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Intraneuronal Amylin Deposition Peroxidative Membrane Injury and Increased IL-1 β Synthesis in Brains of Alzheimer's Disease Patients with Type-2 Diabetes and in Diabetic HIP Rats

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Abstract

Amylin is a hormone synthesized and co-secreted with insulin by pancreatic β -cells that crosses the blood-brain barrier and regulates satiety. Amylin from humans (but not rodents) has an increased propensity to aggregate into pancreatic islet amyloid deposits that contribute to β -cell mass depletion and development of type-2 diabetes by inducing oxidative stress and inflammation. Recent studies demonstrated that aggregated amylin also accumulates in brains of Alzheimer's disease (AD) patients, preponderantly those with type-2 diabetes. Here, we report that, in addition to amylin plaques and mixed amylin-A β deposits, brains of diabetic patients with AD show amylin immunoreactive deposits inside the neurons. Neuronal amylin formed adducts with 4-hydroxynonenal (4-HNE), a marker of peroxidative membrane injury, and increased synthesis of the proinflammatory cytokine interleukin (IL)-1 β . These pathological changes were mirrored in rats expressing human amylin in pancreatic islets (HIP rats) and mice intravenously injected with aggregated human amylin, but not in hyperglycemic rats secreting wild-type non-amyloidogenic rat amylin. In cultured primary hippocampal rat neurons, aggregated amylin increased IL-1 β synthesis via membrane destabilization and subsequent generation of 4-HNE. These effects were blocked by membrane stabilizers and lipid peroxidation inhibitors. Thus, elevated circulating levels of aggregated amylin negatively affect the neurons causing peroxidative membrane injury and aberrant inflammatory responses independent of other confounding factors of diabetes. The present results are consistent with the pathological role of aggregated amylin in the pancreas, demonstrate a novel contributing mechanism to neurodegeneration, and suggest a direct, potentially treatable link of type-2 diabetes with AD.

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SUPPLEMENTARY MATERIAL

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Keywords

Alzheimer's disease; amylin; 4-hydroxynonenal; malondialdehyde; neuroinflammation; type-2 diabetes

INTRODUCTION

Amylin (also known as islet amyloid polypeptide) is a regulatory peptide synthesized and co-secreted with insulin by pancreatic β -cells [1]. The peptide amylin crosses the blood-brain barrier [2] 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 [1]. Elevated human amylin secretion promotes its deposition in the pancreas [1], which induces oxidative stress [3], activation of the NLRP3 inflammasome, and release of interleukin (IL)-1 β [4], a cytokine involved in a plethora of inflammatory responses [5]. Thus, dyshomeostasis of amylin is a critical early contributor to the development of type-2 diabetes (T2D). The pathological effects of hyperamylinemia were thought to be limited to pancreatic islets. However, recent studies [6–11] found amylin deposition in failing kidneys [6] and hearts [7, 8] from patients with obesity or T2D, and in brains [9–11] of individuals with Alzheimer's disease (AD). In the brain, amylin formed both independent deposits [9] and mixed plaques with amyloid- β (A β) [9, 11], a peptide implicated in AD pathology [12]. Amylin accumulation in pancreas, heart, kidneys, and brain was mirrored in a rat model of hyperamylinemia expressing human amylin in the pancreas (HIP rats) [7, 8, 13–16]. HIP rats develop T2D [16], cardiovascular dysfunction [7, 8, 15], neuroinflammation [13], and neurologic deficits [13]. The development of neuroinflammation and neurologic deficits in HIP rats [13] demonstrates that amylin deposition negatively affects brain function. Thus, amylin dyshomeostasis may contribute to some of the pathogenic pathways for both T2D and the co-occurring disorders in the brain. Similar inter-tissue communication was previously [17] shown to connect AD with skeletal muscle disorders in aged humans.

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 [18], which are characteristics of proteins involved in neurodegenerative diseases [19]. Accumulation of aggregated amylin in tissues disrupts membrane integrity, ion homeostasis, and cell function [1, 18–20]. The role of amyloidogenicity in amylin-induced toxicity is supported by the observation [20] that, in contrast to HIP rats, rodents overexpressing rodent amylin, which is not amyloidogenic [1], showed neither accumulation of aggregated amylin nor development of T2D.

