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Hyperamylinemia contributes to cardiac dysfunction in obesity and diabetes - a study in humans and rats:

Despa; Cardiotoxicity of Hyperamylinemia

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Abstract

Rationale—Hyperamylinemia is common in patients with obesity and insulin resistance, coincides with hyperinsulinemia, and results in amyloid deposition. Amylin amyloids are generally considered a pancreatic disorder in type-2 diabetes. However, elevated circulating levels of amylin may also lead to amylin accumulation and proteotoxicity in peripheral organs, including the heart.

Objective—To test whether amylin accumulates in the heart of obese and type-2 diabetic patients and to uncover the effects of amylin accumulation on cardiac morphology and function.

Methods and Results—We compared amylin deposition in failing and non-failing hearts from lean, obese, and type-2 diabetic humans using immunohistochemistry and western blots. We found significant accumulation of large amylin oligomers, fibrils and plaques in failing hearts from obese and diabetic patients, but not in normal hearts and failing hearts from lean, non-diabetic humans. Small amylin oligomers were even elevated in non-failing hearts from overweight/obese patients suggesting an early state of accumulation. Using a rat model of hyperamylinemia transgenic for human amylin, we observed that amylin oligomers attach to the sarcolemma, leading to myocyte Ca²⁺ dysregulation, pathological myocyte remodeling, and diastolic dysfunction, starting from pre-diabetes. In contrast, pre-diabetic rats expressing the same level of wild-type rat amylin, a non-amyloidogenic isoform, exhibited normal heart structure and function.

Conclusions—Hyperamylinemia promotes amylin deposition in the heart causing alterations of cardiac myocyte structure and function. We propose that detection and disruption of cardiac amylin buildup may be both a predictor of heart dysfunction and a novel therapeutic strategy in diabetic cardiomyopathy.

Keywords

hyperinsulinemia/hyperamylinemia; diabetic cardiomyopathy; calcium; HIP rat; UCD-T2DM rat

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One third of adults and 17% of children in the US* are currently obese and at high risk of developing both type-2 diabetes and cardiovascular disease.¹⁻³ Progression to overt type-2 diabetes may accelerate pathological changes in heart structure and function,⁴⁻⁷ independent of confounding factors such as coronary artery disease and hypertension.¹⁻³ It is assumed¹ that increases in body fat can affect the body's response to insulin, potentially leading to insulin resistance and subsequent impaired glucose and lipid homeostasis. As such, insulin resistance is unequivocally associated with heart disease.¹⁻¹⁰ However, the myocardial insulin responsiveness in diabetic patients is surprisingly intact,^{11,12} suggesting that factors secondary to insulin resistance may critically contribute to cardiac dysfunction in type-2 diabetes.^{5,9,10} In addition to hyperglycemia and dyslipidemia, patients with obesity and insulin resistance present also hyperinsulinemia and hyperamylinemia.¹³⁻¹⁵ While the hyperinsulinemic response prevents a large fraction of insulin resistant patients from developing type-2 diabetes¹, the coincident hyperamylinemia leads to proteotoxicity and amyloid deposition.^{13,14} More than 95% of patients with type-2 diabetes stain positive for amylin amyloids in pancreatic islets.¹⁴ Amylin deposition was also found in kidneys of obese and type-2 diabetic patients.¹⁶ Recently,^{17,18} we hypothesized that hyperamylinemia may favor cardiac amylin accumulation¹⁷ causing alterations of myocyte structure and function in ways that may contribute to progressive heart failure.¹⁸

Amylin is a 4 kDa hormone co-expressed and co-secreted with insulin by pancreatic β -cells.^{13,14} Human amylin, also known as islet amyloid polypeptide (IAPP), has aggregation properties similar to prions and amyloidogenic proteins that are associated with neurodegenerative diseases.¹⁹ At high secretion rates, amyloidogenic proteins readily form oligomers, fibrils and amyloid plaques. It is increasingly recognized that soluble oligomers rather than fibrils and plaques are the most toxic species of amyloids.²⁰⁻²⁸ They attach to cellular membranes causing Ca^{2+} dyshomeostasis, cell dysfunction, and apoptosis.²⁰⁻²⁸ Previous data^{27,28} indicated that cardiac myocyte-restricted expression and accumulation of amyloidogenic peptides, such as polyglutamine²⁷ or presenilin²⁸, can induce cytotoxicity and heart failure in mice. Presenilin oligomers coimmunoprecipitated with sarcoplasmic reticulum Ca-ATPase (SERCA) and altered Ca^{2+} handling in cardiac myocytes.²⁸

To clarify whether amylin builds up in the heart and whether this could be associated with cardiac failure in obesity and type-2 diabetes, we assessed amylin deposition in hearts from lean, obese, and type-2 diabetic humans, with and without heart failure. Using a 'humanized' rat model of hyperamylinemia, we examined changes in cardiac structure and function in relation with cardiac amylin accumulation.

Methods

Detailed procedures, description of human tissue specimens and animal models are included in the Supplemental Material.

Human tissue specimens

Failing hearts from obese, type-2 diabetic, and non-diabetic patients were obtained at the time of orthotopic heart transplantation at the Hospital of University of Pennsylvania. Non-failing hearts from obese and lean individuals are from organ donation. Tissue specimens were obtained in accordance with the Institutional Review Board approval. Inclusion in tissue-based studies was not restricted on the basis of age, gender, race or ethnic status. Heart failure etiology, body mass index (BMI), age, gender and state of diabetes with

*National Center for Health Statistics (2009)

respect to dependence on insulin and/or oral hypoglycemic agents of all cases studied here are summarized in the Online Table I.

Experimental animals

Studies were approved by the University of California, Davis Animal Research Committee. Because rodent amylin is not amyloidogenic and rodents do not accumulate amylin amyloids,²⁹ most rodent models of type-2 diabetes are not adequate for this study. We used Sprague-Dawley (SD) rats transgenic for human amylin in the pancreatic β -cells (HIP rats).³⁰ HIP rat breeding pairs were kindly provided by Pfizer. HIP rats show hyperamylinemia, leading to amylin deposits in pancreatic islets and gradual decline in β -cell mass.³¹ They develop insulin resistance at 5 months of age and diabetes by 10 months of age.³¹ As negative controls, we used obese, insulin resistant rats expressing only wild-type, non-amyloidogenic rat amylin, which does not form amyloids (UCD-T2DM rats).³² UCD-T2DM rats were obtained by breeding obese SD rats with Zucker Diabetic Lean rats that lack the leptin receptor defect and have inherent β -cell defects.³² UCD-T2DM rats exhibit insulin resistance prior to the onset of diabetes,³² similar to HIP rats³⁰ and humans.¹ In the present study, we used age matched HIP (N=17) and UCD-T2DM (N=19) rats in the pre-diabetic state, i.e. *non-fasting* blood glucose level in the 150-200 mg/dl range.³³ Wild-type littermates (N=16) served as non-diabetic controls for HIP rats. Age-matched SD rats (N=13, Charles Rivers Laboratory) were controls for UCD-T2DM rats.

Immunohistochemistry

Western blot analysis was performed on left ventricular homogenates, myocyte lysates, and blood serum. Immunohistochemistry was done on thin sections from paraffin blocks.

Insulin Signaling

Myocardial insulin responsiveness was determined by measuring the phosphorylation level of protein kinase B (Akt) and glycogen synthase kinase 3β (GSK 3β) in hearts from rats injected (I.P.) with insulin (10 mU/g body weight) and sacrificed 10 min after injection.

Cardiac myocyte isolation and Ca^{2+} measurements

Rat ventricular myocytes were isolated by perfusion with 1 mg/ml collagenase on a Langendorff apparatus as previously reported.³⁴ Intracellular Ca^{2+} level ($[Ca^{2+}]_i$) was measured with Fura2 or Fluo4.

Activation of Ca^{2+} -dependent hypertrophic pathways

Activation of Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII)-histone deacetylase (HDAC) and calcineurin-nuclear factor of activated T cells (NFAT) hypertrophic pathways was examined by determining the nuclear vs. cytosolic localization of HDAC4 and NFATc4 in cardiac myocytes using immunofluorescence.

In vivo echocardiography and hemodynamics

M-mode echo echocardiography and hemodynamic measurements were performed as described before.³⁵

Electron microscopy

Aliquots of human/rat amylin aggregation reaction were imaged by a Philips CM 12 electron microscope, as previously described.³⁶

Statistical Analysis

Data are expressed as mean \pm SEM. Statistical discriminations were performed using two-tailed unpaired Student's t test, with $P < 0.05$ considered significant. One way analysis of variance (ANOVA) with the Dunnett post hoc test was used when comparing multiple groups.

