Published in final edited form as:

Kidney Int. 2020 January ; 97(1): 143–155. doi:10.1016/j.kint.2019.07.028.

Diabetic microcirculatory disturbances and pathologic erythropoiesis are provoked by deposition of amyloid-forming amylin in red blood cells and capillaries

Nirmal Verma, Ph.D¹, Miao Liu, Ph.D¹, Han Ly, BS¹, Analia Loria, Ph.D¹, Kenneth S. Campbell, Ph.D², Heather Bush, Ph.D³, Philip A. Kern, M.D.⁴, Pedro A. Jose, M.D., Ph.D.⁵, Heinrich Taegtmeyer, M.D., D.Phil.⁶, Donald M. Bers, Ph.D⁷, Sanda Despa, Ph.D¹, Larry B. Goldstein, M.D.⁸, Andrew J. Murray, Ph.D.⁹, Florin Despa, Ph.D^{1,8}

¹Department of Pharmacology and Nutritional Sciences, University of Kentucky, Lexington, KY, USA.

²Department of Physiology, University of Kentucky, Lexington, KY, USA.

³Department of Biostatistics, College of Public Health, University of Kentucky, Lexington, KY, USA.

⁴Division of Endocrinology, Department of Medicine, University of Kentucky, Lexington, KY, USA.

⁵Department of Medicine, Division of Renal Diseases & Hypertension, The George Washington University School of Medicine & Health Sciences, Washington, DC, USA.

⁶Department of Internal Medicine, McGovern Medical School at UT Health, Houston, TX, USA

⁷Department of Pharmacology, University of California, Davis, CA, USA.

⁸Department of Neurology, University of Kentucky, Lexington, KY, USA.

⁹Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge CB2 3EG, UK.

Abstract

In the setting of type-2 diabetes, there are declines of structural stability and functionality of blood capillaries and red blood cells (RBCs), increasing the risk for microcirculatory disturbances.

Correspondence: Florin Despa, Department of Pharmacology and Nutritional Sciences, College of Medicine, University of Kentucky, Wethington Building, Room 459, 900 S. Limestone, Lexington, Kentucky, 40536, USA. Phone: 859-218-0291. Fax: 859-257-3646. f.despa@uky.edu.

Author Contributions: N.V., M.L. and H.L. conducted the experiments; (#) N.V. and M.L. contributed equally; A.L. analyzed the kidney function data; K.S.C. contributed the RBC samples and clinical data stored in the CCTS Biobank; M.L., N.V., H.L., S.D. and F.D. analyzed the data; H.B. provided advice on the statistical methods and interpretation of the statistical analyses; P.A.K., P.A.J., H.T., D.M.B., L.B.G. and S.D. provided advice on the interpretation of the data analyses; A.J.M. designed and assisted with the interpretation of the hypoxia experiments; F.D. conceived the study and wrote the manuscript with contributions from all authors.

Disclosures: The authors have declared that no conflict of interest exists.

Supplementary information is available at www.kidney-international.org

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

Correcting hyperglycemia is not entirely effective at reestablishing normal cellular metabolism and function. Therefore, identification of pathological changes occurring before the development of overt hyperglycemia, may lead to novel therapeutic targets for reducing the risk of microvascular dysfunction. Here we determine whether RBC-capillary interactions are altered by prediabetic hypersecretion of amylin, an amyloid forming hormone co-synthesized with insulin, and is reversed by endothelial cell-secreted epoxyeicosatrienoic acids. In patients, we found amylin deposition in RBCs in association with type-2 diabetes, heart failure, cancer and stroke. Amylin-coated RBCs have altered shape and reduced functional (non-glycated) hemoglobin. Amylin-coated RBCs administered intravenously in control rats upregulated erythropoietin and renal arginase expression and activity. We also found that diabetic rats expressing amyloid-forming human amylin in the pancreas (the HIP rat model) have increased tissue levels of hypoxia-inducible transcription factors, compared to diabetic rats that express non-amyloid forming rat amylin (the UCD rat model). Upregulation of erythropoietin correlated with lower hematocrit in the HIP model indicating pathologic erythropoiesis. In the HIP model, pharmacological upregulation of endogenous epoxyeicosatrienoic acids protected the renal microvasculature against amylin deposition and also reduced renal accumulation of HIFs. Thus, prediabetes induces dysregulation of amylin homeostasis and promotes amylin deposition in RBCs and the microvasculature altering RBC-capillary interaction leading to activation of hypoxia signaling pathways and pathologic erythropoiesis. Hence, dysregulation of amylin homeostasis could be a therapeutic target for ameliorating diabetic vascular complications.

Graphical Abstract

Keywords

Type-2-diabetes; Amylin; Microvascular Disease; Hypoxia; Erythropoiesis

Introduction

Oxygen is essential for cell function and cell survival¹. RBCs deliver oxygen to cells and tissues via mechanisms that involve the passage of RBCs through capillaries. This process is enabled by the viscoelastic properties of the RBCs, which allow them to be deformed within

capillaries². In type-2 diabetes, the oxygen-carrying capacity of RBCs and the integrity and stability of the capillaries decline, exacerbating the risk of tissue hypoxia and end organ malfunction^{3–7}. The underlying mechanisms are complex and incompletely understood.

Physiological responses to low oxygen levels are primarily driven by the stabilization of the α subunits of the hypoxia-inducible transcription factors HIF-1 and 2^{1,8,9}. HIF-2 regulates the hypoxia response by elevating the renal expression of EPO, a hormone that signals an increased demand for RBCs from the bone marrow, which then increases the production of RBCs⁸. Stabilization of HIF-2 α also induces *arginase* expression⁹ in vascular endothelial cells. Because arginase has the same substrate (L-arginine) as nitric oxide (NO) synthase⁹, increased *arginase* production/ activation may reduce NO availability. Depleted NO production impairs relaxation of the blood vessels and affects microvascular autoregulation^{1,8,9}. Thus, increased EPO coupled with arginase-NO dysregulation constitutes one of the multiple¹⁰ molecular derangements linking systemic hypoxia with microvascular dysfunction.

