Diurnal protein expression in blood revealed by high throughput mass spectrometry proteomics and implications for translational medicine and body time of day

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1University Health Network, Toronto, Ontario, and Departments of Medicine, Physiology, and Heart and Stroke Richard Lewar Centre of Excellence, University of Toronto, and 2Toronto Sunnybrook Regional Cancer Centre, Division of Medical Oncology, and Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada; and 3Customized Online Biomathematical Research Applications, Charlottesville, Virginia

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Martino TA, Tata N, Bjarnason GA, Straume M, Sole MJ. Diurnal protein expression in blood revealed by high throughput mass spectrometry proteomics and implications for translational medicine and body time of day. Am J Physiol Regul Integr Comp Physiol 293: R1430–R1437, 2007. First published June 6, 2007; doi:10.1152/ajpregu.00183.2007.—Molecular gene cycling is useful for determining body time of day (BTOD) with important applications in personalized medicine, including cardiovascular disease and cancer, our leading causes of death. However, it impractically requires repetitive invasive tissue sampling that is obviously not applicable for humans. Here we characterize diurnal protein cycling in blood using high-throughput proteomics; blood proteins are easily accessible, minimally invasive, and can importantly serve as surrogates for what is happening elsewhere in the body in health and disease. As proof of the concept, we used normal C57BL/6 mice maintained under regular 24-h light and dark cycles. First, we demonstrated fingerprint patterns in 24-h plasma, revealed using surface-enhanced laser desorption and ionization (SELDI). Second, we characterized diurnal cycling proteins in blood using chromatography and tandem electrospray ionization mass spectrometry. Importantly, we noted little association between the cycling blood proteome and tissue transcriptome, delineating the necessity to identify de novo cycling proteins in blood for measuring BTOD. Furthermore, we explored known interaction networks to identify putative functional pathways regulating protein expression patterns in blood, thus shedding new light on our understanding of integrative physiology. These studies have profound clinical significance in translating the concept of BTOD to the practical realm for molecular diagnostics and open new opportunities for clinically relevant discoveries when applied to ELISA-based molecular testing and/or point-of-care devices.

chronotherapy; biomarkers

The circadian clock in mammals underlies biological rhythms of physiology and couples these to the natural cycling of 24-h light and dark cycles (33, 35). At a molecular level there is a core clockwork mechanism, originally discovered in the suprachiasmatic nucleus of the hypothalamus and now known to be present in virtually every cell, tissue, and organ in the body with the exception of the testes. Core genes and proteins (e.g., Arntl, Clock, Per2, Cry, CK1ε, GSK3β, and Nr1d1) comprise an oscillatory feedback loop that cycles every 24 h to keep time, as has been extensively reviewed (33–35). Recently, temporal changes in gene expression have been applied to the development of a gene-based apparatus measuring body time (40). Oligonucleotide arrays were used to characterize sets of “time-indicating” oscillatory genes in murine liver tissue, demonstrating the feasibility of such an approach and obviating the need for sampling pulsatile neural-regulated hormones such as cortisol and melatonin. However, measuring body time of day (BTOD) as such remains for the most part an unfulfilled dream, since technology involving “genechips” is not easy to implement, especially in routine diagnostic care. Also, this approach requires repetitive invasive tissue sampling, which is clearly not practical for humans.

There is a strong clinical rationale for measuring BTOD in humans. The 24-h diurnal cycling is relevant to the cardiovascular system for the cyclic variation of heart rate and blood pressure (29), timing of endocrine hormone secretion (10), and timing of adverse events (e.g., myocardial infarction, stroke, sudden cardiac death) (12, 28, 29, 42). Experimental studies further indicate that gene expression in the heart is not uniform across 24 h but exhibits diurnal variation like our physiology does (11, 25, 38, 44, 45). Conversely, rhythm disturbance is a major health risk. Disturbed diurnal rhythms demonstrably exacerbate heart disease in aortic-banded pressure-overload mice (26) and reduce longevity in cardiomyopathic hamsters (31). Shift workers and patients with sleep disorders are at increased risk of adverse cardiovascular events and poorer prognosis (6, 15). Having a correlative measure of body time in humans is a novel therapeutic strategy that may significantly benefit cardiac patients in the clinical setting.