Disrupting membrane integrity by incorporation of aggregated amylin could also increase the exposure of unsaturated fatty acids to cytosolic reactive oxygen species (ROS). This process leads to formation of reactive aldehydes [21], such as 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA). While generation of reactive aldehydes is essential for cell survival signaling [21], increased levels of 4-HNE and MDA further elevate ROS and trigger inflammatory responses [21]. 4-HNE and MDA can also non-enzymatically form stable

protein adducts by binding to histidine, lysine, and cysteine side chains [21]. 4-HNE- and MDA-modified proteins have been used as biomarkers for oxidative damage in cells [21]. Here, we assessed the hypothesis that accumulation of aggregated amylin in the brain causes peroxidative membrane injury and aberrant inflammatory responses. To decipher this new contributing mechanism to diabetes complications in the brain, 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.

MATERIALS AND METHODS

Human samples

The brain specimens (temporal lobe; Brodmann areas 21/22) included in the present study were also used in the cohorts reported in our previous publication [9] and provided by the Alzheimer's Disease Center at University of California Davis. We investigated brain tissues from late-onset dementia patients (>70 years old) who suffered from AD and T2D ($n = 4$). Brain tissues from age-matched non-diabetic individuals, without AD, served as controls ($n = 4$).

Experimental animals

Rats that express human amylin in the pancreatic β -cells on the insulin II promoter (HIP rats, $n = 25$; Charles River Laboratory) were used as T2D animals with pancreatic amylin deposition. Pancreatic deposition of amylin leads to a gradual decline of β -cell mass and development of T2D [16]. HIP rats used in this study were ~10 months of age and displayed non-fasting blood glucose levels (morning time) in the 300–400 mg/dl range. T2D rats expressing only the native rat amylin, generated following the breeding protocol described in [22] (UCD rats, $n = 12$), were used as diabetic controls. Therefore, by comparing age- and glucose-matched HIP and UCD rats, we can discriminate the deleterious effects induced by amylin dyshomeostasis from other confounding pathological factors in T2D. Age-matched wild-type (WT, $n = 30$) littermates were used as non-diabetic controls. C57BL/6 mice were intravenously injected (via tail vein) with either aggregated human amylin (2 μ g/g body weight; $n = 8$) or saline ($n = 8$) for 5 days (b.i.d.) followed by 14 days of intraperitoneal injection.

Study approval

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.

For human samples, the protocol concerning the use of biopsy from patients was approved in agreement with Institutional Review Board approval and informed consent was obtained prospectively in all cases.

Cell isolations

Neurons were isolated from hippocampi of one-day-old rats by digestion with 0.25% Trypsin-EDTA followed by repeated trituration. Neurons were then plated on poly-L-lysine-coated coverslips, cultured in N21 (AR008; R&D systems; MN) Neurobasal medium as described in [23], and used in experiments after 10 days in culture. Primary neuronal cells were detected with mouse anti-MAP2 antibody (NB300-213; Novus Biologicals; CO).

Immunofluorescence

Thin sections of paraffin-embedded tissues (brain and pancreas) were de-paraffinized, blocked in blocking solution (10% goat serum+5% BSA+0.5% triton X-100) and then incubated with primary antibodies at 4°C overnight. In the immunofluorescence measurements, a combination of anti-amylin (SC-377530; Santa Cruz biotech; TX, raised in mouse) and anti-4-HNE (ab46545; Abcam; MA, raised in rabbit), anti-MDA (ab94671; Abcam; MA, raised in rabbit), or anti-IL-1 β (ab9722; Abcam; MA, raised in rabbit) primary antibodies were used. After washing, 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). Immunofluorescence measurements were also done on fixed neurons incubated with anti-IL-1 β primary and Alexa Fluor 488 conjugated secondary antibodies. Immunofluorescence staining for amylin was verified with a second anti-amylin antibody (T-4157; Bachem-Peninsula Laboratories; San Carlos; CA, raised in rabbit; Supplementary Figure 1). Tissue autofluorescence and elastin autofluorescence were blocked with 1% Sudan black (Supplementary Figure 2).

Immunohistochemistry

Anti-human amylin (T-4157; Bachem-Peninsula Laboratories; San Carlos; CA, raised in rabbit) and anti-beta amyloid (6E10, Biolegend, CA, raised in mouse) were the primary antibodies for the immunohistochemistry experiments. Biotinylated goat anti-rabbit IgG and biotinylated goat anti-mouse IgG were used as secondary antibodies for human amylin and A β , respectively. Sections were incubated with HRP conjugated avidin-biotin complex (ABC kit; PK-6100; Vectastain laboratories, CA) as per manufacturer instructions. Section were further incubated with 3,3'-diaminobenzidine (DAB; K3468; Dako; CA) or 3-amino-9-ethylcarbazole (AEC; K3461; Dako; CA) to visualize amylin and A β , respectively.

