A BRIEF COMMUNICATION

Longitudinal Ultrastructure Study of Islet Amyloid in the HIP Rat Model of Type 2 Diabetes Mellitus

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In 2004, the human islet amyloid polypeptide (HIP) rat model was created by transfecting the Sprague-Dawley rat with the human islet amyloid polypeptide (hIAPP)-amylin gene. The objective of this study is to utilize the transmission electron microscope to study the longitudinal cellular and extracellular morphological changes within the islets of this model at 4, 8, and 14 months of age. It has been previously demonstrated that the 2-, 5-, and 10-month HIP models have no diabetes, impaired fasting glucose, and diabetes, respectively. The 4-month HIP model (FBS 123 mg/dl) demonstrated an abundance of β-cells and insulin secretory granules with significant pericapillary and inter-β-cell islet amyloid deposition. The 8-month model (FBS 187 mg/dl) demonstrated extensive islet amyloid deposition and marked changes of β-cell apoptosis. The 14-month-old model (FBS 244 mg/dl) demonstrated islet and β-cell atrophy with even greater amounts of extracellular islet amyloid compared to the 4-month-old and 8-month-old models. Functional beta cells were sparse and were associated with intra islet adipose deposition. These findings of ultrastructure cellular and extracellular morphological longitudinal remodeling changes in this novel animal model of type 2 diabetes may provide investigators with a better understanding regarding the role of islet amyloid in human islet transplantation and type 2 diabetes mellitus. Exp Biol Med 232:772–779, 2007

Key words: apoptosis; B cell; islet structure; islet cell pathology; reactive oxygen species

Introduction

The pandemic of obesity, metabolic syndrome, insulin resistance, type 2 diabetes mellitus (T2DM), and their resultant multiple end-organ complications merit a better understanding of underlying pathophysiologic mechanisms, which contributes to deteriorating pancreatic β-cell function. It is also critical to better understand the longitudinal morphological changes of islet amyloid deposition and associated ultrastructural subcellular and extracellular defects within the endocrine pancreas, which will lead to a better understanding of the pathophysiology of these conditions. The second metabolically active β-cell derived hormone amylin–islet amyloid polypeptide (IAPP) and the amylin derived islet amyloid story have emerged over the past two decades. Their importance in the metabolic syndrome and T2DM have grown exponentially during the past decade due largely to the availability of animal models utilized to study this exciting area of research (1–9).

Human, feline, and nonhuman primates are the only species that develop T2DM spontaneously (8–10). These three species are also known to have the only natural occurring β-cell derived, amyloidogenic amylin–IAPP, which is the precursor for the mutant-insoluble protein conformation responsible for the development of the major endocrine pancreatic morphological abnormality found at human autopsy: islet amyloid (11, 12).

Rodent species do not possess this amyloidogenic

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protein due to proline substitutions at positions 25, 28, and 29 of the 37 amino acid primary structure of islet amyloid and do not develop T2DM spontaneously (12). In 2004, the Sprague-Dawley rodent model was transfected with the human amylin hIAPP gene and the human islet amyloid polypeptide (HIP) rat model was created (4). The heterozygous model develops impaired fasting glucose at 5 months of age and overt T2DM between 6 and 10 months of age on an *ad libitum* diet of Rodent Chow diet 8604 (50% carbohydrate, 24% protein and 4% fat) (5). This is the first animal model that parallels both the functional metabolic and the structural islet amyloid morphological abnormalities typical of human T2DM. Indeed, the endocrine islets in human T2DM are characterized by islet amyloid deposition and approximately a 60% loss of β-cells due to increased apoptosis (13). These parallel findings are present in the HIP rat model of T2DM (1, 4–6).

In this investigation, the HIP rat model was utilized to better understand the progressive morphological islet changes in this new monomeric transgenic rat model of T2DM as determined by the longitudinal analysis with the transmission electron microscope (TEM).

Material and Methods

Animals and Treatments. The generation, housing, diet, metabolic collection, sacrifice techniques, and the utilization and treatment of animals have been previously described in detail (4–6). The University of California Los Angeles Institutional Animal Care and Use Committee approved all surgical and experimental procedures (4, 5).

Animal Tissue Samples. Following harvesting, the tail sections of pancreatic tissue in 4-month-old Sprague-Dawley control rats and the 4-, 8-, and 14-month-old male HIP rat models were thinly sliced and placed immediately in standard EM fixative and shipped to our electron microscopic core facility at the University of Missouri, Columbia, Missouri. Standard electron microscopic tissue preparation, fixation, and staining were employed to study these tissues with the TEM as previously described (14).

Results

Blood Glucose Values of the Study Animals. Fasting plasma blood sugars were <100, 123, 187, and 244 mg/dl in the Sprague-Dawley control (2–18 months), 4-month, 8-month, and 14-month HIP rat models, respectively, that were imaged in this TEM study. Detailed weights, metabolic parameters, statistical analyses, and light microscopic morphological changes have been previously described in detail regarding this model at ages 2, 5, and 10 months of age (4–6). Weights, blood pressures, and insulin levels were unavailable for the HIP rat models imaged in this paper; however, we do know from previous studies that weights increased to approximately 500 grams at age 5 months and remained constant to age 18 months, mean fasting glucose levels were approximately 100 mg/dl until age 5 months, 198 mg/dl at 10 months, and 300 mg/dl at 18 months (4).

