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Platelet proteome changes associated with diabetes and during platelet storage for transfusion

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Abstract

Human platelets play a key role in homeostasis and thrombosis and have recently emerged as key regulators of inflammation. Platelets stored for transfusion produce pro-thrombotic and pro-inflammatory mediators implicated in adverse transfusion reactions. Correspondingly, these mediators are central players in pathological conditions including cardiovascular disease, the major cause of death in diabetics. In view of this, a mass spectrometry based proteomics study was performed on platelets collected from healthy and type-2 diabetics stored for transfusion. Strikingly, our innovative and sensitive proteomic approach identified 122 proteins that were either up- or down-regulated in type-2 diabetics relative to non-diabetic controls and 117 proteins whose abundances changed during a 5-day storage period. Notably our studies are the first to characterize the proteome of platelets from diabetics before and after storage for transfusion. These identified differences allow us to formulate new hypotheses and experimentation to improve clinical outcomes by targeting “high risk platelets” that render platelet transfusion less effective or even unsafe.

Keywords

Proteomics; platelet storage; diabetes; transfusion; mass spectrometry

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Introduction

Circulating blood platelets are small anucleate cells derived from cytoplasmic fragmentation of bone marrow megakaryocytes. Platelets play a key role in hemostasis and thrombosis, as well as in diseases such as myocardial infarction, stroke and deep vein thrombosis. Indeed millions of Americans are disabled or die each year as a consequence of pathologic platelet activation. Recently, it was recognized that platelets are key regulators of inflammation due to their release of potent proinflammatory and prothrombotic mediators such as CD40 ligand (CD154; CD40L) thromboxane A_2 (TXA_2), IL-1, etc.[1–5] Further interest in platelets arises from the fact that millions of doses of platelets are transfused to patients with cancer, blood diseases, trauma, and those undergoing cardiac surgery.

There is an emerging realization that platelets from type-2 diabetics circulate in a partially activated state, which may predispose diabetics to vascular disease.[6, 7] Furthermore, while blood donors with diabetes are routinely accepted for donation for transfusion, there are no data to determine whether platelets from diabetic donors are more or less efficacious or safe for recipients. Platelets can be stored for up to five days prior to transfusion. However, this results in a "platelet storage lesion" that includes partial platelet activation and release of bioactive proteins and lipid mediators (i.e., CD40L, IL-1, PPARγ, and TXA_2).[1–4, 8] These protein and lipid changes have been partially characterized and may contribute to serious post-transfusion adverse effects such as potentially fatal transfusion-related acute lung injury (TRALI) or multi-organ failure.[9] Thus, there is a great need to better understand platelet function and to develop new insights into their proteome, ultimately leading to the development of new therapeutic strategies and disease biomarkers.

Even though platelets lack nuclear DNA they have a substantial and diverse transcriptome derived from the progenitor megakaryocyte and contain rough endoplasmic reticulum and polyribosomes, thereby retaining the capacity for protein biosynthesis from existing mRNA[10–13]. While quiescent platelets exhibit only limited translational activity, platelet activation leads to signaling-dependent translation of existing mRNA.[14]. Modern mass spectrometry based proteome capabilities allow for characterization of very large numbers of cellular proteins simultaneously.[15, 16] Our strategy for analysis of human platelet proteins involves fractionation and purification of platelets, solublization of all proteins, trypsin digestion, chromatographic separation with direct coupling through electro-spray ionization into a tandem mass spectrometer,[17–20] followed by statistical evaluation of ion current measurement to estimate changes in protein abundances relative to the appropriate controls.[21] While our proteomics approach is excellent for identifying a large number of proteins and their variation in our samples, information regarding the functional status of these proteins is difficult to extrapolate from this data. Thus, comprehensive characterization of our proteomic results will be integrated with other experimental methods to correlate our proteomic data to biological activity. This novel approach allowed us to identify for the first time not only proteomic differences in normal versus platelets from type-2 diabetics, but also to determine how these proteins changed during the typical 5-day platelet storage.

Materials and methods

Platelet collection and purification

Whole blood was obtained (500 mL) according to an IRB approved protocol in the University of Rochester Blood Bank from male and female donors (ages 20–70) that were free of drugs known to alter platelet function, such as aspirin or non-steroidal anti-inflammatory agents, for two weeks prior to donation. Normal donors had a body mass index (BMI) ≤25 and were well-characterized with regard to normal platelet aggregation and coagulation profiles and other mediators indicative of general good health. Diabetic

patients had a body mass index (BMI) ≥30 and at least one of the following criteria: random blood glucose > 200 mg/dL, elevated fasting glucose >106 mg/dL or impaired glucose tolerance during an oral glucose tolerance test. We considered donors whose physicians diagnosed them “diabetic” and if they also had at least two fasting glucose measurements that were over either 126 mg/dl (two samples) or 106 mg/dl (single donor). Both criteria are in use.

Blood was collected by venipuncture into a citrate phosphate dextrose adenine solution containing collection bag (Baxter Fenwal, Round Lake, IL). Platelets were prepared and stored by the whole blood method as used prior to transfusion in our Blood Bank/ Transfusion Medicine Unit. In brief, platelet-rich plasma (PRP) was obtained by centrifugation (all centrifugations at 20–24°C) at 2,000 × g for 15 min. Platelet concentrates (PCs) were prepared by mechanically expressing the supernatant into an integrally attached plastic bag after centrifugation. The platelet rich plasma was then centrifuged at 4,300 × g for 10 min to form a platelet pellet. Residual plasma was expressed to leave approximately 50–70 mL for resuspension of the platelets by gentle mechanical agitation after a one hour rest period. Platelet concentrates were maintained at room temperature (22–26°C) throughout the storage period (5 days) with gentle agitation. Residual leukocytes were reduced to 5×10^6 or less by filtration (Pall Purecell LRF, Pall Corporation, East Hills, NY) and the platelet concentrate was gamma irradiated at 2,500 rads (CIS-US IBL 437 Blood Irradiator) prior to storage to mimic typical clinical practice in our FDA registered transfusion service.

Preparation of platelet lysates

A 15 mL aliquot of stored platelet concentrate was removed at days 0 (4–6 h post venipuncture), and 5 and centrifuged (950 × g for10 min). The platelet concentrate supernatant was removed and stored at −80°C. The platelet pellet was gently washed in Krebs-Ringer Bicarbonate Buffer (KRB) (Sigma, St. Louis, MO) pH 7.4 containing 15 mM sodium bicarbonate and 19 mM sodium citrate, centrifuged (950xg/10 min), and the wash saved. The platelet pellet was resuspended in KRB pH 7.4 and platelets were counted on an Abbott Cell-Dyn 1700 (Abbott Park, IL). The purity of the platelets was approximately 99.9% of cells present. The final platelet suspension was centrifuged (950 × g for10 min) and the second wash removed and saved. Platelet pellets were lysed using nonidet P-40 lysis buffer containing a protease inhibitor cocktail (4-(2-aminoethyl)-benzenesulfonyl fluoride, pepstatin A, transepoxysuccinyl-L-leucylamido (4-guanidino) butane, bestatin, leupeptin, and aprotinin; Sigma). Total protein was quantified with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL).

Integrin $\alpha_2\beta_1$ Analysis

Stored platelet concentrate samples were collected and centrifuged at 1000 × g for 5 min., and the platelets resuspended in PAB (1x PBS/1% BSA/0.1% sodium azide). Platelets were fluorescently labeled with anti-CD42a (BD Biosciences, San Jose, CA) and anti-integrin α_2/ β_1 (Abcam, Cambridge, MA) antibodies and relative expression of each molecule assessed on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar, Inc., Ashland, OR). Platelets were identified by their relative size and CD42a positive expression, then the intensity of the integrin α_2/β_1 expression calculated using the analysis software.

Platelet Spreading

Glass cover-slips were pre-treated with a suspension of fibrinogen (100 μg/mL) in phosphate buffered saline (PBS) for 1 hr at room temperature, washed with PBS and blocked with BSA (5mg/mL in PBS) for 1 h. Stored platelet concentrate samples were collected and applied

directly to the coated cover-slips for 45 min at 37°C, washed as above and fixed with 4% PFA. Platelet spreading was imaged using Normarski differential interference contrast optics with an Olympus BX51 microscope (Melville, NY). Photographs were taken using a SPOT camera with SPOT RT software (New Hyde Park, NY).

