Hyperamylinemia Increases IL-1β Synthesis in the Heart via Peroxidative Sarcolemmal Injury

Running title: Role of Amylin in Diabetic Heart Disease

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Abstract
Hypersecretion of amylin is common in individuals with pre-diabetes, causes amylin deposition and proteotoxicity in pancreatic islets and contributes to the development of type-2 diabetes. Recent studies identified amylin deposits also in failing hearts from patients with obesity or type-2 diabetes and demonstrated that hyperamylinemia accelerates the development of heart dysfunction in rats expressing human amylin in pancreatic β-cells (HIP rats). To further determine the impact of hyperamylinemia on cardiac myocytes, we investigated human myocardium, compared diabetic HIP rats with diabetic rats expressing endogenous (non-amyloidogenic) rat amylin, studied normal mice injected with aggregated human amylin and developed in vitro cell models. We found that amylin deposition negatively affects cardiac myocytes by inducing sarcolemmal injury, generating reactive aldehydes, forming amylin-based adducts with reactive aldehydes and increasing synthesis of the pro-inflammatory cytokine interleukin (IL)-1β independently of hyperglycemia. These results are consistent with the pathological role of amylin deposition in the pancreas, uncover a novel contributing mechanism to cardiac myocyte injury in type-2 diabetes and suggest a potentially treatable link of type-2 diabetes with diabetic heart disease. While further studies are necessary, these data also suggest that IL-1β might function as a sensor of myocyte amylin uptake and potential mediator of myocyte injury.
Amylin (also known as islet amyloid polypeptide) is a regulatory peptide synthesized and co-secreted with insulin by pancreatic β-cells and is believed to participate in promoting satiety by slowing gastric emptying (1). Like insulin, amylin is oversecreted in patients with obesity or pre-diabetic insulin resistance, i.e., hyperinsulinemia coincides always with hyperamylinemia. Elevated human amylin secretion promotes its deposition in the pancreas (1), which induces oxidative stress (2), activation of the NLRP3 inflammasome and release of IL-1β (3), a cytokine involved in a plethora of inflammatory responses (4). Thus, hyperamylinemia is a critical early contributor to the development of type-2 diabetes. The pathological effects of hyperamylinemia were thought to be limited to pancreatic islets. However, recent studies (5-10) found amylin deposition in failing kidneys (5) and hearts (6,7) from patients with type-2 diabetes, and in brains (8-10) of diabetic patients with Alzheimer’s disease. In the heart, significant amylin deposits were identified in areas of cardiac myocyte injury (6,7) and in myocyte lysates (7), suggesting a role in the pathogenesis of diabetic heart disease. Amylin deposition in pancreas and extra-pancreatic tissues, including the heart, was mirrored in a rat model of type-2 diabetes expressing human amylin in the pancreas (HIP rats) (6,7,11-16). HIP rats develop type-2 diabetes (14) cardiovascular dysfunction (6,7,12-14), neuroinflammation (11,15) and neurologic deficits (11). Particularly, the HIP rats show diastolic dysfunction (6), cardiac hypertrophy (7) and dilation (7), which resemble some of the pathogenic mechanisms of diabetic cardiomyopathy in humans (17). Thus, hyperamylinemia contributes to pathogenic pathways for both type-2 diabetes and the co-occurring cardiac disease. Similar inter-tissue communication was previously (18) shown to connect Alzheimer’s disease with skeletal muscle disorders in aged humans.

The toxicity of amylin from humans (and a few other species) (1) was linked to amyloidogenicity (1) and increased propensity to aggregate and interact with cellular membranes
Aggregated amylin disrupts membrane integrity, ion homeostasis and cell function (19-21). The role of amyloidogenicity in amylin-induced toxicity is supported by the observation (21) that, in contrast to HIP rats, rodents overexpressing rodent amylin, which is not amyloidogenic (1), showed neither amylin deposition nor development of type-2 diabetes.

Disrupting membrane integrity by incorporation of aggregated amylin could also increase the exposure of unsaturated fatty acids to cytosolic ROS. This process leads to formation of reactive aldehydes (22), such as 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA). While generation of reactive aldehydes is essential for cell survival signaling (22), increased levels of 4-HNE and MDA further elevate ROS and trigger inflammatory responses (22). 4-HNE and MDA can also non-enzymatically form stable protein adducts by binding to histidine, lysine, and cysteine side chains, i.e. Maillard reaction (22). 4-HNE- and MDA-modified proteins have been used as biomarkers for cell oxidative damage (22). We therefore hypothesize that myocardial amylin deposition destabilizes the sarcolemma and generates reactive aldehydes that perturb intracellular homeostasis, leading to increased synthesis of IL-1β. To test this hypothesis, we analyzed tissue specimens from humans, compared human amylin-expressing (HIP) rats with age- and glucose-matched diabetic rats expressing only endogenous non-amyloidogenic rat amylin, studied mice injected with aggregated human amylin and developed in vitro cell models.
**Research Design and Methods**

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Institutional Animal Care and Use Committees at University of Kentucky.

The protocol concerning the use of biopsy from patients was approved by the Institutional Review Board at the Hospital of the University of Pennsylvania and informed consent was obtained prospectively in all cases.

**Human Samples**

Human specimens used in this study were also used in the cohorts reported in our previous publications (6,7). Myocardial samples were obtained from obese individuals (body mass index, BMI, $\geq 30$) with non-ischemic heart failure (O-HF; n=7) and patients with type-2 diabetes and non-ischemic heart failure (D-HF; n=5). Patients in the O-HF group developed overt type-2 diabetes during the early post-transplantation period. Hence, we speculated that these patients were in pre-diabetes at the time of heart transplantation. Non-failing hearts from lean, non-diabetic donors formed the control group (Ctl; n=7).