Proximity ligation assay

For the Duolink *in situ* proximity ligation assay (PLA), sections were incubated for 90 min 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.

Western blot analysis

Western blot analysis was performed on brain tissue homogenate. 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-1 β (all three as above), β -actin (PIMA515739; Fisher; PA; loading control for brain samples). Of note, the amylin antibody (T-4157; Bachem-Peninsula Laboratories; San Carlos; CA) used here recognizes both human and rat amylin (the latter with higher avidity; [7]). The specific staining of protein bands was verified as previously described [7–9].

In some experiments, 4-HNE or MDA was immunoprecipitated using same antibodies as above and immobilized protein by A/G resin slurry (20421; Thermofisher; IL). Brain homogenates were prepared from 10 mg of brain tissue. 4-HNE or MDA was immunoprecipitated from 1 mg of total brain homogenate. ELISA was used to measure human amylin concentration in 4-HNE (MDA) enriched tissue homogenate.

Slot blot analysis

Reactivity of human amylin antibody against human amylin peptide (hAmylin; American peptide company, CA) and human CGRP peptide (hCGRP; Sigma aldrich, MO) was tested by slot blot (Supplementary Figure 3). Human amylin and hCGRP were diluted in 50 mM/L sodium carbonate buffer (pH 9.6), and applied (5 ng and 10 ng hAmylin and 10 ng hCGRP) to nitrocellulose membranes using Slot Blot blotting manifold (PR648, Hoefer Inc., MA). The membranes were boiled in microwave and blocked in nonfat dry milk for 1 h followed by incubation with anti-hAmylin antibody overnight at 4°C. Reactivity was visualized with HRP-conjugated secondary antibodies and chemiluminescence detection.

Quantitative real-time PCR was used to assess the mRNA level of IL-1 β in the brain as previously described [9].

HPLC

Human brain tissues (50 mg wet weight) were homogenized with PBS and boiled in 10 volumes of 1M acetic acid containing 20 mM HCl for 10 min, as described in [24]. The homogenates were centrifuged at 20,000 *g* for 30 min. The supernatant was dry overnight and was then reconstituted with H₂O containing 0.1% trifluoroacetic acid (TFA). Analysis of amylin was carried out using a Waters 717 plus HPLC system (Phenomenex C18 column; 5 μ m; 4.6 mm $\times$ 5 cm). A 15-min linear gradient from 5% to 75% mobile phase B (0.1% TFA in acetonitrile) was used for separation. The mobile phase A was 0.1% (v/v) TFA in water and the flow rate was set at 1 ml/min. Amylin was detected with a diode array detector at 214 nm and fractions were collected. The fractions of amylin were confirmed by comparison with an amylin standard.

LC-MS/MS

For testing the presence of amylin human brain specimens, the amylin-containing tissue lysate fractions were analyzed by LC-MS/MS using 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]. Samples

were separated by reversed phase cHiPLC (ChromXP C18 column; 75 μ m I.D. $\times$ 15 cm length; Eksigent cat# 804-00001). A 24-min linear gradient from 3% to 40% mobile phase B (0.1% (v/v) formic acid in acetonitrile) was used for separation. The mobile phase A was 0.1% (v/v) formic acid in water and the flow rate at 300 nl/min. LC-MS/MS 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 = 976.23) was extracted from the total ion spectrum. The MS/MS fragments of amylin were confirmed by comparison with an amylin standard.

For measuring MDA and 4-HNE-GSH by LC-MS/MS, tissue 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 GSH-HNE 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 (MRM) mode. MDA-DNPH and GSH-HNE were analyzed using a Machery-Nagel Nucleodur C8 Gravity column, 5 μ m, 125 mm $\times$ 2.0 mm.

Measurement of lipid peroxidation in cultured neurons

Primary cultured neurons were loaded with the fluorescent probe 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a, 4a-diaza-s-indacene-3-carboxylic acid (C₁₁-BODIPY^{581/591}; D3861; Invitrogen; OR) and imaged with a fluorescence microscope (Live5; Zeiss; Germany). Upon peroxidation, the fluorescence emission peak of C₁₁-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. A previous study [27] reported the tendency of this fluorescent dye to overestimate lipid peroxidation and to inhibit oxidative damage, but confirmed its capability to detect oxidative stress conditions at the membrane level.