Sample preparation for MS

A 100 μg aliquot of platelet protein from each sample in 100 μl of 0.1mM ammonium bicarbonate buffer was desalted on Zeba spin columns (Pierce); this procedure removed excess detergent, protease inhibitors and salts.[19] The resulting eluates were made up to 4 M urea and warmed to 37°C for 10 min. Dithiothreitol was added to 10 mM final concentration and iodoacetamide added (0.25 mM final concentration) and the samples incubated for 1 h. The samples were diluted (1:4) with water to give 1.0 M urea, trypsin was added at 1:50 (wt:wt; trypsin:platelet protein) and the samples incubated at 37°C for 1 h. These samples were stored at −80°C prior to the next purification.[17, 22] The peptide samples were thawed, acidified with formic acid to pH 4.0, applied to a strong cation exchange column and eluted with methanol:water:ammonium hydroxide (80:15:5). The eluting peptides were collected and evaporated to approximately 50 μL and 10 μL of 100 mM ammonium bicarbonate buffer was added. The samples were brought to 100 μL with deionized water, pH 7.0 and stored at −80°C prior to analysis by mass spectrometry. Protein and peptide concentrations were determined using the BCA assay using BSA as the standard. Completeness of the tryptic digestion was confirmed using 4–12% Bis-Tris PAGE gel separation.

Capillary LC-MS/MS spectrometry

The trypsin digested platelet samples were analyzed using an automated custom-built capillary HPLC system linked to an LTQ Orbitrap mass spectrometer (ThermoElectron, San Jose, CA) equipped with an electrospray ionization interface[20]. A reversed phase capillary column was prepared from 3μm Jupiter C18 particles (Phenomenex, Torrence, CA) into 75 μm i.d. × 70 cm fused silica capillary (Polymicro Technologies, Pheonix, AZ). The mobile phase consisted of 0.2% acetic acid and 0.05% TFA in water (A) and 0.1% TFA in 90% ACN/10% water (B)[18]. Samples (2.5 μg total peptide mass) were analyzed over a mass (m/z) range of 400–2000. The six most abundant ions from MS analysis were selected for MS/MS analysis using a collision energy setting of 35%. Dynamic exclusion was used to discriminate against previously analyzed ions.

After MS analysis the data were processed by SEQUEST version v.27 (rev. 12) using a 36 node cluster and the human IPI fasta protein list (v3.39, 2/07/2008).[23, 24] The settings included: dynamic oxidation of methionine (+15.9949 Da), ±3 Da parent mass window, ± 1.0 Da fragment ion tolerance, up to 4 missed internal proteolytic cleavages, and no proteolytic enzyme requirement. SEQUEST results were filtered according to the criteria established by Yates et al.[25–27] Peptides singly charged must have an Xcorr greater than 1.9 and doubly and triply charged peptides must have Xcorr equal to or greater that 2.2 and 3.75, respectively. Peptide identifications were imported into a Microsoft SQL database and peptide masses re-calculated according to IUPAC values (including modifications when identified). High resolution MS level data deisotoped was employed using an in-house implementation of the THRASH algorithm (Decon2LS, see http://omics.pnl.gov/software/ for details). Normalized elution times (NET) were calculated according to neural net algorithm previously described[28, 29] and used to enhance peptide identifications. LC-MS features identified via the in-house VIPER software[30] such that mass and NET values for eluting peptides are recorded along with their integrated abundance values. LC-MS features were then compared back to previously identified peptide sequences to provide sequence and protein information for each. Peptides and their associated proteins were collated into spreadsheet format. Protein redundancies from the IPI fasta were handled by associating

peptides with the first occurrence of a given protein in the file. In those cases where uniquely identifying peptides were found, the protein associated with that peptide was chosen for grouping.[31] This list of peptides, proteins, and their abundances were subsequently grouped for statistical evaluation.

Statistical methods

The biological study featured 45 MS analyses from 6 subjects including 3 non-diabetics, and 3 diabetics prepared fresh (Day 0), or after 1, 3, or 5 days of storage (Table 1). The samples from treatment groups were analyzed in 8 blocks (5 or 6 samples per block). This organization provided data for samples from both non-diabetic and diabetic individuals within each block allowing for control of LC-MS processing effects as well as the effects for time of data acquisition. Mean protein abundances were extracted from peptide ion current data using a mixed-effects linear statistical model.[21] Since the sample abundance distributions varied over analysis time from the overall mean abundance, with a slight downward trend over time, sample abundances were adjusted by subtracting the difference between a sample's geometric mean abundance and the overall geometric mean abundance calculated from abundance values for the 252 peptides observed in 43 or more of the 45 LC-MS injections. The LC-MS data set was parsed into protein-specific subsets. A two-stage fit of a mixed effects linear model was applied to each subset to estimate a protein's relative concentrations using methods that we reported previously.[21] The model included diabetes, sample storage time and peptide identity as fixed effects factors and also diabetes by sample storage time interactions. Previously calculated LC-MS whole sample effects were included as covariate while blocking was considered a random effect. Using the mixed effects model and restricted maximum likelihood estimation (REML), a two stage fit accounted for the variability among a protein's abundances with parameters associated with either diabetes, sample storage time, or LC-MS processing. Criteria for significantly up- and down-regulated proteins included: 1) differences in means at a $P < 0.05$ level, and 2) elimination of proteins with 95% confidence intervals that include 1.0 (either above or below). Proteins listed in the tables meet both of these criteria.

Results

Overall description

There were 844 protein identifications using our mass spectrometry based proteomics approach after removal of redundancies. Sufficient data was obtained for statistical analysis of 537 of these proteins using the mixed-effects linear model (i.e., at least 9 peptide observations for any given protein identification; Table 2). The data were partitioned to identify those proteins with significantly altered abundances ($P \leq 0.05$) with respect to diabetes or storage time. This approach resulted in identification of 122 proteins that were related to diabetes and 117 proteins that changed during the 5-day storage period.

Protein changes attributable to diabetes

Since peptide ion current data were normalized to the appropriate non-disease controls, proteins that were up- or down-regulated could be identified. This approach identified 25 up-regulated and 97 down-regulated proteins in the disease group (Table 2). The cellular location of these proteins were determined using UniProt and are summarized in Figure 1. The proteins were associated with many subcellular locations with the largest number associated with the cytoplasm, membrane, nuclear (in anucleated cells), cytoskeleton and extracellular compartments. For the diabetic group 34 proteins possessed enzymatic activity and approximately 53% of these were localized to the cytoplasm. Thirty one percent of the 38 membrane associated proteins contained transmembrane (TM) domains; most were

single pass proteins. The cellular location for 8 of the 122 proteins with altered abundance is unknown.

When the 122 diabetes related proteins were evaluated using information from UniProt, several protein groups were apparent (Table 3). First the relative concentrations of several hemoglobin subunits were elevated in diabetics by as much as 28.9 fold (abundance ratio range, 5.7–28.9). This was seen in all samples from diabetics and not seen in those from non-diabetics suggesting that it is an effect of diabetes. Another group of proteins, associated with carbohydrate metabolism, localized to both the cytoplasm and mitochondria. Three proteins from this group (glycogen phosphorylase, down-0.41; pyruvate kinase, down-0.67; 6-phosphofructokinase, up-1.54) are allosterically regulated enzymes that catalyze rate limiting steps in carbohydrate metabolism. Other down-regulated proteins involved in glycolysis included lactate dehydrogenase, triosephosphate isomerase, enolase, and transaldolase from the pentose-phosphate pathway. Similarly isocitrate dehydrogenase, ATP synthase and malate dehydrogenase, all mitochondrial enzymes involved in energy production, were down-regulated. Platelet glycoprotein 1b, a platelet membrane receptor comprised of alpha and beta chains that participate in the initial adhesion to the subendothelium through binding to von Willebrand factor, was also down–regulated.[32] Other groups of protein included those that bind nucleotides, proteins that are involved in complexes, proteins that bind to actin, and proteins that inhibit proteases.

