Figure 1. Human amylin and redox stressors activate the cytotoxic ASK1/JNK signaling cascade in insulinoma and primary human islet cells

Two pancreatic cell lines, RINm5-F cells (A, D) and INS832/13 cells (B) were treated with human amylin (hA), rat amylin (rA), or redox stressors H₂O₂ and diamide and changes in phospho-ASK1 Ser966 and Thr 845 levels, reflecting ASK1 activation, were detected with phospho-ASK1 Ser966 specific ELISA (A–B) and confocal microscopy (D). Note a significant ~40–60% decrease in pASK1 Ser966 levels induced by amylin and pro-oxidants as compared to the control, indicative of ASK1 activation (A–B). (C) Western blot analysis depicts a progressive intracellular accumulation of hA and increase in phospho-ASK1 Thr845 and phospho-JNK levels in cultured human islet cells following exposure to cytotoxic hA preparation. Significance was established at ** p<0.01 and *** p<0.001, control vs. treatments n=3, ANOVA followed by Tukey's post hoc comparison test (A–B).

Figure 2. Human amylin activates redox-sensitive ASK1/JNK signaling complex in insulinoma and human islet cells

(A) The effect of hA and H₂O₂ in the presence and absence of antioxidant NAC on reduced glutathione (GSH) levels in RIN-m5F cells is shown. Significance was established at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, control vs. treatments; and, ## $p < 0.01$ and ### $p < 0.001$, hA vs. treatments $n=3$, ANOVA followed by Tukey's post hoc test. (B) The regulatory effect of NAC and the other disulfide-reducing agent, β -mercapthoethanol (BME), on hA-evoked ASK1 and JNK activation in human islets is depicted. A representative western blot from three independent experiments is shown.

Figure 3. Selective ASK1 inhibitor NQDI1 prevents human amylin-induced ASK1 activation and proteotoxicity in insulinoma and human islet cells

(A) The effect of ASK1 inhibitor NQDI1 on hA-induced ASK1 activation (phosphorylation) in RIN-m5F cells was analyzed by confocal microscopy. Note a complete inhibition of hA- and stress-induced phosphor-ASK1 Thr845 signal in majority of cells (>90%) by NQDI. Bar, 10 μ m. (B–C) Inhibition of ASK1 phosphorylation by NQDI1 significantly reduce hA-induced cytotoxicity as demonstrated by MTT stress assay in RIN-m5F cells (B) and by PARP cleavage in cultured human islets detected with western blot (C). Significance was established at * $p < 0.05$ and *** $p < 0.001$, $n = 3$, ANOVA followed by Tukey's post hoc test (B).

Figure 4. ASK1 activation is required for hA-induced cellular stress and apoptosis in human islets

Human islets were treated with amylin in the absence or presence of specific MAP kinase inhibitors for 24h and their modulatory effects on ASK1/JNK phosphorylation/activation states determined by western blot (A, C) and phospho-JNK specific ELISA (B). (A) Western blot analysis of effect of Ask1 inhibitor NQDI1 on hA and rA induced ASK1 activation in human islets is shown. (B) ELISA depicts effects of hA, rA and NQDI1 on phospho-JNK levels in human islets. Significance was established at *** $p < 0.001$, $n = 3$, ANOVA followed by Tukey's post hoc test. (C) Modulatory effects of NQDI1 and JNK specific inhibitor (JK1) on hA-evoked ASK1 and JNK activation in human islets are shown. Both MAP kinase

inhibitors reversed hA-induced toxicity as revealed by PARP cleavage assay. Representative western blots from three independent experiments are shown (A, C).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5. β -cell specific expression of human amylin induces ASK1 activation in the islets of hA transgenic mice

(A) Non-fasting blood glucose profile in wt, hemizygous ($hA^{+/-}$) and homozygous ($hA^{+/+}$) transgenic mice. A severe hyperglycemia was observed only in homozygous transgenic mice. Significance was established at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, wt or hemizygous ($hA^{+/-}$) vs. homozygous ($hA^{+/+}$) mice, $n=4$, ANOVA followed by Tukey's post hoc comparison test. (B) Glycated hemoglobin levels in hA transgenic and wt mice. Significance established at ** $p < 0.01$ and *** $p < 0.001$, 54wks wt or 40wks $hA^{+/-}$ vs. 54wks $hA^{+/+}$ mice, $n=4$, ANOVA followed by Tukey's post hoc comparison test. (C) Immunohistochemical analysis of hA and insulin expression and localization in hA