**Experimental Animals**

Rats that express human amylin in the pancreatic $\beta$-cells (HIP rats, n=25; Charles River Laboratory) were used as type-2 diabetes animals with myocardial amylin deposition (6,7). They were generated from Sprague-Dawley (SD) rats by expressing human amylin on the insulin promoter (16). HIP rats used in this study were $\sim$10 months of age and displayed non-fasting blood glucose levels (morning time) in the 300-400 mg/dl range. Age-matched wild-type (WT; n=30) littermates were used as non-diabetic controls. Ideal diabetic control rats for this study
should have a SD genetic background and develop type-2 diabetes spontaneously (with regular diet) on the same timeline as HIP rats. However, such a model is not feasible because rats do not develop type-2 diabetes spontaneously (1). We therefore used UCD rats (n=12), which have half of SD genes and develop type-2 diabetes (7,23). The other half of their genes are from the Zucker Diabetic-lean rat, a rat model that displays a proinsulin defect in pancreatic β-cells and intact leptin receptor function (24). Changes of blood insulin and amylin in HIP and UCD rats with the transition from normal to pre-diabetes and full-blown diabetes were published elsewhere (7,11,23).

C57BL/6 mice were intravenously injected (via tail vein) with either aggregated human amylin (2 µg/g body weight; n=8) or saline (n=8) for 5 days (b.i.d.) followed by 14 days of intraperitoneal injection.

Proteomics

Amylin-positive protein fractions from myocardial lysates were isolated using high-performance liquid chromatography (HPLC) and further analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). A Waters 717 plus HPLC system (Phenomenex C18 column; 5 um; 4.6 mm x 5 cm) with a 15 minute linear gradient from 5% to 75% mobile phase B (0.1% TFA in acetonitrile) was used for separation of amylin-positive fractions. The mobile phase A was 0.1% (v/v) TFA in water and the flow rate was set at 1ml/min. Amylin was detected with a diode array detector at 214 nm and fractions were collected. Amylin-containing tissue fractions were confirmed by comparison with an amylin standard.

For the LC-MS/MS test, we used an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled with an Eksigent Nanoflex cHiPLC™ system (Eksigent, Dublin, CA) through a nano-electrospray ionization source (25). Data were acquired in an automated data
dependent acquisition mode consisting of an Orbitrap MS scan (300–1800 m/z, 60,000 resolutions) followed by MS/MS for fragmentation of the 7 most abundant ions with the collision induced dissociation method. The 4+ ion of amylin peptide (m/z = 976.22) was extracted from the total ion spectrum. The MS/MS fragments of amylin were confirmed by comparison with an amylin standard.

**Metabolomics**

Heart homogenates were derivatized with 2,4-Dinitrophenylhydrazine (DNPH; for MDA analysis), extracted with ethyl acetate, dried under nitrogen and reconstituted with acetonitrile (26). Analysis of MDA-DNPH and 4-HNE-GSH was carried out using a Shimadzu UFLC coupled with an AB Sciex 4000-Qtrap hybrid linear ion trap triple quadrupole mass spectrometer in multiple reaction monitoring mode. MDA-DNPH and GSH-HNE were analyzed using a Machery-Nagel Nucleodur C8 Gravity column, 5µm, 125 mm × 2.0 mm.

**Immunohistochemistry**

Thin sections of paraffin-embedded tissues were incubated with a combination of anti-human amylin (SC-377530; Santa Cruz biotech; TX) and anti-4-HNE (ab46545; Abcam; MA), anti-MDA (ab94671; Abcam; MA) or anti-IL-1β (ab9722; Abcam; MA) primary antibodies. After washings, sections were incubated with Alexa Fluor 488 conjugated anti-mouse IgG (A11029; Invitrogen; NY) and Texas red conjugated anti-rabbit IgG (SC-2780; Santa Cruz biotech; TX) secondary antibodies. The sections were then stained with DAPI (ab 104139, Abcam; MA) and imaged with a laser-scanning confocal microscope (Live5; Zeiss; Germany). Elastin autofluorescence was blocked with 1% Sudan black. Immunochemistry measurements were also done on fixed myocytes incubated with anti-IL-1β primary and Alexa Fluor 488 conjugated secondary antibodies. Western blot analysis was performed on tissue homogenates and lysates.
from cardiac myocytes. After electrophoresis, blotting and blocking, membranes were incubated with primary antibodies for amylin (T-4157; Bachem-Peninsula Laboratories; San Carlos; CA), 4-HNE, MDA, IL-1β (all three as above), TNF-α (ab1793; Abcam; MA), IL-6 (ab9324; Abcam; MA), IL-10 (ab25073; Abcam; MA) or GAPDH (ab8245; Abcam; MA; loading control for heart samples). The specific staining of protein bands was verified as previously described (6–8). Total amylin level in plasma was assessed by ELISA (EZHAT-K51K; Millipore; MA).

In some experiments, 4-HNE or MDA were immunoprecipitated using same antibodies as above and immobilized protein A/G resin slurry (20421; Thermofisher; IL).

**Quantitative real-time PCR** was used to assess the mRNA level of IL-1β in myocardial tissues as previously described (8).