Using information from UniProt and RNA array databases,[33–35] we identified proteins from the diabetes group that have been shown to be associated with platelets (Table 4). One of these, extracellular matrix protein-1, was up-regulated in the diabetes group by 85% (abundance ratio 1.85) and functions to positively regulate I-kappaB kinase. Further, many proteins involved in blood coagulation were consistently down-regulated by approximately 25%. This included transmembrane receptors known as integrins (alpha-6 isoform) that complex with fibrinogen and other blood clotting factors that are involved in cell adhesion.

Protein changes attributable to storage time

The cellular locations for the 117 proteins with altered abundance from the 5-day storage group were similar to those of the diabetics group with the exception that there were fewer proteins associated with the nuclear and mitochondrial compartments (Figure 1). Twenty nine percent of the proteins with altered abundance were up-regulated (34) whereas 83 were down-regulated (Table 2). When UniProt information was used to help classify the 117 proteins, several differences were observed with respect to the diabetes group (Table 5). Most notably there were far fewer enzymes involved in carbohydrate metabolism (i.e., 5 vs. 14); all of these proteins were down-regulated in the storage group compared to 14% (2 of 14) that were up-regulated in the diabetes group. Additionally, there were no storage time changes in the three allosterically regulated enzymes (glycogen phosphorylase, pyruvate kinase, and 6-phosphofructokinase) that catalyze rate limiting steps in carbohydrate metabolism. Other groups of proteins that were unchanged with respect to the diabetes group included those that are involved in complexes, inhibit proteases, and bind to nucleotides or actin.

Using information from UniProt and RNA array databases for the 117 proteins with altered abundance from the 5-day storage group,[33–35] we identified 22 proteins that are known to be associated with platelets (Table 4). These proteins are involved in cell adhesion, blood coagulation, and platelet activation and signaling processes. Seven platelet-specific proteins have receptor activity or are associated with receptor complexes. It is noteworthy that nearly two thirds of the platelet-specific proteins identified as unique to storage time are known to be associated with the platelet alpha granule membrane. This observation supports platelet degranulation as a major factor in changes seen in stored platelets.[36–39] We also observed

that half of the platelet-specific proteins for the storage group localized to the extracellular compartment and 10 proteins are integral to the plasma membrane (i.e., contain one or more transmembrane domains). Three proteins (macrophage migration inhibitory factor, platelet factor 4, and platelet basic protein precursor) have chemokine, cytokine, and/or growth factor activities. One (i.e., CD9) is a platelet cell surface antigen.

Enrichment analysis

The MetaCore™ software package (GeneGO, Inc St Joseph, MS) was used to discover the gene ontology (GO) processes that were significantly enriched by platelet-related proteins (see Table 4) whose abundance is affected by either diabetes or storage for 5-days. Based on enrichment statistics, the 10 most likely GO processes associated with platelet proteins up- and down-regulated by diabetes are shown in Table 6. The first 6 are also the most likely GO processes associated with platelet proteins affected by 5 days of storage; however, the last 4 GO processes in Table 6 are not significantly enriched by proteins changed by storage. The main distinction between the enrichment analyses of the two groups of platelet proteins appears to be that proteins associated with tissue remodeling are more strongly correlated with diabetes (vs normal phenotype) than they are with the 5-day storage (vs fresh phenotype). The disease specific GO processes in Table 6 are related to abundance changes observed for extracellular matrix protein-1, integrin alpha-6, and junctional adhesion molecule A, which were not affected by storage.

We are aware that due to the small number of differentially abundant platelet-related proteins, biological mechanisms suggested by variations in enrichment statistics must be viewed with caution. Their greatest value will be in the formulation of new hypotheses that can be tested by additional experiments. With this in mind, we obtained and stored platelets from two additional normal and two diabetic donors to measure the levels of $\alpha_2\beta_1$ integrin. This receptor is involved in platelet adhesion to collagen,[40, 41] and functions in close concert with the central collagen receptor, GPVI, in the initial step of the hemostatic cascade to activate coagulant activity.[42] It was previously shown that patients with type-2 diabetes have enhanced platelet surface expression of GPVI compared with non-diabetic individuals.[43] Our preliminary experimental findings demonstrated that there was only a slight increase in the surface expression of $\alpha_2\beta_1$ in healthy platelets over the storage period, suggesting that storage time does not significantly effect this integrin (Figure 2). In contrast, platelets from diabetics exhibited an increase in $\alpha_2\beta_1$ surface expression at days 3–5 of storage. This result is interesting as our data revealed that the α_2 subunit of this integrin is down-regulated in both diabetic and stored platelets (0.68 and 0.71, respectively), while the β_1 subunit was up-regulated in stored platelets only (1.33). Our findings demonstrated that the changes in α_2 and β_1 subunit composition effected platelet from diabetics surface expression of $\alpha_2\beta_1$ compared to healthy platelets, and the change in surface expression of platelets from diabetics is at a pinnacle when platelets are typically transfused to patients (3–5 days). Future transfusion and mechanistic studies will be critical in determining the biologic and clinical significance of our findings. We will use the valuable information obtained from this experiment to direct our studies.

To begin investigating diabetic versus storage-specific effects, we used a static measure of cell adhesion and spreading. Since our results demonstrated integrin β_3 is down-regulated in the diabetes and storage groups (Tables 3 and 5), we used additional normal and diabetic donor platelets stored for 5 days (Figure 3) to look at platelet spreading on fibrinogen which requires the major platelet integrin, $\alpha_{IIb}\beta_3$.[44] We observed that normal healthy platelets prepared for storage have a reduced capacity to spread (compared to normal washed platelets - data not shown), and although this level of spreading is not significantly reduced over time, less platelets appear to adhere to the matrix (Figure 3, compare Normal-D0 to Normal-D5). In contrast, the D0 platelets from diabetics are beginning to form aggregates

(Figure 3, Diabetic-D0, arrow) and few are spread. By day 5, there was virtually no platelet spreading in the platelets from diabetics, just clusters of aggregates (Figure 3, Diabetic-D5, white squares), and like the normal platelets, adhesion appeared to be impaired. Future examination of platelet adhesion under flow conditions and using different matrixes will help to decipher the specific pathways involved in diabetic and/or storage mechanisms.

Discussion

This report describes a mass spectrometry based approach to platelet proteomics and additional investigational strategies into the role of transfusion storage, including donor glucose dysregulation (diabetes) effects on the platelet proteome. Technically, our approach enabled detection with great sensitivity of a variety of molecular species in stored and/or platelets from diabetics. However as a caution it should be remembered that our MS approach involves a bottoms-up characterization of trypsin digested proteins and that it is not a direct measurement of biological activity. Previous studies of platelet storage have employed other approaches, including sonication rather than detergent for lysing platelets, and restricting proteomic methods to those that are gel based.[45] These approaches have the limitation that they cannot be employed to characterize membrane molecules. Nonetheless, it is reassuring that previously reported increased abundance of septin and actin with storage was replicated in our findings. In a more recent publication, use of mass spectrometry and detergent lysis confirmed the original description of increases in septin with storage and also described increases in superoxide dismutase, Rho-GDP dissociation inhibitor, and zyxin, as well as enzymes involved in glucose metabolism (G6PD, hexokinase).[8]

New findings in our study show an increase in detectable molecular species, almost doubling the number previously reported. The variety of cell membrane species showing abundance changes in our study is noteworthy (Tables 3, 4 and 5). Furthermore, the presence of altered abundance of molecular species of nuclear origin confirms our previous observation that transcription factors are present in platelets, and play previously unsuspected roles in platelet biology. Our group recently demonstrated that platelets contain and release in platelet microparticles (PMPs), the transcription factor, peroxisome proliferator-activated receptor gamma (PPARγ) and its heterodimeric partner retinoid X receptor (RXR). PPARγ ligands attenuate platelet release of pro-inflammatory and pro-coagulant mediators including soluble CD40L (sCD40L) and thromboxane A_2 (TXA_2), and elicit transcellular effects.[46, 47] This provides evidence that transcription factors are capable of biologic activity and have important roles in platelet physiology.