Results

Patients with obesity and type-2 diabetes present cardiac amylin accumulation

We examined left ventricular tissue from 53 human hearts divided in five pathologically distinct groups (Online Table I). These included failing hearts from patients with type-2 diabetes (N=25) and obese patients that developed overt type-2 diabetes within one year post-transplantation (N=8). These hearts were expected to show significant amylin accumulation. To uncover the early stage of amylin buildup in the heart, a third group included non-failing hearts from overweight/obese humans (N=8). Lastly, non-failing hearts from lean, healthy patients (N=5) and failing hearts from lean patients without diabetes (N=7), which should not accumulate amylin, served as negative controls.

To assess the level and size distribution of cardiac amylin aggregates, we performed western blots with an anti-amylin antibody on left ventricular protein homogenates. We found molecular weight bands corresponding to amylin trimers (12 kDa), tetramers (16 kDa) and two additional larger molecular weight structures at ~ 32 kDa (octamers) and ~ 64 kDa (16-mers) (Fig. 1A-C). Negative controls showed that these bands are specific (Online Fig. I). Intensity signal analysis (Fig. 1D-F) indicated that amylin oligomer accumulation is markedly larger in failing hearts from patients with type-2 diabetes and overweight/obesity than in normal hearts and failing hearts from patients without diabetes (controls). Intriguingly, large amylin oligomers, i.e. >32 kDa, are abundant in *failing* hearts from diabetic and obese patients (Fig. 1A,B,F), but not in *non-failing* hearts from overweight/obese individuals (Fig. 1C,F). Smaller amylin oligomers were already elevated in non-failing hearts from overweight/obese patients (Fig. 1C-E) indicating an early stage of cardiac amylin accumulation. The results are consistent with the idea that accumulation of large amylin oligomers can induce deleterious cardiac effects. Amylin tetramers are also present in failing hearts from non-diabetic patients (Fig. 1E), which might indicate undiagnosed insulin resistance in those patients (as commonly seen in aging).

Immunohistochemistry with an anti-amylin antibody shows large amylin deposits in failing hearts from type-2 diabetic patients (Fig. 2A-D), similar to those found in the pancreas of type-2 diabetic patients (Fig. 2F). Amylin plaques (Fig. 2A,C, D) and fibrillar tangles (Fig. 2B) are scattered through the entire heart. Amylin deposits are often seen at sites with myocyte multinucleation, variation in nuclear size and infiltrating cells, which usually occur with fibrotic and infiltrative diseases. In contrast, sections from normal hearts (Fig. 2E) do not show amylin deposition and structural abnormalities. To quantify the extent of amylin deposition in large plaques and fibrils, pellets from heart protein homogenates were treated with formic acid and guanidine hydrochloride to partially break down the amylin oligomers. Dot blots showed significantly increased amylin levels (Online Fig. II) indicating that fragmenting of large amylin aggregates enhanced detection by the anti-amylin antibody. This also implies that blots exhibiting higher order oligomers (Fig. 1) likely underestimate the amount of amylin in these aggregates.

Human but not rat amylin alters cardiac myocyte structure and function *ex vivo*

Amylin oligomers elevate $[Ca^{2+}]_i$ in pancreatic β -cells leading to cellular dysfunction and apoptosis.³⁷ Similarly, β -amyloid oligomers induce neuron dysfunction and death in

Alzheimer's disease through a mechanism involving increased $[Ca^{2+}]_i$.^{20,24,25} Thus, we examined Ca^{2+} cycling in cardiac myocytes incubated with exogenous amylin oligomers. Rat cardiac myocytes were incubated for ~2 hours with 5 or 50 $\mu\text{mol/L}$ exogenous human (amyloidogenic) or rat (non-amyloidogenic) amylin. Electron microscopy showed that at 50 $\mu\text{mol/L}$ and 2 hours incubation time, human but not rat amylin forms oligomers (Online Fig. III). 50 $\mu\text{mol/L}$ human amylin significantly increased Ca^{2+} transient amplitude (Fig. 3A,C). In contrast, same concentration of non-amyloidogenic rat amylin had only a modest, not significant effect (Fig. 3B). Incubation with either human or rat amylin (50 $\mu\text{mol/L}$) did not alter Ca^{2+} transient decay and diastolic $[Ca^{2+}]_i$ (Online Fig. IV). This suggests that amylin does not directly affect SERCA function.

To determine if amylin oligomers elevate $[Ca^{2+}]_i$ by increasing sarcolemmal Ca^{2+} permeability, we measured the effect of human amylin oligomers on the passive sarcolemmal Ca^{2+} leak. We measured the initial rate of $[Ca^{2+}]_i$ decline upon reducing $[Ca^{2+}]_0$ from 1 to 0 mmol/L, with the SR, Na^+/Ca^+ exchanger and sarcolemmal Ca^{2+} -ATPase blocked (Fig. 3D). Trans-sarcolemmal Ca^{2+} leak was significantly larger in myocytes pre-incubated with 50 $\mu\text{mol/L}$ human amylin vs. control (Fig. 3E), suggesting alteration of sarcolemmal processes. Incubation of isolated myocytes with fluorescent human amylin showed that amylin attaches to the sarcolemma (Online Fig. V-B). Human amylin monomers, dimers, and trimers were present in lysates of myocytes pre-incubated with 50 $\mu\text{mol/L}$ human amylin (Online Fig. V-A), in agreement with human amylin attachment to sarcolemma. Thus, amylin oligomers attach to sarcolemma and raise cellular Ca^{2+} load in cardiac myocytes, an effect generated also in neurons²⁵ and pancreatic β -cells.^{26,37}

Cardiac amylin accumulation alters Ca^{2+} cycling in HIP rats

To test whether *in vivo* cardiac accumulation of human amylin affects Ca^{2+} cycling, we used pre-diabetic HIP rats. Age-matched, pre-diabetic UCD-T2DM rats expressing only the native, non-amyloidogenic rat amylin were used as negative control. Using pre-diabetic rats has the advantage that one can dissociate the effect of cardiac amylin accumulation from other confounding factors that affect cardiac Ca^{2+} cycling during late diabetes.^{7,38,39} Immunohistochemistry (Fig. 4A) and dot blots (Fig. 4B) with an anti-amylin antibody that recognizes both human and rat amylin (the latter with higher avidity) show that amylin significantly accumulates only in HIP rat hearts. Western blots on left ventricular homogenates and cardiac myocyte lysates from HIP rats (Fig. 4C) show amylin multimers similar to those detected in humans (Fig. 1A-C) in all groups. These data indicate that amylin oligomer accumulation in HIP rat hearts starts from pre-diabetes. The presence of amylin oligomers in cardiac myocyte lysates suggests that they attach to sarcolemma and/or enter the myocytes.

To test whether cardiac amylin accumulation affects myocardial insulin responsiveness, we compared the activation status of Akt and GSK-3 β , key components of the cardiac insulin signaling pathway, in HIP, littermate control, and UCD-T2DM rats. For this test, HIP and UCD-T2DM rats were matched for age and *non-fasting* blood glucose level (Fig. 4D). The ratio between basal levels of phosphorylated Akt and total Akt is not statistically different among the three groups (Fig. 4E,F). Whereas insulin stimulation significantly increased the phosphorylation of Akt in all rats, no statistical difference among HIP, UCD-T2DM, and control rat groups was observed (Fig. 4E). GSK-3 β displays a similar response to stimulation by insulin (Online Fig. VI).

Cardiac amylin accumulation in pre-diabetic HIP rats alters myocyte Ca^{2+} cycling (Fig. 5). At low stimulation frequencies, Ca^{2+} transient amplitude is significantly larger (4.7 ± 0.5 vs. 3.5 ± 0.3 at 0.5 Hz) in myocytes from pre-diabetic HIP rats versus control rats (Figs. 5A,D).