Metabolic derangements that occur before the development of overt hyperglycemia may induce microvascular dysfunction¹¹. In prediabetes, pancreatic β -cells compensate for insulin resistance by increasing the secretion of insulin (hyperinsulinemia)¹². Amylin (also known as islet amyloid polypeptide; IAPP), is a 37-amino acid peptide synthesized and co-secreted with insulin in response to physiological stimuli^{13–15}. It is normally soluble, crosses the blood brain barrier and binds to neurons in the feeding centers participating in the regulation of gastric fluxes¹⁶. Amylin from humans and a few other species, including cats, dogs and monkeys, but not rodents, has an increased propensity to aggregate, forming amyloid¹⁷ (i.e., amylin dyshomeostasis). This triggers β -cell apoptosis by mechanisms involving incorporation of aggregated amylin into cellular membranes^{18–20}. We^{21–26} and others^{27–31} have shown the presence of amylin deposition in failing hearts^{21–23} and kidneys²⁷ of patients with type-2 diabetes and in brains^{28–31} of humans with dementia³². We also showed that rats that develop type-2 diabetes linked to amylin dyshomeostasis (i.e. the HIP rat model for type-2 diabetes³³) develop heart dysfunction^{21,23} and neurological deficits^{25,34} sooner than age- and blood glucose-matched rats that develop type-2 diabetes in the absence of amylin dyshomeostasis (i.e. the UCD rat model for type-2 diabetes³⁵). The upregulation of epoxyeicosatrienoic acids (EETs) in endothelial cells appeared to protect against cardiac amylin accumulation in HIP rats, which correlated with improved heart function²². Although we interpret these findings to support the hypothesis that reducing amylin dyshomeostasis may ameliorate diabetic vascular complications, the impact of elevated blood levels of amylin on blood cells and the microvasculature remains unknown.

Here we tested the hypothesis that systemic amylin dyshomeostasis alters the interaction between RBCs and capillaries leading to hypoxic-ischemic tissue injury. To test this hypothesis, we measured the amylin levels in RBCs from humans with and without diabetes, and used transgenic rats, RBC transfusions and pharmacological tools for mechanistic studies.

Results

Humans with type-2 diabetes have amylin deposition in RBCs

We assessed the relationship between HbA1c level (the common marker of hyperglycemia) and amylin concentration in RBC lysates from patients with type-2 diabetes or diseases that are commonly associated with insulin resistance, including heart failure, cancer and stroke. Western blot analysis of matched plasma, RBC lysate and white blood cell (WBC) lysate from a human with type-2 diabetes (the positive control for amylin dyshomeostasis) detected both monomeric amylin (Figure 1a) and amylin-positive higher molecular weight bands (Supplementary Figure 1). RBC lysates from individuals with a primary diagnosis of type-2 diabetes (T2D) (without heart failure, cancer or stroke) had higher amylin concentration than those from healthy individuals (H) and patients with type-1 diabetes (T1D group; the negative control for amylin) (Figure 1b). Patients with a primary diagnosis of heart failure (HF), cancer (C), or stroke (S) also had elevated RBC amylin levels independent of type-2 diabetes as a secondary diagnosis (Figure 1b). Lighter colored symbols in the HF and C groups indicate heart failure or cancer without type-2 diabetes. RBC amylin and HbA1c levels were highly variable in all groups (Figure 1c–i), except in patients with HF and type-2 diabetes in whom higher HbA1c levels correlated with RBC amylin accumulation. There were non-significant inverse correlations between HbA1c and RBC amylin levels in the HF without diabetes (Figure 1f), cancer with diabetes (Figure 1g) and stroke (Figure 1i) groups.

These results suggest that type-2 diabetes and diseases associated with insulin resistance such as heart failure, cancer and stroke promote amylin accumulation in RBCs in humans.

Amylin deposition in RBCs results from hypersecretion of amyloid-forming human amylin

The HIP rat is a unique animal model for late-life onset type-2 diabetes as it is characterized by pancreatic expression of the human (amyloid-forming) variant of amylin³³, whereas other rodent models for type-2 diabetes express only the native, non-amyloid forming amylin¹⁷. As in humans¹⁴, the development of type-2 diabetes in HIP rats is associated with pancreatic amyloid³³ (Supplementary Figure 2). We compared the amylin content in RBCs, WBCs, and plasma from diabetic HIP rats and non-diabetic WT littermates (Figure 2a). Amylin was concentrated in RBCs and the *difference* in amylin levels between WT and diabetic HIP rats was greater in RBCs than in plasma or WBCs (Figure 2a).

Analysis of freshly packed RBCs by flow cytometry (Figure 2b) and amylin ELISA (Figure 2c) revealed a correlation between RBC amylin levels and different levels of non-fasted blood glucose (normal, ≤ 11 mM, 6–8 months old; prediabetic, 11–14 mM on two consecutive measurements separated by >3 days, 10–12 months old; and diabetic, >14 mM on two consecutive measurements, separated by >3 days; 14–16 months old).

Confocal microscopy analysis of RBCs that were double-stained for amylin and hemoglobin showed amylin deposition on RBCs from HIP rats (Figure 2d). Amylin appeared to co-localize with glycophorin A (Figure 2e), a membrane protein that is specific to RBCs, suggesting amylin accumulation on the RBC membrane. The analysis of RBCs co-stained for amylin and glycophorin A using super-resolution imaging (STORM) showed the presence of amylin within the RBC membrane with some dense patches on the outer part of

the cell membrane (Figure 2f). Electron microscopic examination of immunogold-labeled thin sections of epoxy resin-embedded RBCs indicated the presence of amylin within the cell membrane (Supplementary Figure 3; arrowhead pointing to amylin deposits).

The results demonstrate that hypersecretion of the amyloid-forming human variant of amylin leads to amylin deposition within circulating RBCs.