Other novel findings include the first data on differences between the proteome of platelets from diabetics versus those from healthy individuals, as well as the effects of diabetes on the proteome of platelets prepared and stored for blood transfusion. Most notable is that fresh platelets from diabetic patients in otherwise good health demonstrated almost as many abundance changes as stored platelets from healthy donors (well over 100). Most of these changes are in the same direction, namely a decrease from normal abundance. However, the most striking deviations quantitatively are increases in the abundance of hemoglobin subunits. While these may represent contaminants due to sample red cell lysis during preparation, this increased abundance of hemoglobin species was seen in all platelet sample from diabetics and was not seen in the three platelet samples from non-diabetics. Consistent with our findings, globin and ferritin mRNAs have been identified as abundant transcripts in platelets by two independent groups.[35, 48] While these two facts taken together imply that platelets from diabetics have increased synthesis of these proteins, further investigation will be necessary to determine whether this is true or whether they take up increased amounts of hemoglobin from plasma or internalize it from interactions with red cells *in vivo*.

Interestingly, platelets from diabetics had lower abundance of several adhesion molecules including a number of integrins and PECAM. Diabetic and stored platelets were both characterized by an increased abundance of an endopeptidase inhibitor and decreased abundance of fibrinogen receptors and thrombospondin. Our supporting experiments reinforced these findings as diabetic and stored platelets had impaired ability to spread on fibrinogen (Figure 3). In contrast, increased abundance has been observed for some key collagen receptors. For example, the hemostatic cascade is initiated at sites of vascular injury by exposed extracellular matrix (ECM) and collagen, a key constituent. We found that extracellular matrix protein (ECM)-1 is significantly elevated in platelets from diabetics. Interestingly, ECM1 functions in the regulation of blood vessel formation and maintenance. It has been suggested that the influence on angiogenesis exerted by ECM1 may be important in wound repair[49] and diabetic retinopathy.[50] Moreover, it was previously shown that expression of the collagen-binding GPVI, a major platelet receptor involved in the development of thrombosis and atherosclerosis, is elevated in diabetics.[43] Excitingly, our supporting studies herein provided corroborating data showing that integrin $\alpha_2\beta_1$, central for stable adhesion to collagen following GPVI activation,[42, 51] has diabetic-specific effects (Figure 2).

Platelet storage over a five day period prior to transfusion, the FDA permissible duration, was characterized by some shared abundance changes with platelets from diabetics, and other unique changes in abundance. GPIX, an adhesion molecule, increased in abundance with storage, but most other receptors and adhesion molecules decreased in abundance, including GPIb, GPV, and GPIV. These findings suggest storage may decrease the functional capabilities of both diabetic and stored platelets, and impair interactions with endothelium and hemostatic molecules such as fibrinogen. These findings will need to be confirmed by techniques employing functionally viable platelets as we demonstrated here with our initial two supporting studies (Figure 2 and 3). This combined approach will assist in constructing a thorough understanding of the differences between diabetic and normal platelet physiology, and the role of storage in the “platelet storage lesion”.

Conclusion

In summary, these new findings describe a powerful new approach to assess the platelet proteome as it changes with disease and storage for transfusion. These findings delineate for the first time similarities of platelets from diabetics and after storage for transfusion that reflect altered platelet hemostatic and immunologic function. Given that diabetics have an increased risk of thrombosis, inflammatory disorders and decreased resistance to infection, the changes we describe suggest innovative mechanisms by which platelet function is altered in hyperglycemic states. It will be desirable to further investigate the suitability of diabetics as blood donors, and to evaluate their risks as transfusion recipients, given the evidence of alterations in multiple immunologic and hemostatic mediators seen even in freshly prepared platelets from diabetics. Similar to platelets from diabetics, platelets from healthy donors stored for several days also exhibit changes in abundance of multiple hemostatic and immunologic mediators. These findings are likely of clinical relevance, as platelet transfusions are associated with thrombosis, mortality[52] as well as inflammation.[1, 9]

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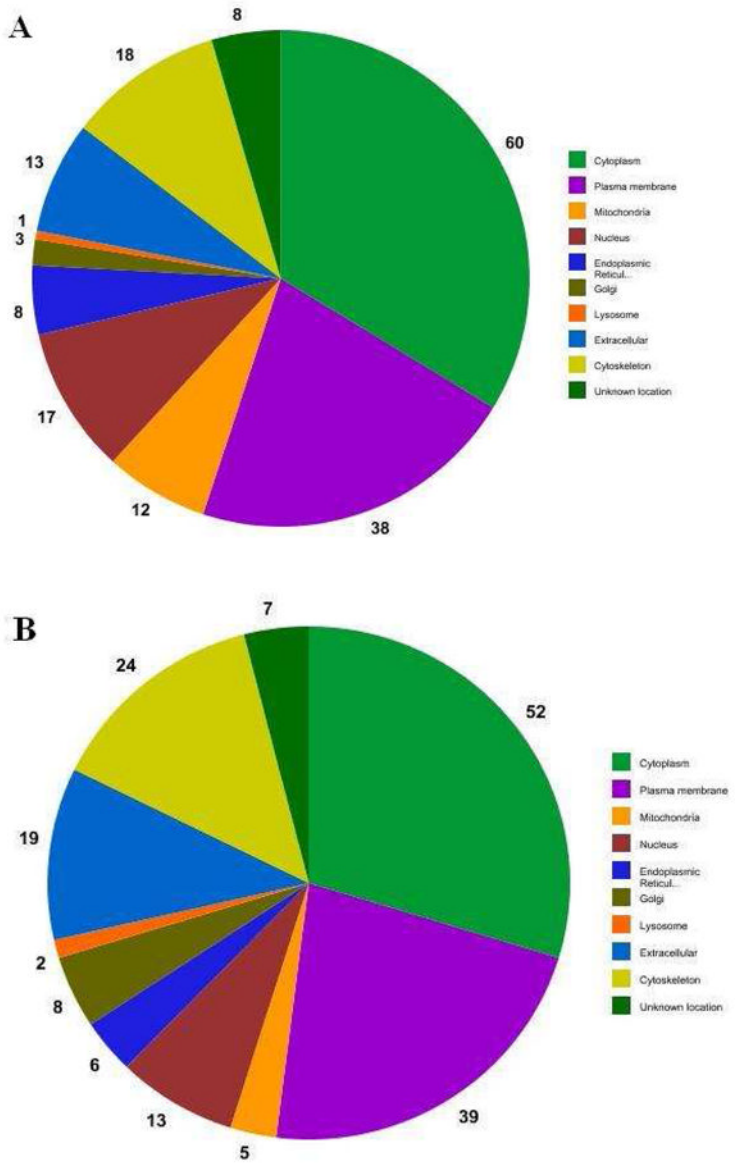

Figure 1.
Cellular locations of proteins observed with differential abundance due to diabetes (A) and after 5-days of storage (B).

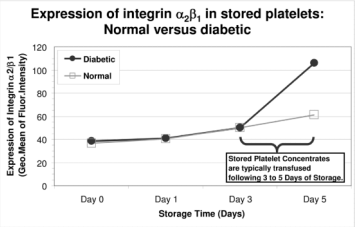

Figure 2. Stored diabetic platelets have increased levels of the collagen binding integrin, $\alpha_2\beta_1$

Platelet samples were collected at days 0, 1, 3, and 5 and analyzed by flow cytometry to determine the levels of the $\alpha_2\beta_1$ integrin. An increase in $\alpha_2\beta_1$ integrin levels was apparent between days 3 and 5 of storage relative to that for similarly stored platelets from healthy non-diabetics. The data shown are representative of two normal and two diabetic donors.

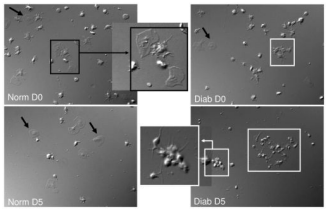

Figure 3. Normal and platelets from diabetics stored for transfusion have platelet spreading defects

Platelets from normal and diabetic donors were stored for transfusion and allowed to spread on fibrinogen-coated coverslips (100 μg/mL) blocked with 0.5% BSA. The coverslips were fixed in 2.5% glutaraldehyde and processed for imaging by DIC (100x). Black arrows illustrate a fully spread platelet. White squares indicate platelet aggregates. Center images show magnification of spread platelets (black square) and a platelet aggregate (white square). The results are representative of two normal and two diabetic donors.

