Altered Extracellular Vesicle Concentration, Cargo and Function in Diabetes Mellitus

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ABSTRACT
Type 2 diabetes mellitus is a chronic age-associated degenerative metabolic disease that reflects relative insulin deficiency and resistance. Extracellular vesicles (EVs; exosomes, microvesicles and apoptotic bodies) are small (50-400 nM) lipid-bound vesicles capable of shuttling functional proteins, nucleic acids, and lipids as part of intercellular communication systems. Recent studies in mouse models and in cell culture suggest that EVs may modulate insulin signaling. Here, we designed cross-sectional and longitudinal cohorts of euglycemic, pre-diabetic and diabetic participants. Diabetic individuals had significantly higher levels of EVs in their circulation than euglycemic controls. Using a cell-specific EV assay, we identified that erythrocyte-derived EVs are higher with diabetes. We found that insulin resistance increases EV secretion. Furthermore, the levels of insulin signaling proteins were altered in EVs from individuals with high levels of insulin resistance and β-cell dysfunction. Moreover, EVs from diabetic individuals were preferentially internalized by circulating leukocytes. Cytokine levels in the media and in EVs were higher from monocytes incubated with diabetic EVs. Microarray of these leukocytes revealed altered gene expression pathways related to cell survival, oxidative stress and immune function. Collectively, these results suggest that insulin resistance increases the secretion of EVs, which are preferentially internalized by leukocytes and alters leukocyte function.
INTRODUCTION

One of the most important medical challenges is the epidemic of diabetes mellitus type 2. It is estimated that 9.4% of the United States population (~30 million) are affected by this disease (1), while older individuals and different racial groups are affected disproportionately. Individuals with diabetes have an increased risk for age-associated comorbidities including hypertension, dementia, renal disease, and chronic heart disease (2). Therefore, it is important to understand how diabetes contributes to the development and severity of these age-associated diseases. Furthermore, diabetic individuals have impaired cellular insulin signaling and accumulating data suggest that circulating factors may also contribute to the disease.

Recent data indicate that membrane-bound extracellular vesicles (EVs; 30-400 nm) are important mediators of intercellular communication among different tissues and organs. EVs, found in most bodily fluids, contain proteins, RNA and lipids, and can be characterized into at least one of three groups. Exosomes are released through the fusion of multivesicular bodies with the plasma membrane. Microvesicles are released by budding of the plasma membrane and apoptotic bodies are shed from dying cells (3). EVs have a role in physiological and pathological intercellular signaling and in a wide spectrum of biological functions (3).

While EVs have been studied in the context of many diseases (4), little is known about EVs in the context of human diabetes mellitus. Studies in the db/db diabetic mouse model demonstrated that EVs from adipose tissue activate macrophages and promote expression of IL-6 and TNF-α (5), suggesting that EVs from diabetic mice may convey inflammatory signals. EVs from insulin resistant mice modulate insulin signaling in skeletal muscle (6) and pancreatic β-cells (7) further suggesting a role for EVs in insulin
signaling in mice. Recent evidence shows that adipose tissue, macrophage-derived exosomes may modulate insulin resistance in mice through transfer of specific miRNAs (8).

Studies investigating the interplay between EVs and diabetes mellitus in humans have been predominantly *in vitro* studies. EVs derived from adipocyte cell lines or explants *ex vivo* were incubated with a hepatocyte and skeletal muscle cell line. However, the results have not confirmed the effects on insulin signaling (9). Additionally, EVs from human adipocyte-explants have been shown to modulate the release of inflammatory cytokines in macrophages (10).

Thus far, human data has primarily investigated the relationship between larger microparticles isolated at slow centrifugation speeds or by FACS (11) and Type 2 diabetes (12-14). For example, circulating endothelium-derived microparticles (EMPs) (15) increase with type 2 diabetes (12-14). However, EVs in the circulation are derived from many different cell types and vary extensively in size. Specifically, EVs encompassing the size range of exosomes, microvesicles and apoptotic bodies have not been investigated extensively in the context of diabetes in humans.

To investigate whether these previously reported changes *in vitro* and in animal models were consistent with changes in diabetic individuals, we examined different EV characteristics in cross-sectional and longitudinal cohorts of both euglycemic and diabetic individuals.

**Research Design and Methods**

**Clinical Study Participants**
Three sub-cohorts of euglycemic and diabetic individuals were selected from the Healthy Aging in Neighborhoods of Diversity across the Life Span (HANDLS) study of the National Institute on Aging Intramural Research Program, National Institutes of Health (NIH). HANDLS has been approved by the Institutional Review Board of the National Institute of Environmental Health Science, NIH. All participants provided written informed consent. HANDLS is a longitudinal study comprised of community-dwelling participants investigating the role of race and socioeconomic status on the development of age-associated health disparities. Diabetic individuals met one of the following three criteria: (1) previous diagnosis by health care provider, (2) currently taking medication for diabetes, or (3) fasting serum glucose >125mg/dL. Plasma samples were collected after overnight fasting.

Cross-sectional cohort 1 comprised of 16 euglycemic and 22 diabetic individuals and cross-sectional cohort 2 comprised obesity-matched euglycemic and diabetic individuals (n=30/group) (Table 1, Fig. 1A,B). Individuals were classified based on BMI as underweight/normal (<25), overweight (25 - <30), obese class I (30 - <35) and obese class II/III (35+). Underweight and normal individuals were grouped together due to a low number of underweight participants. We chose 58 obesity-matched individuals for the longitudinal cohort with blood samples at two times ~5 years apart (4.95 ± 0.23) and were grouped as follows: euglycemic at both times (n=19), euglycemic at time 1 and diabetic at time 2 (n=19) and pre-diabetic at time 1 and diabetic at time 2 (n=20) (Table 2, Fig. 1C).