**Cell isolation** was performed as previously described (6, 7, 27).

**Measurement of lipid peroxidation in isolated cells**

Isolated myocytes were incubated for 2 h under control conditions, with aggregated amylin and/or 400 mg/dl glucose, with 50 µM poloxamer 188 for 2 h followed by aggregated amylin, and with 5 mM NAC for 30 minutes followed by aggregated amylin. After incubation, myocytes were loaded with the fluorescent probe 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C_{11}-BODIPY^{581/591}; D3861; Invitrogen; OR) and imaged with a confocal microscope (Live5; Zeiss; Germany). Upon peroxidation, the fluorescence emission peak of C_{11}-BODIPY^{581/591} shifts from 590 nm (red) to 510 nm (green). Thus, lipid peroxidation was measured as the ratio between the average fluorescence intensity in the green and red channels.

**Proximity Ligation Assay (PLA)**
For the Duolink in situ PLA, sections were incubated for 90 minutes with oligonucleotide-conjugated anti-mouse IgG MINUS (DUO92004; Sigma; MO) and anti-rabbit IgG PLUS (PLA probes; DUO92002; Sigma; MO) diluted 1:6 in Tris-buffered saline at 37°C. Amplified DNA strands were detected with oligonucleotides conjugated to a fluorophore (Duolink In Situ Detection Reagents Red, DUO92008, Sigma; MO). They were cover-slipped with mounting medium DAPI (DUO82040; Sigma; MO) and analyzed by confocal microscopy.

**Langendorff perfusion**

To assess the dynamics of cardiac amylin deposition, isolated mouse hearts (n=4) were perfused on a Langendorff apparatus with 10 µM biotinylated human amylin in Tyrode’s solution for 2 hours followed by 10 minutes washout with Tyrode’s solution. Experiments were repeated with 10 µM of aggregated amylin vs. saline.

**ROS production** was measured as previously described (7).

**Treatment**

Poloxamer 188 is a surfactant that stabilizes lipid bilayers through hydrophobic interactions (28) and was used to block amylin-induced sarcolemmal damage in isolated cardiac myocytes (50 µM; 2 hours prior application of amylin). N-acetyl cysteine (NAC) was used to quench basal ROS production in control myocytes (5 mM; 30 minutes prior application of amylin).

**Statistical analysis**

Data are presented as mean ±standard error. Statistical differences between groups were determined using (i) the unpaired 2-tailed student’s t-test when comparing 2 groups and (ii) one-way ANOVA with Bonferroni’s post hoc test when comparing multiple groups, and were considered significant when P<0.05.
Results

Amylin deposition in human hearts

Amylin and insulin have similar diurnal variation (29,30) and are elevated in individuals with obesity or pre-diabetic insulin resistance (31,32). Compared to controls, patients in the O-HF group showed elevated blood levels of amylin and insulin (Figure 1A). Western blot analysis of blood, heart and pancreas lysates identified multiple amylin aggregates and only a faint 4 kDa band corresponding to the monomeric amylin (Figure 1B). Furthermore, aggregated amylin with similar molecular weights are seen in both blood and cardiac myocyte lysates. These results are consistent with our previous data from HIP rats (7) and suggest that circulating aggregated amylin may promote myocardial amylin deposition. (Additional data are provided in the Supplementary Figure S1).

Next, we used proteomics to test the presence of amylin deposition in the heart. Based on the retention time of the standard amylin peptide, as derived from the HPLC chromatogram (Figure 1C), we initially collected four different cardiac lysate fractions. These fractions were immunoblotted to identify the amylin (Figure 1C; inset). The lysate fraction positive for amylin by Western blot showed also the presence of amylin by LC-MS/MS (Figure 1D).

4-HNE-amylin adducts and elevated synthesis of IL-1β in hearts of diabetic patients

To test the formation of amylin-4-HNE and amylin-MDA adducts, we immunoprecipitated 4-HNE and MDA from cardiac myocyte lysates and used an ELISA to measure the amylin content in 4-HNE/MDA enriched fractions. Amylin-4-HNE and amylin-MDA complexes were significantly elevated in hearts from the O-HF group (Figure 2A). Thus, incorporation of aggregated amylin in cardiac myocytes is associated with elevated markers of peroxidative membrane injury.
Furthermore, immunoconfocal microscopy analysis demonstrated that areas of myocyte amylin deposition were positive for both 4-HNE and MDA (Figure 2B). In contrast, amylin-4HNE and amylin-MDA adducts were undetectable in myocardial tissue from the control group. Pancreatic tissue from a diabetic patient (positive control for amylin deposition) also showed amylin-4-HNE/MDA co-localization (Figure 2C).

Although cardiac myocytes are not major contributors to myocardial inflammation, they participate in the establishment of a pro-inflammatory environment under stress conditions by releasing cytokines and chemokines (33). IL-1β is part of the signaling pathway within myocytes that controls communication with leucocytes (33). The density of myocytes showing both amylin and IL-1β immunoreactivities was significantly higher in O-HF hearts compared to controls (Figure 2D), suggesting that myocyte uptake of amylin could trigger stress responses leading to exacerbated IL-1β synthesis, similar to amylin-mediated pathology in the pancreas (3).

We previously showed that failing hearts from patients with obesity or type-2 diabetes contain comparable levels of aggregated amylin (6). Amylin in myocardial fractions enriched in 4-HNE and MDA, formation of amylin-4-HNE and amylin-MDA adducts and IL-1β synthesis are all higher in the D-HF group compared to controls (Figure 3). This result suggests that obese individuals that developed type-2 diabetes shortly after heart transplant and patients with pre-existing type-2 diabetes present similar relationships between amylin deposition and levels of reactive aldehydes and IL-1β.