In contrast, cardiac myocytes from age-matched, pre-diabetic UCD-T2DM rats show no change in Ca^{2+} transient amplitude (Fig. 5G and Online Fig. VII). Thus, cardiac amylin accumulation may be the cause for the larger Ca^{2+} transient amplitude in pre-diabetic HIP rats, in agreement with our results using exogenous human amylin oligomers (Fig. 3). Different from littermate controls, the amplitude of Ca^{2+} transients in myocytes from pre-diabetic HIP rats decreases with increasing the stimulation frequency (negative staircase), so that at 2 Hz the amplitude is similar to that recorded in control rats (Fig. 5B,D). This is probably due to deficiencies in Ca^{2+} re-uptake into the SR. Indeed, Ca^{2+} transient decline, which is mostly due to SR Ca^{2+} re-uptake via the SR Ca-ATPase (SERCA), is significantly slower in pre-diabetic HIP rats vs. control ($\tau=0.71\pm 0.07$ vs. 0.55 ± 0.04 s at a stimulation rate of 0.5 Hz; Fig. 5C,E). In contrast, Ca^{2+} transient decay remains unchanged in myocytes from age-matched, pre-diabetic UCD-T2DM rats (Fig. 5H). Despite slower Ca^{2+} transient relaxation, the SR Ca^{2+} load, assessed as the amplitude of Ca^{2+} transient produced by 10 mmol/L caffeine, is similar in myocytes from control and pre-diabetic HIP rats paced at 2 Hz ($\Delta F/F_0=8.5\pm 0.4$ vs. 8.6 ± 0.5). However, the slower Ca^{2+} transient relaxation in pre-diabetic HIP rats results in elevated diastolic $[\text{Ca}^{2+}]_i$ at higher pacing rates (Fig. 5B,F). Diastolic $[\text{Ca}^{2+}]_i$ is unaltered in pre-diabetic UCD-T2DM rats (Fig. 5I). Similar to myocytes incubated with human amylin, the sarcolemmal Ca^{2+} leak was significantly larger in pre-diabetic HIP rats vs. control (41.6 ± 4.5 vs. 29.2 ± 2.4 $10^4 \cdot \Delta F_{340}/F_{380} \cdot \text{s}^{-1}$, $P<0.05$). We infer that amylin oligomers can acutely increase Ca^{2+} leak into myocytes, causing elevated diastolic $[\text{Ca}^{2+}]_i$ and Ca^{2+} transients, but that reduced SERCA function may be a longer term effect, as in heart failure.

HDAC and NFAT translocation in pre-diabetic HIP rats and myocytes incubated with human amylin

Larger Ca^{2+} transients and elevated diastolic $[\text{Ca}^{2+}]_i$ may activate Ca^{2+} -dependent hypertrophic signaling, such as CaMKII-HDAC and calcineurin-NFAT pathways.^{40,41} High $[\text{Ca}^{2+}]_i$ activates CaMKII, which phosphorylates HDAC. Normally, HDAC represses transcriptional activation. However, HDAC phosphorylation causes its export from the nucleus, which activates hypertrophic gene expression. In the calcineurin-NFAT pathway, upon activation by Ca^{2+} /calmodulin, calcineurin dephosphorylates NFAT, causing NFAT import into the nucleus, which activates hypertrophic gene transcription. Using immunofluorescence, we found lower nuclear-to-cytosolic ratio of HDAC4 in myocytes from pre-diabetic HIP vs. littermate controls (Fig. 6A,C), indicating nuclear HDAC export. In contrast, the nuclear-to-cytosolic ratio of NFATc4 is elevated in pre-diabetic HIP rats (Fig. 6B,D), suggesting the nuclear import of NFAT. Thus, both CaMKII-HDAC and calcineurin-NFAT hypertrophic pathways may be activated in pre-diabetic HIP rats.

NFATc4 was also translocated to the nucleus in control isolated rats myocytes incubated with 50 $\mu\text{mol/L}$ human amylin for 2 h (Online Fig. VIII). At this concentration, human amylin forms oligomers and fibrils and elevates Ca^{2+} transients, as discussed in above. The distribution of HDAC4 was not altered by this acute amylin exposure (Online Fig. VIII). We conclude that Ca^{2+} -dependent nuclear signaling initiated by amylin oligomers is capable of inducing hypertrophic transcriptional effects.

Cardiac amylin accumulation accelerates cardiac hypertrophy and remodeling

Elevated natriuretic peptide levels are thought to reflect cardiac dysfunction and have been used as a “biomarker” of cardiac hypertrophy.^{42,43} We found that the level of brain natriuretic peptide (BNP) is elevated (by $100\pm 30\%$) in hearts from pre-diabetic HIP rats vs. littermate controls and further increases with diabetes development (Fig. 6E). This suggests hormonal alterations specific to the onset of cardiac hypertrophy in HIP rats. In contrast, the BNP level is not altered in pre-diabetic UCD-T2DM rats and only increases after the full

development of diabetes (Online Fig. IX-A). Of note, a previous study found that external human amylin induces hypertrophy in isolated cardiac myocytes.⁴⁴ However, the heart weight/body weight ratio in pre-diabetic HIP rats (2.72 ± 0.22 g) versus control rats (2.71 ± 0.2 g) did not change, showing the lack of overt cardiac hypertrophy in this early disease state.

Activation of Ca^{2+} -dependent transcriptional pathways may also alter the transcription of key Ca^{2+} transport and regulatory proteins, which cause further alterations in Ca^{2+} cycling. Thus, we measured the protein expression of SERCA, phospholamban (the endogenous SERCA inhibitor), and $\text{Na}^+/\text{Ca}^{2+}$ exchanger, the main Ca^{2+} extrusion pathway in HIP rats (Fig. 6F). We found that SERCA expression is reduced by 20% and 30% in pre-diabetic and diabetic HIP rats, respectively (Fig. 6F). In contrast, SERCA expression was unchanged in pre-diabetic UCD-T2DM rats (Online Fig. IX-B). Protein expressions of phospholamban and $\text{Na}^+/\text{Ca}^{2+}$ exchanger are unaltered in pre-diabetic HIP rats (Fig. 6F).

Pre-diabetic HIP rats show diastolic dysfunction

To determine how amylin accumulation affects cardiac function, we performed *in vivo* echocardiography and hemodynamic measurements on pre-diabetic HIP rats (Table 1). We found significantly slower relaxation (reduced $-\text{dP}/\text{dt}_{\text{min}}$ values) in pre-diabetic HIP rats *vs.* control. This suggests that cardiac amylin oligomer accumulation may accelerate the occurrence of heart dysfunction, particularly diastolic dysfunction, a typical sign of diabetic cardiomyopathy.^{2-10,38,39} Furthermore, the left ventricular end diastolic volume is increased in pre-diabetic HIP rats, which, combined with the unchanged fractional shortening, suggests dilation of the heart (Table 1).

Discussion

We found significant accumulation of large amylin oligomers (>octamers, Figs. 1A,B,F), fibrillar tangles (Fig. 2B), and plaques (Fig. 2A,C,D) in failing hearts from patients with obesity and type-2 diabetes, but not in normal hearts and failing hearts from lean humans without diabetes (Figs. 1C,F). Small amylin aggregates are even elevated in non-failing hearts from overweight/obese patients (Fig. 1C-E), suggesting that cardiac amylin buildup starts in the early state of insulin resistance/pre-diabetes. HIP rats, which express human amylin in the pancreas, accumulate amylin oligomers in the heart (Fig. 4A-C) starting also in pre-diabetes. In pre-diabetic HIP rats, the interaction of amylin oligomers with cardiac myocytes results in larger sarcolemmal Ca^{2+} leak and Ca^{2+} transients (Figs. 5A,D) leading to activation of Ca^{2+} -mediated hypertrophic pathways (Fig. 6A-D), pathological heart remodeling (Fig. 6E,F), and diastolic dysfunction (Fig. 5 6E,F, Table 1). In contrast, UCD-T2DM rats, which are matched for age, blood glucose level (Fig. 4D), and myocardial insulin responsiveness (Fig. 4E,F), but lack amylin deposition (Fig. 4A,B), have normal cardiac structure (Fig. 4A,B) and function (Fig. 5G-I). These results suggest that cardiac dysfunction in HIP rats is most likely an amylin-mediated effect. Hence, hyperamylinemia and consequent amylin deposition, a toxic effect generally assumed to contribute to pancreatic β -cell dysfunction and development of type-2 diabetes,^{13,14,31,45,46} may also be causally implicated in cardiac dysfunction.