HOMA-B and HOMA-IR were calculated based on fasting serum glucose and fasting insulin levels (16), and were divided into quintiles for our analysis.
ExoQuick\textsuperscript{TM} EV Isolation

A fasting plasma sample was obtained as detailed previously (17). Plasma EVs were isolated from either 0.5 ml or 0.25 ml (cross-sectional cohort 2) using ExoQuick\textsuperscript{TM} Exosome Precipitation Solution (System Bioscience Inc.) as described (17) and resuspended in either 0.5 ml or 0.25 ml, respectively. This isolation method provides more reproducible results than either differential ultracentrifugation or size exclusion columns (17) and also is more suitable for EV isolation from large human cohorts.

Differential Ultracentrifugation EV Isolation

EVs were isolated from plasma and conditioned media via differential ultracentrifugation as described (18) and detailed in the Supplemental Materials.

Immunoblotting

EVs (10\mu g) samples lysed in MPER\textsuperscript{TM}, 3T3-L1 pre-adipocyte cell line lysate, and two plasma ExoQuick\textsuperscript{TM} EV-depleted supernatant samples were subjected to SDS-PAGE and immunoblotted with antibodies described in the Supplemental Materials.

Electron Microscopy

Electron Microscopy was performed as described in Supplemental Materials.

Nanoparticle Tracking Analysis
Isolated EV samples were diluted 1:300 and 1:50 in 0.2 µM filtered PBS for EVs isolated by ExoQuick™ and differential ultracentrifugation, respectively. Size distribution and concentration was analyzed using nanoparticle tracking analysis (NTA) on a NanoSight NS500 (Malvern Instruments Ltd.). Samples were recorded at camera level=14, detection level=3 and 5 videos of 20 seconds with a coefficient of variance <20% were used for analysis. To ensure measurement accuracy all samples were analyzed during the same time frame for each cohort/experiment on a single instrument by the same operator. All analyses were performed blinded to disease status. Total EV concentration from plasma was calculated as previously described (17).

**Cell-specific EV Assay**

Streptavidin-coated black plates (ThermoFisher) were coated with biotin-labeled primary antibodies (5 µg/ml) against cell-specific markers (see Supplemental Materials) in PBS/0.5% BSA at 4°C overnight. After blocking in PBS/5% BSA, plates were washed with PBS. Plasma EVs from cross-sectional cohort 2 were labeled with 0.1 µM (f.c.) PKH26 (Cat#1077 Sigma-Aldrich) and excess dye was removed as previously described (17). The remaining volume (150 µl) was diluted 1:3 in PBS and 40 µl was added to each well and incubated for 2 hrs at room temperature. After washing 3X with PBS, the PKH26 signal was read by a fluorescent plate reader (Excitation=540nm, Emission=570nm). Each sample was performed in duplicate and the average intensity was used for analysis.
Primary Neuronal Cell Culture

Cortical neurons were dissected from embryonic Sprague Dawley rats (E18) (Animal Protocol # 263-LNS-2019) as previously described (19). This research was approved by the NIA Animal Care and Use Committee and was performed according to guidelines in the NIH Guide for the Care and Use of Laboratory Animals. Cells were maintained in Neuralbasal medium containing 1 mM d-glucose and B27 without insulin (Thermo Scientific). On either Day In Vitro (DIV) 6 or 7 cells were treated with either media containing 200 nM Novolin R100 insulin or no insulin for 48 or 24 hours. Media was collected and fresh media either containing 200 nM insulin or no insulin was added for 30 minutes, after which media was collected. Wortmannin was added for both the 24 hr and 30 min time points. Cells were washed with PBS, collected and pelleted at 500 X g for 10 minutes and lysed with MPER™.

ELISAs

Equal volume of lysed EVs were used for quantitative protein measurement by the following Meso Scale kits (Meso Scale Discovery): Insulin Signaling Panel Phospho Protein (Cat#K15151A-1), AKT Signaling Panel Whole Cell Lysate Kit (Cat#K15177D-1), and Phospho-IRS-1 Base Kit (Cat#K150HLA-1). Leptin receptor was analyzed by a kit from R& D systems (Cat#DY389).

PBMC Internalization Assay

Human peripheral blood was collected by the Health Apheresis Unit and the Clinical Core Laboratory, the NIA, under Human Subject and Tissue Procurement Protocols. All
participants provided written consent and the protocols have been approved by the IRB of the NIEHS, NIH.

Peripheral blood mononuclear cells (PBMCs) were incubated with PKH26-labeled plasma EVs for 24 hours and cells were sorted as previously described (17). For the experiments in Figure 5 and Figure S3, two PBMC donors were used aged 47 and 60 respectively. They were both female, non-allergic and one was a smoker.

For gene expression changes, PBS or PKH26-labeled EVs from diabetic or euglycemic individuals were pooled together and incubated with PBMCs (female, 38 yr old with no allergies) for 24 hours. Following the incubation, monocytes were isolated using the Monocyte Isolation Kit II (Miltenyi Biotec). RNA was isolated with Trizol (Thermo Fisher Scientific) including a DNase treatment.