4-HNE-amylin adducts in hearts of human amylin-expressing diabetic rats

HIP rat hearts show higher levels of 4-HNE and MDA compared to WT littermate rats (Figure 4A). In contrast, cardiac 4-HNE and MDA in age- and glucose-matched UCD rats were comparable to those in the WT rats, indicating that in the absence of amylin deposition,
hyperglycemia does not cause significant lipid peroxidation in the heart. Using the same antibodies as in above, we identified amylin-4-HNE adducts in HIP rat hearts, but not UCD rat hearts (Figure 4B). The PLA signal shows an overall consistency with 4-HNE-amylin appearing in clusters in HIP rat hearts, consistent with the formation of adducts (Figure 4C). Furthermore, Western blot analysis of heart lysates that were enriched in 4-HNE (and MDA) by immunoprecipitation demonstrated that amylin deposits in HIP rats underwent modifications by 4-HNE and MDA (Figure 4D). Specifically, amylin-4-HNE and amylin-MDA complexes remained insoluble to sodium dodecyl sulfate and dithiothreitol, which were present in the gel and buffer, indicating that amylin forms intermolecular bonds with 4-HNE and MDA. The higher avidity of amylin from humans compared to rodent amylin to bind 4-HNE and MDA is likely due to the larger number of amino acids with binding affinity to reactive aldehydes (K, 2C and H in human amylin vs. only K and 2C in rat amylin). Additional data showing the anatomical localization of amylin-4HNE adducts are included in the Supplementary Figure S2.

**IL-1β activation in hearts of human amylin-expressing diabetic rats**

IL-1β immunoreactivity was detected in HIP rat heart tissue, but not UCD rats (Figure 5A). These results correlate with a greatly increased IL-1β transcript in HIP rat hearts (Figure 5B), suggesting that cardiac incorporation of aggregated amylin induces expression of IL-1β. Western blot analysis showed increased IL-1β levels in HIP rat hearts (Figure 5C). In contrast, hearts of UCD and WT rats displayed similar IL-1β levels. HIP rats showed also elevated TNF-α, but unchanged levels of IL-6 and IL-10. Compared to WT rats, UCD rats present no significant alteration of cardiac TNF-α, IL-6 and IL-10 cytokine levels (Figure 5C). Thus, the association of aggregated amylin with peroxidative membrane injury and IL-1β synthesis in hearts of patients in the O-HF group is mirrored in HIP rats, but absent in UCD rats.
Systemic effects following intravenous injection of aggregated amylin in mice

Aggregated amylin was found in human pancreas, blood and heart (Figure 1B). Using the Langendorff system and intravenously injected aggregated human amylin in normal mice, we tested the hypothesis that circulating aggregated amylin promotes myocardial amylin accumulation, generation of reactive aldehydes and IL-1β synthesis.

Perfusion of isolated hearts with amylin led to amylin deposition on the sarcolemma and in myocardial interstices (arrows; Figure 6A). Western blots further confirmed incorporation of aggregated amylin in cardiac tissue (Figure 6B) and formation of amylin-4-HNE and amylin-MDA adducts (Figure 6C). The IL-1β level was similar to that in saline-perfused hearts (Figure 6D). These results demonstrate a direct and rapid effect of circulating aggregated amylin to generate reactive aldehydes in cardiac tissue and suggest that the inflammation response involving IL-1β is downstream of lipid peroxidation.

In contrast to controls, mice injected with aggregated human amylin contained amylin-4-HNE and amylin-MDA adducts in cardiac myocyte lysates (Figure 6E). The IL-1β level was also increased in this animal group (Figure 6F). These results confirm that circulating aggregated amylin can induce cardiac peroxidative injury and elevate IL-1β synthesis independently of hyperglycemia.

Mechanism for amylin-induced peroxidative membrane injury and IL-1β expression

To further understand this novel mechanism of sarcolemmal injury, we used an in vitro system where isolated control myocytes were incubated for 2 hours with 50 µM human amylin or/and 400 mg/dl glucose. Exposure to 50 µM human amylin for 2 hours results in the same level of
amylin uptake in myocytes as in HIP rats (7). The lipid peroxide level was measured in single cells using the fluorescent probe C_{11}-BODIPY^{581/591}. Compared to WT rats, the lipid peroxide level was increased in myocytes from HIP rats, but not UCD rats (Figure 7A). Furthermore, incubation of control rat myocytes with human amylin (50 µM for 2 hours) increased lipid peroxidation (Figure 7A). This result is further supported by Western blot analysis (Figure 7B). In contrast, incubation for the same duration with 400 mg/dl glucose had no effect on the lipid peroxidation level (Figure 7, A and B). Incorporation of aggregated amylin in cell membranes was then prevented by pre-treating isolated control myocytes with 50 µM poloxamer 188 surfactant. Surfactant molecules (S) blocked amylin-induced lipid peroxidation (Figure 7C; magenta bar). In a separate experiment, we quenched basal ROS production in control myocytes by pre-incubation with NAC, a scavenger of H_{2}O_{2} and HO^*. Pre-treatment with NAC prevented the ignition of the lipid peroxidation chain reaction which is induced by the incubation with human amylin (Figure 7C; green bar). Thus, impeding the incorporation of aggregated amylin in cellular membranes (e.g. by membrane sealants), or quenching the basal ROS, blocked amylin-induced lipid peroxidation in isolated myocytes.