Pathologically important form of amylin

Conditions underlying amylin oligomerization^{13-15,45,46} and proteotoxicity^{21,22} are complex and only poorly understood. Amyloidogenicity of human amylin promotes the attachment to the sarcolemma (Online Fig. V-B) and oligomer formation, two apparently independent processes. Small oligomers develop rapidly at the sarcolemma, i.e. in only 1-2 hours (Online Fig. V), which correlates with an increase in sarcolemmal permeability to Ca^{2+} (Fig. 3D,E) and elevated Ca^{2+} transients (Fig. 3A,C), with consequent activation of Ca^{2+} -mediated

hypertrophic pathways (Online Fig. VIII). In contrast, rat amylin, a non-amyloidogenic isoform of amylin²⁹ (Online Fig. III), does not affect Ca²⁺ cycling when incubated with cardiac myocytes (Fig. 3B). These results suggest that the oligomers may be the pathologically important forms of amylin. Amylin oligomerization at the sarcolemma may act as seeds for further amyloid growth. Fibril growth at the membrane amplifies structural alteration of the membrane²² and Ca²⁺ dysregulation, aggravating the deleterious effects in the heart. This might be the case for the large amylin oligomers in the 32-64 kDa size range that are abundant in failing hearts from diabetic and obese patients (Figs. 1A,B,F) and in HIP rat hearts (Fig.4C).

There is increasing support for the toxic oligomer hypothesis in amyloid-related diseases,²⁰⁻²⁸ including cardiomyopathies caused by other amyloidogenic proteins that infiltrate the heart, i.e. transthyretin, immunoglobulin light chain, and serum amyloid.⁴⁷ Data^{48,49} suggest that the infiltration of amyloidogenic proteins in the heart may induce cardiotoxicity even before amyloid fibril formation. Moreover, intracellular accumulation of amyloid oligomers, such as those formed by polyglutamine²⁷ or presenilin²⁸, induced cytotoxicity and heart failure in mice. Presenilin oligomers coimmunoprecipitated with SERCA and altered Ca²⁺ handling.²⁸ Our data from human and HIP rat hearts suggest that amylin oligomer accumulation in the heart, which is an outside-inside cardiac event, is cardiotoxic and may represent an early pathogenic mechanism linking type-2 diabetes with cardiac dysfunction.

Cardiac amylin accumulation and altered myocyte Ca²⁺ cycling

Our data show that the primary effect of cardiac amylin oligomer accumulation is an increase in myocyte Ca²⁺ and Ca²⁺ transients (schematic in Fig. 7). This effect was observed both in cardiac myocytes exposed acutely to human amylin and in pre-diabetic HIP rats, which accumulate amylin oligomers in the heart, but not in pre-diabetic UCD-T2DM rats that express only non-amyloidogenic rat amylin and thus lack cardiac oligomeric amylin accumulation. Such an effect agrees well with previous data showing that the toxicity associated with amyloidogenic proteins is mediated by an initial increase in [Ca²⁺]_i.^{20,25,37} While the mechanisms underlying the increase of [Ca²⁺]_i are not fully elucidated, our data suggest that an augmented passive trans-sarcolemmal Ca²⁺ flux is partly responsible. Amylin oligomers may also modulate the function of Ca²⁺ channels, as proposed in the pathology of Alzheimer's disease.^{20,50} Elevated [Ca²⁺]_i is involved in transcriptional regulation and hypertrophic signaling in the heart.^{40,41} Both CaMKII-HDAC and calcineurin-NFAT hypertrophic pathways are activated in cardiac myocytes from pre-diabetic HIP rats (Fig. 6A-D). Moreover, the calcineurin-NFAT pathway can be activated even by acute exposure of isolated myocytes to human amylin oligomers (Online Fig. VIII). These data implicate the amylin oligomers as a trigger of hypertrophic and remodeling maladaptive changes in the heart (Fig. 7). Pre-diabetic HIP rats show SERCA downregulation (Fig. 6F), a common occurrence in diabetic cardiomyopathy,⁵¹ and increased level of the pro-hypertrophic hormone BNP (Fig. 6E). SERCA downregulation causes further alterations in cardiac myocyte Ca²⁺ cycling by impairing Ca²⁺ transient relaxation, which leads to negative force-frequency relationship (Fig. 5D) and elevated diastolic [Ca²⁺]_i (Fig. 5F). Slower Ca²⁺ transient relaxation and elevated diastolic [Ca²⁺]_i may further activate the CaMKII-HDAC and calcineurin-NFAT transcriptional regulation/hypertrophic pathways and thus aggravate the cardiac hypertrophy and remodeling (Fig. 7). Furthermore, impaired Ca²⁺ transient relaxation and elevated diastolic [Ca²⁺]_i cause diastolic dysfunction in pre-diabetic HIP rats (Table 1). None of these alterations were present in pre-diabetic UCD-T2DM rats. Alterations in function and/or expression of proteins involved in cardiac Ca²⁺ cycling, including SERCA, and diastolic dysfunction have been reported in other rodent models of type-2 diabetes, but only after the onset of full-

blown type-2 diabetes.^{7,38,39} Thus, our data indicate that cardiac amylin oligomer accumulation accelerates the occurrence of cardiac dysfunction and remodeling in diabetes.

Amylin oligomer-induced cardiac phenotype

At the whole heart level, HIP rats show pathological signs of an infiltrative disease⁵² (Table 1). This is characterized by diastolic dysfunction (significantly reduced $-dP/dt_{min}$) and unchanged fractional shortening, which is one type of diastolic heart failure.⁵² Diastolic heart failure often progresses to systolic failure. Hemodynamics and echocardiographic measurements in HIP rats show indeed significantly reduced maximum rate of pressure fall ($-dP/dt_{min}$), an index of diastolic dysfunction, along with unchanged fractional shortening (Table 1). The left-ventricular maximum systolic pressure is also significantly reduced in HIP rats (Table 1). Hence, pre-diabetic HIP rats display cardiac changes resembling the cardiac infiltrative disease in humans.⁵² Diastolic dysfunction is also important in the pathogenesis of diabetic cardiomyopathy.^{2-10,38,39} The molecular mechanisms underlying diastolic dysfunction in diabetic patients are poorly understood.^{2-10,38,39} Present data suggest that cardiac amylin oligomer accumulation is linked to Ca^{2+} dysregulation and pathological cardiac hypertrophy, which may accelerate the onset of diastolic dysfunction in diabetes.

The pathogenesis of diabetic cardiomyopathy is multifactorial and includes metabolic components^{2-10,38,39} which have not been studied here. Additional work to address any potential influences of the amylin oligomers on key metabolic processes in the heart is needed.

In conclusion, our data show that patients with obesity and type-2 diabetes accumulate amylin oligomers in the heart and suggest that this accelerates the development of cardiac dysfunction. We propose that detection and disruption of cardiac amylin buildup may be a predictor of myocardial dysfunction and a novel therapeutic target in diabetic cardiomyopathy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

BMI	body mass index
BNP	brain natriuretic peptide
HDAC	histone deacetylase
HF	heart failure
HIP rats	type-2 diabetic rat model transgenic for human amylin
IAPP	islet amyloid polypeptide
NF	non-failing
NFAT	nuclear factor of activated T cells
OB	obese
OW	overweight
PD	pre-diabetic
UCD-T2DM rats	type-2 diabetic rat model expressing only wild-type rat amylin

Novelty and Significance

What is known?

- Patients with obesity and insulin resistance have elevated circulating levels of amylin, an amyloidogenic hormone co-expressed and co-secreted with insulin by pancreatic β -cells.
- At increased concentrations, amylin readily form amyloids, which are cytotoxic and contribute to the development of type-2 diabetes.
- The most toxic species of amylin amyloids are the soluble oligomers, which attach to cellular membranes causing Ca^{2+} dyshomeostasis, cell dysfunction, and apoptosis.

What new information does this article contribute?

- Amylin oligomers accumulate in the heart and are associated with cardiac failure in patients with obesity and type-2 diabetes.
- Amylin oligomer buildup in the heart of rats transgenic for human amylin is linked to myocyte Ca^{2+} dysregulation, pathological cardiac hypertrophy and remodeling, and diastolic dysfunction.
- Cardiac amylin accumulation accelerates the onset of diabetic cardiomyopathy.

Summary

Obesity and insulin resistance increase the risk for both type-2 diabetes and cardiac disease, but the underlying mechanisms remain poorly understood. In addition to hyperglycemia and dyslipidemia, patients with obesity and insulin resistance present also hyperinsulinemia and hyperamylinemia. While the hyperinsulinemic response prevents a large fraction of insulin resistant patients from developing type-2 diabetes, the coincident hyperamylinemia leads to proteotoxicity and amyloid deposition in pancreatic islets. Here we show that amylin oligomers, fibrils and plaques also accumulate in failing hearts from obese and diabetic patients, but not in non-failing hearts and failing hearts from lean, non-diabetic humans. Using rats transgenic for human amylin, we show that cardiac amylin oligomer accumulation causes myocyte Ca^{2+} dysregulation, activation of Ca^{2+} -dependent pathological cardiac hypertrophy and remodeling, and diastolic dysfunction. Our data suggest that cardiac amylin accumulation accelerates the onset of diabetic cardiomyopathy. The present results show for the first time that amylin oligomers are a direct pathogenic link between pancreatic and cardiac disorders and an independent contributor to the multifactorial pathogenesis of diabetic cardiomyopathy. We propose that detection and disruption of cardiac amylin buildup may be a predictor of myocardial dysfunction and a novel therapeutic target in diabetic cardiomyopathy.