Amylin-coated RBCs have lower deformability and functional (non-glycated) hemoglobin

Next, we investigated pathophysiological characteristics of RBCs from diabetic HIP rats and RBCs from diabetic rats without amylin dyshomeostasis (UCD rats) and non-diabetic WT rats, as they express only the non-amyloid forming rat amylin. Compared to RBCs from WT rats, RBCs from HIP rats contained less functional (non-glycated) hemoglobin (Figure 3a), whereas there was no difference between UCD and WT rats. RBCs of HIP, UCD and WT rats (~ 12 months old rats) had similar oxygen dissociation curves (Figure 3b) indicating that the affinity of hemoglobin for oxygen, the release of bound oxygen and the partial oxygen pressure for maintaining oxygen saturation are not significantly affected by amylin dyshomeostasis or hyperglycemia.

In flow cytometry experiments, the distribution of the forward scattering (FSC) signals by RBCs from WT rats (Figure 3c; orange) was bimodal, which reflects the biconcave disk shape of normal RBCs. In the case of amylin-coated RBCs from diabetic HIP rats, the FSC distribution was monomodal (Figure 3c; pink), indicating changes in RBC morphology towards a more spherical shape. The Pearson coefficient of dissymmetry (PCD), which indicates the departure from sphericity, suggested distinct morphological changes in RBCs from HIP rats compared to WT and UCD rats (Figure 3c). Incubation of RBCs from WT rats with aggregated human amylin for 4 hours (as described in our previous studies^{26,36}) replicated the morphological change observed in HIP rat RBCs (Supplementary Figure 4). Altered shape of RBCs from HIP and UCD rats did not affect the response to an osmotic resistance test, as the propensity for hemolysis in hypo-osmotic solutions was comparable for RBCs from all three rat groups (Figure 3d).

These results show that amylin deposition in RBCs is associated with lower hemoglobin concentrations and reduced RBC deformability, independently of effects of chronic glucose levels. At an early stage of diabetes, the cumulative effects of amylin dyshomeostasis and hyperglycemia (as in HIP rats) or hyperglycemia alone (as in UCD rats) do not appear to induce significant changes in hemolysis and oxygen dissociation.

The microvasculature is disrupted in kidneys of rats with amylin dyshomeostasis

Next we investigated the structural integrity of the capillary network and the stability of the capillary beds in kidneys from age- and blood glucose-matched HIP and UCD rats. We used immunohistochemistry with antibodies against amylin (brown) and collagen IV (Col IV; green), a component of the basement membrane, to anatomically localize amylin deposition with respect to the vasculature. In HIP rat kidneys, there were patches of amylin deposits in arterioles, interstitial tissue between the tubules (Figure 3e) and glomeruli (Figure 3f). Vascular amylin deposition correlated with accumulation of macrophages, as indicated by co-staining for amylin and the ionized calcium-binding adapter molecule 1 (IBA1), a marker

of macrophage activation (Figure 3g). There were macrophages in areas of amylin deposition, which may indicate a potential role for these cells in the clearance of vascular amylin deposition. Staining with ED1 (Figure 3h), an antibody against the cluster of differentiation (CD) 68 protein (that is highly expressed by circulating macrophages), supports the increased activity of macrophages in areas of vascular amylin deposition. In contrast, vascular amylin deposits and macrophage infiltration were not found in kidneys from diabetic UCD rats.

These data suggest that amylin dyshomeostasis injures the capillaries, which may be associated with systemic inflammatory responses leading to macrophage infiltration that may exacerbate ischemic vascular injury in HIP rats.

Amylin dyshomeostasis activates renal hypoxia signaling pathways

The kidney is a critical component of a regulatory feedback loop that controls the hematocrit via EPO production⁸. Both HIP and UCD rats had renal dysfunction, as indicated by polyuria and albuminuria (Supplementary Figures 5a and 5b). Creatinine clearance was elevated in diabetic HIP rats compared with WT littermates and diabetic UCD rats (Supplementary Figure 5c). The plasma level of EPO, the hormone that signals an increased demand for RBCs to the bone marrow, was higher in diabetic HIP rats than in WT littermates (Figure 4a) and tended to be higher in age-matched diabetic UCD rats vs. WT rats ($P = 0.27$). The reticulocyte count was also higher in HIP than in WT rats (Figure 4b). Despite elevated plasma EPO levels, the average hematocrit was not different in HIP rats compared with WT littermates, but was lower in HIP compared to UCD rats (Figure 4c). The spleen, a major blood reservoir, had lower weight in diabetic HIP rats compared to age-matched WT rats (Supplementary Figure 6). Compared with diabetic UCD rats and healthy WT rats, diabetic HIP rats had elevated levels of HIF-1 α and HIF-2 α in whole kidney tissue homogenates (Figure 4d and 4e). Consistent with elevated HIF-2 α , HIP rat kidneys also had upregulated arginase-1 and 2 proteins (Figure 4f and 4g) and greater arginase activity (Figure 4h). Increased stabilization of HIF α units in HIP rat kidney tissue correlated with a trend towards downregulation of the von Hippel-Lindau (vHL) tumor suppressor protein (Supplementary Figure 7) suggesting impaired degradation of HIF α units.

These results suggest that activation of hypoxia signaling in kidneys and downstream upregulation of EPO are associated with pathologic erythropoiesis and amylin deposition in RBCs.

Blocking of adhesion proteins in endothelium reverses amylin dyshomeostasis and HIF activation

The adhesion of RBCs to cultured vascular endothelial cells (ECs) under flow condition tended to be greater for HIP rat RBCs than RBCs from UCD rats ($P = 0.17$) and from WT rats ($P = 0.17$) (Figure 5a). Greater adhesion of RBCs from HIP rats to ECs was found in experiments without flow condition (Figure 5b) in an adhesion test in which RBCs from WT rats that were incubated with 50 μ M synthetic human amylin for 4 hours is shown in Figure 5c. Analysis of kidney capillary lysates by Western blot (A) and ELISA (B) showed a trend towards upregulation of the expression of vascular cell adhesion molecule 1 (VCAM-1) in

HIP rats (Supplementary Figure 8). Taken together, these results suggest that the cell membrane adhesion proteins may play a role in amylin accumulation at the RBC-capillary interface. To test this hypothesis, we used EETs, which are primarily expressed by vascular ECs³⁷ and RBCs³⁸ and are known to downregulate the expression of VCAM-1 in endothelium³⁹. We found that *ex vivo* incubation with ($\pm$)14(15)-EET reduced the adhesion of HIP rat RBCs to cultured endothelial cells (Figure 5d). Upregulation of EETs by treatment with an inhibitor of soluble epoxide hydrolase, the enzyme that degrades endogenous EETs³⁷, was associated with lower amylin deposition in renal microvasculature (the HIP-T group; Figure 5e). The treatment lowered renal accumulation of HIF-2 α (Figure 5f) and HIF-1 α (Figure 5g) and had variable effects on arginase expression and arginase activation (Supplementary Fig 9) in HIP rat kidneys.