A strong rationale is indicated for cancer treatment as well. Multiple rodent and human studies have shown that the timing of chemotherapy impacts on toxicity and activity (2, 3). This is explained to some extent by the impact of drug timing on pharmacokinetics and pharmacodynamics (5, 16). Furthermore, cell cycle, apoptosis, angiogenesis, and gene expression are controlled by the clock (4, 14, 21, 23, 27). These pathways are critical in cancer biology and involve multiple rhythmic targets for novel drugs currently in clinical use and in clinical trials. Although most of the research on clock-controlled pathways has been done in rodents, there is increasing data from human studies. For example, a circadian rhythm has been demonstrated in cell-cycle progression in human bone marrow...
(37), skin (7), and gastrointestinal tract mucosa (4, 8, 24), the organs where toxicity most frequently manifests itself after cancer therapy. By developing novel approaches for identifying de novo diurnal indicators of time in blood, we can improve treatment efficacy and significantly impact on health care in clinical settings.

In this study we set out to characterize diurnal protein cycling in blood, an easily accessible and minimally invasive tissue, which can serve as a surrogate for what is happening elsewhere in the body in health and disease. High-throughput mass spectrometry (MS) provides a means for de novo characterization of time-indicating oscillatory proteins in blood. This effectively allows for the concept of BTOD to be carried into the realm of molecular and clinical diagnostics.

MATERIALS AND METHODS

Animals and plasma sample collection. Six-week-old male C57Bl/6 mice (Jackson) were entrained to a 12-h:12-h light-dark cycle for 2 wk. Plasma was carefully collected from mice, anesthetized with ketamine-xylathane using Na2-heparin coated syringes, and then poured into sample tubes on ice. All blood-handling procedures were done in the same manner (by T. A. Martino and N. Tata) to ensure reproducibility between samples and at all time points. Samples were centrifuged, aliquoted, frozen, and used only once. The protein content of each sample was determined using the Bradford Assay method in accordance with the manufacturer’s specifications (Bio-Rad). Experiments were performed in triplicate (n = 3 mice/time point). All animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care. This study was approved by the Animal Care Committee at the Toronto General Hospital.

Surface-enhanced laser desorption ionization. Samples were analyzed by surface-enhanced laser desorption ionization (SELDI) using the Ciphergen Biosystems weak cation exchange protein chip (Ciphergen Biosystems, Palo Alto, CA). Blood samples (5 μg/sample) were loaded onto the chips in acetate buffer (pH 5.5) in accordance with the manufacturer’s specifications. Samples were dried on the chips and then treated with α-cyano-4-hydroxycinnamic acid. Samples were analyzed by PBS II SELDI-MS software (Ciphergen).

Bioinformatics and statistical analyses. For relative quantitative analyses, SELDI-MS data were visualized using the “peak view.” The area under the curve for each protein/peptide bands of interest was quantified in conjunction with ImageJ 1.33u software (National Institutes of Health). The data were analyzed using one-way ANOVA probabilities, as well as the nonparametric Kruskal-Wallis (PK-W) ANOVA equivalent (where the PK-W value is comparable with the parametric ANOVA value). The particular pairwise points that show significant differences in abundance were determined by the application of the Student-Newman-Keuls or Dunn’s post hoc test. Microsoft Excel was used to visualize protein abundance over 24-h diurnal cycles.

Chromatography and SDS-PAGE and silver staining. Samples were prefractionated by column chromatography using HiQ ion exchange resin. Columns were assembled and run in microcolumn format (100 μl bed volume). Resin was added, and the columns were equilibrated with 50 mM phosphate buffer (pH 5.5). Samples diluted in binding buffer were loaded onto columns and then eluted in 50 mM phosphate buffer with 250 mM NaCl. Three fractions were subsequently analyzed on 10–20% tricine gels (Invitrogen): flow through, 250 mM salt elution, and retentate. Silver staining was performed in accordance with standard procedures. Gels were stored in 1% acetic acid-H2O until needed.

Trypsin digestion and MS/MS by liquid chromatography-electron spray ionization ion trap. Protein bands were prepared for MS/MS from the gel by excision and digestion overnight in 50 μl buffer containing 50 mM NH4HCO3, 10% acetonitrile, and 10 ng/μl sequencing-grade trypsin (Roche). The MS/MS analysis of digested peptides was performed using a LCQ DECA XP ion trap (Thermo Finnigan). Buffer A for liquid chromatography (LC) contained 0.1% formic acid, and buffer B contained 99.9% acetonitrile and 0.1% acetic acid. Samples were injected at a rate of 10 μl/min onto a Vydac reverse-phase column (0.2 × 150 mM, Grace Vydac, Hesperia, CA) over 5 min in 5% buffer B using an Agilent 1100 Series CAP-LC (Palo Alto, CA). Peptides were eluted over a 10–65% buffer B gradient for 40 min at a rate of 2 μl/min for detection by LCQ DECA XP MS. Sequencing data generated from the MS/MS ion trap were searched against a murine database using SEQUEST.