transgenic mice. (D) Representative confocal light and fluorescent images of pancreatic sections from non-transgenic wt mice (top panel) and homozygous ($hA^{+/+}$) transgenic (middle and bottom panels) mice stained for amylin (red), pASK1 (green), and the nucleus with DAPI (blue). Scale bar, 100 μ m. Human amylin expression levels in mouse islets of wt (n=4) and 40 and 54 weeks old homozygous mice (n=4) is shown (D, top panel). Significance established at ** $p<0.01$ and *** $p<0.001$, 54wks wt vs. 40wks $hA^{+/-}$ or 54wks $hA^{+/-}$ mice, n=4, ANOVA followed by Tukey's post hoc comparison test. Phospho-Ask1 levels in mouse islets of wt (n=4) and 40 and 54 weeks old homozygous mice (n=4) is shown (D, bottom panel). Significance established at ** $p<0.01$ and *** $p<0.001$, n=4, ANOVA followed by Tukey's post hoc comparison test. n, indicates number of mice per experimental group. (E) Confocal microscopy analysis of amyloid formation and hA and insulin expression in hA transgenic mice. Islet amyloid aggregates (white areas) were detected using amyloid-specific dye, thioflavin-S (Th-S). Significance established at ** $p<0.01$ and *** $p<0.001$, n=4, ANOVA followed by Tukey's post hoc comparison test

Figure 6. Human amylin induces expression of cytosolic and plasma-membrane associated NOX subunits in insulinoma cells

(A–B) RT-QPCR analysis of NOX isoforms expression in RINm5F and INS-832/13 cells is shown. Modulatory effect of hA on NOX1 mRNA levels over time in two cell lines are depicted (A–B, insets). Data are expressed as mean $\pm$ SEM of two independent experiments with three samples per experimental group. Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n=6$).

(C) Confocal microscopy depicts progressive stimulatory effect of toxic concentration (30 μ M) of hA on expression of cytosolic regulatory subunits, p47^{phox} and p67^{phox} in RINm5F cells. Bars, 10 μ m.

Figure 7. Effect of NOX1 inhibitor on amylin-induced cellular stress

MTT stress assay depicts toxic effect of hA on insulinoma cells in the absence and in the presence of NOX1 specific inhibitor, ML171. Data are expressed as mean $\pm$ SEM of two independent experiments and sample size of 6. One-way ANOVA followed by Tukey's post-hoc test (***) $p < 0.001$, $n=6$).

Figure 8. NOX1 silencing reduces amylin cytotoxicity

The changes in protein levels of NOX1 were detected by western blot analysis 48 hours post-transfection with NOX1-siRNA or Silencer™ Negative siRNA (20 nM, upper panels). Percentage of MTT reduction in NOX1-silenced and control cells exposed to 30μM human amylin for 24 hour is shown (lower panel). One-way ANOVA followed by Tukey's post-hoc test (**p < 0.01, n=6).

Figure 9. NOX1 is not involved in amylin-induced redox stress and MAP kinase activation

(A) Effects of hA and NOX inhibitors, apocynin and ML171, on ROS and SO production in insulinoma cells. Data are expressed as mean $\pm$ SEM of three independent treatments. One-way ANOVA and followed by Tukey's post-hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$, $n = 6$, Ctrl. vs. treatments). (B) Western blot analysis of JNK and NOX protein expression/activation in RINm5F cells in the absence (control) and presence of hA and/or NOX1 inhibitor, ML171, is depicted. A representative western blot from three independent experiments is shown.

Table 1

List of primers used for qPCR studies. r represents rat NOX gene. Primer sequences were designed using NCBI-Primer-BLAST software.

Gene	Forward (5'>3')	Reverse (5'>3')	Accession No.
rNOX1	TTCTAGAATAGCTACTGCCCACC	AGGCTTTTGGCAAAGTCGGAG	NM053683.1
rNOX2	TGACAATGCCACCAGTCTGAG	GGTCTGCGAACCACTCAAAA	NM023965.1
rNOX3	AGGTCGCATCATTCGAGGTC	TTCGTCGAAGTGGTCTCTGC	NM001004216.1
rNOX4	TTCGGGTGGCTTGTGAAGT	TGGGGTCCGGTTAAGACTGA	NM053524.1