Taken together, these results indicate that 1) reduced capillary RBC flux owing to amylin deposition on RBCs and the capillary wall likely contributes to tissue hypoxia in HIP rats and 2) EETs reduce this effect by downregulation of adhesion proteins in the vascular endothelium.

Transfused amylin-coated RBCs upregulate EPO through HIF-2 α activation in kidneys

RBCs act as both oxygen carriers and mediators of oxygen sensing and signaling pathways within ECs^{8,9}. To determine whether amylin deposition on RBCs activates hypoxia signaling pathways in tissues, amylin-coated RBCs from HIP rats were administered to WT rats. Rats were given 300 μ l freshly packed RBCs/day for seven days. WT rats given similar volumes of RBCs from diabetic UCD rats were used as positive controls for the possible effects of hyperglycemia on RBC function, whereas WT rats receiving RBCs from WT rats served as negative controls. Plasma levels of EPO were elevated in WT rats receiving amylin-coated RBCs from HIP rats compared to WT rats that received either RBCs from diabetic UCD rats or WT rats (Figure 6a). Kidneys of WT rats receiving HIP rat RBCs had increased stabilization of HIF-1 α (Figure 6b) but no difference in HIF-2 α levels (Figure 6c), compared with rats in the two control groups.

To examine further a possible ‘amylin stress’ on oxygen-sensing pathways, aggregated human amylin (hA) (0.08 μ g/g body weight) was intravenously administered to diabetic UCD rats, daily, for one week. Intravenous infusion of aggregated human amylin in UCD rats led to amylin deposition on RBCs (Figure 6d). This acute ‘amylin stress’ provoked an increase in plasma EPO level (Figure 6e; the ‘after hA inj’ rat group) and no difference in renal HIF-2 α and HIF-1 α levels in whole kidney tissue homogenate (Figure 6f). Arginase 1 protein levels were elevated in kidney tissue homogenates from amylin-infused UCD rats, whereas there was no significant change of arginase 2 protein expression (Figure 6g).

These data indicate elevated EPO and accumulation of HIFs in kidneys as a direct response to circulating amylin-coated RBCs (Figure 6h).

Discussion

We found that hypersecretion of human amylin is associated with amylin deposition in the microvasculature and RBCs leading to impaired RBC-capillary interaction and activation of

hypoxia signaling pathways. Impairment of tissue oxygen-sensing found in diabetic HIP rats was mirrored in control rats that were given amylin-coated RBCs intravenously and in diabetic UCD rats that were given human amylin intravenously. These results indicate that amylin-coated RBCs are a trigger of hypoxia signaling pathways. These deleterious effects result in part from a reduced flux of amylin-coated RBCs through the capillaries that may involve adhesion proteins. Blocking the expression of adhesion proteins in the vascular endothelium by upregulation of EETs reduces amylin dyshomeostasis and HIF activation. Future studies need to identify the ligand pairs for the amylin-mediated adhesive interaction between vascular ECs and RBCs. Candidate ligand pairs may include the VCAM-1- $\alpha 4\beta 1$ -integrin pair of proteins that appears to be involved in sickle cell disease⁴⁰.

This link between prediabetes-induced systemic amylin dyshomeostasis and tissue hypoxia has broad implications for health care because: 1) accumulating evidence demonstrates the presence of amylin deposition in vital organs^{21–31} and RBCs (as shown by the present human data) that may provide a therapeutic target reducing vascular injury prior the development of overt diabetes, 2) the public health impact of type-2 diabetes; and 3) the lack of drugs that counter amylin dyshomeostasis.

Glycated hemoglobin has a higher affinity for oxygen than adult hemoglobin A (functional hemoglobin), and can disrupt the supply of oxygen to tissues⁴¹. Indeed, RBCs from hyperglycemic UCD rats have decreased deformability and functional hemoglobin compared with WT rats. RBC deformability and hemoglobin levels are even lower in HIP rats, which may explain, in part, the higher hypoxic response in control WT rats receiving RBCs from HIP rats compared to control rats receiving similar volumes of RBCs from UCD rats.

Elevated EPO and microvascular dysfunction identify diabetic patients with increased risk of death^{42–44}. In diabetic HIP rats, elevated plasma EPO does not correlate with an increase in hematocrit. HIP rat kidneys have amylin deposits in the microvasculature that co-localized with macrophage activation. Taken together, the results suggest that systemic amylin dyshomeostasis may upregulate eryptosis via macrophage activation. The results also indicate that amylin dyshomeostasis injures capillaries and is associated with inflammatory responses exacerbating ischemic vascular injury.

There are inherent limitations to our study of the interaction between pancreatic amylin secretion and RBCs. In the human study, we were not able to control for the potential effects of glucose, steroids, anti-diabetic drugs or specific forms of anesthesia given to individual patients, which can affect pancreatic function including amylin and insulin secretion.

In conclusion, systemic amylin dyshomeostasis promotes amylin deposition in RBCs and the microvasculature associated with pathologic erythropoiesis, macrophage activation and macrophage accumulation in blood vessels. Future studies should determine whether the RBC amylin levels affect the course of microvascular complications.

Methods

Detailed Methods are included in Supplementary Materials.