Statistics

Data are presented as mean $\pm$ standard error. Statistical differences between groups were determined using the unpaired 2-tailed student's *t*-test or one-way ANOVA, as appropriate, and were considered significant when *p* < 0.05.

RESULTS

We previously [9] showed that brains of AD patients with T2D display large amylin immunoreactive deposits in both blood vessels and brain parenchyma. A large fraction of brain amylin positive plaques stained also for A β [9]. The amylin-A β deposits comprised amylin at both the core and surround of the plaque [9]. Amylin deposition was not due to production of amylin within the brain, as amylin mRNA in brains from both AD patients and healthy individuals was nearly undetectable [9]. To unambiguously validate the presence of amylin in the brain, tissue specimens from the same cohort of AD patients were now analyzed by reverse-phase high performance liquid chromatography (HPLC) and liquid chromatography tandem mass spectrometry (LC-MS/MS). Based on the retention time of

the standard amylin peptide as derived from the HPLC chromatogram (Fig. 1A), four different fractions of brain tissue lysates were collected. These brain lysate fractions were immunoblotted to verify the presence of amylin (Fig. 1B). The identity of the amylin peptide was further tested by LC-MS/MS. The results (Fig. 1B–D) convincingly demonstrate that amylin is contained in brain lysates from AD patients with T2D.

Intraneuronal 4-HNE-amylin adducts in diabetic patients with AD

To detect the anatomical localization of the brain amylin deposition, brain specimens (T2D-AD-1 to T2D-AD-4) were investigated by immunohistochemistry and immunofluorescence with an anti-amylin antibody. The amylin antibody used in this study does not recognize A β , as demonstrated in our prior work [9]. Pancreatic tissue from diabetic patients was the positive control for amylin. In addition to amylin deposition in brain blood vessels and parenchyma (Fig. 2A), diabetic patients with AD show amylin immunoreactive deposits within neurons (Fig. 2B–D). Neuron immunoreactivity for amylin may result from amylin deposition around them, as suggested by Fig. 2B and D. However, some neurons (Fig. 2C, D, arrows) appear to accumulate cytosolic amylin immunoreactive inclusions. In contrast, brain sections of non-diabetic cognitively normal individuals lack cytosolic amylin immunoreactive inclusions (Fig. 2E). Neuron amylin uptake may occur via specific amylin receptors [28], or/and by incorporation of aggregated amylin into cellular membranes, a process common to all amyloidogenic proteins [29]. Additional data showing amylin-positive neurons by using immunoconfocal microscopy are displayed in Supplementary Figure 1.

Cytosolic amylin immunoreactive inclusions are positive for both 4-HNE (Fig. 3A) and MDA (Fig. 3B). In contrast, amylin-4-HNE (MDA) adducts were undetectable in brain tissue from control, non-diabetic individuals (Fig. 3A, B). The analysis of pixel-by-pixel covariance of amylin and 4-HNE (and MDA) immunoreactivity (Pearson's correlation coefficient; PCC) showed greater co-localization of amylin and 4-HNE (and MDA) in diabetic human brains compared to controls (Fig. 3A, B; bar graphs). Pancreatic tissue from a diabetic patient also showed amylin-4-HNE co-localization (Fig. 3C). To further test the interaction of amylin with reactive aldehydes, we immunoprecipitated 4-HNE and MDA from brain homogenates and used an ELISA to measure the amylin content in these 4-HNE/MDA enriched fractions. In agreement with co-localization data, amylin-4-HNE and amylin-MDA complexes were significantly elevated in brains from AD patients with T2D compared to specimens in the control group (Fig. 3D). Thus, incorporation of aggregated amylin in neurons is associated with elevated products of peroxidative membrane injury, similar to A β pathology [30–32].

Elevated IL-1 β levels in brains of diabetic AD patients

In the brain, IL-1 β is expressed in glia and contributes to the pathophysiology of neuronal injury [33]. However, neurons also produce and respond to IL-1 β [33]. The density of neurons staining for both amylin and IL-1 β was substantially higher in brain tissues from diabetics compared to controls (Fig. 4). Interstitial deposits of IL-1 β can also be seen surrounding amylin positive neurons. Thus, incorporation of aggregated amylin in neurons

in T2D patients with AD is associated with increased IL-1 β synthesis, consistent with the pathological role of aggregated amylin in pancreatic islets [1, 3, 4].