Consistent with our previous data showing that incubation of isolated cardiac myocytes with aggregated amylin increases ROS production (7), we found significantly higher H_{2}O_{2} levels in cardiac myocytes from HIP rats compared to UCD rats (Figure 7D). ROS are considered the proximal signals for activation of the IL-1β-processing inflammasome in pancreatic islets (3) and heart (33). Intriguingly, recent experimental evidence (34) points to 4-HNE as another activator of signaling cascades underlying IL-1β synthesis and activation. We therefore hypothesized that amylin-mediated peroxidative membrane injury increases IL-1β synthesis. Indeed, incubation of control myocytes (Figure 7E) with aggregated amylin (50 µM for 2 hours) resulted in robust IL-
1β synthesis. Preventing amylin-induced lipid peroxidation by impeding amylin incorporation with a membrane stabilizer (S; magenta bars) or by quenching the basal ROS with NAC (green bars) blocked IL-1β synthesis in cardiac myocytes (Figure 7E). This result is further supported by Western blot (Figure 7F). In contrast, incubation for the same duration with 400 mg/dl glucose had no effect on IL-1β (Figure 7F).

Based on these data, we propose (Figure 7G) that myocardial amylin deposition destabilizes the sarcolemma and generates reactive aldehydes that perturb intracellular homeostasis, leading to increased synthesis of IL-1β. Blocking either myocyte amylin uptake (by surfactants; S), or the lipid peroxidation chain reaction (by NAC), demonstrated that peroxidative membrane injury is upstream of IL-1β increased synthesis (Figure 7G).
Discussion

The link of diabetes with co-occurring disorders in the heart involves complex multifactorial pathways (17,35) that are still incompletely understood. Prior studies (17,36-40), including our work (36), demonstrated heart dysfunction in rodent models of type-2 diabetes that lack amylin deposition. However, we previously (6,7) showed that HIP rats develop diastolic dysfunction and cardiac hypertrophy even in pre-diabetes, pointing to amylin deposition as a factor that accelerates the development of diabetic heart disease. From our prior work (6,7) and present results, it appears that the amylin-specific deleterious effects on the heart involve the destabilization of the sarcolemma. We have previously demonstrated that amylin deposition induces sarcolaminal Ca\(^{2+}\) leak leading to increased myocyte cytosolic Ca\(^{2+}\) and activation of Ca\(^{2+}\)-mediated hypertrophy and remodeling signaling pathways (6). Conversely, reducing myocardial amylin deposition improved myocyte Ca\(^{2+}\) handling and heart function in HIP rats (7). We now report that hearts accumulating aggregated amylin (i.e., HIP rat hearts) are prone to generation of reactive aldehydes (4-HNE and MDA) and formation of amylin-HNE and amylin-MDA adducts. The levels of 4-HNE and MDA in diabetic hearts that lack amylin deposition (i.e., UCD rat hearts) are comparable to those in hearts from wild type non-diabetic rats, despite a pronounced ROS production (Figure 7D). Furthermore, HIP rat hearts showed hypersynthesis of IL-1β, which is linked to the increased production of reactive aldehydes, as suggested by previous results (34) and our experiments in isolated cells (Figure 7). This pathologic change is not seen in UCD rat hearts supporting a role of amylin deposition in inducing sarcolemmal injury. Because generation of reactive aldehydes, formation of amylin adducts and increased IL-1β synthesis in human cardiac tissue appear in association with amylin deposition (Figure 2 and 3), HIP rats are clinically relevant animal models to mechanistically understand this novel
pathologic link between type-2 diabetes and heart disease. In Table 1, we summarize alterations to cardiac function/structure associated with amylin deposition in the HIP rat model.

Intriguingly, several bands corresponding to aggregated amylin are seen in cardiac myocytes, but not in blood (Figure 1B), suggesting that amylin may aggregate \textit{in situ}, within myocytes. This result begs questions regarding the ability of myocytes to degrade intracellular amylin and whether implicated mechanisms are impaired under conditions underlying the amylin incorporation in cardiac myocytes. Therefore, future studies should focus on identifying ways to augment the clearance of cytosolic amylin before its aggregation. Furthermore, the formation of amylin-4-HNE and amylin-MDA adducts suggests the hypothesis that hyperamylinemia increases the production of reactive aldehydes (as demonstrated by data displayed in Figure 4A and Figure 6E) which, in turn, may accelerate myocardial amylin deposition via adduct formation. This mechanism is common to Aβ pathology in brains of Alzheimer’s disease patients (41), but it needs clarification in relation to amylin deposition in the heart. Amylin-induced generation of reactive aldehydes may be of further relevance to various changes in heart metabolism attributed to peroxidative injury (22,38-40). Antioxidants and surfactants inhibited the lipid peroxidation chain reaction and incorporation of aggregated amylin into cellular membranes, respectively. The use of NAC and a membrane stabilizer was intended to demonstrate a proof of concept and provide insights into a potential mechanism. Additional studies are needed to assess the mechanisms of action, formulations and potential side effects.