Human studies

This research employed de-identified blood specimens matched with medical record data obtained from the biobank of the Center for Clinical and Translational Science at University of Kentucky. Sample collection and storage were approved by the Institutional Review Board. We assessed the relationship between HbA1c level and amylin concentration in RBC lysates from individuals with primary diagnosis of type-2 diabetes or diseases that are commonly associated with insulin resistance, including heart failure, cancer and stroke. RBC specimens were divided into groups based on the primary diagnosis of type-2 diabetes, heart failure, cancer or stroke. Most humans in the heart failure and cancer groups had type-2 diabetes as a second diagnosis. The negative control for amylin was RBC lysates from patients with over 15 years of type-1 diabetes (and, therefore, depleted β -cell mass) who were otherwise healthy. The healthy control group included individuals without diabetes, heart failure, stroke or cancer. Exclusion criteria were based on conditions that may affect the pancreatic secretion of amylin such as transplant recipients (less than 6 months after the transplant), patients with liver disease, patients with HIV and pregnant or lactating women. Table 1 summarizes the diabetes status, co-morbidities, age, sex and body mass index (BMI) of the patients who provided the blood used in these analyses.

Experimental Animals

All animal experiments conform to the NIH guide for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee at University of Kentucky. We compared rats that develop type-2 diabetes linked to expression of human amylin in the pancreas (HIP rats; n=65), with rats that develop type-2 diabetes in the absence of amyloid, as they express the non-amyloidogenic rat amylin (UCD rats; n=40) and control, non-diabetic rats (WT rats; n=53).

Treatment—We performed a retrospective analysis of kidney tissue from a prior study²² in which HIP rats were treated for 10 weeks with a soluble epoxide inhibitor APAU (UC1153) added to their drinking water in order to upregulate the endogenous EET levels. APAU was formulated in polypropylene glycol at a concentration of 10 mg/mL with 30 minutes sonication. The APAU aliquot was added to the drinking water at a final content of 1%.

Renal function analysis—Urine and blood were collected from rats in metabolic cages. Creatinine concentration in plasma and urine was measured using the kinetic Jaffé method⁴⁵. Creatinine clearance (Ccr) was calculated using the formula: $Ccr \text{ (ml/min)} = (\text{urine creatinine/plasma creatinine}) \times \text{urine flow rate}$. Urinary albumin was measured by ELISA.

Tissue extraction and RBC isolation—For measurements of hypoxia markers (hypoxia-inducible transcription factors 1α , HIF1- α , and 2α , HIF2- α , arginase 1, arginase 2 and von Hippel-Lindau factor, vHL) and arginase activity, kidney tissue was extracted in PBS with protease and phosphatase inhibitors, followed by three consecutive cycles of freezing to -80°C and thawing. For other biochemical assays, tissues were extracted in homogenization buffer containing Triton X-100, SDS and protease and phosphatase inhibitors.

RBCs were isolated immediately after blood collection by centrifugation at 1,000g. For some experiments, RBCs were lysed in lysis buffer containing 10% NP-40.

RBC transfusion—Isolated RBCs were combined with storage solution (150 mM HCl, 45 mM dextrose, 45.5 mM mannitol, and 2.2 mM adenine) in a 3:1 v/v ratio, transferred to sterile tubes and stored at 4°C in the dark before injection. Rats received 300 µL of pre-warmed (at 37° C) RBC solution once daily for 7 days via tail vein injection.

Hematocrit and hemoglobin measurements—Hematocrit was measured with an i-STAT analyzer using i-STAT CG8+ cartridges according to the manufacturer's protocol. Hemoglobin content was calculated from the optical density at 405 nm.

Assessment of RBC shape and amylin deposition on RBCs by flow cytometry—RBCs were incubated with an anti-amylin antibody followed by incubation with an anti-rabbit Alexa Fluor® 488 secondary antibody. Flow cytometry was performed with a Becton Dickinson LSRII instrument. To assess cell shape, RBCs were first gated on a forward scatter (FSC)/side scatter (SSC) plot. The region 1 (R1) events were visualized using a FSC-A/FSC-H dot plot. For detecting amylin deposited on RBCs, cells were first gated on a forward scatter (FSC)/side scatter (SSC) plot. RBCs were further gated to determine the amylin signal (Alexa 488), using negative control (no antibody) and positive control (RBCs incubated with 50 µM of synthetic amylin peptide) to set the upper and lower boundaries.

Immunofluorescence—Isolated RBCs were incubated with primary antibodies against human amylin and glycophorin A, followed by incubation secondary antibodies, and imaged with a Nikon A1R confocal microscope. For hemoglobin staining, blood smears on glass slide were fixed and incubated with primary antibodies against hemoglobin and human amylin. Smears were then incubated with secondary antibodies, mounted in mounting media and imaged.

Biochemical assays—ELISA for human amylin, erythropoietin, HIF1-α, HIF2-α, arginase 1, arginase 2 and vHL were performed according to the manufacturer's protocols. Arginase activity was measured in kidney homogenates using a colorimetric assay. Western blots were performed on plasma, WBC lysates, RBC lysates from humans and using a primary antibody against amylin and on kidney capillary lysates from rats using a primary antibody against VCAM-1 (1: 1000, ab134047, Abcam).

Immunohistochemistry—Immunohistochemical staining was performed on rat kidney slices using antibodies against amylin, collagen IV, IBA-1, and ED1). The staining area for amylin was analyzed in ImageJ.

Statistical analysis—Statistical differences between groups were determined using Student's t-test, one-way ANOVA or two-way ANOVA, as appropriate. Multiple comparison and P values were calculated by Bonferroni correction for human data. P value for each regression analysis was calculated by the Spearman correlation test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Sources of Funding: This research was supported by National Institutes of Health HL118474 (F.D.), HL135000 (S.D.), AG057290 (F.D.) and AG053999 (F.D.), American Heart Association 16GRNT310200 (F.D.) and 18PRE33990154 (H.L.) and Alzheimer's Association VMF-15–363458 (F.D.). The University of Kentucky CCTS Biostatistics, Epidemiology & Research Design (BERD) and Biospecimens Cores are funded by the CTSA grant UL1 TR001998.