Pathway analysis. Interaction maps (interactomes) were created using the Ingenuity Pathways Knowledge Base. This is a well-documented and comprehensive knowledge database used to construct pathways and function-based modules (9, 22). An interactome was constructed by J) importing the blood proteins by name and 2) importing the canonical circadian rhythm components. The shortest pathways between each were determined on the basis of direct interactions only and are supported by literature online. Nodes for the
resultant interactome are displayed by their designated cellular location.

RESULTS AND DISCUSSION

Gene expression cycles across daily 24-h periods have been known for some time. Genomewide microarrays have been used to identify circadian and diurnal-regulated genes in the murine central clock in the suprachiasmatic nucleus of the hypothalamus and in major peripheral organs including heart, liver, aortic vasculature (25, 30, 36, 38, 39), and others. Recently, it was demonstrated that this information can be used to create a molecular timetable (40): a hepatic gene expression-based approach whereby body time can be accurately measured, thus showing potential for transforming a basic discovery tool into one that can prove useful in clinical medicine. Although elegant in design and proof of concept, it is important to note that a timetable based on liver gene expression is not easily accessible for routine or point-of-care testing. Protein cycling has also been recently demonstrated using a global approach but, as with the studies noted above, only in body tissue and, in particular, in the liver (32). Global cycling as a useful measure of time requires a practical means for sample taking, other than testing and retesting body organs. In contrast, blood is easily obtained in clinical settings and is rich in proteins. Many proteins in blood are effused from the liver, so sampling here may be consistent with current paradigms of hepatic processes reflecting body time. Furthermore, it is in keeping with the notion that diagnostic chips can be subsequently developed and used for rapid point-of-care testing.

In this study we set out to characterize protein cycling in blood by quantifying expression across 24-h cycles. The experiment was performed using Ciphergen Biosystems weak cation exchange protein chips. Blood plasma proteins with molecular weights of 5,121, 5,946, and 8,336 Da show significant differences in abundance across the 6 available time points, whereas proteins with 6,779 and 7,884 do not. Parametric ANOVA (PANOVA) results are consistent with the nonparametric Kruskal-Wallis (PK-W) results. *P ≤ 0.05. PANOVA, parametric ANOVA; SNK, Student-Newman-Kuels post hoc test; PK-W, nonparametric Kruskal-Wallis.

Table 1. Significant differences in protein abundance in blood over 24-h time period

<table>
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<tr>
<th>Protein Mass</th>
<th>PANOVA</th>
<th>PK-W</th>
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<tr>
<td>P5121</td>
<td>0.0050*</td>
<td>0.0225*</td>
</tr>
<tr>
<td>P5946</td>
<td>0.0141*</td>
<td>0.0494*</td>
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<tr>
<td>P8336</td>
<td>0.0004*</td>
<td>0.0139*</td>
</tr>
</tbody>
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Surface-enhanced laser desorption and ionization (SELDI) mass spectrometry was performed using Ciphergen Biosystems weak cation exchange protein chips. Blood plasma proteins with molecular weights of 5,121, 5,946, and 8,336 Da show significant differences in abundance across the 6 available time points, whereas proteins with 6,779 and 7,884 do not. Parametric ANOVA (PANOVA) results are consistent with the nonparametric Kruskal-Wallis (PK-W) results. *P ≤ 0.05.
performed in the diurnal setting of light and dark cycling because we are ultimately interested in applications for clinical diagnostics, that is, to consider the light-dark environment in which humans normally exist. Ultimately, cycling patterns for normal humans in regular 24-h light-dark environments will be compared with those that are not necessarily so: clinical patients, shift workers, etc. It is this specific difference, occurring in health versus disease, that we anticipate to be important for clinical applicability.

Fig. 3. Characterization of proteins in blood over 24-h time period using proteomic tools. Samples were prefractionated using ion exchange resin with binding conditions of 50 mM phosphate buffer (pH 5.5). Three fractions, flow through (A), 250 mM salt elution (B), and retentate (C) were collected and analyzed on 10–20% tricine gels. One representative gel is shown for each of the different fractions, with arrows denoting bands with varying intensity over 24 h. Bands were excised, trypsin digested, and analyzed by mass spectrometry (MS/MS). M, marker; 17–23, ZT23, ZT23 (duplicate) ZT03, ZT07, ZT11, ZT15, ZT19, respectively. D: representative MS/MS spectrum for transthyretin. E: output of the complementary database search showing two sequence data matches with transthyretin. Multiple peptides were sequenced for each protein and are listed in Table 2. AA, amino acid; i–v, specific bands.
The first approach for examining protein cycling in blood used SELDI MS. Proteins were retained on solid-phase chromatographic surfaces, or “chips,” then subsequently ionized, and detected on the basis of mass-to-charge ratio. Ion exchange chips were used here; many additional profiles can be obtained using chips with other base biochemical properties as desired (e.g., hydrophobic, anionic, metal ion, hydrophilic, activated surface, antibody/antigen, receptor-ligand, etc.). Figure 1 shows diurnal protein cycling in blood, with data visually displayed in gel view. Profiles were collected across a wide mass range. The low molecular mass range (1–10 kDa) classically contains many bioactive peptides, whereas midrange (10–50 kDa) and larger (>50 kDa) proteins often reflect those involved in cell structure/function processes. Thus SELDI allows for rapid profiling of proteins and essentially creates a “fingerprint” that may ultimately be used as indicative of BTOD.