HIP rat models became significantly insulin deficient between the ages of 10–18 months compared with the Sprague-Dawley control models (4, 5). Islet amyloid and the extent of amyloid within the islet by percentage increased progressively to reach a plateau by age 10 months in the HIP model of T2DM (4). Islet amyloid was not present in any of the control models because they do not have an amyloidogenic amylin.

Longitudinal Ultrastructural Evaluation by TEM at 4, 8, and 14 Months. *Four-Month Sprague-Dawley Control (SDC) Model.* The image of the SDC model will assist in the understanding of overall morphology in the islet and provide a comparison of what is normal to the HIP rat model of T2DM being studied in this longitudinal study. Additionally, images of this SDC model will aid in the understanding of the impaired insulin secretory granule (ISG) trafficking, docking, and absorption morphology. Figure 1 demonstrates the normal anatomical relationship of the β-cell and how it aligns adjacent to the endothelial cell. Figure 1B through D demonstrates the movement (trafficking) and docking of the ISG to the capillary endothelial cell.

Four-Month HIP Model (Stage of Pericapillary and Inter β-cell Islet Amyloid Deposition). The islets of the 4-month-old HIP rat model (prior to the development of impaired fasting glucose of the 5-month-old HIP rat model) were larger as compared with the 8- and 14-month HIP rat models evaluated in this longitudinal study. Additionally, the β-cells appeared larger and contained greater numbers ISGs compared to the 8- and 14-month-old models. The fasting plasma blood glucose was 123 mg/dl in this model.

Even at this earlier stage, there were considerable amounts of islet amyloid being deposited around the islet capillaries within the islets (Figs. 2 and 3A). We have described this pericapillary hIAPP deposition as the "flowering or blooming stage" of islet amyloid as it resembles the petals of flowers emanating from the capillary. This pericapillary deposition of islet amyloid may result in an early endothelial β-cell (EβC) structural uncoupling as a result of the structural diffusion barrier created by the deposition of islet amyloid (Figs. 2 and 3A). The capillary endothelial cell basement membrane-islet amyloid interface is intimately involved (Fig. 3B). This tight association is primarily due to the strong noncovalent bonds between perlecan of islet amyloid and the heparan sulfate proteoglycans within the basement membrane of the capillary endothelial cell (2, 10–11). Currently, it is not known if the prevention of the deposition of islet amyloid would prevent the impaired fasting glucose findings in the 5-month HIP rat model (4–6). Individual and clusters of β-cells completely surrounded by a sea of hIAPP islet amyloid were also apparent (Fig. 3C and D). These images make it difficult to structurally understand how the ISGs can navigate their way (trafficking) to the islet capillaries for islet docking and