**Microarray and RT-qPCR**

Total RNA quantity and quality was tested using the Agilent Bioanalyzer RNA 6000 Chip (Agilent, Santa Clara, CA). Monocyte gene expression was analyzed by microarray using Illumina HumanHT-12 v4 (San Diego, CA). Microarray was performed and analyzed as previously described (20) and available at GEO: GSE105167.

For RT-qPCR analysis, two PBMC donors were used aged 38 and 46. They were both female, non-allergic and one was a smoker. PBMCs were incubated for 6 hrs with euglycemic or diabetic EVs pooled from donors from cross-sectional cohort 2. Monocytes were sorted as above and then incubated in media containing EV-depleted FBS (17). After 12 hrs, media and monocytes were separated by centrifuging at 500 x g. Monocyte total RNA was isolated and reverse transcribed using random hexamers and
reverse transcriptase and RT-qPCR was performed using gene-specific primers (Supp. Table S1) and SYBR green master mix on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Gene expression was normalized to the average of *HPRT* and *UBC*.

Media from above was collected after the 2,500 x g centrifugation and EVs were then isolated by ultracentrifugation as described in the Supplemental Materials. EVs were lysed in M-PER. Cytokines from EVs and media were measured on a Human Pro-inflammatory Panel I (K15049D; MesoScale Diagnostics) according to manufacturer’s instructions.

**Statistics**

Statistical analyses were performed using R version 3.3.2 (21). Concentration values were positively skewed and thus log (natural logarithm) transformed for testing in linear models. An ANOVA was used to test the mean log concentration for the cross-sectional cohort. Linear mixed models, accounting for the matching across BMI groups, were used to examine the longitudinal cohort and included age, sex and race.

**RESULTS**

**Higher EV concentration with type 2 diabetes mellitus**

To assess differences in circulating EVs with type 2 diabetes mellitus, we designed cross-sectional and longitudinal cohorts of both euglycemic and diabetic individuals (Table 1-2, Fig. 1). Isolated plasma EVs were validated according to the International Society of Extracellular Vesicles guidelines (22). Known EV markers were present in each of the EV samples and absent in EV-depleted plasma (Fig. 2A). The electron microscopy image
shows intact, round vesicles of approximately 50-200 nm (Fig. 2B). A similar size
distribution was also confirmed by nanoparticle tracking analysis (NTA) with a peak
around 175nm (Fig. 2B,C). These data confirm the size, morphology and protein markers
that are characteristic of EVs.

We sought to determine whether there were differences in plasma EV
concentration and size between euglycemic and diabetic individuals. An analysis of a
cross-sectional cohort consisting of both groups revealed a significantly higher EV
concentration in diabetic individuals compared to euglycemic individuals ($P=0.022$; Fig.
2D). To further validate these results and to exclude the possibility that obesity
contributes to higher EVs levels in diabetic individuals, we designed a second cross-
sectional cohort matched on obesity status (Table 1). A linear mixed model regression
analysis of EV concentration revealed a significant interaction between diabetes and race,
such that diabetic individuals had significantly higher levels of circulating EVs than
euglycemic individuals among white participants (Fig. 2E).

Although lipoproteins may co-precipitate with EVs, the significant relationship of
diabetes and race on EV concentration was unaffected by LDL or HDL levels in the
model. This suggests that the relationship between diabetes and EV concentration is
stronger than what could be explained by differences between lipoprotein levels.
Additionally, neither HDL nor LDL levels were significantly greater in diabetic
individuals in any of the cohorts (Table 2), suggesting that lipoproteins do not contribute
significantly to EV concentration.

To determine whether the onset of diabetes was associated with changes in EV
concentration, we examined EV concentration in our longitudinal cohort (Fig. 1C).
Participants who developed diabetes over the time period had elevated EV plasma concentration on average compared to the euglycemic group (Fig. 2F). The group that had pre-diabetes and developed diabetes had the highest EV concentration over time compared to controls ($P=0.01$). There were no significant changes in EV size between diabetic and euglycemic individuals.

To further confirm our results obtained using precipitation isolation methods, we used a protocol recently reported by Kowal et al. to compare EVs recovered from different steps of the differential ultracentrifugation protocol (18). Diabetic individuals had higher concentration of EVs isolated at both medium ($10,000 \times g$) and ultracentrifugation ($120,000 \times g$) speeds (Fig. 2G). Thus, diabetes status is associated with higher levels of circulating EVs that include both classically-defined exosomes and microvesicles according to isolation procedure.

Since plasma EVs are a heterogeneous population and little is known about the cell-origin of plasma EVs, we wanted to determine whether diabetes results in higher levels of EVs-derived from specific cells. To do this, we developed an antibody-based assay to compare cell-specific marker expression on the surface of intact EVs. We found that diabetic individuals have significantly higher levels CD235a-positive (erythrocyte) EVs and a trend towards higher CD68-positive (leukocytes) and CD62p-positive (platelet/endothelial cells) EVs (Fig. 2H). Levels of CD146-positive EVs (endothelial cells) were comparable between the two groups.

**Diabetes alters insulin signaling proteins in EVs**
We used ELISAs to measure proteins from lysed EVs. We identified 12 proteins (phospho-p70S6K(Thr389), phospho-S6RP(Ser240/244), phospho-GSK3β(Ser9), phospho-AKT(Ser473), phospho-insulin receptor(IR;Tyr), phospho-IRS1(Ser312), tyr-phospho-IRS1, phospho-IGF-1R(Tyr), Leptin Receptor, and FGF21) involved in cellular insulin signaling in EVs. Leptin receptor and phospho-IR levels were decreased in EVs of diabetic individuals (Fig. 3A).