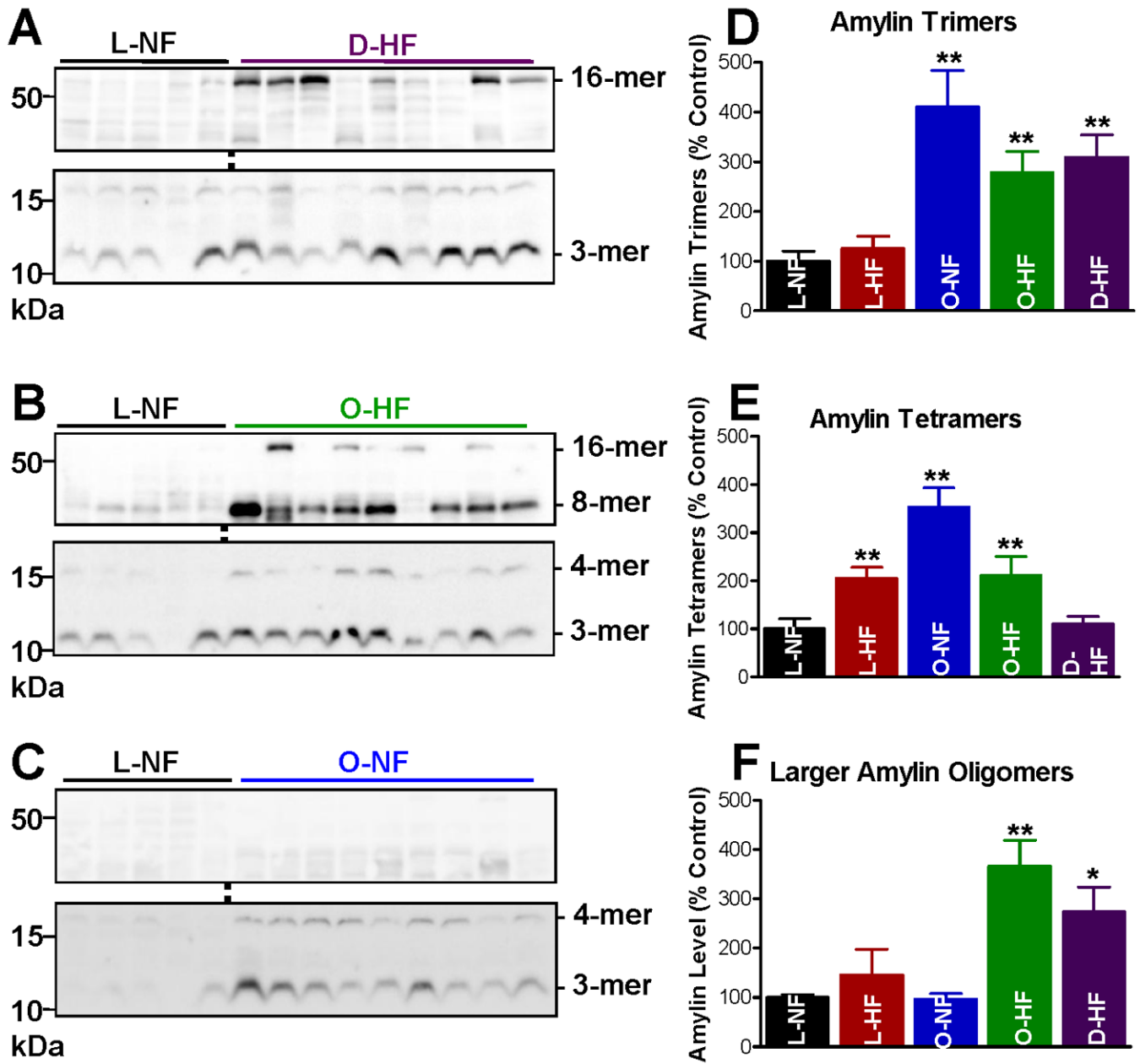


Figure 1.

(A-C) Amylin oligomer size distribution in failing (HF) and non-failing (NF) hearts from diabetic (D) and overweight/obese (O) patients vs. lean (L) controls. Representative (of ≥ 4 experiments) western blots with anti-amylin antibody of left ventricle protein homogenates from hearts in the D-HF (A), O-HF (B) and O-NF (C) groups. In each case, comparisons are made with hearts from the L-NF group. Specific molecular weight bands correspond to amylin trimers (12 kDa), tetramers (16 kDa) and two larger molecular weight structures at ~ 32 kDa (octamers) and ~ 64 kDa (16-mers). (D-F) Intensity signal analysis of the 12, 16 and the integrated 32-64 kDa bands. Heart samples in the D-HF (N=25), O-HF (N=8) and O-NF (N=8) groups contain markedly higher amylin oligomer levels than the controls L-NF

(N=5) and L-HF (N=7). Large size amylin octamers and 16-mers are abundantly present only in the failing heart O-HF and D-HF groups. *P < 0.05; **P < 0.01.