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

Rodent amylin is neither amyloidogenic [34] nor cytotoxic [20]. Therefore, we employed T2D rats that express human amylin specifically in pancreatic β -cells (HIP rats) to study mechanistically the impact of amylin dyshomeostasis on the brain. Using mass spectrometry, we found that brains from diabetic HIP rats have higher levels of 4-HNE and MDA compared to control WT rats (Fig. 5A). To assess the role of amylin deposition in inducing lipid peroxidation in the brain, we performed similar measurements in age- and glucose-matched diabetic rats expressing only endogenous rat amylin (UCD rats). The amounts of 4-HNE and MDA in brains from diabetic UCD rats were comparable to those in the WT rats (Fig. 5A), suggesting that in the absence of amylin buildup, T2D does not cause significant lipid peroxidation. Similar to diabetic subjects with AD, brains of HIP rats contain amylin-4-HNE and amylin-MDA adducts (Fig. 5B). These pathological changes are virtually absent in UCD rats (Fig. 5C). To further investigate the 4-HNE-amylin co-localization in brains from HIP rats, we employed an *in situ* proximity ligation assay (PLA) (Fig. 5D). In the PLA test, anti-amylin and anti-4-HNE primary antibodies are detected with oligonucleotide-conjugated secondary antibodies that generate an amplifiable circular DNA only if both target proteins are in close proximity. The PLA signal shows an overall consistency with 4-HNE-amylin appearing in clusters, consistent with the formation of adducts. Furthermore, western blot analysis of brain fractions that were enriched in 4-HNE/MDA by immunoprecipitation demonstrated that amylin deposits in HIP rats underwent modifications by reactive aldehydes (Fig. 5E). 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 is likely due to the larger number of amino acids with binding affinity to 4-HNE (K, C, C, and H in human amylin versus only K, C and C in rat amylin). Thus, induced “human” hyperamylinemia in rodents triggers intracellular accumulation of amylin-based lipid peroxidation adducts in the brain, similar to diabetic AD humans (Fig. 3).

Accumulation of IL-1 β in brains of human amylin-expressing diabetic rats

We compared the synthesis of IL-1 β in the brain in HIP, WT, and UCD rats using fluorescence imaging, western blot, and quantitative reverse transcription real-time PCR (qRT-PCR).

Confocal microscopy analysis showed elevated IL-1 β immunoreactivity in HIP rat brain, particularly in amylin positive neurons (Fig. 6A), but not in brain from UCD rats (Fig. 6B). Compared to WT rats, IL-1 β synthesis was elevated in the brains of HIP rats (Fig. 6C). In contrast, brains of UCD and WT rats displayed similar IL-1 β levels (Fig. 6C). These results correlate well with a greatly increased IL-1 β transcript in HIP rat brains compared to UCD rats (Fig. 6D). Thus, the association of aggregated amylin with peroxidative membrane injury and IL-1 β activation in the brain of patients with T2D is mirrored in HIP rats, but

absent in UCD rats. Taken together, these data suggest that the intracellular uptake of amylin increases IL-1 β synthesis in neurons, in T2D.

Systemic effects following intravenous injection of aggregated amylin in mice

We previously demonstrated the absence of amylin transcript in brains from both humans [9] and HIP rats [13]. This means that amylin deposited in the brain originates from the pancreas, the only known source of amylin [1], and reaches extra-pancreatic tissues by circulating in the blood. The brain uptake of amylin appears to increase when amylin circulates in aggregated form, as we have shown recently [13] in the HIP rat model. Aggregated amylin is present also in the blood of diabetic AD patients [9]. Therefore, we investigated whether increased circulating levels of aggregated amylin can induce brain peroxidative injury and IL-1 β synthesis in mice. C57BL mice were injected intravenously with aggregated human amylin (2 μ g/g per body weight) for five days, then by intraperitoneal delivery for 2 weeks. Animals in the amylin treatment group and saline-injected controls were sacrificed to collect the brain. Brain lysate fractions were enriched in 4-HNE/MDA by immunoprecipitation and then analyzed by western blot with an amylin antibody. Mice injected with aggregated amylin showed elevated levels of amylin-4-HNE and amylin-MDA adducts (Fig. 7A) along with increased synthesis of IL-1 β (Fig. 7B). These results indicate that elevated circulating levels of aggregated amylin can promote peroxidative injury and IL-1 β synthesis in the brain, independent of hyperglycemia.