References

1. Semenza GL. Life with oxygen. *Science*. 2007;318:62–64. [PubMed: 17916722]
2. Mohandas N, Gallagher PG. Red cell membrane: past, present, and future. *Blood*. 2008;112:3939–3948. [PubMed: 18988878]
3. McMillan DE, Utterback NG, La Puma J. Reduced erythrocyte deformability in diabetes. *Diabetes*. 1978;27: 895–901. [PubMed: 689301]
4. Tonelli M, Sacks F, Arnold M, et al. Relation between Red Blood Cell Distribution Width and Cardiovascular Event Rate in People with Coronary Disease. *Circulation*. 2008;117:163–168. [PubMed: 18172029]
5. Thomas MC. Anemia in diabetes: marker or mediator of microvascular disease? *Nat. Clin. Pract. Nephrol* 2007;3:20–30. [PubMed: 17183259]
6. Maddox TM, Stanislawski MA, Grunwald GK, et al. Nonobstructive coronary artery disease and risk of myocardial infarction. *JAMA*. 2014;312:1754–1763. [PubMed: 25369489]
7. Barrett EJ, Liu Z, Khamaisi M, et al. Diabetic Microvascular Disease: An Endocrine Society Scientific Statement. *J Clin Endocrinol Metab*. 2017;102:4343–4410. [PubMed: 29126250]
8. Semenza GL. Involvement of oxygen-sensing pathways in physiologic and pathologic erythropoiesis. *Blood*. 2009;114:2015–2019. [PubMed: 19494350]
9. Samanta D, Prabhakar NR, Semenza GL. Systems biology of oxygen homeostasis. *Wiley Interdiscip. Rev. Syst. Biol. Med* 2017;9: e1382.
10. Wong BW, Marsch E, Treps L, et al. Endothelial cell metabolism in health and disease: impact of hypoxia. *EMBO J*. 2017;36:2187–2203. [PubMed: 28637793]
11. Gerstein HC, Bosch J, Dagenais GR, et al. ORIGIN Trial Investigators. Basal insulin and cardiovascular and other outcomes in dysglycemia. *N Engl J Med*. 2012;367:319–328. [PubMed: 22686416]
12. Dankner R, Chetrit A, Shanik MH, et al. Basal state hyperinsulinemia in healthy normoglycemic adults heralds dysglycemia after more than two decades of follow up. *Diabetes. Metab. Res. Rev* 2012;28:618–624. [PubMed: 22865584]
13. Butler PC, Chou J, Carter WB, et al. Effects of meal ingestion on plasma amylin concentration in NIDDM and nondiabetic humans. *Diabetes*. 1990;39:752–756. [PubMed: 2189768]
14. Westermark P, Andersson A, Westermark GT. Islet amyloid polypeptide, islet amyloid, and diabetes mellitus. *Physiol. Rev* 2011;91:795–826. [PubMed: 21742788]
15. Kahn SE, D'Alessio DA, Schwartz MW, et al. Evidence of cosecretion of islet amyloid polypeptide and insulin by beta-cells. *Diabetes*. 1990;39:634–638. [PubMed: 2185112]
16. Lutz TA. The role of amylin in the control of energy homeostasis. *Am. J. Physiol. Regul. Integr. Comp. Physiol* 2010;298:R1475–R1484. [PubMed: 20357016]
17. Westermark P, Engstrom U, Johnson KH, et al. Islet amyloid polypeptide: pinpointing amino acid residues linked to amyloid fibril formation. *Proc. Natl. Acad. Sci. USA* 1990;87:5036–5040. [PubMed: 2195544]
18. Janson J, Ashley RH, Harrison D, et al. The mechanism of islet amyloid polypeptide toxicity is membrane disruption by intermediate-sized toxic amyloid particles. *Diabetes*. 1999;48:491–498. [PubMed: 10078548]

19. Zraika S1, Hull RL, Udayasankar J, et al. Oxidative stress is induced by islet amyloid formation and time-dependently mediates amyloid-induced beta cell apoptosis. *Diabetologia*. 2009;52:626–635. [PubMed: 19148619]
20. Huang CJ, Haataja L, Gurlo T, et al. Induction of endoplasmic reticulum stress-induced beta-cell apoptosis and accumulation of polyubiquitinated proteins by human islet amyloid polypeptide. *Am. J. Physiol. Endocrinol. Metab* 2007;293:E1656–E1662. [PubMed: 17911343]
21. Despa S, Margulies KB, Chen L, et al. Hyperamylinemia contributes to cardiac dysfunction in obesity and diabetes: A study in humans and rats. *Circ. Res* 2012;110:598–608. [PubMed: 22275486]
22. Despa S, Sharma S, Harris TR, et al. Cardioprotection by controlling hyperamylinemia in a “humanized” diabetic rat model. *J. Am. Heart. Assoc* 2014;3:pii: e001015. [PubMed: 25146704]
23. Liu M, Verma N, Peng X, et al. Hyperamylinemia increases IL-1 β synthesis in the heart via peroxidative sarcolemmal injury. *Diabetes*. 2016;65:2772–2783. [PubMed: 27335231]
24. Jackson K, Barisone GA, Diaz E, et al. Amylin deposition in the brain: A second amyloid in Alzheimer disease? *Ann. Neurol* 2013;74:517–526. [PubMed: 23794448]
25. Verma N, Ly H, Liu M, et al. 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. *J. Alzheimers. Dis* 2016;53:259–272. [PubMed: 27163815]
26. Ly H, Verma N, Wu F, et al. Brain microvascular injury and white matter disease provoked by diabetes-associated hyperamylinemia. *Ann. Neurol* 2017;82:208–222. [PubMed: 28696548]
27. Gong W, Liu ZH, Zeng CH, et al. Amylin deposition in the kidney of patients with diabetic nephropathy. *Kidney Int.* 2007;72:213–218. [PubMed: 17495860]
28. Fawver JN, Ghiwot Y, Koola C, et al. Islet amyloid polypeptide (IAPP): A second amyloid in Alzheimer’s disease. *Curr. Alzheimer. Res* 2014;1:928–940.
29. Oskarsson ME, Paulsson JF, Schultz SW, et al. In vivo seeding and cross-seeding of localized amyloidosis: A molecular link between type 2 diabetes and Alzheimer disease. *Am. J. Pathol* 2015;185:834–846. [PubMed: 25700985]
30. Schultz N, Byman E, Fex M, et al. Amylin alters human brain pericyte viability and NG2 expression. *J. Cereb. Blood Flow Metab* 2007;37:1470–1482.
31. Schultz N, Byman E, Netherlands BB, et al. Levels of retinal IAPP are altered in Alzheimer’s disease patients and correlate with vascular changes and hippocampal IAPP levels. *Neurobiol. Aging* 2018;69:94–101. [PubMed: 29864717]
32. Biessels GJ, Despa F. Cognitive decline and dementia in diabetes mellitus: mechanisms and clinical implications. *Nat. Rev. Endocrinol* 2018;14:591–604. [PubMed: 30022099]
33. Butler AE, Jang J, Gurlo T, et al. Diabetes due to a progressive defect in beta-cell mass in rats transgenic for human islet amyloid polypeptide (HIP Rat): a new model for type 2 diabetes. *Diabetes*. 2004;53:1509–1516. [PubMed: 15161755]
34. Srodulski S, Sharma S, Bachstetter AB, et al. Neuroinflammation and neurologic deficits in diabetes linked to brain accumulation of amylin. *Mol. Neurodegener* 2014;9:30. [PubMed: 25149184]
35. Cummings BP, Digitale EK, Stanhope KL, et al. Development and characterization of a novel rat model of type 2 diabetes mellitus: the UC Davis type 2 diabetes mellitus UCD-T2DM rat. *Am. J. Physiol. Regul. Integr. Comp. Physiol* 2008;295:R1782–R1793. [PubMed: 18832086]
36. Liu M, Hoskins A, Verma N, et al. Amylin and diabetic cardiomyopathy - amylin-induced sarcolemmal Ca²⁺ leak is independent of diabetic remodeling of myocardium. *Biochim. Biophys. Acta. Mol. Basis Dis* 2018;1864:1923–1930. [PubMed: 29066284]
37. Imig JD. Epoxides and soluble epoxide hydrolase in cardiovascular physiology. *Physiol Rev*. 2012;92:101–130. [PubMed: 22298653]
38. Jiang H, Anderson GD, McGiff JC. Red blood cells (RBCs), epoxyeicosatrienoic acids (EETs) and adenosine triphosphate (ATP). *Pharmacol Rep*. 2010;62:468–474. [PubMed: 20631410]
39. Node K, Huo Y, Ruan X, et al. Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. *Science*. 1999;285:1276–1279. [PubMed: 10455056]