Table 1

MS analysis frequency across disease and platelet storage time

Disease State	Subject	Sample Prep Day				Total
		0	1	3	5	
Non- Diabetic	1	3	1	2	2	8
	2	4	2	1	2	9
	3	3	2	1	2	8
Diabetic	4	1	1	1	1	4
	5	4	1	1	2	8
	6	3	2	1	2	8
Total		18	9	7	11	45

Table 2

Summary of MS identified platelet proteins following statistical analysis to identify proteins that were either up- or down-regulated proteins in sample from diabetics or following 5-days of platelet storage. A total of 844 proteins were identified by our MS based proteomics approach; for 537 of these proteins there was sufficient data for statistical evaluation.

		Diabetes	Storage time
Identifications		122	117
Abundance	-Up	25	34
	-Down	97	83

P≤ 0.05 was used to identify proteins with significantly altered abundance.

Table 3

Proteins with significantly altered abundances which are attributable to type-2 diabetes. D/N indicates the abundance ratio for diabetics to non-diabetics. Statistical significance ($p \leq 0.05$) was determined using the mixed-effects linear method as described in the methods section. Also shown are the number of peptide(s) seen by the MS and the number of times each peptide(s) was observed out of a total of 45 MS runs.

Uniprot	ProteinDescription	D/N	95% Conf. Int.	Peptides	# MS Runs	Location
Q9Y6D8	Mutant beta-globin	28.9	27.0–30.1	2	45	
P68871	Hemoglobin subunit beta	21.7	10.6–44.4	12	45	
P02008	Hemoglobin subunit zeta	21.3	17.1–26.4	3	45	
P02042	Hemoglobin subunit delta	19.2	11.3–32.5	15	45	
P69905	Hemoglobin subunit alpha	18.8	7.8–45.3	19	45	
P02100	Hemoglobin subunit epsilon	5.70	2.4–13.4	1	45	
Q9H532	Solute carrier family 17 (sodium phosphate)	3.15	2.0–4.8	1	17	Pm, ER
Q92673	Sortilin-related receptor precursor	3.14	2.1–4.8	1	13	Pm
Q9P173	Serine-type endopeptidase inhibitor, PRO2275	2.62	2.2–3.2	4	32	Ec
Q6PIH6	Immunoglobin kappa variable 1–5	2.44	1.4–4.3	3	17	Ec
Q6N097	Putative uncharacterized protein DKFZp686H20196	2.18	1.8–2.7	7	38	Ec
P54727	UV excision repair protein RAD23 homolog B	2.10	1.4–3.1	2	18	Cy, N
O43266	Extracellular matrix protein 1	1.85	1.3–2.6	1	15	Ec
P09972	Fructose-bisphosphate aldolase C	1.72	1.3–2.3	3	27	Cs
P07437	Tubulin beta chain	1.61	1.2–2.2	8	41	Cy, Cs
Q01813	6-phosphofructokinase type C	1.54	1.1–2.2	7	25	Cy
P02787	Serotransferrin precursor	1.48	1.1–2.0	7	17	Ec
P00918	Carbonic anhydrase 2	1.41	1.2–1.7	8	45	Cy
O00161-1	Synaptosomal-associated protein 23, isoform SNAP-23a	1.38	1.2–1.6	2	34	Pm
Q06323	Proteasome activator complex subunit 1	1.37	1.2–1.6	3	38	Cy
P37840-1	Alpha-synuclein, isoform 1	1.37	1.2–1.6	7	44	Cy, N
P47756	F-actin-capping protein, subunit beta	1.27	1.1–1.4	2	20	Cy, Pm
Q13404-4	Ubiquitin-conjugating enzyme E2 variant 1, isoform 4	1.24	1.2–1.3	2	42	Cy, N
Q59F97	Putative uncharacterized protein DKFZp686I04222	1.16	1.0–1.3	2	31	Cy
O00499-1	Myc box-dependent-interacting protein 1, isoform IIA	1.12	1.1–1.2	2	27	Cy, Cs, N
P68402	Platelet-activating factor acetylhydrolase IB subunit beta	0.90	0.9–0.9	2	42	Cy
P45880-1	Voltage-dependent anion-selective channel protein 2	0.89	0.8–1.0	2	39	M

Uniprot	ProteinDescription	D/N	95% Conf. Int.	Peptides	# MS Runs	Location
Q15257-3	Serine/threonine-protein phosphatase 2A regulatory subunit B, isoform 3	0.88	0.8–0.9	2	24	Cy
Q9BYZ2	L-lactate dehydrogenase A-like 6B	0.87	0.8–1.0	2	27	Cy
P47756-1	F-actin-capping protein subunit beta, isoform 1	0.87	0.8–1.0	2	38	Cy, Pm
O75083-2	WD repeat-containing protein 1, isoform 2	0.85	0.7–1.0	25	44	Cy, Cs
P48735	Isocitrate dehydrogenase [NADP]	0.84	0.7–1.0	16	43	M
Q5VVY1	Similar to Y74C9A.3	0.83	0.7–1.0	1	41	-
P35241	Radixin isoform b	0.82	0.7–1.0	9	44	Cy, Cs, Pm
P07737	Profilin-1	0.82	0.7–1.0	14	45	Cy, Cs, N
P25705	ATP synthase subunit alpha	0.82	0.7–0.9	18	44	M
O00299	Chloride intracellular channel protein 1	0.82	0.7–0.9	10	43	Cy, Pm
P60174-2	Triosephosphate isomerase, isoform 2	0.82	0.7–1.0	2	42	Cy
P12814	Alpha-actinin-1	0.82	0.7–0.9	61	45	Cy, Cs
Q9Y490	Talin-1	0.81	0.7–0.9	155	45	Cy, Cs, Pm
P35442	Thrombospondin-2 precursor	0.81	0.8–0.8	2	37	Ec
P18054	Arachidonate 12-lipoxygenase	0.80	0.7–0.9	10	43	Cy
Q14568	Heat shock protein 90 kDa alpha class A member 2 (Fragment)	0.80	0.7–1.0	2	36	Cy
P30740	Leukocyte elastase inhibitor	0.80	0.7–1.0	11	42	Cy
P48426	Phosphatidylinositol-5-phosphate 4-kinase type-2 alpha	0.79	0.6–1.0	4	43	-
P55072	Transitional endoplasmic reticulum ATPase	0.78	0.6–1.0	23	42	Cy, ER, N
P07947	Proto-oncogene tyrosine-protein kinase Yes	0.78	0.7–0.9	2	35	Cy, Pm
P02679-1	Fibrinogen gamma chain precursor, isoform Gamma-B	0.78	0.6–0.9	29	43	Ec, Pm
P34931	Heat shock 70 kDa protein 1L	0.77	0.6–1.0	4	37	Cy
Q59GD5	Actin-related protein 3-beta isoform 1	0.76	0.6–1.0	2	36	Cs
P13224	Glycoprotein Ib beta	0.76	0.6–0.9	5	44	Pm
P61586	Transforming protein RhoA precursor	0.76	0.6–0.9	4	32	Cy, Cs, Pm
P30101	Protein disulfide-isomerase A3 precursor	0.76	0.7–0.9	28	44	ER
P52566	Rho GDP-dissociation inhibitor 2	0.76	0.6–0.9	13	45	Cy, Cs, Pm
P27797	Calreticulin precursor	0.75	0.6–1.0	14	42	Ec, Cy, ER
P60953-1	Cell division control protein 42 homolog precursor, isoform 1	0.74	0.6–0.9	3	43	Cy, Pm
Q13418	Integrin-linked protein kinase	0.74	0.6–0.9	26	45	Cy, Pm
P30405	Peptidyl-prolyl cis-trans isomerase	0.73	0.6–1.0	4	43	M