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

Aggregates formed by amylin from humans interact with and incorporate into cellular membranes [1, 18]. Based on prior studies [18, 20] and the current results, we hypothesize that accumulation of aggregated amylin disrupts membrane integrity in neurons, which exposes unsaturated lipids to basal ROS and increases production of reactive aldehydes. The resulting 4-HNE (and MDA) interacts with incorporated amylin to form intracellular adducts and can also exacerbate IL-1 β synthesis, as suggested by recent studies [35]. To test this hypothesis, we used an *in vitro* system where cultured primary hippocampal rat neurons were incubated for 2 h with 50 μ M human amylin. The lipid peroxide level was measured in single cells using the fluorescent probe C₁₁-BODIPY^{581/591}, whose emission peak shifts from 590 nm (red) to 510 nm (green) upon peroxidation (Fig. 8A). Incorporation of aggregated amylin in cell membranes was then prevented by pre-treating isolated control neurons with 50 μ M poloxamer 188, a surfactant that stabilizes the lipid bilayer through hydrophobic interactions [36, 37]. Surfactant molecules (S) blocked amylin-induced lipid peroxidation (Fig. 8A; magenta bar). In a separate experiment, control neurons were pre-incubated with N-acetyl cysteine (NAC; 5 mM for 30 min), a H₂O₂ and HO* scavenger, to quench basal ROS production. Pre-treatment with NAC prevented the ignition of the lipid peroxidation chain reaction which is induced by the incubation with human amylin (Fig. 8A; green bar). Thus, impeding the incorporation of aggregated amylin in cellular membranes (e.g., by membrane stabilizer) or quenching the basal ROS (by pre-treatment with NAC) blocked lipid per-oxidation in cultured neurons.

ROS are considered the proximal signals for activation of the IL-1 β -processing inflammasome in pancreatic islets [4] and brain [33]. Intriguingly, recent experimental evidence [35] points to 4-HNE as another potential activator of signaling cascades underlying IL-1 β synthesis and activation. We therefore hypothesized that amylin-mediated peroxidative membrane injury can increase IL-1 β synthesis. Indeed, incubation of cultured neurons (Fig. 8B) with aggregated amylin (50 μ M for 2 h) resulted in robust IL-1 β expression. Preventing amylin-induced lipid peroxidation by impeding amylin incorporation with a membrane stabilizer (S; magentabars) or by quenching the basal ROS with NAC (green bars) blocked IL-1 β synthesis in neurons (Fig. 8B). These *in vitro* data support the hypothesis that aggregated amylin induces peroxidative membrane injury leading to an increase synthesis of IL-1 β in the brain.

DISCUSSION

The link of diabetes with co-occurring disorders in the brain involves complex and multifactorial pathways [38–39]. In diabetic AD patients, we found amylin deposition in the brain (Figs. 1 and 2), which correlated with elevated intracellular levels of amylin-4-HNE/MDA adducts (Fig. 3A, B) and IL-1 β (Fig. 4). Such cellular and molecular changes were absent in age-matched healthy individuals (Figs. 3 and 4). Accumulation of amylin-4-HNE/MDA adducts and increased synthesis of IL-1 β in brain were mirrored in T2D rats that express human amylin in pancreatic β -cells (Figs. 5 and 6) and non-diabetic mice intravenously injected with aggregated amylin (Fig. 7). In contrast, age- and glucose-matched diabetic rats expressing non-amyloidogenic rat amylin lacked inflammation and lipid peroxidation in the brain (Figs. 5 and 6). These results demonstrate that accumulation of aggregated pancreatic amylin in the brain results from diabetes and is a pathological substrate that predisposes to intracellular oxidative stress and aberrant inflammatory responses. So, lowering the circulating level of aggregated amylin should promote brain health.

Our study indicates that mechanisms underlying deleterious effects of aggregated amylin in the brain likely involve amylin-mediated peroxidative membrane injury, in line with previous results demonstrating that aggregated amylin injures cellular membranes in pancreatic β -cells [1–18]. Acute exposure to aggregated amylin also increased lipid peroxidation in cultured neurons (Fig. 8A). Thus, amylin dyshomeostasis induces systemic peroxidative injury independent of hyperglycemia. The link of amylin dyshomeostasis with increased lipid peroxidation may be of further relevance via its role in enzyme activities, cell signaling and gene expression [31].