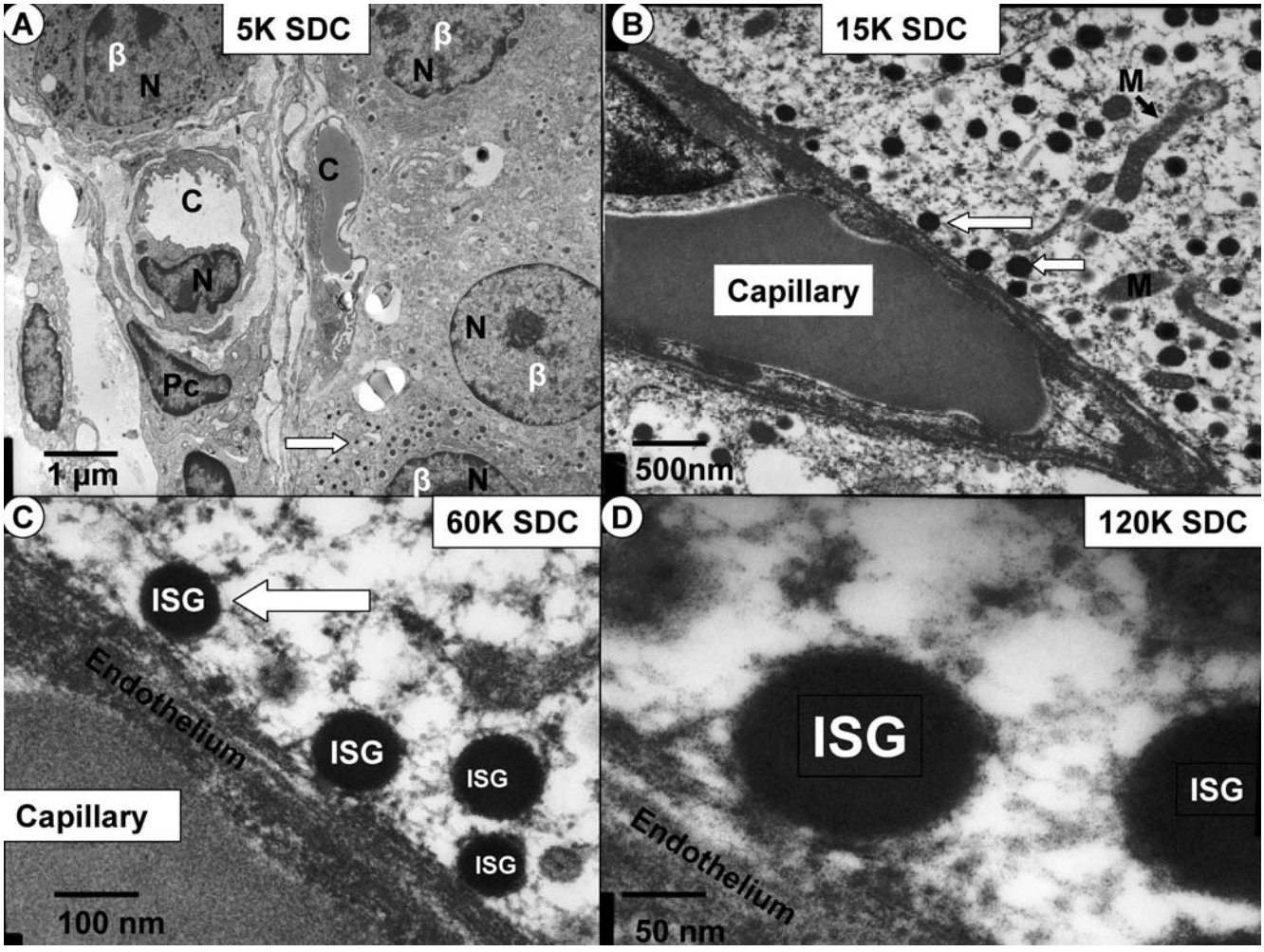

Figure 1. Normal trafficking and docking of insulin secretory granules in control male animals. (A) Four β-cells (β) aligning immediately adjacent to capillaries with minimum separation for proper docking and absorption of the insulin secretory granules (ISG) (white arrow) in the 4-month-old male Sprague-Dawley control (SDC) model at ×5000 magnification. Just below the one endothelial cell of the capillary there is a pericyte (Pc), which protect endothelial cells. N = nucleus, K = 1000, and bar = 1μm. (B) A central capillary endothelial cell in the same model with the β-cell abutting it at ×15,000 magnification. Note how the ISG (white arrows) appear to be trafficking and docking with the endothelial cell of the capillary. M = mitochondria and bar = 500nm. (C) Higher magnification (×60,000) of this trafficking and docking sequence of ISG (white arrow) in this same model. Bar = 100nm. (D) An ISG at ×120,000 magnification actually docking with the capillary endothelial cell in the same model. Bar = 50nm.

absorption mechanics and subsequently be delivered to the insular-acinar-portal pathway to the liver, and hence systemically at this young age in the HIP rat model. This structural remodeling of the endocrine pancreas could certainly help explain the delay in the first phase insulin secretory response noted in the 5-month-old HIP rat model (5). Additionally, it is important to note that ISG docking with the endothelium was not observed in any of the images where pericapillary islet amyloid formation was present (Figs. 2 and 3A) as compared to the SDC (Fig. 1B–D).

Eight-Month HIP Rat Model (Stage of Apoptosis). The 8-month-old rat model islets were markedly more involved with amyloid deposition consuming up to 60%–70% of the islets in the TEM grids that were examined as compared to the 4-month-old model and mean blood sugar was 244 mg/dl.

This model demonstrated multiple β-cells actively undergoing apoptosis; nevertheless, these β-cells still had ISG present within but were markedly reduced as compared to the SDC and 4-month-old HIP rat model. Islet amyloid was present in vast amounts and the few remaining β-cells were considerably distanced from the local absorptive capillaries within these islets. Small-soluble oligomers of hIAPP are felt to be responsible for inducing these apoptotic findings (4–6); however, one cannot overlook the structural changes and wonder if the remaining β-cells are also undergoing ischemic necrosis in these images due to the vast amount of amyloid and the distancing from capillaries (Fig. 4). Additionally inter-β-cell amyloid deposition was also a prominent finding (Fig. 4C) and it was difficult to locate functioning β-cells in the 8-month HIP rat model.