Next, we examined the relationship between EV proteins and homeostatic model assessment (HOMA) in this cohort. HOMA-B and HOMA-IR quantitatively measure β-cell function and insulin resistance, respectively. Linear mixed model regression revealed that HOMA-B and HOMA-IR were significantly associated with EV concentration and with various insulin signaling proteins found in EVs (Fig. 3B). Examination of cross-sectional changes at time 2 revealed that higher HOMA-B levels were significantly associated with lower levels of phospho-S6RP, phospho-GSK3β and phospho-AKT in EVs. Higher HOMA-IR was associated with lower phospho-S6RP in the cross-sectional analysis and higher FGF21 levels in the longitudinal and cross-sectional analyses (Fig. 3B). This indicates that EV cargo is affected by the different components of diabetes and may reflect the nature of the disease.

**Insulin resistance increases EV secretion**

Higher levels of circulating EVs in diabetic individuals can be a consequence of several pathological causes. We wanted to test whether insulin resistance was related to this increase in circulating EVs. We chose to use primary neuronal cells as a model because accumulating evidence links insulin resistance in the brain to neurodegenerative disorders.
including Parkinson’s and Alzheimer’s diseases (23; 24). Moreover, little is known about the underlying factors that contribute to neuronal insulin resistance. AKT signaling was increased by acute insulin treatment (30 min) (Fig. 4A). Consistent with insulin resistance, neurons pre-treated for 48 hours with insulin and then treated with an acute insulin treatment had reduced capability to activate AKT signaling (Fig. 4A).

To examine the effects of prolonged insulin exposure on EV secretion from cortical neurons, we collected conditioned media after 30 mins from each of the treatment groups. EVs were isolated and purity was assessed by immunoblotting for known EV markers as well as a negative control Calnexin (Fig. 4B). NTA of EVs showed a peak around 140 nm (Fig. 4C). EV concentration was significantly higher in the media collected from cells exposed to insulin for 48 hours then treated with an acute insulin treatment (Fig. 4D). Insulin treatment for 48 hours alone did not alter EV concentration (Fig. 4D). Insulin-signaling protein levels were not significantly altered in the neuronal EVs after chronic or acute exposure to insulin (Fig. S1). To exclude the effects of apoptotic bodies in the media, we assessed cell viability under different treatment conditions and found no significant differences (Fig. S2). Collectively, these data indicate that hyperinsulinemia induces insulin resistance in cortical neurons leading to increased EV secretion.

Given that autophagy may play a role in neuronal insulin signaling in response to hyperinsulinemia (25), we treated insulin resistant primary cortical neurons with wortmannin as it is a PI3K/AKT class III inhibitor and other autophagy inhibitors can increase autophagy under nutrient-stressed conditions (26). Cells exposed to hyperinsulinemic conditions for 24 hours showed higher levels of the autophagy marker
LC3 II than untreated cells and this was attenuated by treatment with wortmannin (Fig. 4E). Wortmannin significantly decreased EV secretion in insulin resistant neurons under these conditions (Fig. 4F), suggesting that autophagy may be important for the increased neuronal EV secretion in response to insulin resistance.

**EVs from diabetic individuals are preferentially internalized by circulating leukocytes**

Diabetes is often associated with a heightened immune response, thus we wanted to explore if EVs contributed to this pathology. Previously, we developed a FACS-based method to quantify EV internalization in cells that interact with circulating EVs: monocytes and B cells (17). Here we chose to use this method to identify whether EVs from euglycemic or diabetic individuals are differentially internalized by leukocytes and alter signaling pathways. Monocytes (defined as classical (CD14++CD16PKH⁺), non-classical (CD14CD16⁺PKH⁺) and intermediate (CD14⁺⁺CD16⁺PKH⁺)) and B cells (CD19⁺PKH⁺) internalized EVs from diabetic individuals more readily than EVs from euglycemic individuals (Fig. 5A-D, Fig. S3-S6). EVs from diabetic individuals did not significantly alter the surface levels of activation markers for B cells (CD25, CD80, MHC-II) and monocytes (MHC-II, CD80) (Fig S4-S7).

To test whether other inflammatory signals are altered by internalization of diabetic EVs, we isolated RNA from monocytes incubated with EVs from euglycemic or diabetic individuals. Cells not treated with EVs were also used as an additional control. We performed a genome-wide microarray to assess gene expression changes and analyzed biological pathways. All significant pathways are included in Fig. S8, while we
focused primarily on pathways related to cell survival, oxidative stress and immune functions as these occupy critical niches in type 2 diabetes (Fig. 6A). EVs from diabetic participants increased genes that are related to immune function and inflammation (Fig 6A). Apoptosis (CRADD, DDIT3, BBC3 and DEDD2) and oxidative stress pathways and genes (GSTP1, SOD2, NCF1 and PARK7) were downregulated in monocytes exposed to diabetic EVs (Fig 6A,B).

To validate these results, we analyzed monocyte expression of selected genes by RT-qPCR. To confirm changes in inflammatory pathways, we collected the media and EVs from monocytes. Incubation of monocytes with EVs from diabetic individuals decreased expression of apoptosis and oxidative stress-related genes and increased the levels of several cytokines in the media and in the EVs (Fig. 6C-D). Overall, these data indicate that EVs are internalized and affect signaling pathways in circulating leukocytes.