Furthermore, blocking either amylin incorporation or the lipid peroxidation chain reaction demonstrated that peroxidative membrane injury is upstream of IL-1 β increased synthesis (Fig. 8). The same conclusion emerged from human amylin injected mice, which demonstrated a direct and rapid effect of circulating aggregated amylin to increase IL-1 β in the brain. Therefore, peroxidative membrane injury and an aberrant inflammatory response are intrinsic progressive effects induced by the interaction of aggregated amylin with neurons, consistent with the amylin pathology in pancreatic islets [4].

Antioxidants and membrane stabilizers can inhibit the incorporation of aggregated amylin into cellular membranes and the lipid peroxidation chain reaction (Fig. 8). 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. Thus, besides determining responses to aggregated amylin in neurons, our results demonstrate that preventing/-reducing amylin dyshomeostasis is protective against aberrantly induced IL-1 β expression. Amylin dyshomeostasis may prove a valid therapeutic target to reduce systemic inflammation in diabetic patients.

Amylin pathophysiology is notoriously species-specific [1]. Amylin from rodents does not form amyloid due to proline substitutions at positions 25, 28, and 29 [34]. Therefore, all previous studies using rodent models of neurologic disorders [40, 41] were hampered by the absence of amylin deposition in the brain. Here, we exploited the innate properties of human amylin and rodent amylin species to assess mechanistically the impact of a “human” amylin dyshomeostasis on the brain in a rat model transgenic for human amylin (the HIP rat). Not surprisingly, the amylin pathology in the brain in diabetic AD patients is similar to diabetic HIP rats. Using the HIP rat model increases the translational potential of the results.

In conclusion, we showed that the dyshomeostasis of the pancreatic hormone amylin is a link of type-2 diabetes with co-occurring disorders in the brain. Aggregated amylin incorporates in neurons causing intracellular oxidative stress and aberrant inflammatory responses, consistent with the pathological role of amylin in the pancreatic islets in individuals with T2D. Future studies need to elucidate the mechanism(s) of amylin uptake intracellularly in neurons and therapies to reverse or prevent the process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Testing the amylin deposition in human brain specimens by HPLC-MS/MS. A) Typical reverse phase HPLC chromatogram of human amylin in human amylin standard and human brain lysates. B) Fractions of human brain lysates collected by HPLC were immunoblotted with a rabbit anti-amylin antibody. 10 ng of recombinant human amylin (H-Amy) was loaded as positive control. A representative blot from 3 independent experiments is shown. C) Fractions collected from HPLC (the amylin positive fractions) were analyzed by LC-MS/MS. The representative mass spectrum of peptides eluted at 27.54 min shows the 4+ ion of amylin (m/z = 976.23). D) The tandem MS/MS spectrum of amylin. The fragments corresponding to the b ions (amylin ions) are labeled proving that this peptide is amylin. E) Tandem MS/MS spectra of amylin in three additional brain samples from T2D-AD group. The amylin peak m/z = 976.23200 ± 10 ppm is extracted and intensity is shown in each spectrum.

Fig. 2.

Amylin deposition in brain specimens from the T2D-AD patient group demonstrated by immunohistochemistry with an anti-amylin antibody on thin tissue sections. A) Representative image of amylin deposition in brain blood vessels and parenchyma. B–D) Neurons showing immunoreactivity for amylin. Neuron amylin immunoreactivity may result from amylin deposition around neurons, as suggested by images in B and D (arrows). Some neurons (D, arrows) appear to accumulate cytosolic amylin immunoreactive inclusions. Red arrows in C point to A β deposits (red) which form mixed plaques with amylin (brown), as demonstrated by prior data [9–11]. Brain sections of non-diabetic cognitively normal individuals lack cytosolic amylin immunoreactive inclusions (E). Scale bars, 200 μ m in A and 20 μ m in B–E.

Fig. 3.

Incorporation of aggregated amylin in brains of humans is associated with peroxidative membrane injury. A–C) Dual immunofluorescence staining of amylin (green) and 4-HNE (MDA) (red) in the brain (A and B) and pancreas (C) of patients with T2D and non-diabetic controls (Ctl). Nuclei are shown in blue in the overlay images. Bar graphs display the mean pixel-by-pixel covariance in amylin and 4-HNE(MDA) images (Pearson's correlation coefficient; PCC) in brains from patients with T2D versus Ctl. Scale bars, 20 μ m. 10 sections per sample from $n = 4$ individuals in each group were investigated. In (D), 4-HNE and MDA were immunoprecipitated from brain homogenates. The level of amylin in the fractions enriched in 4-HNE and MDA was then quantified by ELISA. Data are presented as mean $\pm$ standard error. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 4.