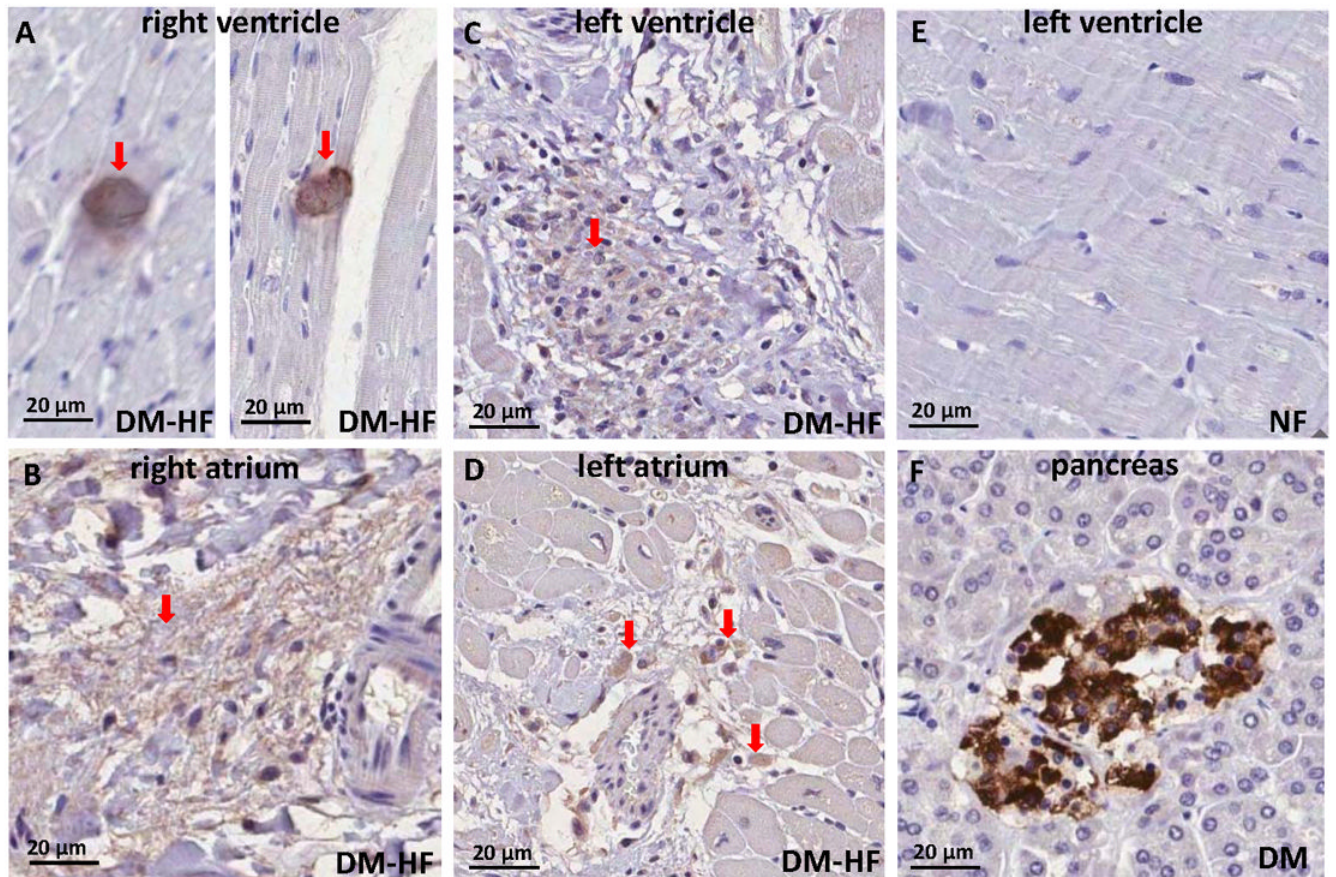


Figure 2. Amylin deposition in failing diabetic hearts demonstrated by immunohistochemistry with an anti-amylin antibody on thin heart sections. Amylin plaques (**A**, **C**, and **D**, arrows) and tangles (**B**, arrow) are scattered through the entire heart. (**E**) Left ventricle section from a non-failing heart; no amylin deposits are revealed. (**F**) Positive control for amylin deposition in a pancreas from a diabetic patient.

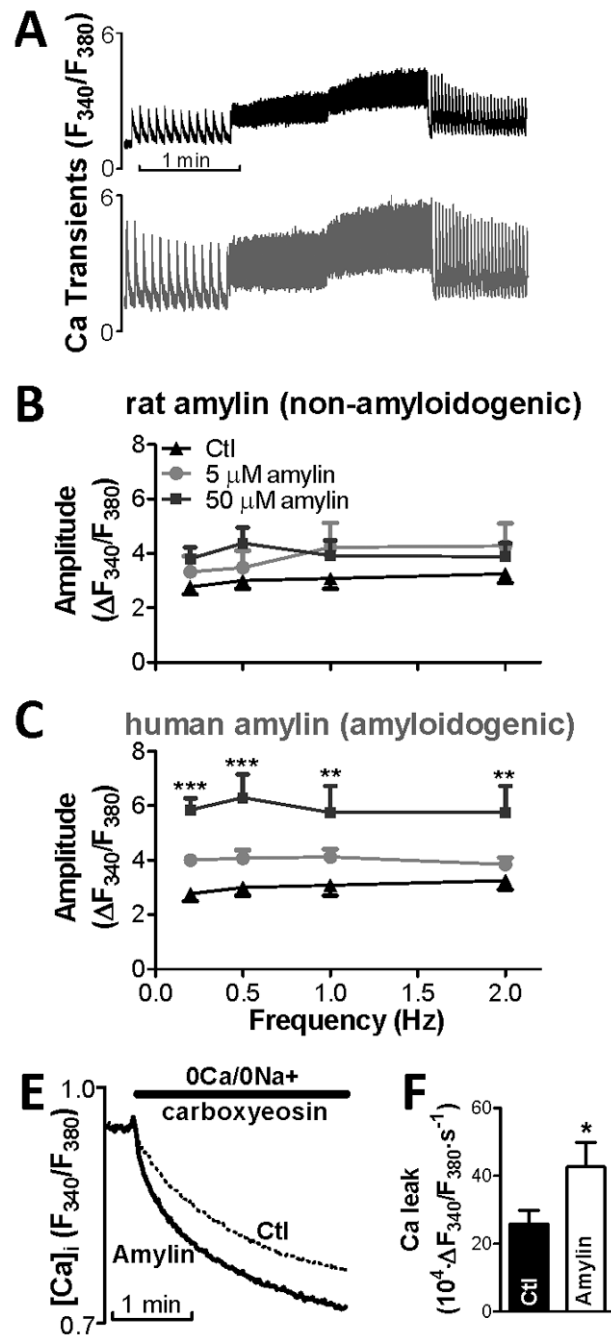


Figure 3.

Exogenous human amylin oligomers increase Ca^{2+} transient amplitude in isolated rat cardiac myocytes. **(A)** Representative Ca^{2+} transient measurements in a control (*top panel*) cell and a myocyte pre-incubated with $50 \mu\text{mol/L}$ human amylin (*bottom panel*). **(B-C)** Effect of 5 and $50 \mu\text{mol/L}$ of rat **(B)** and human **(C)** amylin on Ca^{2+} transient amplitude. Oligomerization of *human* amylin resulted in a marked Ca^{2+} transient increase. **(D)** Passive sarcolemmal Ca^{2+} leak measurements as initial slope of $[Ca^{2+}]_i$ decline upon reducing external $[Ca^{2+}]$ from 1 mmol/L to 0 , with the SR, Na/Ca exchanger and sarcolemmal Ca^{2+} -ATPase blocked by pre-treatment with thapsigargin, $0Na^+/0Ca^+$ solution and $20 \mu\text{mol/L}$ carboxyeosin, respectively. **(E)** Passive trans-sarcolemmal Ca^{2+} leak is significantly larger

in myocytes pre-treated with 50 $\mu\text{mol/L}$ human amylin vs. control. For each group, measurements were done on ≥ 6 myocytes from 3 different rats.