**DISCUSSION**

Several studies have characterized the role that EVs play in modulating insulin signaling in mouse models (7; 27; 28) and in vitro studies (29-31); however, few studies examine EVs in type 2 diabetes using human cohorts. Here we report differences in EV concentration, cargo and function in three cohorts of diabetic and euglycemic individuals.

In our cross-sectional analyses, we observed higher levels of plasma EVs from diabetic individuals compared to euglycemic controls. Longitudinally, individuals who were diagnosed as pre-diabetic during visit 1 and developed diabetes by visit 2 had a greater plasma EV concentration than participants who were euglycemic at both visits. These data corroborate previously reported findings that have shown that diabetic
individuals have higher levels of EMPs, a subset of large EVs with endothelium specific markers, in the circulation than euglycemic controls (12-14). Importantly, our assay examining cell-specific markers indicate that diabetic individuals have higher levels of circulating CD235a-positive EVs. A trend towards higher levels of CD68-positive and CD62p-positive EVs was also observed. These data enhance available information about the variety of cell types that contribute to EVs in the circulation and how these EVs change with diabetes mellitus.

In humans, we found that EV concentration was positively correlated with HOMA-IR suggesting that insulin resistance in vivo may contribute to higher EV levels. To test these findings, we exposed primary neurons to hyperinsulinemic conditions. In response to prolonged exposure to insulin, cells developed reduced insulin signaling and increased EV secretion. These data indicate that insulin resistance contributes to the higher EV concentration associated with diabetes. We also found that autophagy may play a role in this pathway.

EVs are readily internalized by cells (Fig. S9) and elicit functional changes in target tissues and modulate insulin signaling in other tissues (6). Of the 12 insulin-signaling proteins we measured, the levels of the leptin receptor and phospho-IR were decreased longitudinally in EVs from participants who progressed from euglycemic to diabetic compared to euglycemic controls. It has been previously reported that diabetes status is positively correlated with levels of soluble insulin receptor (SIR) in the blood (32). Although this may appear contradictory to the data reported here, it is difficult to know how changes in SIR relate to the changes in phospho-IR within EVs. Consistent
with our data, an inverse correlation between soluble leptin receptor levels and the risk of developing type 2 diabetes has previously been reported (33).

Phospho-AKT and phospho-GSK3β were negatively associated with HOMA-B while phospho-S6RP was negatively associated with both HOMA-B and HOMA-IR in our cross-sectional analysis of time 2 (Fig. 3). Consistent with our findings in EVs, levels of activated proteins involved in AKT signaling are decreased in insulin resistant tissue (34). It is possible that the changes in proteins shuttled within EVs in the circulation directly reflects changes in the cells of origin in response to insulin resistance and β-cell function.

EVs from diabetic individuals were preferentially internalized by monocytes and B cells compared to euglycemic individuals. Gene expression analysis showed exposure to diabetic EVs resulted in upregulation of anti-apoptotic genes in monocytes. As apoptosis regulation is a major factor in monocyte differentiation to dendritic cells (35), decreased apoptosis driven by diabetic EVs may result in increased monocyte differentiation.

Diabetic EVs also appeared to dampen processes related to oxidative stress response. Our data show a decrease in biological processes related to the regulation of cell redox homeostasis, glutathione metabolism and response to hydrogen peroxide. Genes involved in oxidative stress management were decreased in our analysis. Low levels of the antioxidant SOD2 in monocytes have previously been shown in individuals with type 2 diabetes (36). These data suggest that internalization of EVs from diabetic individuals inhibits oxidative stress response pathways in monocytes, which may affect phagocytosis function.
Previous studies have implicated EVs as a possible mediator of inflammation and immune crosstalk in diabetic murine models (5) and insulin resistant tissue (30; 31). Specifically, adipose-derived EVs from both mouse and human explants increased IL-6 production in macrophages (5; 30). Here, we observed an increase in IL-2, IL-4 and IL-12p70 in EVs and IL-2 in the media from monocytes treated with EVs from diabetic individuals. These data suggest that EVs from diabetic individuals may affect inflammatory pathways.

These results demonstrate that EV concentration is higher in diabetic individuals and EVs may be important signaling mediators in type 2 diabetes. Further defining the mechanism, we found that insulin resistance drives EV secretion. We have also shown alterations in the protein content of EVs that may serve as possible biomarkers for this chronic disease. EVs from diabetic individuals are more readily internalized and affect monocyte function. Collectively, these data highlight the importance that EVs play as diagnostic tools as well as mediators of type 2 diabetes.

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AUTHOR CONTRIBUTIONS. NNH, EE, DF conceived and designed the study with help from MPM and MKE. DF, NNH, EE and JG executed the experiments. MB performed PBMC EV internalization assay. EL, YZ and KGB performed and analyzed gene expression microarrays. NM and ABZ performed statistical analysis. ABZ and MKE are co-principal investigators for HANDLS. Research was conducted in the laboratories of AB, MPM and MKE. DF and NNH wrote the manuscript with input from all the authors. MKE is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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**Figure Legends**

**Figure 1.** Cohort design. (A,B) Cross-sectional cohort 1 and 2 of euglycemic and diabetic individuals. (C) Longitudinal cohort design of euglycemic, pre-diabetic and diabetic individuals.