Incorporation of aggregated amylin in the brains of humans is associated IL-1 β accumulation. Immunofluorescence staining of amylin (green) and IL-1 β (red) in the brain specimens from diabetic humans. The bar graph shows the number of neurons that are positive for both amylin and IL-1 β in a $134 \times 134 \mu\text{m}$ area in brain sections from patients in the diabetic group versus controls. Scale bars, $20 \mu\text{m}$. 10 sections per sample from $n = 4$ individuals in each group were investigated. Data are presented as mean $\pm$ standard error. *** $p < 0.001$.

Fig. 5.

Aggregated amylin induces peroxidative membrane injury in brain in diabetic rats expressing human amylin in the pancreas. A) A LC-MS/MS method was applied to measure MDA in brain homogenates using derivatization of MDA with 2,4-dinitrophenylhydrazine (DNPH). Same method was applied to measure GSH-HNE in rat brain homogenates. B) Dual-immunofluorescence staining of amylin (green) and 4-HNE (or MDA; red) in brain tissue sections from HIP rats. Bar graphs display the mean pixel-by-pixel covariance in amylin and 4-HNE(MDA) images (PCC) in brains from HIP versus WT rats. C) Same as in above for UCD rats (negative controls). D) Proximity ligation assay (PLA) was performed with anti-amylin and anti-4-HNE antibodies on brain sections. PLA signal shows an overall consistency with 4-HNE-amylin appearing in clusters. Nuclei are shown in blue in the overlay image. 10 sections per sample from $n = 4$ rats in each group were investigated. Scale bars, 20 μ m. E) 4-HNE or MDA was immunoprecipitated from brain homogenates obtained from HIP and WT rats and the level of amylin in fractions enriched in 4-HNE/MDA was quantified by western blot with an amylin antibody. A representative blot from 2 independent experiments is shown. Data are presented as mean $\pm$ standard error. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 6.

Aggregated amylin increases IL-1 β levels in the brains of rats expressing human amylin in the pancreas. A) Brain tissues from HIP rats were analyzed by immunofluorescence imaging with amylin and IL-1 β antibodies. Neurons staining for both amylin (green) and IL-1 β (red) are readily observed. The bar graph shows the number of neurons that are positive for both amylin and IL-1 β in a $134 \times 134 \mu\text{m}$ area in brain sections from HIP versus WT rats. B) Same as in above for UCD rats (negative controls). Scale bars, $20 \mu\text{m}$. C) Relative IL-1 β protein level in whole brain homogenates from age-matched WT, UCD and HIP rats assessed by western blot. D) Relative IL-1 β mRNA level in whole brain homogenates from HIP versus UCD rats measured by qRT-PCR. $n = 7$ samples/group. A representative blot from 2 independent experiments is shown. Data are presented as mean $\pm$ standard error. ** $p < 0.01$, *** $p < 0.001$.

Fig. 7.

Intravenous injections of mice with aggregated amylin leads to formation of amylin-4-HNE/MDA adducts and IL-1 β accumulation in the brain. C57BL/6 mice were intravenously injected with 2 μ g/g body weight aggregated human amylin ($n = 4$; Inj) or saline ($n = 4$; Ctl). A) The level of amylin in fractions from brains that were enriched in 4-HNE/MDA by immunoprecipitation was quantified by western blot. B) IL-1 β levels in brain lysates from mice injected with human amylin versus control. A representative blot from 2 independent experiments is shown. Data are presented as mean $\pm$ standard error. * $p < 0.05$, ** $p < 0.01$.

Fig. 8.

Amylin-mediated peroxidative membrane injury increases IL-1 β synthesis in cultured neurons. A) Lipid peroxidation measurements with C₁₁-BODIPY^{581/591} in cultured primary hippocampal rat neurons incubated for two hours under control conditions (Ctl), with aggregated amylin (Amy), with 50 μ M poloxamer 188 followed by aggregated amylin (S $\rightarrow$ Amy), and with 5 mM NAC for 30 min followed by aggregated amylin (NAC $\rightarrow$ Amy). B) IL-1 β level, assessed by immunofluorescence, in cultured primary hippocampal rat neurons incubated under the four conditions described in panel A. Neuronal cells were identified with mouse anti-MAP2 antibody. 10 neurons/rat; $n=5$ rats/group. Data are presented as mean $\pm$ standard error. ** $p < 0.01$, *** $p < 0.001$.