In conclusion, we demonstrated that hyperamylinemia and subsequent elevated blood levels of aggregated amylin promote incorporation of aggregated amylin within cardiac myocytes, which destabilizes the sarcolemma and generates reactive aldehydes that perturb intracellular homeostasis leading to increased IL-1β synthesis. Thus, exacerbated synthesis of IL-1β is
revealed as a critical stress-activated signaling pathway in response to the interaction of aggregated amylin with myocytes. Given that aggregated amylin triggers IL-1β release in pancreatic islets (3), present results suggest that pancreatic amylin pathology may be linked with diabetic myocyte injury by amylin deposition in the heart. Future studies need to determine whether exacerbated IL-1β synthesis in myocytes accumulating aggregated amylin results from activation of the NLRP3 inflammasome, which thus might function as a sensor of myocyte amylin uptake and potential mediator of myocyte injury.
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Author Contributions. ML carried out experiments that include immunoblotting, HPLC, LC-MS/MS, lipid peroxidation for myocytes, animal injection and helped with manuscript editing. NV performed all double immunofluorescence staining, confocal imaging and RT-PCR experiments, including data analysis. XLP and SS performed animal surgeries and metabolic characterization of the animals. AM conducted the LC-MS/MS analysis for assessing lipid peroxidation in animal models. MC and LHB performed and analyzed HPLC experiments. JC and HZ performed and analyzed proteomic experiments. KBM isolated and prepared the human cardiac tissue and contributed to study design. MGN contributed to designing of the inflammation study. SD performed the ROS experiments in isolated cardiac myocytes and contributed to study design. FD conceived the project and wrote the manuscript with assistance from the other authors. FD is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
References


Figure legends

**Figure 1. Testing the amylin deposition in human hearts.** (A) Blood amylin and insulin levels in patients with obesity and non-ischemic heart failure (O-HF) and non-diabetic control (Ctl) were measured by ELISA (n=7 samples/group). (B) Top panel shows Western blot analysis of amylin in pancreas (Pan), blood, and cardiac tissue from humans. Freshly solubilized recombinant human amylin (Amy; 50 ng) and pancreatic tissue from a type-2 diabetes patient were positive controls for monomeric amylin and aggregated amylin, respectively. In the bottom panel, we compared aggregated amylin in blood and cardiac myocytes from the O-HF group by Western blot. (C) Typical reverse phase HPLC chromatogram of human amylin standard human cardiac tissue. In the inset, selected fractions according to the retention time of human amylin standard were tested for the presence of amylin by Western blot. 10 ng of recombinant human amylin (H-Amy) was loaded as positive control. A representative blot from 3 independent experiments is shown. (D) Fractions collected from HPLC (the amylin positive fractions) were analyzed by LC-MS/MS. The representative mass spectrum of peptides eluted at 27.40 min shows the 4+ ion of amylin (m/z = 976.22). Right panel is the corresponding tandem MS/MS spectrum of amylin. The fragments corresponding to the b ions are labeled, clearly proving that this peptide is amylin.

**Figure 2. Failing hearts from obese patients display amylin-4-HNE and amylin-MDA adducts and increased IL-1β synthesis.** In (A), 4-HNE and MDA were immunoprecipitated from heart homogenates. The level of amylin in the fractions enriched in 4-HNE and MDA was then quantified by ELISA. (B-C) Dual immunofluorescence staining of amylin (green) and 4-HNE (MDA) (red) in the heart (B) and pancreas (C) of patients with
obesity and non-ischemic heart failure (O-HF; B), type-2 diabetics (C) and non-diabetic controls (Ctl; B). Bar graphs in (B) display the mean pixel-by-pixel covariance in amylin and 4-HNE or MDA staining (Pearson’s correlation coefficient; PCC) in hearts (B) from patients with O-HF vs. Ctl. Scale bars, 20 µm. 10 sections per sample from n=4 individuals in each group were investigated. (D) Immunofluorescence staining of amylin (green) and IL-1β (red) in the heart specimens from O-HF patients and Ctl. Bar graphs show the number of myocytes that are positive for both amylin and IL-1β in a 134x134 µm area in heart sections from patients with O-HF vs. Ctl. Scale bars, 20 µm. 10 sections per sample from n=4 individuals in each group were investigated. Data are presented as mean ± standard error. ***P<0.001.

Figure 3. Failing hearts from patients with type-2 diabetes display amylin-4-HNE and amylin-MDA adducts and increased IL-1β synthesis. (A) Western blot analysis of enriched 4-HNE and MDA fractions immunoprecipitated from left ventricles of patients with heart failure and type-2 diabetes (D-HF) vs. non-diabetic controls (Ctl). (B) First three images show dual immunofluorescence staining of amylin (green) and 4-HNE (red) on transverse sections from left ventricle tissue of patients with heart failure and type-2 diabetes (D-HF) vs. non-diabetic controls (Ctl). Arrows indicate amylin incorporation within the sarcolemma and subsequent formation of adducts with 4-HNE. Next three images are cross sections of cardiac tissue showing the formation of amylin-MDA adducts within cardiac myocytes. Scale bars, 20 µm. (C) Western blot analysis of IL-1β in left ventricle tissue of patients with heart failure and type-2 diabetes (D-HF) vs. non-diabetic controls (Ctl). A representative blot from 2 independent experiments is shown. Data are presented as mean ± standard error.*P<0.05.
Figure 4. Aggregated amylin induces peroxidative membrane injury in the heart in a diabetic rat expressing human amylin in the pancreas (the HIP rat). (A) A LC-MS/MS method was applied to measure MDA in rat heart homogenates using derivatization of MDA with 2,4-dinitrophenylhydrazine (DNPH). Same method was applied to measure GSH-HNE in rat heart homogenates. (B) Dual-immunofluorescence staining of amylin (green) and 4-HNE (red) in heart tissue sections from HIP rats, UCD rats and WT rats (negative controls). (C) Proximity ligation assay (PLA) was performed with anti-amylin and anti-4-HNE antibodies on heart sections from WT, HIP and UCD rats. PLA signal shows an overall consistency with 4-HNE-amylin appearing in clusters. 10 sections per sample from n=4 rats in each group were investigated. Scale bars, 20 µm. (D) Co-IP/IB assay of 4-HNE (MDA) with amylin from WT, HIP and UCD rat heart homogenates. (Input) IB for amylin, 4-HNE (MDA) and GAPDH respectively, in the heart homogenates. (IP) Anti-4-HNE (anti-MDA) antibody pulled down more amylin from HIP compared to WT and UCD heart homogenates. A representative blot from 2 independent experiments is shown. Data are presented as mean ± standard error. *P<0.05, not statistically significant (n.s.).