Uniprot	ProteinDescription	D/N	95% Conf. Int.	Peptides	# MS Runs	Location
P50552	Vasodilator-stimulated phosphoprotein	0.73	0.6–0.9	12	44	Cy, Cs
P40227	T-complex protein 1 subunit zeta	0.73	0.6–1.0	6	21	Cy
O15143	Actin-related protein 2/3 complex subunit 1B	0.73	0.6–0.9	4	33	Cy
Q9GZV4	Eukaryotic translation initiation factor 5A-2	0.73	0.7–0.8	2	44	Cy
Q86UX7-2	Unc-112-related protein 2, isoform 2	0.72	0.6–0.9	38	45	Cy, Cs
P05106-1	Integrin beta-3 precursor, isoform Beta-3A	0.72	0.6–0.8	3	43	Pm
P99999	Cytochrome c	0.72	0.6–0.9	4	33	Cy, N, M
O95837	Guanine nucleotide-binding protein alpha-14 subunit	0.72	0.7–0.8	2	41	Pm
P08107	Heat shock 70 kDa protein 1	0.72	0.6–0.8	7	35	-
Q14315-1	Filamin-C, isoform 1	0.72	0.5–1.0	7	44	Cy, Cs, Pm
Q16843	Thromboxane A synthase 1	0.71	0.6–0.9	5	36	Pm
P14174	Macrophage migration inhibitory factor	0.71	0.7–0.7	2	27	Ec
Q9Y251	Heparanase precursor	0.71	0.6–0.8	4	45	Ec, Pm, L
O95866-1	Protein G6b precursor, isoform B	0.70	0.6–0.8	4	43	ER, G, Pm
Q15365	Poly(rC)-binding protein 1	0.70	0.6–0.8	3	38	Cy, N
P23229-1	Integrin alpha-6 precursor, isoform Alpha-6X1X2B	0.70	0.6–0.8	11	42	Pm
A8K1Z2	Glycerol-3-phosphate dehydrogenase, isoform 1	0.70	0.6–0.8	5	22	M
P07384	Calpain-1 catalytic subunit	0.69	0.6–0.8	9	41	Cy, Pm
Q9Y624	Junctional adhesion molecule A precursor	0.69	0.6–0.9	5	43	Pm
Q9NRW1	Ras-related protein Rab-6B	0.69	0.6–0.9	4	27	G, Pm
P17301	Integrin alpha-2 precursor	0.68	0.6–0.8	8	27	Pm
P23219-1	Cyclooxygenase 1b3	0.68	0.5–1.0	4	38	Cy, N, ER, Pm
P51452	Dual specificity protein phosphatase 3	0.68	0.5–0.9	3	19	N
Q9UJC5	SH3 domain-binding glutamic acid-rich-like protein 2	0.68	0.5–0.9	2	32	N
P48059	Similar to LIM and senescent cell antigen-like domains 1	0.67	0.5–0.9	1	43	-
P14618-1	Pyruvate kinase isozymes M1/M2, isoform M2	0.67	0.6–0.8	3	43	Cy
Q15404	Ras suppressor protein 1	0.67	0.5–0.9	18	45	-
P40926	Malate dehydrogenase	0.66	0.5–0.9	10	43	M
P10720	Platelet factor 4 variant precursor	0.66	0.6–0.7	2	45	Ec
P61604	Heat shock protein, 10 kDa	0.66	0.6–0.8	4	41	M
O14556	Glyceraldehyde-3-phosphate dehydrogenase	0.66	0.5–0.8	1	44	Cy

Uniprot	ProteinDescription	D/N	95% Conf. Int.	Peptides	# MS Runs	Location
P61158	Actin-related protein 3	0.66	0.6–0.8	6	36	Cy
P31146	Coronin-1A	0.66	0.5–0.8	9	42	Cy, Cs, Pm, N
P50395	Rab GDP dissociation inhibitor beta	0.65	0.5–0.9	3	43	Cy, Pm
Q9Y277-1	Voltage-dependent anion-selective channel protein 3, isoform 1	0.65	0.5–0.9	4	29	M
Q53FI7	Four and a half LIM domains 1 variant	0.65	0.5–0.8	3	36	Cy, N
Q9UI15	Transgelin-3	0.65	0.6–0.7	3	15	-
Q14019	Coactosin-like protein	0.64	0.5–0.8	8	41	Cy, Cs
O43707	Alpha-actinin-4	0.64	0.5–0.8	12	39	Cy, Cs, N
XP_064265	Similar to Myosin light polypeptide 6	0.64	0.6–0.7	2	45	-
P04632	Calpain small subunit 1	0.64	0.5–0.8	5	39	Cy, Pm
Q5SQ64	Lymphocyte antigen 6 complex locus protein G6f	0.63	0.5–0.8	3	39	Pm
P48047	ATP synthase subunit O	0.63	0.5–0.8	7	29	M
P09104	Gamma-enolase	0.62	0.6–0.7	5	44	Cy, Pm
P09486	SPARC precursor	0.62	0.5–0.8	4	36	Ec
P51659	Peroxisomal multifunctional enzyme type 2	0.61	0.5–0.7	3	21	M
O75915	PRA1 family protein 3	0.60	0.4–0.9	4	31	Cy, ER, Pm
Q9BSJ8-1	Extended-synaptotagmin-1, isoform 1	0.60	0.5–0.8	5	26	Pm
O75558	Syntaxin-11	0.60	0.5–0.8	4	34	G, Pm
P37837	Transaldolase	0.59	0.4–0.8	3	26	Cy
P30040	Endoplasmic reticulum protein ERp29 precursor	0.58	0.5–0.7	3	36	ER
O43396	Thioredoxin-like protein 1	0.58	0.5–0.7	1	35	Cy
P10124	Serglycin precursor	0.57	0.5–0.7	5	42	Ec
A6NMR3	Uncharacterized protein PECAM1	0.57	0.5–0.7	5	36	Pm
P38646	Stress-70 protein	0.56	0.4–0.8	1	25	Cy, M
P61088	Ubiquitin-conjugating enzyme E2 N	0.56	0.6–0.6	2	27	Cy, N
P16284-1	Platelet endothelial cell adhesion molecule precursor, isoform Long	0.53	0.4–0.6	9	37	Pm
Q96RI3	Short heat shock protein 60 Hsp60s2	0.52	0.4–0.7	2	19	-
P62979	UBB ubiquitin and ribosomal protein S27a precursor	0.50	0.4–0.7	2	28	Cy
Q9ULV4	Coronin-1C_i3 protein	0.49	0.3–0.8	6	23	Cs
Q9BVC6	Transmembrane protein 109 precursor	0.46	0.3–0.7	1	24	N
P11216	Glycogen phosphorylase	0.41	0.3–0.5	14	36	Cy

Uniprot	ProteinDescription	D/N	95% Conf. Int.	Peptides	# MS Runs	Location
P30443	HLA class I histocompatibility antigen	0.35	0.3–0.4	1	33	Cy, Pm
P21291	Cysteine and glycine-rich protein 1	0.30	0.2–0.4	2	29	N

The last column shows the sub-cellular location including: Cy = cytoplasm, Cs = cytoskeleton, Ec = extracellular, ER = endoplasmic reticulum, G = golgi, L = lysosome, M = mitochondria, N = nucleus, Pm = plasma membrane, - = unknown.

Table 4

Using information from mRNA databases and UniProt, proteins known to be associated with platelets were identified. These proteins were classified as either unique to diabetes, unique to storage or common to diabetes and storage. D/N indicates the abundance ratio for diabetics to non-diabetic, whereas D5/D0 is the abundance ratio for platelets stored for 5-days relative to those from freshly collected platelets. Statistical significance ($p \leq 0.05$) was determined using the mixed-effects linear method.