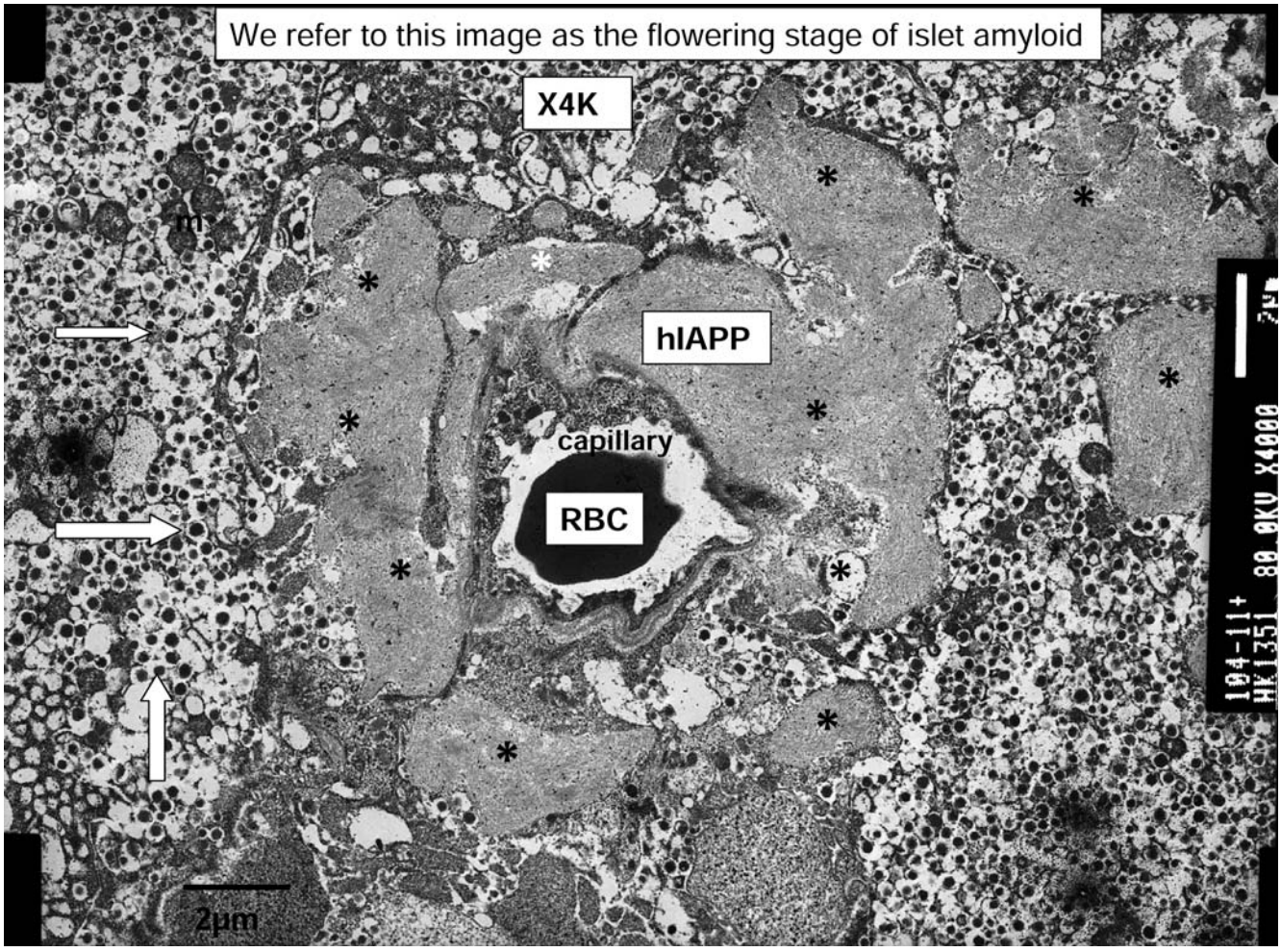

Figure 2. Pericapillary islet amyloid. This panel represents the initial stage of pericapillary islet amyloid (hIAPP) deposition denoted by asterisks (*) in the 4-month-old male HIP rat model at ×4000 magnification. A higher power view of islet amyloid (×120,000) is presented in Figure 4D with discussion. Note the abundance of insulin secretory granules (ISG), which are very small (electron dense) black dots measuring 100–200 nm in diameter (white arrows) and containing insulin and human islet amyloid polypeptide (hIAPP)-amylin monomers. Additionally, note the absence of normal ISG docking with the endothelial cell of the centrally located capillary. Islet amyloid appears to structurally impair ISG docking to the endothelium. Bar = 2μm.

Fourteen-Month HIP Rat Model (Stage of Intra-Islet Adipogenesis). The 14-month-old HIP rat model demonstrated almost complete involvement of the islets with islet amyloid and appeared smaller when compared to the 4- and 8-month-old models. When islets with functional β-cells were identified, they were typically associated with intra-islet adipose tissue deposition. These adipocytes appeared to be synthetically active and most displayed an extensive amount of rough endoplasmic reticulum (Fig. 5).

Fibrosis was an anticipated structural finding; however, when this rare finding was noted, it was only noted in areas associated with adipocyte formation (Fig. 5A). These findings may suggest these synthetically active intra-islet preadipocytes provide signaling mechanisms for the development of intra-islet fibrosis, which may only become significant in older models such as the 18- and 20-month-old HIP rat model deserving further evaluation.

β-Cell Apoptosis and β-Cell Failure. β-cell apoptosis is absolutely critical to the development of T2DM in humans as well as animal models of T2DM (4–6). Because β-cell apoptosis was so prevalent in the 8-month-old HIP model (Fig. 4A and B) it was decided to include a special section regarding β-cell apoptosis.