Figure 5. Aggregated amylin increases IL-1β synthesis in the hearts of rats expressing human amylin in the pancreas. (A) Cardiac tissue from HIP rats was analyzed by immunofluorescence imaging with an IL-1β antibody. Myocytes staining for both amylin (green) and IL-1β (red) are readily observed. Scale bars, 20 µm. In contrast, UCD rats and WT rats (negative controls) lacked IL-1β immunoreactivity signal in cardiac myocytes. Scale bars, 20 µm. (B) qRT-PCR data showed elevated IL-1β mRNA levels in heart specimens from HIP vs. UCD rats and WT rats. n=7 samples/group. Data are presented as mean ± standard error. (C)
Western blot analysis of IL-1β, TNF-α, IL-6 and IL-10 in hearts from HIP, UCD and WT rats. A representative blot from 2 independent experiments is shown. *P<0.05, **P<0.01, ***P<0.001, not statistically significant (n.s.).

**Figure 6. Langendorff perfusion of mice with aggregated amylin leads to formation of amylin-4-HNE/MDA adducts and IL-1β activation.** (A) 10 µM biotinylated human amylin was recirculated in an isolated mouse heart on a Langendorff apparatus for 2 hours. Deposition of amylin was assessed using FITC-avidin and fluorescence imaging. (B-D) Isolated mouse hearts were perfused with 10 µM aggregated human amylin (n=3; Inf. Amy) or Tyrode’s solution (n=1; Ctl) for 2 hours on a Langendorff system, followed by 10 minutes washout. (B) Western blot measurement of amylin incorporated in the Langendorff perfused hearts. (C) 4-HNE and MDA were immunoprecipitated and amylin level in the immuneprecipitate was measured by Western blot. (D) Western blot analysis of IL-1β in Inf. Amy vs. Ctl hearts. (E) C57BL/6 mice were intravenously injected with 2 µg/g per body weight aggregated human amylin (n=4; Inj. Amy) or saline (n=4; Ctl). Co-IP/IB assay of 4-HNE (MDA) with amylin from amylin injected and control mice heart homogenates. (Input) IB for amylin, 4-HNE (MDA) and GAPDH respectively, in the heart homogenates. (IP) Anti-4-HNE (anti-MDA) antibody pulled down more amylin from amylin injected (Inj. Amy) compared to control (Ctl) mice heart homogenates. (F) IL-1β level in hearts from mice injected with human amylin vs. control. A representative blot from 2 independent experiments is shown. Data are presented as mean ± standard error.*P<0.05.

**Figure 7. Amylin-mediated peroxidative membrane injury exacerbates ROS production and increases IL-1β synthesis in isolated cardiac myocytes.** (A) Measurement of lipid
peroxidation with $\text{C}_{11}\text{-BODIPY}^{581/591}$ in cardiac myocytes from WT, HIP and UCD rats, as well as in myocytes incubated with aggregated amylin and/or 400 mg/dl glucose. (B) Western blot analysis of 4-HNE in myocytes incubated with aggregated amylin (Amy) or 400 mg/dl glucose. (C) Lipid peroxidation measurements with $\text{C}_{11}\text{-BODIPY}^{581/591}$ in myocytes incubated for two hours under control conditions (Ctl), with aggregated amylin (Amy), with 50 µM poloxamer 188 followed by aggregated amylin (S→Amy), and with 5 mM NAC for 30 minutes followed by aggregated amylin (NAC→Amy). (D) ROS production, measured with the fluorescent indicator CM-H$_2$DCFDA, was compared in cardiac myocytes from WT, UCD and HIP rats. (E) IL-1β level, assessed by immunofluorescence, in isolated control myocytes incubated under the four conditions described in panel C. (F) Western blot analysis of IL-1β in myocytes incubated with aggregated amylin (Amy) or 400 mg/dl glucose. 10 myocytes/rat; n=5 rats/group. A representative blot from 2 independent experiments is shown. Data are presented as mean ± standard error. *P<0.05, **P<0.01, ***P<0.001. (G) Proposed mechanism for amylin-induced lipid peroxidation and IL-1β activation.
Table 1. A summary of alterations to cardiac function/structure associated with amylin deposition in the HIP rat model.

<table>
<thead>
<tr>
<th>Cardiac Function/Structure Alteration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diastolic dysfunction</td>
<td>6</td>
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<tr>
<td>Eccentric hypertrophy</td>
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<tr>
<td>Dilation</td>
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<td>Arrhythmia</td>
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<tr>
<td>Myocyte Ca(^{2+}) dysregulation</td>
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<td>Impaired protein biosynthesis</td>
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<td>Amylin deposition</td>
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<tr>
<td>Sarcolemmal lipid peroxidation</td>
<td>present results</td>
</tr>
<tr>
<td>Inflammation</td>
<td>present results</td>
</tr>
</tbody>
</table>
Figure 1 (revised)

Mass Spectrum @ elution time 27.40 min

Tandem MS/MS of Amylin (Amylin^4+ m/z=976.22)

Pan Heart Blood Amylin

HPLC Chromatogram

Dual MS/MS of Amylin

ELISA
Figure 2 (revised)