**Figure 2.** Higher plasma EV concentration in diabetic individuals. (A) Plasma-derived EVs isolated from 6 individuals (2 from each longitudinal group), two EV-depleted plasma samples and cell lysate from 3T3-L1 cells were subjected to SDS-PAGE and probed for EV-enriched proteins (B) Electron microscopy of EVs isolated from plasma exhibit expected morphology and size. Scale bar=500 nm. (C,D) EVs were isolated from the cross-sectional diabetes cohort and concentration and size distribution were analyzed using nanoparticle tracking analysis (NTA). Size distribution was averaged for each group. The area under the curve in (C) is shown in (D). $P<0.022$ by linear mixed model regression. (E) EV concentration for cross-sectional cohort 2. $P<0.016$ between white euglycemic (Eu) and diabetic (DM) individuals by linear mixed model regression. AA=African American (F) EV concentration in a longitudinal cohort showed a significant difference between the Eu $\rightarrow$ Eu and PreDM $\rightarrow$ DM groups ($P=0.01$) ( Eu=Euglycemic, PreDM=prediabetic and DM=diabetes mellitus). The histogram in (D) and line in (F) represent the predicted value from linear mixed model regression. P value was determined by linear mixed model regression on log transformed values. (G) NTA analysis of plasma EVs isolated from euglycemic (n=6) and diabetic (n=6) individuals from cross-sectional cohort 1 using differential ultracentrifugation. EVs were isolated from both the 10K and 120K fractions as indicated, **$P<0.01$. (H) Antibodies against the
cell-specific markers were used to capture intact euglycemic and diabetic (n=22/group) PKH-labeled EVs from the cross-sectional cohort 2. Fluorescent intensity was measured and log transformed values are shown. Dashed white line indicates average IgG signal (n=3) for each assay. $P<0.05$ by Student’s t-test.

**Figure 3.** Insulin signaling proteins are shuttled in EVs and are affected by diabetes status. (A) EV protein levels of the leptin receptor and phospho-IR were measured using ELISAs and the lines represent the predicted values from linear mixed model regression. $P=0.01$ for leptin receptor and $P=0.051$ for pIR for Eu→Eu group (n=19) compared to the Eu→DM (n=19) group (Eu=Euglycemic PreDM=prediabetic and DM=diabetes mellitus). (B) EV protein levels and concentration were quantified in the longitudinal cohort at both times (Eu→Eu n=19 and Eu→DM n=19). A cross-sectional analysis was performed using time 2 (Eu=19, DM=39). The relationship with HOMA-B and HOMA-IR were analyzed using linear mixed model regression. Significant changes are indicated and the direction of change are indicated by the up and down arrows.

**Figure 4.** Prolonged exposure to insulin impairs insulin signaling in primary cortical neurons and increases EV secretion. Neurons were untreated, pre-treated with insulin (200 nM) for 48 hrs and then either untreated or treated for 30 min with insulin (200 nM) or treated only for 30 min with insulin as indicated. (A) Neurons were lysed and immunoblotted with anti-phosphorylated AKT (p-AKT), total AKT or actin antibodies. (B) The conditioned media was collected from treated neurons and the EVs were isolated by differential ultracentrifugation. EVs were lysed and analyzed by SDS-PAGE along
with cell lysate from primary cortical neurons. Samples were probed using antibodies for positive and negative EV markers. (C, D) NTA was used to analyze the size distribution and concentration of EVs isolated from conditioned media from treated and untreated neurons. (n=4). (E) Neurons treated as above were also incubated with 200nM wortmannin. Cell lysates were collected and immunoblotted with an anti-LC3 antibody. Arrow indicates LC3 II and numbers indicate the ratio of LCII/LC1. Actin was used as a protein loading control. (F) EVs were isolated from the conditioned media and the concentration was measured using NTA (n=3). All histograms represent the mean ± S.E.M. ** P < 0.01, *P < 0.05 by Student’s t-test

**Figure 5.** EVs from diabetic individuals are preferentially internalized by circulating leukocytes. (A) Plasma EVs (6 x 10^8) from diabetic (n=39) and euglycemic (n=19) individuals from the longitudinal cohort at time 2 were incubated with PBMCs (~200,000 cells/well) for 24 hours. Cells positive for EV internalization (PKH^+) were sorted into B cells (CD19^+PKH^+) (B) Classical monocytes (CD14^{++}CD16^{PKH^+}) (C) Non-classical monocytes (CD14^{CD16^{++}}PKH^+) and (D) Intermediate monocytes (CD14^{++CD16^{PKH^+}}). The histograms represent means ± S.E.M. Statistical significance was assessed by linear mixed model regression on the log transformed values to account for skewness of the data. ***P < 0.001.

**Figure 6.** EVs from diabetic individuals alter gene expression in monocytes. (A) Plasma EVs were pooled (4.5 x 10^{11}) from several individuals from the longitudinal cohort at time 2 and grouped as either diabetic (n=2; DM) or euglycemic (n=3; EU). Cells not
treated with EVs were used as another control (UNT). EVs were incubated with PBMCs for 24 hours and monocytes were isolated. Gene expression was assessed by microarray and Gene Ontology analysis was performed. Heat map shows significant pathways (P<0.01) related to apoptosis, immune response, oxidative stress and vesicle formation. (B) Top 20 significant downregulated and upregulated genes from the pathways in A are shown. A p-value cutoff of <0.05 was used for significance. Labeling represents control group vs. treatment group. (C,D) After incubation with euglycemic or diabetic EVs from cross-sectional cohort 2 (n=3/group), total RNA and media was collected from the monocytes. Gene-specific primers were used for RT-qPCR analysis of genes from the microarray (C). (D) EVs were isolated from the media using ultracentrifugation, lysed and run along with EV-depleted media on a cytokine panel. Histograms represent the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 by Student’s t-test.
Table 1. Clinical Characteristics of Cross-Sectional Cohorts