β-Cell Apoptosis May Involve a Hypothetical "Triple-Hit" Phenomenon. The first-hit may result from the excess demand of β-cells to meet the demands for increased insulin production inducing chronic endoplasmic reticulum (ER) stress and result initially in β-cell dysfunction, and eventually a reduction in β-cell mass via ER stress induced apoptosis (12, 15–17). β-cell apoptosis is the most striking and prevalent finding in the 8-month-old HIP rat model, in addition to the progressive and diffuse nature of islet amyloid deposition (Fig. 4A and B).

The second-hit may be the involvement of plasma membrane toxicity due to the vesicle formation due to soluble oligomers of hIAPP or protofibrils forming

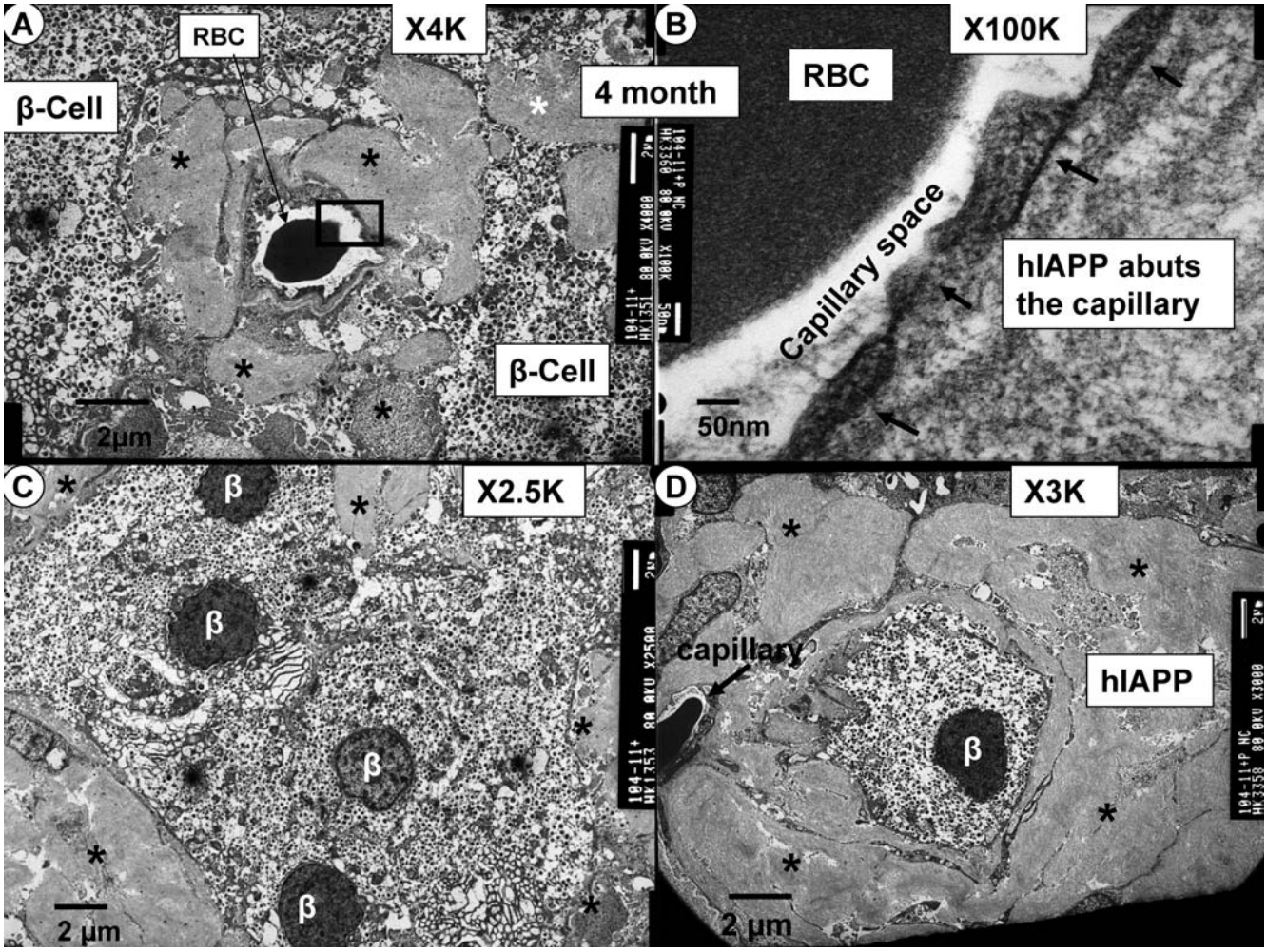

Figure 3. The 4-month-old male HIP rat model. (A) Pericapillary islet amyloid denoted by asterisks (*) in the 4-month-old male HIP rat at ×4000 magnification, discussed in Figure 2. Bar = 2μm. (B) The capillary endothelial cell basement membrane-islet amyloid interface at higher magnification of ×100,000. Note the complete absence of ISG trafficking and docking to the endothelium. There seems to be a predilection for islet amyloid to form at the pericapillary area adjacent to the endothelial cell in the earliest phases of islet amyloid deposition. The next most common area for the deposition of islet amyloid is the basement membrane of the mother β cells forming inter-β-cell islet amyloid (Figure 4C). Bar = 50 nm. (C) A cluster of β-cells (β) surrounded by islet amyloid (*). It is difficult to visualize at this low-power magnification (×2500); however, there are abundant ISGs (very small black dots) containing insulin and the hIAPP-amylin monomers. Bar = 2 μm. (D) A single β-cell (β) with abundant ISGs embedded within a sea of islet amyloid. In Figures 4A and B and 6A through D, one can see that this β-cell is destined to undergo apoptosis. Bar = 2 μm.