40. White J, Lancelot M, Sarnaik S, et al. Increased erythrocyte adhesion to VCAM-1 during pulsatile flow: Application of a microfluidic flow adhesion bioassay. *Clin. Hemorheol. Microcirc* 2015;60:201–13 [PubMed: 24898561]
41. Jain SK, McVie R, Duett J, et al. Erythrocyte membrane lipid peroxidation and glycosylated hemoglobin in diabetes. *Diabetes*. 1989;38:1539–1543. [PubMed: 2583378]
42. Wagner M, Alam A, Zimmermann J, et al. Endogenous erythropoietin and the association with inflammation and mortality in diabetic chronic kidney disease. *Clin. J. Am. Soc. Nephrol* 2011 6:1573–1579. [PubMed: 21734083]
43. Thomas MC. Type 2 Diabetes and Heart Failure: Challenges and Solutions. *Curr. Cardiol. Rev* 2016;12:249–255. [PubMed: 27280301]
44. Thomas MC, Brownlee M, Susztak K, et al. Diabetic kidney disease. *Nat. Rev. Dis. Primers* 2015; 1:15018. [PubMed: 27188921]
45. Peake M, Whiting M. Measurement of serum creatinine- current status and future goals. *Clin Biochem Rev*. 2006;27:173–84. [PubMed: 17581641]

Translational statement

Amylin accumulation in RBCs induces hypoxic-ischemic tissue injury. Detection of amylin accumulation in human RBCs, in combination with current American Heart Association guidelines to define cardiovascular risk, could result in better risk stratification for microvascular complications, improve the ability to predict progression of diabetic microvascular complications by taking a precision medicine approach, and better rationalize therapeutic strategies and response to treatment.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 1. Amylin-coated RBCs in human pathology.

(a) Representative Western blots showing the amylin monomer in matched plasma, RBC and white blood cell (WBC) lysates from an individual with type-2 diabetes. Recombinant human amylin served as control. (b) Whisker box plots comparing the concentration of amylin, measured by ELISA, in RBCs from healthy individuals (h; dark green; n=66) versus individuals with various diseases or combination thereof, including type-2 diabetes (T2D; blue; n=69), heart failure (HF) with diabetes (HF-T2D; dark red; n=49) and without diabetes (HF w/o T2D; light red; n=59), cancer with diabetes (C-T2D; black; n=33) and without diabetes (C; gray; n=58), and stroke (S; yellow; n=13). RBC lysates from patients with type 1 diabetes (T1D; black; n=5) are the negative control for amylin. Statistical significance of the differences in amylin level was assessed using One-way ANOVA with the Bonferroni post-test for comparing all pairs of columns. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. The correlation between RBC amylin and HbA1c in groups of healthy (c) and diseased individuals (d-i) described in (b). Out-of axis amylin-HbA1c levels: 25.2–5.8 and 12.2–4.6 in (d); 16.3–5.5 in (f) and 10.7–5.3 and 13.1–5.1 in (i), respectively. The Spearman

nonparametric correlation analysis was performed in GraphPad and the values for the Spearman r and P are indicated on the plots.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 2. Accumulation of amyloid-forming human amylin in RBCs from diabetic rats.