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cross-Sectional Cohort 1</th>
<th>Cross-Sectional Cohort 2</th>
<th>P-Value</th>
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<td></td>
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<tr>
<td>N</td>
<td>16</td>
<td>22</td>
<td>30</td>
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<tr>
<td>Age</td>
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<tr>
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<tr>
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<td>9 (56.2%)</td>
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Mean ± SD is shown for continuous variables and analyzed by one-way ANOVA. Chi-squared goodness of fit test was used to analyze differences for categorical variables.
Table 2. Clinical Characteristics of Longitudinal Cohort

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Mean ± SD is shown for continuous variables and analyzed by one-way ANOVA. Chi-squared goodness of fit test was used to analyze differences for categorical variables.
Figure 1

A **Cross-sectional Cohort 1**
- Euglycemic (N=16)
- Diabetic (N=22)

1. Isolate EVs from plasma
2. NTA

B **Cross-sectional Cohort 2**
- Euglycemic (N=30)
- Diabetic (N=30)

1. Isolate EVs from plasma
2. NTA, Cell-type specific EV assay, PBMC internalization

C **Longitudinal**

**Visit 1**
- Euglycemic (N=19)
- Euglycemic (N=19)
- Pre-Diabetic (N=20)

5 Years

**Visit 2**
- Euglycemic
- Diabetic
- Diabetic

1. Isolate EVs from plasma
2. NTA, Protein Analysis, PBMC Internalization
**Figure 2**

A. Western blot analysis showing the expression of various proteins in different conditions.

B. Electron micrograph depicting nanoparticles.

C. Size distribution graph showing the particle size in nanometers.

D. Bar graph representing the particle concentration in parts per million.

E. Box plot showing the variation in log concentration among different groups.

F. Line graph depicting the predicted log concentration over age.

G. Bar graphs showing the particle concentration in 10k and 120k fractions.

H. Bar charts illustrating the log intensity of different markers.

References:

- Lysate
- EV Depleted
- Eu→Eu
- Eu→DM
- PreDM→DM

Proteins detected:

- ALIX
- Flot1
- TSG101

Markers analyzed:

- CD35a
- CD146
- CD88
- CD62p
Figure 3

A

Log Leptin Receptor vs. Age

Eu → Eu
Eu → DM
PreDM → DM

B

Cross-sectional at Time 2

↓ pS6RP
↓ pGSK3β
↓ pAKT

Longitudinal
↑ Concentration

Cross-sectional at Time 2

↓ pS6RP
↑ FGF21
↑ Concentration

Longitudinal
↑ FGF21
↑ Concentration
Figure 4

A

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<td>AKT</td>
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<td>Actin</td>
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B

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C

Size (nm) vs. Particles/mL

D

Size (nm) vs. Particles/mL

E

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<tr>
<td>Actin</td>
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<td>+</td>
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F

Size (nm) vs. Particles/mL

**Diabetes**
Figure 6

A. Relative Protein

B. Z-Score

C. Relative Expression

D. Relative Protein and Media

** Positive Regulation of Anti-Apoptosis
Regulation of Apoptosis
Inactivation of MAPK Activity
JAK STAT Cascade
Inflammatory Response
Cytokine Production
T Cell Differentiation
Positive Regulation of T Cell Activation
T Cell Activation
Lymphocyte Activation
Macrophage Activation
Positive Regulation of B Cell Differentiation
Positive Regulation of IL-2 Biosynthesis
Positive Regulation of IL-6 Biosynthesis
Regulation of Cell Redox Homeostasis
Glutathione Metabolic Process
Response to Hydrogen Peroxide
Response to DNA Damage Stimulus
Endocytosis
Vesicle Fusion
Transcription
Translation

** Relative Expression

** Relative Protein and Media

** Z-Score

** Relative Expression

** Relative Protein and Media
Online Supplemental Materials Data

Altered Extracellular Vesicle Concentration, Cargo and Function in Diabetes Mellitus

David W. Freeman, Nicole Noren Hooten, Erez Eitan, Jamal Green, Nicolle A. Mode, Monica Bodogai, Yongqing Zhang, Elin Lehrmann, Alan B. Zonderman, Arya Biragyn, Josephine Egan, Kevin G. Becker, Mark P. Mattson, Ngozi Ejiogu, and Michele K. Evans