invaginations in the cell membranes, thereby allowing for calcium leakage into the β-cells promoting apoptosis (4–6, 18). Figure 4B demonstrates the plasma membrane vesicles in the apoptotic β-cell in the upper right-hand corner.

The third-hit may involve islet metabolic toxicity resulting initially from increased glucose autooxidation and generation of ROS. In the presence of glucotoxicity, lipotoxicity may add to the oxidative stress and ROS production via an increase in oxidative stress, beta-oxidation of free fatty acids, and ceramide-associated lipotoxicity in redox stress-associated β-cell apoptosis (Fig. 4B) (19, 20).

Oligomers of soluble hIAPP are known to induce apoptosis and the replicating β-cells are known to be more susceptible to the effects of hIAPP (5, 6, 21). In contrast, the insoluble-mature hIAPP aggregates-fibrils of hIAPP are not toxic to the β-cell; however, they may create a diffusion barrier, a secretory and absorptive defect, which could literally starve the remaining β-cells (Figs. 3D, 4A, and 5A–B). This structural defect could permit β-cells to undergo ischemic necrosis-apoptosis by impairing the transport of oxygen and allowing the accumulation of toxic metabolic by-products of metabolism. Even though the latter has not been proven, the ultrastructural images presented certainly support this notion in the longitudinal study of the HIP rat model (Fig. 4A and B) (18, 22–23).

Discussion

This longitudinal morphological study of the HIP rat model at 4, 8, and 14 months of age is intended to provide representative ultrastructural images that may aid in the better understanding of how hIAPP is deposited in the islets