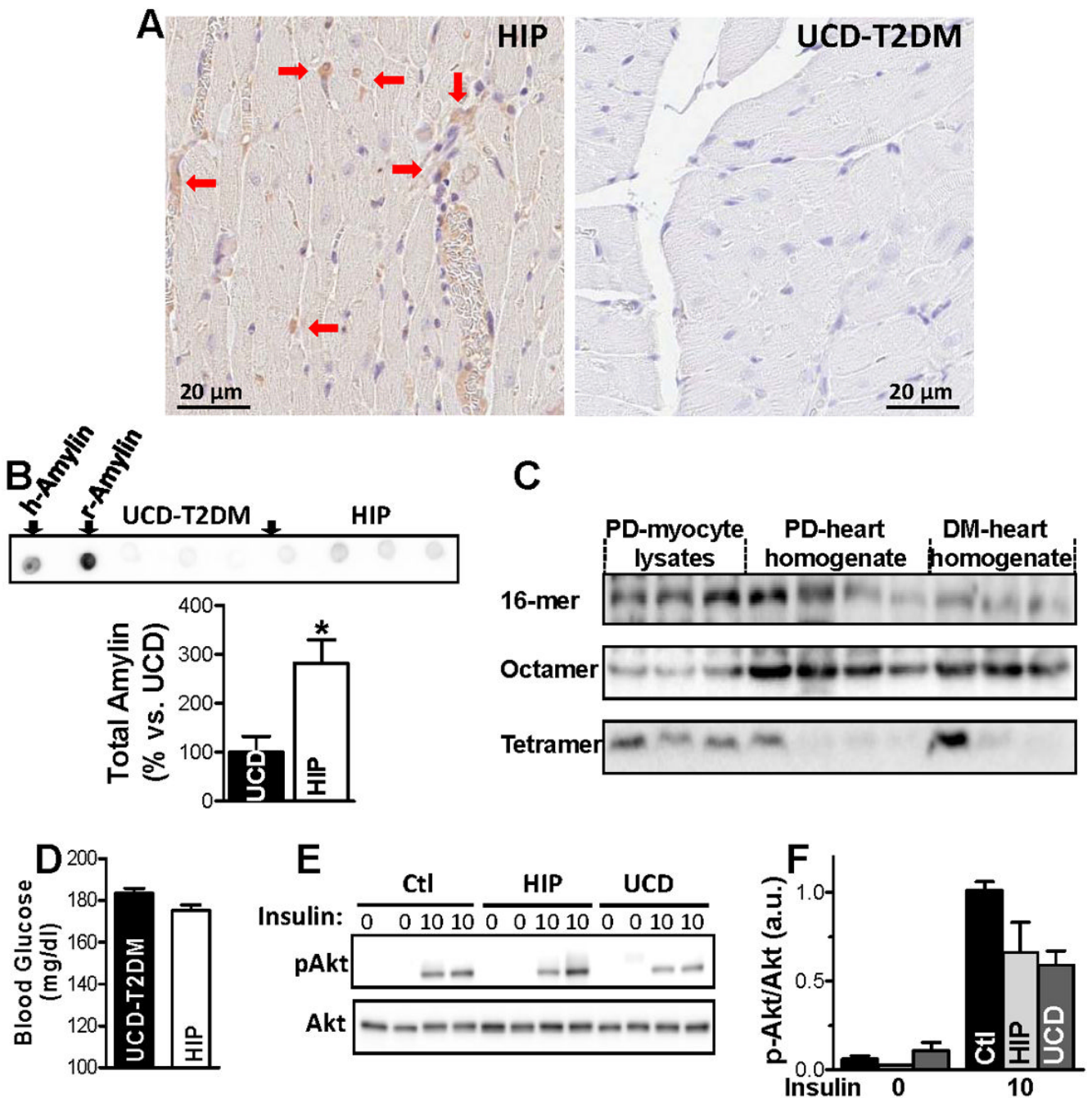
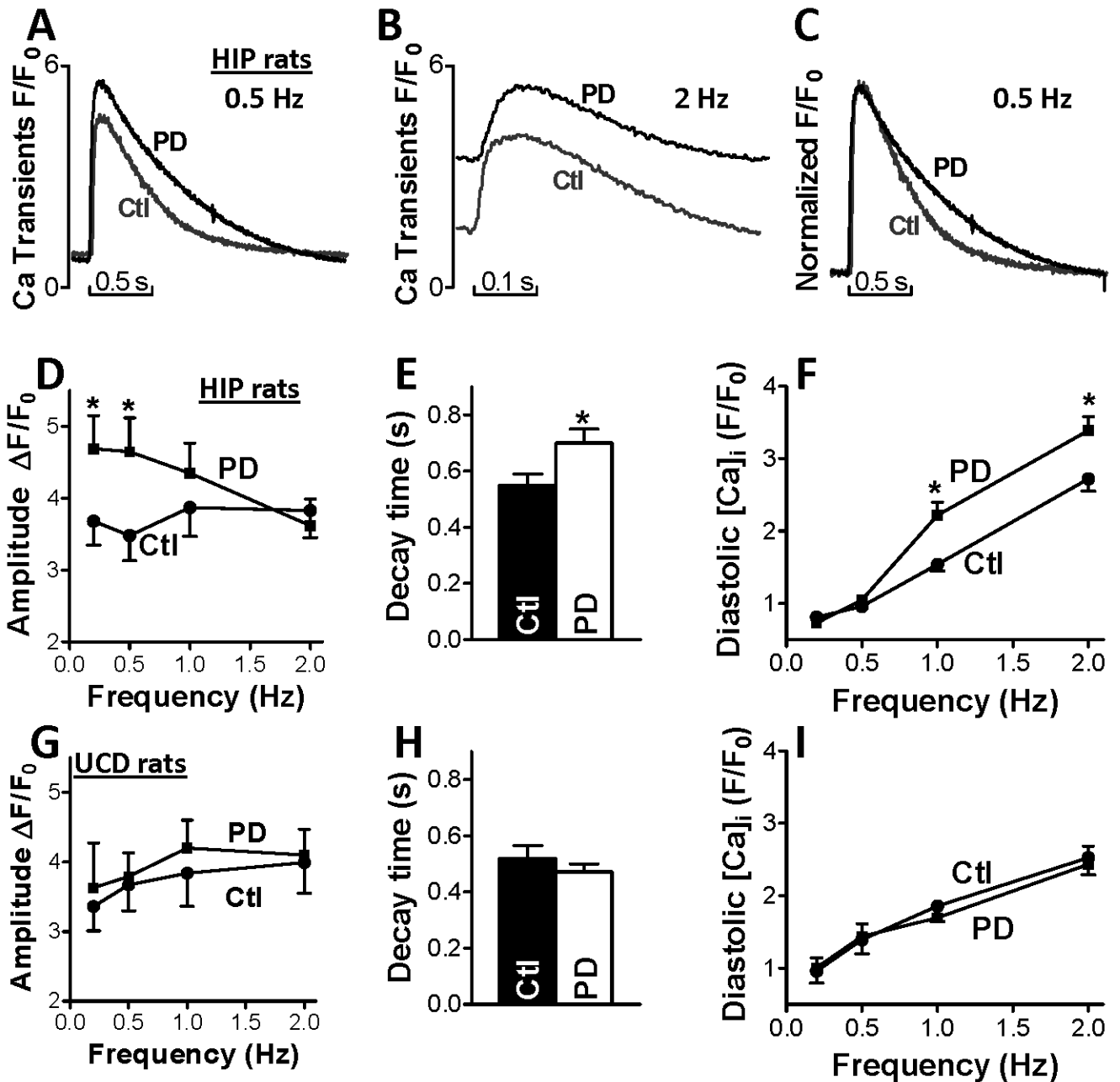


Figure 4.

(A) Immunohistochemistry with an anti-amylin antibody on thin heart sections demonstrating amylin deposition in cardiac tissues from pre-diabetic HIP but not UCD-T2DM rats (20X). (B) Dot blots with the anti-amylin antibody comparing total amylin level in HIP vs. UCD-T2DM rats. The first two dots on the left show positive controls using 5 ng of recombinant human (h-Amylin) and rat (r-Amylin) amylin. The antibody binds r-Amylin with significantly higher affinity than h-amylin; The bottom panel shows the average signal intensity in hearts from pre-diabetic HIP vs. UCD-T2DM rats. The experiment was performed in triplicate. (C) Representative western blot with anti-amylin primary antibody on ventricular myocyte lysates from pre-diabetic HIP rats, and left ventricle protein

homogenates from pre-diabetic (PD) and diabetic (DM) HIP rats. **(D)** Blood glucose levels in age-matched pre-diabetic HIP rats (N=14) and UCD-T2DM rats (N=16). **(E-F)** Akt phosphorylation in hearts from pre-diabetic HIP and UCD-T2DM rats and littermate controls under basal conditions (0 insulin) and following stimulation with insulin (10 mU/g body weight). Representative example **(E)** and mean values for the ratio between phosphorylated and total Akt **(F)**. N=3 rats for each group.

**Figure 5.**

Altered Ca²⁺ cycling in cardiac myocytes from pre-diabetic HIP but not pre-diabetic UCD-T2DM rats. Representative Ca²⁺ transients in myocytes from control (Ctl) and pre-diabetic (PD) HIP rats paced at 0.5 Hz (A) and 2 Hz (B). (C) Normalized Ca²⁺ transients in myocytes from control and pre-diabetic HIP rats (0.5 Hz) indicate slower Ca²⁺ transient relaxation in pre-diabetic HIP rats vs. control. (D) Mean amplitude of Ca²⁺ transients recorded in cardiac myocytes from control rats (20 myocytes, 4 rats) and pre-diabetic HIP rats (18 cells, 4 rats) paced at 0.2, 0.5, 1 and 2 Hz. At 0.2 and 0.5 Hz, Ca²⁺ transient amplitude is significantly larger in myocytes from pre-diabetic HIP rats vs. control. This difference disappears at higher stimulation frequencies. (E) Ca²⁺ transient decay time in cardiac myocytes from control and pre-diabetic HIP rats paced at 0.5 Hz. (F) Diastolic

$[Ca^{2+}]_i$ in cardiac myocytes from control rats and pre-diabetic HIP rats paced at 0.2, 0.5, 1 and 2 Hz. At higher frequencies, diastolic $[Ca^{2+}]_i$ is significantly higher in myocytes from pre-diabetic HIP vs. control rats. **(G)** Mean amplitude of Ca^{2+} transients in myocytes from control rats (22 myocytes, 6 rats) and pre-diabetic UCD-T2DM rats (21 cells, 4 rats) paced at 0.2, 0.5, 1 and 2 Hz. **(H)** Ca transient decay time in myocytes from control and pre-diabetic UCD-T2DM rats paced at 0.5 Hz. **(I)** Diastolic $[Ca^{2+}]_i$ in myocytes from control and pre-diabetic UCD-T2DM rats paced at 0.2, 0.5, 1 and 2 Hz. * $P < 0.05$