(a) Amylin concentration, measured by ELISA, in matched plasma and lysates of WBCs and RBCs from HIP rats and WT littermates (n=4/group). (b) Representative flow cytometry graphs (upper) and mean intensity (lower) for amylin/Alexa Fluor 488 fluorescence in RBCs from healthy, prediabetic and diabetic HIP rats (n=5 rats/group). (c) Amylin concentration in RBC lysates from 16 months old WT rats (n=7) and healthy (n=6), prediabetic (n=15) and diabetic (n=16) HIP rats measured by ELISA. (d and e) Representative images of co-staining for amylin and hemoglobin (d) and amylin and glycophorin A (e) in RBCs from age-matched WT and diabetic HIP rats (n=3 rats/group). Scale bar, 10 μ m (top row) and 5 μ m (middle and bottom rows). (f) Representative STORM images showing RBCs from HIP and WT rats stained for human amylin (red) and Glycophorin A (green). (Scale bar 2 μ m). (n=3 for each rat group). *P < 0.05; **P < 0.01 by t-test (a) and One-way ANOVA (b and c).

Fig. 3. Pathophysiological changes induced by amyloid-forming amylin in RBCs.

(a) Hemoglobin levels in RBCs from WT, HIP and UCD rats (n=11 rats/group). (b) Oxygen dissociation curve for RBCs from WT, HIP and UCD rats (n=5 rats/group). (c) Representative example of cell shape distribution of RBCs from WT (orange), UCD (blue) and HIP (pink) rats (n=6 rats/group) and the Pearson coefficient of dissymmetry (PCD) calculated from these data. (d) Percentage of RBC hemolysis in hypo-osmotic solutions. The NaCl concentration for 50% hemolysis of WT, HIP and UCD RBCs is indicated in the inset. (n=4 rats/group). (e and f) Representative images of co-staining for amylin and collagen IV (Col IV) in kidney tissue sections showing amylin deposition in arterioles and interstitial tissue (e) and in the glomerulus (f) in diabetic HIP rats but not in diabetic UCD rats (n=3 rats/group). (g) Representative images of co-staining for amylin and ionized calcium binding adaptor molecule 1 (IBA1) in kidney tissue sections from diabetic HIP and diabetic UCD

rats (n=3 rats/group). (h) Representative images of staining for the cluster of differentiation 68 (CD68; ED1) in kidney tissue section from diabetic HIP and diabetic UCD rats (n=3 rats/group). (Scale bar 50µm) *P<0.05; **P<0.01 by One-way ANOVA with Tukey's post-test (a and c).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 4. Modulation of renal hypoxia markers by amylin dyshomeostasis.

(a) EPO levels in plasma from 16 months WT rats (n=17), diabetic UCD rats (n=8) and diabetic HIP rats (n=17). (b) Percentage number of reticulocytes over total numbers of RBCs in blood of WT, HIP and UCD rats (n=3 for each rat group). (c) Hematocrit levels in diabetic HIP and UCD rats and WT controls (n=6 rats/group). (d-h) Protein levels of HIF-1 α (d), HIF-2 α (e), arginase-1 (f) and arginase-2 (g) and arginase activity (h) measured by ELISA in renal tissues from 16 months old WT rats and diabetic UCD and HIP rats (n=10 rats/group). *P < 0.05; **P < 0.01; ***P < 0.001 by One-way ANOVA with Tukey's post-test.

Fig. 5. Effect of increasing endogenous EETs on RBC-capillary coupling and renal hypoxia signaling.

(a) Average numbers of RBCs adhered to cultured vascular endothelial cells when fixed hematocrit of RBCs flowed over cultured EC at constant rate for constant time of 20 minutes (n=3/group). (b) Analysis of the adhesion of RBCs isolated from HIP rats and WT littermates to cultured vascular endothelial cells (n=8/group). (c) Adhesion of WT rat RBCs to cultured vascular endothelial cells with/without incubation with recombinant human amylin (50 μM) for 2 hours (n=5/group). (d) Attachment of RBCs from WT and diabetic HIP rats to vascular endothelial cells in the absence or in the presence of various amounts of

EETs (n=8 rats/group). (e) Representative images of staining for amylin and Col IV in kidney sections from diabetic HIP rats (HIP-UT) and diabetic HIP rats with pharmacologically upregulated EETs (HIP-T). The scatterplot shows the percentage of the tissue area that is positive for amylin (n=3 rats/group). (f-g) Protein levels of HIF-2 α (f) and HIF-1 α (g) in whole kidney tissue homogenate from rats in the HIP-UT and HIP-T groups (n=4 rats/group). *P <0.05; **P <0.01; ***P <0.001 by t-test.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 6. Altered oxygen sensing in kidneys following transfusion with amylin-loaded RBCs

(a) Plasma EPO levels in WT rats transfused with RBCs from i) WT rats (n=7); ii) diabetic UCD rats (n=7) and iii) diabetic HIP rats (n=7). (b and c) Protein levels of HIF-1α (b) and HIF-2α (c) in renal tissue homogenate from the rats described in (a). (d) Representative images of amylin and glycophorin A co-staining (left panel) and of amylin and hemoglobin co-staining (right panel) in RBCs from UCD rats infused with aggregated human amylin (daily injection of 0.08 μg/g body weight for 7 days; n=3 rats/group). (e) Plasma EPO levels in diabetic UCD rats (n=3) at baseline and at the end of the acute intravenous treatment with

aggregated human amylin (hA). (f-g) Protein levels of HIF-2 α and HIF-1 α (f) and arginase-1 and arginase-2 (g) in renal tissue homogenates from diabetic UCD rats injected with human amylin versus non-injected diabetic UCD control rats (n=3 rats/group). (h) Cartoon illustrating the proposed mechanism through which “human” hyperamylinemia exacerbates hypoxia signaling in diabetic rats with pancreatic expression of non-amyloid forming amylin rat amylin (UCD rats). *P <0.05; **P <0.01; ***P <0.001 by One-way ANOVA with Tukey’s post-test (a-c) or two-tailed t-test (e-g).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1.

Characteristics of the individuals providing the RBC samples, including age, sex, body

Sample size	n = 353	
	Disease	Healthy (n=66)
Age	55±1 years	43±2 years
Female sex	127(45%)	29(45%)
Obesity (BMI>30)	116(41%)	14(21%)
Heart Failure	108(38%)	
Type-2 Diabetes	69(24%)	
Cancer	91(32%)	
Stroke	13(5%)	
Type-1 Diabetes	5(2%)	

Body mass index (BMI), diabetes status and co-morbidities.