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Figure S1. EV neuronal cargo after insulin treatment. (A) EVs were isolated from primary cortical neurons treated with either no insulin (untreated), insulin for 48 hours (chronic), insulin for 48 hours followed by 30 minutes with fresh insulin (chronic + acute) or no insulin for 48 hours followed by 30 minutes with fresh insulin (acute). (B) Primary cortical neurons were incubated with fresh media for 48 hours either containing no insulin (untreated) or 200nM of insulin (chronic). The vesicles were lysed and protein content was analyzed using the AKT signaling panel II and insulin signaling kits from MesoScale Diagnostics. The histograms represent the mean + SEM (n=4 for A and n=3 for B).
**Figure S2.** Cell viability of primary cortical neurons. **(A)** Primary cortical neurons were treated with insulin (200nM) for the indicated times. Cell viability was measured using a MTT assay. **(B)** Neurons were treated as in (A) in the absence or presence of wortmannin. Each experiment was performed in triplicate. The histogram represents the mean + SEM from 3 independent experiments.
Figure S3. PBMC internalization of EVs from diabetic and euglycemic individuals. (A) PBMCs (~200,000 cells) were incubated with plasma EVs (3 x 10⁸) from diabetic (DM, n=39) and euglycemic (Eu, n=19) for 24 hours. Cells were sorted by FACS into B cells that had internalized EVs (CD19⁺PKH⁺) (B) classical monocytes that had internalized EVs (CD14⁺⁺CD16⁻PKH⁺) (C) non-classical monocytes that had internalized EVs (CD14⁻CD16⁺PKH⁺) and (D) intermediate monocytes that had internalized EVs (CD14⁺⁺CD16⁺PKH⁺). The histograms represent the mean ± S.E.M. The data was log transformed and significance was calculated using linear mixed model regression taking into account matching. ***P<0.001
Figure S4. FACS gating for EV internalization assays. PBMCS were incubated with PKH26-labeled EVs as described in Research Design and Methods. Cells were sorted into PKH+ and PKH- T Cells (CD3+), B Cells (CD19+), intermediate monocytes (CD14++CD16+), classical monocytes (CD14++CD16-) and non-classical monocytes (CD14-CD16+PKH+). Representative FACS plots are shown.
Figure S5. FACS sorting controls for EV internalization assay. (A,B) Representative FACS plots of B Cells (CD19⁺) sorted for either PKH-26, CD80, CD25 or MHC-II are shown. Samples were stained for IgG control (A) or specific antibodies (B). (C-E) Representative FACS plots of monocytes (CD14⁺) sorted into (C) classical monocytes (CD14⁺⁺CD16⁻), (D) intermediate monocytes (CD14⁺⁺CD16⁺) or (E) non-classical monocytes (CD14⁻CD16⁺). Cells were incubated with either PBS and stained with a CD14 isotype control or incubated with PKH-labeled EVs and stained with a Pacific Blue conjugated CD14 antibody as denoted on the plots.
Figure S6. Monocyte internalization of diabetic and euglycemic EVs from the microarray experiment. (A) PKH26 labeled EVs from several individuals were pooled (4.5 x 10^{11}) and grouped as either diabetic (DM, n=3) or euglycemic (Eu, n=3) and incubated with PBMCs for 24 hours. PBS with PKH26 was used as a negative control (UNT, n=3). A small aliquot was taken for FACS analysis while RNA from the remaining cells was isolated and used for microarray. The FACS aliquot was sorted into B cells that had internalized EVs (CD19^{+}PKH^{+}) (B) classical monocytes that had internalized EVs (CD14^{++}CD16^{-}PKH^{+}) (C) non-classical monocytes that had internalized EVs (CD14^{+}CD16^{+}PKH^{+}) and (D) intermediate monocytes that had internalized EVs (CD14^{++}CD16^{+}PKH^{+}). Histograms represent the mean + SEM. Statistical significance was calculated using student’s T test. *P<0.05.
**Figure S7.** PBMC activation levels following EV internalization. PBMCs (~200,000 cells) were incubated with plasma EVs (3 x 10^8) from diabetic (DM, n=39) and euglycemic (Eu, n=19) for 24 hours and cells were sorted by FACS. (A) B cells that had internalized EVs expressing either CD25 (CD19+PKH+CD25+), CD80 (CD19+PKH+CD80+) or MHC-II (CD19+PKH+MHC-II+). (B-D) Monocytes were subdivided into either (B) classical monocytes that had internalized EVs (CD14++CD16−PKH+CD25+) (C) non-classical monocytes that had internalized EVs (CD14−CD16+PKH+) and (D) intermediate monocytes that had internalized EVs (CD14++CD16−PKH+) and the mean fluorescent intensity (MFI) for MHC-II and CD80 were measured.
Figure S8. Significant biological pathways altered in monocytes by EVs from diabetic individuals. Plasma EVs (4.5 x 10^{11}) from diabetic (DM, n=2) or euglycemic (Eu, n=3) individuals were incubated with PBMCs for 24 hours or untreated (UNT). Monocytes were isolated using FACS and gene expression was assessed via microarray. Significant biological pathways (P<0.01) are visualized on the heat map.
Figure S9. EVs are internalized by cells. Similar EV internalization assays were performed as we previously reported (Eitan E. et al., NPJ Aging Mech. Dis. 2016; Zhang, S. et al., Neuro. Biol. of Aging 2018). Primary cortical neurons were incubated with PKH26-labeled EVs in a ratio ~300 vesicles per neuron for 2 hours. Neurons were washed twice with PBS and fixed in 4% paraformaldehyde/PBS for 20 min. Fixed cells were incubated in blocking solution (0.3% Triton X-100 and 10% normal goat serum in PBS) for 30 min, and then incubated overnight at 4°C with antibodies against the neuronal marker MAP2 (Hm2, Sigma M9942). Cells were washed three times and incubated with Alexa Fluor 488 tagged anti-mouse secondary antibodies (Invitrogen) in blocking solution for 1 hr. The cells were then washed twice with PBS and nuclei were stained with DAPI (Sigma #32670). Coverslips were washed and mounted on slides in an anti-fade medium (Vector Laboratories, Burlingame, CA, USA). Images were acquired using a Zeiss LSM 510 confocal microscope with a 40 × objective. A representative confocal image of EV internalization is shown.