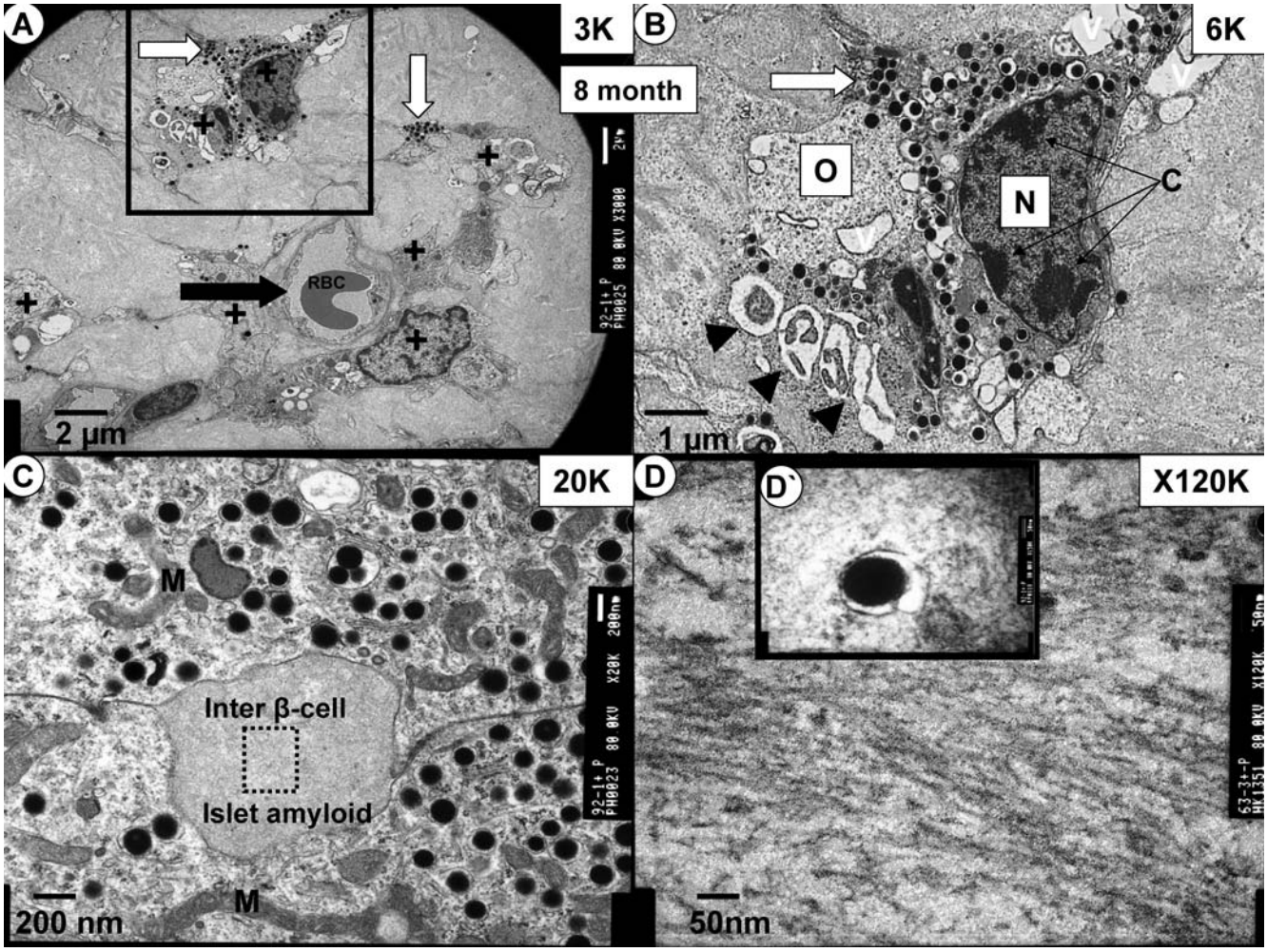

Figure 4. The 8-month-old HIP rat model. (A) The abundance of islet amyloid (the entire background of this image) and the prominent feature of β-cell apoptosis (×3000 magnification). Note the seven β-cells undergoing apoptosis (+) and the paucity of functioning β-cells. Also note the capillary (black arrow) and at least 3 pericapillary apoptotic β-cells. The very small black dots (ISG) remain in some of the apoptotic β-cells (white arrows). The boxed-in area is depicted at higher magnification in Panel B. (B) An enlarged view (×6000) of two β-cells undergoing apoptosis. Note the small numbers of ISG (white arrows) present, the condensation of chromatin material (black arrows) within the nucleus (N), the loss of cytoplasmic organelles (O), cellular vesiculation (V), and the formation of classic apoptotic bodies (arrowheads) in the β-cells undergoing apoptosis. (C) Inter-β-cell islet amyloid formation at ×20,000 magnification. When functioning β-cells were found in this age group, there were varying degrees of inter β-cell amyloid deposition. These cells had greater numbers of ISG than β-cells undergoing apoptosis in Panels A and B. Tubular and elongated mitochondria (M) are more prevalent in the β-cells of the islet than in other cells and tissues. The boxed area is represented at higher magnification in Panel D. Bar = 200nm. (D) Noncrossed banded parallel arrays and interlacing disordered fibrils with a diameter of 7–10 nm of islet amyloid in contrast to collagen types I and III, which are banded with a diameter 10-fold larger. X-ray diffraction reveals the adjacent amyloid fibrils to be organized as antiparallel crossed beta-pleated sheet conformations. Inset (D′) depicts an isolated ISG (150 nm) buried deep within islet amyloid. Bar = 50nm.

in relation to other islet constituents such as the β-cell and islet capillaries. We noted no evidence of fibrosis except for an occasional appearance in the intra-islet adipogenic regions in the 14-month-old animal.

A potential weakness of this longitudinal study is that only three snapshots in time (4, 8, and 14-month-old images) of islet amyloid deposition were studied. It is possible that by studying earlier and later models (2- and 18–20-month-old models) our current understanding might be further improved.

In summary, pericapillary hIAPP deposition is a prominent feature in the 4-month-old HIP rat model. This pericapillary barrier may help to explain the first phase insulin secretory defect and deficiency of insulin sensitivity of 40% in the 5-month-old model (4–6). Interestingly, the structural fibrotic changes in the Zucker obese model of T2DM contribute to a similar decrease in first phase insulin secretion and impaired insulin sensitivity, which was restored with angiotensin-converting enzyme inhibitors and angiotensin receptor blockers (24).

Progressive hIAPP deposition and apoptosis are prominent features of the 8-month-old HIP rat model, and further progression of hIAPP deposition and intra-islet adipogenesis are prominent features of the 14-month-old HIP rat model. Additionally, this longitudinal study has allowed for a closer morphological examination of β-cell apoptosis. These images of the ultrastructural morphological