Identifier	Description - Function	D/N	D5/D0	Location
	Proteins Unique to Diabetes			
O43266	Extracellular matrix protein-1 - Signal transducer	1.85	NA	Ec
P60953-1	Cell division control protein 42 homolog Isoform 1 - GTPase	0.74	NA	Pm, Cy
P50552	Vasodilator-stimulated phosphoprotein - Actin binding	0.73	NA	Cs, Cy
P23229-1	Integrin alpha-6 Isoform a-6X1X2B - Receptor, calcium binding	0.70	NA	Pm
Q9Y624	Junctional adhesion molecule A - Protein binding	0.69	NA	Pm
P16284-1	Platelet endothelial cell adhesion-Long - Protein binding	0.53	NA	Pm
	Proteins Unique toStorage			
A6NBZ8	Uncharacterized protein ALB - Transport, apoptosis	NA	2.56	Ec
P14770	Platelet glycoprotein IX precursor - Cell adhesion, blood coagulation	NA	1.67	Pm
P14174	Macrophage migration inhibitory factor - Inflammation, prostaglandin biosynthesis, receptor	NA	0.89	Ec
P16671	Platelet glycoprotein IV - Cell adhesion, blood coagulation, lipoprotein transport	NA	0.82	Pm
P21926	CD9 antigen - Cell adhesion and motility, platelet activation	NA	0.74	Pm
P40197	Platelet glycoprotein V - Cell adhesion, blood coagulation, CD42D antigen	NA	0.41	Pm
P02775	Pro-platelet basic protein - Cytokine, growth factor, chemotaxis, glucose transport	NA	0.40	Ec
Q13201	Multimerin-1 precursor - Cell adhesion, blood coagulation	NA	0.36	Ec
P02671-1	Fibrinogen a Isoform 1 - Receptor	MA	0.67	Pm, Ec
P02675	Fibrinogen b - Receptor	NA	0.54	Cs, Cy
P07996	Thrombospondin-1 - Peptidase inhibitor; signal transducer; structural	NA	0.48	Ec
	Proteins Common to Diabetes and Storage			
Q9P173	Uncharacterized PRO2275 - Endopeptidase inhibitor	2.62	3.54	Ec
P13224	Glycoprotein Ib b - Receptor	0.76	1.53	Pm
P68402	Platelet-activating factor acetylhydrolase IB-b - Esterase	0.90	0.85	Cy
O95866-1	Protein G6b Isoform B - Receptor, Heparin binding	0.70	0.83	Pm, ER, G
P17301	Integrin alpha-2 precursor - Cell adhesion, integrin-mediated signaling, blood coagulation	0.68	0.71	Pm
P02679-1	Fibrinogen γ Isoform γB - Receptor; Calcium binding	0.78	0.63	Pm, Ec

Identifier	Description - Function	D/N	D5/D0	Location
P05106-1	Integrin beta 3 Isoform 3A - Receptor, integrin binding	0.72	0.58	Pm
P35442	Thrombospondin-2 - Structural; Calcium binding	0.81	0.42	Ec
P09486	Secreted protein acidic and rich in cysteine - Copper, calcium collagen binding	0.62	0.41	Ec
P10720	Platelet factor 4 variant - Chemokine activity, heparin binding	0.66	0.38	Ec
P10124	Serglycin - Protein binding	0.57	0.33	Ec, G

The last column shows the sub-cellular location including: Cy = cytoplasm, Cs = cytoskeleton, Ec = extracellular, ER = endoplasmic reticulum, G = golgi, L = lysosome, M = mitochondria, N = nucleus, Pm = plasma membrane, - = unknown.

Table 5

Proteins with significantly altered abundances that are attributable to the 5-days of storage. D5/D0 indicates the abundance ratio for platelets stored for 5-days relative to fresh platelets. Statistical significance ($p \leq 0.05$) was determined using the mixed-effects linear method as described in the methods section. Also shown are the number of peptide(s) seen by the MS and the number of times each peptide(s) was observed out of a total of 45 MS runs.

Identifier	ProteinDescription	D5/D0	95% Conf. Int.	Peptides	NJobs	Location
Q6PIH6	IGKV1-5 protein	4.03	2.3–7.2	3	17	Ec
Q9P173	Uncharacterized PRO2275	3.54	2.9–4.3	4	32	Ec
Q9H532	Solute carrier family 17 (sodium phosphate)	3.11	2.0–4.7	1	17	Pm, ER
Q6N097	Putative uncharacterized protein DKFZp686H20196	2.70	2.2–3.4	7	38	Ec
A6NBZ8	Uncharacterized protein ALB	2.56	2.2–3.0	32	45	Ec
P02787	Serotransferrin precursor	2.13	1.6–2.9	7	17	Ec
Q9BQB6-2	Vitamin K epoxide reductase, isoform 2, subunit 1	2.09	1.6–2.8	1	35	Ec
Q9ULV4	CORO1C 18 kDa protein	2.05	1.4–2.9	1	28	Cs
O75369-1	Filamin-B, isoform 1	1.87	1.0–3.4	5	21	Cy, Cs
O15144	Actin-related protein 2/3 complex, subunit 2	1.82	1.4–2.4	5	28	Cy, Cs
Q13404-4	Ubiquitin-conjugating enzyme E2 variant 1, isoform 4	1.71	1.6–1.8	2	42	N
Q15833	Syntaxin-binding protein 2	1.70	1.2–2.3	11	36	-
P14770	Platelet glycoprotein IX precursor	1.67	1.2–2.3	5	38	Pm
P54727	UV excision repair protein RAD23 homolog B	1.67	1.1–2.4	2	18	N, Cy
P13224	Glycoprotein Ib beta	1.53	1.3–1.8	5	44	Pm
Q9NVA2	Septin-11	1.47	1.2–1.8	5	28	N
P59998	TTLL3 Actin-related protein 2/3 complex subunit 4	1.46	1.3–1.7	2	43	Cy, Cs
Q59GD5	Actin-related protein 3-beta, isoform 1	1.46	1.2–1.8	2	36	Cs
P06753-1	Tropomyosin 3 isoform 1	1.44	1.2–1.7	3	38	Cy, Cs
Q9Y427	Tropomyosin 1 alpha chain isoform 2	1.42	1.2–1.7	11	45	Cy, Cs
Q9Y277-1	Voltage-dependent anion-selective channel protein 3, isoform 1	1.36	1.0–1.8	4	29	M
P05556-3	Integrin beta-1 precursor, isoform beta-1C	1.33	1.2–1.5	8	41	Pm, ER
P45880-1	Voltage-dependent anion-selective channel protein 2	1.30	1.2–1.4	2	39	M
O15511-2	Actin-related protein 2/3 complex subunit 5, isoform 2	1.30	1.1–1.5	1	40	Cy
P62158	Calmodulin	1.29	1.1–1.5	2	31	Cy, Pm

Identifier	ProteinDescription	D5/D0	95% Conf. Int.	Peptides	NJobs	Location
P00403	Cytochrome c oxidase subunit 2	1.28	1.2–1.4	2	41	M
Q15257-3	Serine/threonine-protein phosphatase 2A regulatory subunit B, isoform 3	1.28	1.2–1.3	2	24	Cy
P06753-2	Tropomyosin alpha-3 chain, isoform 2	1.27	1.1–1.5	6	44	Cy, Cs
Q9UI15	Transgelin-3	1.27	1.2–1.4	3	15	-
P51572	B-cell receptor-associated protein 31	1.22	1.1–1.4	5	43	G, Pm, ER
P12814	Alpha-actinin-1	1.21	1.1–1.4	61	45	Cy, Cs
O95837	Guanine nucleotide-binding protein alpha-14 subunit	1.20	1.2–1.2	2	41	Pm, G
P25705	ATP synthase subunit alpha	1.20	1.1–1.4	18	44	M
Q9Y6D8	Mutant beta-globin	1.15	1.1–1.2	2	45	-
P61088	Ubiquitin-conjugating enzyme E2 N	0.89	0.9–0.9	2	27	Cy, N
P60842	Eukaryotic initiation factor 4A-I	0.89	0.8–1.0	2	34	Cy
P14174	Macrophage migration inhibitory factor	0.89	0.9–0.9	2	27	Ec
P68402	Platelet-activating factor acetylhydrolase IB subunit beta	0.85	0.8–0.9	2	42	Cy
P35241	Radixin isoform b	0.83	0.7–1.0	9	44	Cy, Cs, Pm
O95866-1	Protein G6b precursor, isoform B	0.83	0.7–0.9	4	43	ER, G, Pm
P16671	Platelet glycoprotein 4	0.82	0.7–1.0	9	43	Pm
O00299	Chloride intracellular channel protein 1	0.81	0.7–0.9	10	43	Cy, Pm
P30740	Leukocyte elastase inhibitor	0.80	0.7–1.0	11	42	Cy
P18054	Arachidonate 12-lipoxygenase	0.80	0.7–0.9	10	43	Cy
Q9GZV4	Eukaryotic translation initiation factor 5A-2	0.79	0.7–0.9	2	44	Cy
P26038	Moesin	0.79	0.7–0.9	33	43	Cy, Cs, Pm
P37802	Transgelin-2	0.79	0.7–0.9	16	45	-
P47756-1	F-actin-capping protein subunit beta, isoform 1	0.78	0.7–0.9	2	38	Cy, Pm
O00499-1	Myc box-dependent-interacting protein 1, isoform IIA	0.78	0.8–0.8	2	27	Cy, Cs, N
P43304-1	Glycerol-3-phosphate dehydrogenase, isoform 1	0.78	0.7–0.9	5	22	M
P06733-1	Enolase, isoform alpha	0.77	0.6–1.0	19	45	Cy, Pm, N
P01111	GTPase NRas precursor	0.76	0.6–0.9	2	43	G, Pm
P09104	Gamma-enolase	0.76	0.7–0.8	5	44	Cy, Pm
Q13418	Integrin-linked protein kinase	0.76	0.6–1.0	26	45	Cy