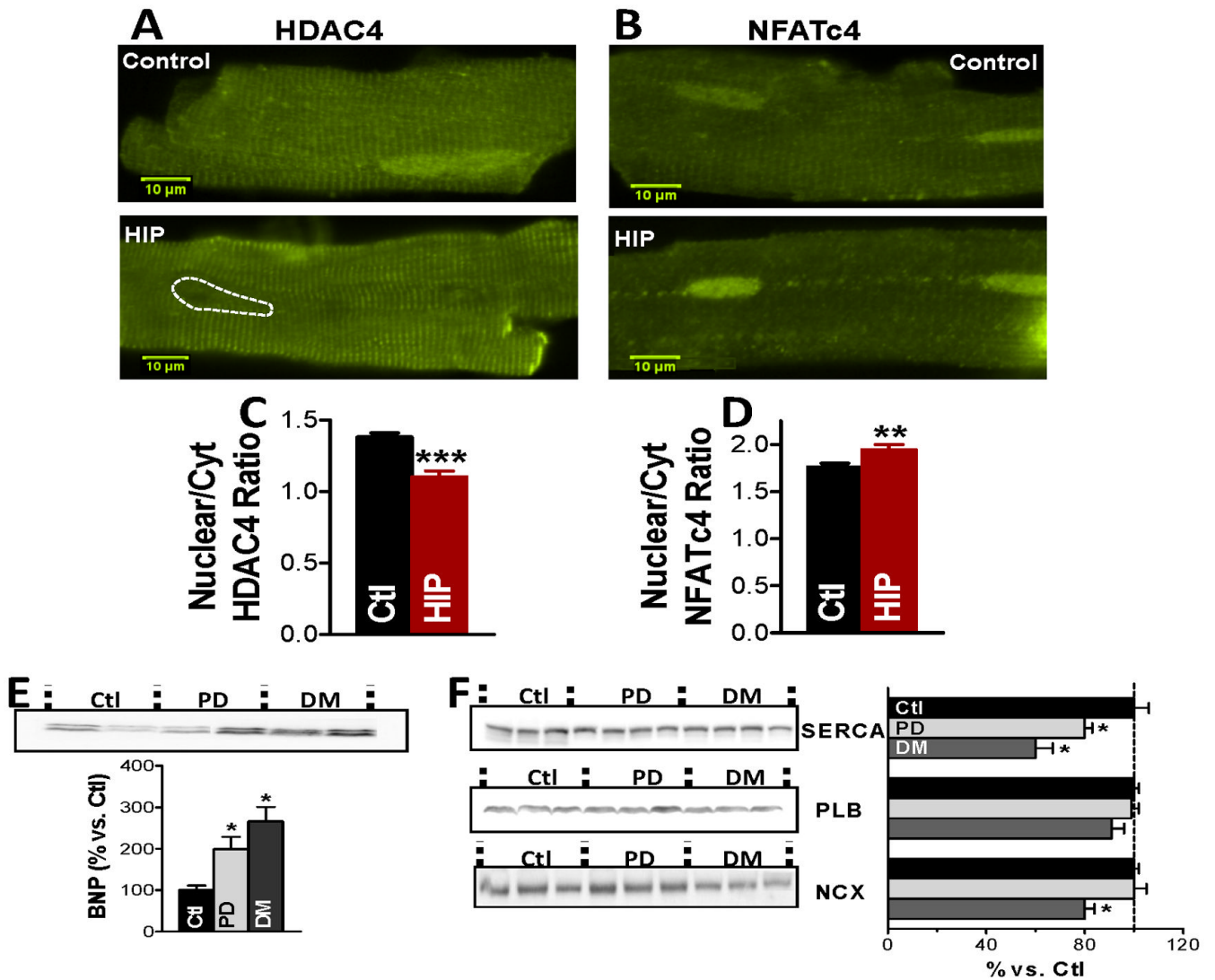


Figure 6.

HDAC4 nuclear export, NFATc4 nuclear import, reduced SERCA and increased BNP in hearts from pre-diabetic HIP rats. (A-B) Representative immunofluorescence images showing the distribution of HDAC4 and, respectively, NFATc4 in myocytes from pre-diabetic HIP rats and age-matched WT rats. (C-D) The nuclear-to-cytoplasmic ratio of HDAC4 (C) and NFATc4 (D) demonstrates the nuclear export of HDAC4 and nuclear import of NFATc4 in pre-diabetic HIP rats. Experiments were done on more than 15 cells from 3 rats for both groups. (E) Increased expression of the hypertrophic marker BNP in hearts from pre-diabetic (PD) and diabetic (DM) HIP rats. (F) Alterations in the protein expression of SERCA, phospholamban and Na/Ca exchanger in hearts from pre-diabetic and diabetic HIP rats vs. control, non-diabetic rats (Ctl). Ctl – 5 hearts; PD – 5 hearts, DM – 5 hearts.

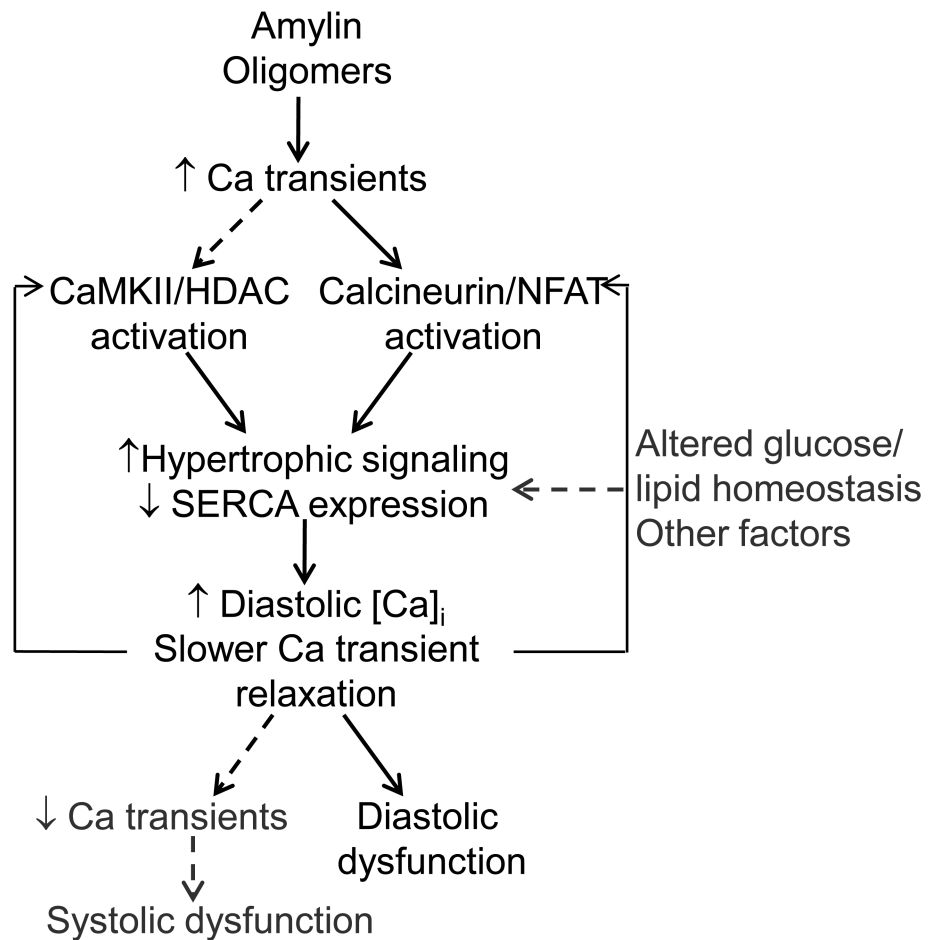


Figure 7.

Proposed mechanism for amylin oligomer-induced cardiac dysfunction. Amylin oligomers elevate Ca^{2+} transients, which results in activation of Ca^{2+} -dependent CaMKII-HDAC and calcineurin-NFAT transcriptional regulation/hypertrophic pathways. This may reduce SERCA expression, which further alters myocyte Ca^{2+} cycling by impairing Ca^{2+} transient relaxation leading to higher diastolic $[\text{Ca}^{2+}]_i$. Impaired Ca^{2+} transient relaxation and elevated diastolic $[\text{Ca}^{2+}]_i$ may further activate the Ca^{2+} -dependent transcriptional regulation/hypertrophic pathways (positive feedback) and cause diastolic dysfunction in HIP rats. With the advancement of the disease, reduced SERCA function may cause SR unloading, consequent reduction in Ca^{2+} transient amplitude, and systolic dysfunction.

Table 1
Echo and hemodynamic parameters in pre-diabetic HIP rats (n=6) vs. control (n=10).
LVEDD-Left-ventricular end diastolic diameter

	Ctl.	HIP
Heart rate (BPM)	208±14	231±17
Fractional shortening (%)	66.7±3.4	57.8±2.8
LVEDD (mm)	6.4±0.3	7.2±0.2*
dP/dt _{max} (mmHg/s)	7277±247	6597±672
-dP/dt _{min} (mmHg/s)	6237±411	4822±344*
LV _{max} systolic pressure (mmHg)	107±2	99±3*
LV end-diastolic pressure (mmHg)	6.8±0.8	5.8±1.4