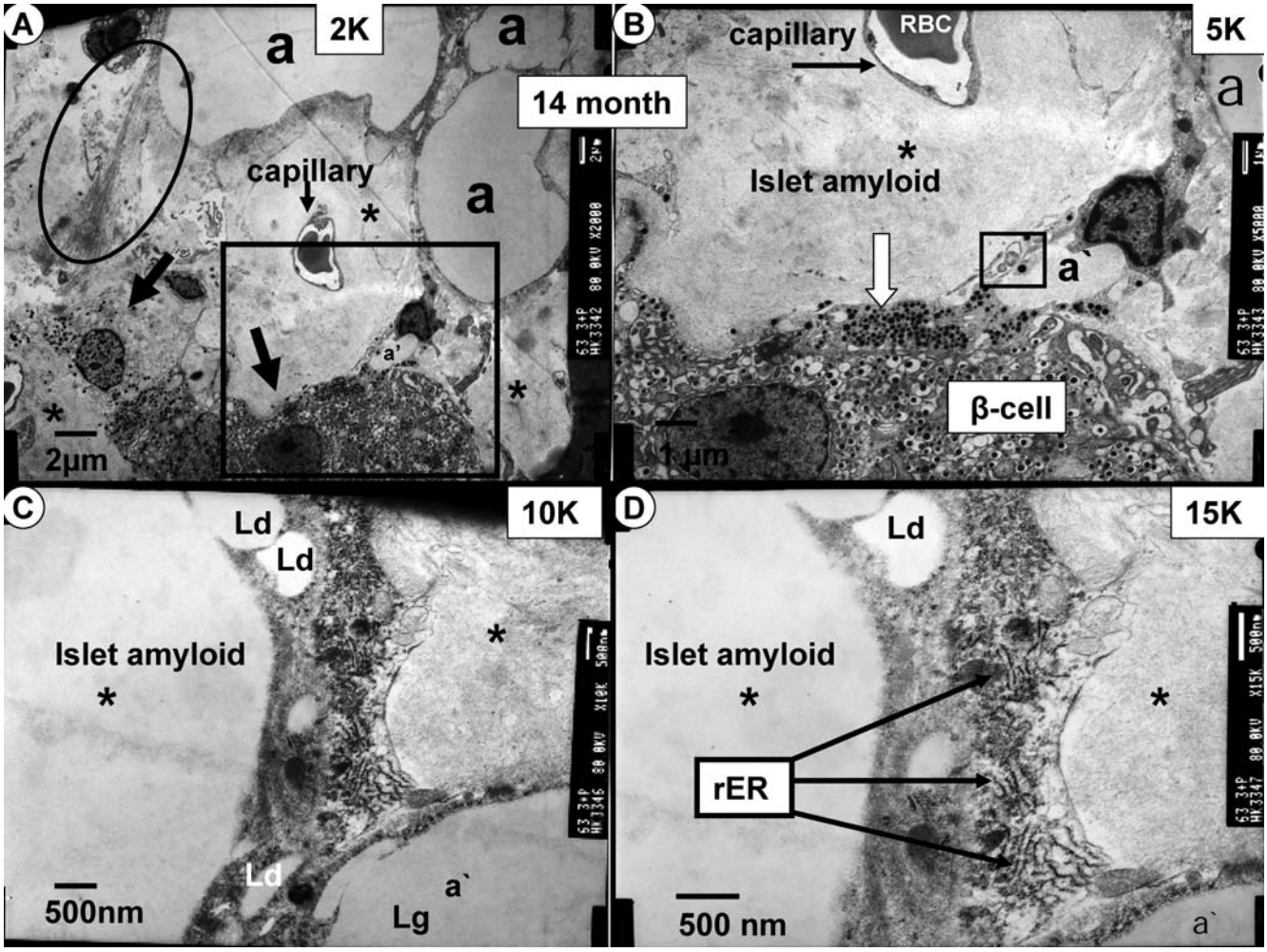

Figure 5. The 14-month-old HIP rat model. (A) β-cells (arrows) in the 14-month model at ×2800. When sparse functioning β-cells were found, they were accompanied with numerous adipocytes (a). Note the ever-present pericapillary islet amyloid (*) and also its presence adjacent to adipocytes. The oval encloses the only fibrotic changes observed in the available models. The boxed area is seen at larger magnification in Panel B. Bar = 2μm. (B) Demonstrates more clearly the proximity of the adipocytes (a–a′) not only to islet amyloid but also the sequestered β-cell at ×5000 magnification. Note the intimate contact between the immature small adipocyte (a′) as if it were attempting to supply nutrient energy to this sequestered β-cell. The boxed area is shown at higher magnification in Panel C. Bar = 1 μm. (C) Demonstrates the small lipid droplets (Ld) in the cytoplasm and the larger lipid granule (Lg) along with the cytoplasmic endoplasmic reticulum at ×10,000 magnification. Bar = 500nm. (D) Demonstrates the rough endoplasmic reticulum (rER, arrows) indicating active protein synthesis in the developing metabolically active immature small adipocyte (a′). Bar = 500 nm.

changes by TEM are also associated with the functional changes that parallel closely the functional and structural changes found in human T2DM (4–6).

Prior to leaving its indelible footprint within the islet and markedly interrupting its morphological structure, the overexpression of the soluble component of hIAPP has done a great deal of damage to the islet β-cells by inducing apoptosis, which has been imaged in morphological detail for the first time to our knowledge in an ultrastructure study of the HIP rat model.

In conclusion, the HIP rat model appears to be an ideal animal model to study and better understand both the functional and structural changes that occur in human T2DM and better understand the progressive nature of this chronic-epidemic disease. This model should allow for the study of future therapeutic treatments to delay or prevent the progressive nature of T2DM, and it is hoped that these initial ultrastructural images might provide a database of knowledge for comparative purposes in the future.

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