Identifier	ProteinDescription	D5/D0	95% Conf. Int.	Peptides	NJobs	Location
Q86UX7-2	Unc-112-related protein 2, isoform 2	0.76	0.6–0.9	38	45	Cy, Cs
Q14568	Heat shock protein 90 kDa alpha class A member 2 (Fragment)	0.76	0.6–0.9	2	36	-
Q9Y251	Heparanase precursor	0.75	0.7–0.8	4	45	Ec, L, Pm
Q2QD09	Triosephosphate isomerase (Fragment)	0.75	0.6–0.9	8	45	-
P34931	Heat shock 70 kDa protein 1L	0.75	0.6–0.9	4	37	Cy
P60174-2	Triosephosphate isomerase, isoform 2	0.74	0.6–0.8	2	42	Cy
P21926	CD9 antigen	0.74	0.6–0.8	2	42	Pm
P37840-1	Alpha-synuclein, isoform 1	0.74	0.6–0.9	7	44	Cy, Pm, N
P30040	Endoplasmic reticulum protein ERp29 precursor	0.73	0.6–0.9	3	36	ER
Q15942	Zyxin	0.72	0.6–0.9	22	45	Cy, Cs, Pm, N
O75830	Serpin I2 precursor	0.72	0.6–0.9	1	43	Ec
P26447	Protein S100-A4	0.71	0.6–0.8	6	45	Cy, N
P30443	HLA class I histocompatibility antigen	0.71	0.6–0.9	1	33	Cy, Pm
P04275	Coagulation factor VIII VWF (von Willebrand factor)	0.71	0.7–0.7	2	26	Ec
P17301	Integrin alpha-2 precursor	0.71	0.6–0.8	8	27	Pm
Q9NRW1	Ras-related protein Rab-6B	0.70	0.6–0.9	4	27	G
P40227	T-complex protein 1 subunit zeta	0.70	0.5–0.9	6	21	Cy
Q01518	Adenylyl cyclase-associated protein	0.70	0.5–1.0	2	41	Pm
P21291	Cysteine and glycine-rich protein 1	0.70	0.6–0.9	2	29	N
Q8N7G1	Similar to purine nucleoside phosphorylase	0.70	0.6–0.9	12	45	-
P07384	Calpain-1 catalytic subunit	0.69	0.6–0.8	9	41	Cy, Pm
P48426	Phosphatidylinositol-5-phosphate 4-kinase type-2 alpha	0.69	0.5–0.9	4	43	-
Q06323	Proteasome activator complex subunit 1	0.68	0.6–0.8	3	38	Cy
O43396	Thioredoxin-like protein 1	0.68	0.6–0.8	1	35	Cy
P46109	Crk-like protein	0.68	0.6–0.7	2	31	Cy, Pm
P02671-1	Fibrinogen alpha chain precursor, isoform 1	0.67	0.6–0.8	35	45	Ec, Pm
Q5SQ64	Lymphocyte antigen 6 complex locus protein G6f	0.66	0.6–0.8	3	39	Pm
P84077	ADP-ribosylation factor 1	0.66	0.5–0.8	10	42	Cy, Pm, G
O95810	Serum deprivation-response protein	0.64	0.5–0.8	17	45	Cy

Identifier	ProteinDescription	D5/D0	95% Conf. Int.	Peptides	NJobs	Location
Q9NVD7-1	Alpha-parvin, isoform 1	0.64	0.5–0.8	1	43	Cy, Cs, Pm
P02679-1	Fibrinogen precursor, isoform gamma-B	0.63	0.5–0.8	29	43	Ec, Pm
Q2WGJ9	Fer-1-like 6	0.62	0.5–0.8	1	42	Pm
P17987	T-complex protein 1 subunit alpha	0.62	0.5–0.8	4	26	Cy
Q70J99-1	Protein unc-13 homolog D, isoform 1	0.62	0.4–0.9	6	31	Cy, Pm
P04632	Calpain small subunit 1	0.62	0.5–0.8	5	39	Cy, Pm
Q15365	Poly(rC)-binding protein 1	0.61	0.5–0.7	3	38	Cy, N
P50395	Rab GDP dissociation inhibitor beta	0.61	0.4–0.8	3	43	Cy, Pm
Q9UJC5	SH3 domain-binding glutamic acid-rich-like protein 2	0.58	0.4–0.8	2	32	N
Q99733	Nucleosome assembly protein 1-like 4	0.58	0.5–0.7	2	38	Cy, N
P05106-1	Integrin beta-3 precursor	0.58	0.5–0.7	3	43	Pm
O14556	Glyceraldehyde-3-phosphate dehydrogenase	0.58	0.4–0.7	1	44	Cy
Q9Y490	Talin 1	0.57	0.5–0.6	4	31	Cy, Cs
P61106	Ras-related protein Rab-14	0.57	0.4–0.8	4	22	Cy, L, Pm, G
P62820-1	Ras-related protein Rab-1A, isoform 1	0.56	0.4–0.7	2	34	ER, G
Q9H4B7	Tubulin beta-1 chain	0.55	0.4–0.7	30	45	Cs
P68371	Tubulin beta-2C chain	0.55	0.3–0.9	18	45	Cs
P02675	Fibrinogen beta chain precursor	0.54	0.4–0.8	26	44	Cy, Cs
Q92673	Sortilin-related receptor precursor	0.53	0.4–0.8	1	13	Ec; Pm
Q9NY65	Tubulin alpha-8 chain	0.51	0.4–0.7	5	31	Cs
P51659	Peroxisomal multifunctional enzyme type 2	0.50	0.4–0.6	3	21	M
A8MW06	Thymosin-like 3	0.49	0.3–0.7	11	45	Cy, Cs
P07996	Thrombospondin-1 precursor	0.48	0.4–0.6	29	45	Ec
P07437	Tubulin beta chain	0.44	0.3–0.6	8	41	Cy, Cs
P61586	Transforming protein RhoA precursor	0.43	0.4–0.5	4	32	Cy, Cs, Pm
P35442	Thrombospondin-2 precursor	0.42	0.4–0.4	2	37	Ec
P09486	Secreted protein acidic and rich in cysteine precursor	0.41	0.3–0.5	4	36	Ec
P40197	Platelet glycoprotein V precursor	0.41	0.3–0.5	15	41	Pm
P02775	Platelet basic protein precursor	0.40	0.3–0.6	6	45	Ec

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Identifier	ProteinDescription	D5/D0	95% Conf. Int.	Peptides	NJobs	Location
P10720	Platelet factor 4 variant precursor	0.38	0.4–0.4	2	45	Ec
Q8N532	TUBA1C protein	0.37	0.3–0.4	5	44	Cs
Q13201	Multimerin-1 precursor	0.36	0.2–0.6	13	33	Ec
P68366	Tubulin alpha-4A chain	0.35	0.3–0.4	20	44	Cs
P10124	Serglycin precursor	0.33	0.3–0.4	5	42	Ec, G

The last column shows the sub-cellular location including: Cy = cytoplasm, Cs = cytoskeleton, Ec = extracellular, ER = endoplasmic reticulum, G = golgi, L = lysosome, M = mitochondria, N = nucleus, Pm = plasma membrane, - = unknown.

Table 6

GO processes associated with proteins differentially abundant in diabetic and stored samples.

GO process	D/N	D5/D0
Blood coagulation	yes	yes
Hemostasis	yes	yes
Response to wounding	yes	yes
Platelet activation	yes	yes
Response to Stress	yes	yes
Cell adhesion	yes	yes
Cell motility	yes	no
Integrin-mediated signaling	yes	no
Cell-matrix adhesion	yes	no
Cell-substrate junction assembly	yes	no