A

Amylin (pM) in 4-HNE/MDA precipitates

B

HUMAN HEART; O-HF

C

HUMAN PANCREAS

D

HUMAN HEART; O-HF

HUMAN HEART; Ctl

AMYLIN

4-HNE

OVERLAY

Amylin (pM) (% vs. Ctl)
Figure 3 (new)

A

AMYLIN

HUMAN HEART; Ctl | HUMAN HEART; D-HF

HUMAN HEART; Ctl | HUMAN HEART; D-HF

IP: 4-HNE

IP: MDA

Amylin precipitates (% vs. Ctl)

HUMAN HEART; D-HF

B

AMYLIN

4-HNE

OVERLAY

AMYLIN

MDA

OVERLAY

HUMAN HEART; D-HF

IL-1β (% vs. Ctl)

GAPDH

37 kDa

C

IL-1β (% vs. Ctl)

kDa

Ctl D-HF

GAPDH

17 37 kDa

Ctl D-HF

Amylin in 4-HNE/MDA precipitates (% vs. Ctl)

Ctl D-HF

4-HNE MDA

***

*

Ctl D-HF

**

*
Figure 4 (revised)

A

Lipid Peroxidation (% vs. WT)

0 100 200 300

WT HIP UCD

n.s.

B

HIP RAT HEART UCD RAT HEART WT RAT HEART

Amylin

4-HNE

4-HNE

4-HNE

OVERLAY

C

HIP RAT HEART UCD RAT HEART WT RAT HEART

Amylin

4-HNE

4-HNE

4-HNE

OVERLAY

D

Amylin

4-HNE

GAPDH

Input

IP: 4-HNE

IP: MDA

WT HIP UCD

WT HIP UCD

WT HIP UCD

WT HIP UCD

WT HIP UCD

WT HIP UCD

WT HIP UCD

WT HIP UCD

WT HIP UCD

WT HIP UCD

WT HIP UCD

WT HIP UCD

WT HIP UCD
Figure 5 (revised)

A HIP RAT HEART UCD RAT HEART WT RAT HEART

AMYLIN AMYLIN AMYLIN

IL-1β IL-1β IL-1β

OVERLAY OVERLAY OVERLAY

B

IL-1β mRNA(%) vs. WT

WT UCD HP

*** **

ns.

C

kDa 28 37 24 37 19 37 17 37

WT HIP WT HIP WT HIP

GAPDH GAPDH GAPDH GAPDH

TNFα IL-6 IL-10 IL-1β

% vs. WT

WT HIP UCD

* ***
Figure 6 (revised)

A

Amylin

Control

20 µm

B

Inf. Amy  Ctl

Amylin

GAPDH

C

Inf. Amy  Ctl

IP: MDA

IP: 4-HNE

IB: Amylin

D

kDa

Inf. Amy  Ctl

IL-1β

GAPDH

E

Input  IP: 4-HNE

Amylin

4-HNE

GAPDH

F

Inf. Amy  Ctl

IL-1β

GAPDH
Figure 7 (revised)

A. Lipid peroxidation (% vs. Ctl)

B. Lipid peroxidation (% vs. Ctl)

C. Lipid peroxidation (% vs. Ctl)

D. ROS (% vs. WT)

E. IL-1β (% vs. Ctl)

F. IL-1β (% vs. Ctl)

G. Aggregated Amylin

4-HNE Adducts

Membrane Damage

ROS

Aggregated Amylin

S

NAC

Membrane Damage

ROS

4-HNE

Adducts

↑ IL-1β
Supplementary Data

**Supplementary Figure S1:** The blood level of aggregated amylin is elevated in patients with obesity and heart failure.

The size distribution of aggregated amylin in blood samples from obese patients with heart failure (O-HF) vs. lean non-failing individuals (Ctl) was assessed by Western blot. Compared to controls, blood specimens from O-HF show a wider distribution of amylin aggregates and overall increased amylin immunoreactivity. A representative blot from 2 independent experiments is shown.

**Supplementary Figure S2:** Anatomical localization of amylin with respect to the sarcolemma.

(A) Western blot analysis of amylin in HIP rat myocytes cytosol (Cyt) fraction and sarcolemma (SL) fraction (top panel). HIP rat pancreas (HIP pan) was used as a positive control. There is
more amylin in the sarcolemma fraction. A representative blot from 2 independent experiments is shown. To separate the SL, myocyte lysates were centrifuged at 1,000 g for 5 min to generate a postnuclear supernatant. The supernatant was further centrifuged at 27,000 g for 35 min and plasma membrane pellet was re-suspended in Laemmli buffer. The protocol results in efficient separation of sarcolemmal (SL) and soluble fractions (Cyt) (bottom panel), i.e. the sarcolemmal protein Caveolin3 is found almost exclusively in the SL fraction while the cytosolic protein GAPDH is highly enriched in the soluble fraction.

(B) Dual immunofluorescence staining of amylin (red) and insulin receptor (green) in isolated HIP rat cardiac myocytes. Consistent with Western blot analysis showing more amylin in SL fraction (see, A), amylin deposits are identified at the sarcolemma in HIP rat cardiac myocytes (Scale bar, 20 µm). The primary antibodies were IR-β antibody (anti-mouse, SC-25103, Santa Cruz biotech; TX) and anti-human amylin antibody (anti-rabbit, T4149, Bachem-Peninsula). The secondary antibodies were Alexa Fluor 488 conjugated anti-mouse IgG (A11029; Invitrogen; NY) and Texas red conjugated anti-rabbit IgG (SC-2780; Santa Cruz biotech; TX).