To verify the possibility of using SELDI analysis for fingerprinting/BTOD, the protein/peptide bands were subsequently quantified, revealing striking temporal protein expression profiles. The five most visible protein bands exhibiting temporal change across the 24-h cycle were selected for analysis. They corresponded to molecular masses of 5,121, 5,946, 6,779, 7,884, and 8,336 Da (defined by the vertical yellow bars; Fig. 2, A–C). Significant and reproducible differences in abundance were observed across the diurnal cycle for three of these proteins at molecular masses of 5,121, 5,946, and 8,336 Da (Table 1). The PK-W analyses were consistent with the parametric ANOVA results ($P < 0.05$); both pointed to the same conclusions regardless of which approach was applied. The results for two of these, 5,121 and 8,393 Da, are illustrated graphically. Pairwise time points that showed significant differences in abundance are also noted (Figs. 2, D and E). Thus these data indicate that SELDI blood profiling is a feasible approach for monitoring protein cycling in blood. Ultimately, a relative measure of candidate fingerprint markers, at one or two select time points, would lead to the development of BTOD measurements.

SELDI is useful for creating a “fingerprint” for BTOD, especially in combination with clinical measures to ensure that the bands chosen are most indicative of rhythmic time. However, one current limitation to this technology and approach is that the identity of the proteins/peptides being measured is not known. Thus we sought to further characterize proteins cycling in blood and to examine whether there were liver secretory products consistent with our hypothesis.

To characterize cycling proteins in blood, samples were first prefractionated. This was done as a first step to remove a majority of albumins and also to concentrate proteins according to characteristic biochemical properties. Here prefractionation was performed by column chromatography using an ion exchange resin; additional resins targeting different biochemical properties might likely elucidate more protein markers of body time as well as those shown here. The column-based approach was chosen since it facilitates high-throughput analyses and de novo marker discovery. Following column chromatography, the proteins comprising effluent, salt elution, and bead retentive fractions were visualized using SDS-PAGE and silver-staining analyses. One representative gel for each of the three chromatography fractions is shown, with proteins ranging from 10–150 kDa (Fig. 3, A–C). Arrows denote protein...
bands varying in intensity in blood over 24 h and targeted for further identification. The identity of these proteins was determined by MS. Gel bands containing proteins of interest were excised, followed by trypsin digestion to generate peptide fragments. The digested peptides, separately for each gel band, were injected by electron spray ionization (ESI) into an LCQ DECA XP ion trap. Protein identity was confirmed by searching against murine databases, by molecular mass comparisons, by detection in multiple timed samples, and from several different gels. An ~150-kDa band was identified by MS/MS as C3, an important component of the innate immune cascade and the alternative pathway of the complement system (20). An ~100-kDa band was identified as plasminogen, the zymogen in circulating blood from which plasmin is formed, for digestion of fibrin in blood clots (1). Interestingly, conversion of plasminogen to plasmin is regulated in part by plasminogen-activated inhibitor 1, a well-characterized output gene of the circadian molecular clock and under rev-erb regulation (41). An ~15-kDa band was determined to be transthyretin, a plasma homotetramer of 55 kDa that binds and transports thyroxin (T4) and retinol (vitamin A) (13, 18). Nuclear hormones and receptors, including those that interact with transthyretin and associated-binding components, are also important in cell clock mechanisms (43). Also identified were apolipoproteins important in lipid and cholesterol regulation. That these have a temporal profile is anticipated; they are key regulators in metabolic pathways that in turn have an obvious bias toward the times when animals are active and awake (here, nocturnal mice = dark period). Typical MS/MS spectra from the LC-ESI ion trap are shown in Fig. 3D, and the complementary output from database searching showing the sequence data match to transthyretin is shown in Fig. 3E. Thus these results demonstrate the discovery process and identification of specific proteins cycling in blood. Ultimately, predefined cycling markers coupled with an ELISA-based diagnostic device would allow for the development of a practical and user-friendly test.

Although we are examining protein cycling in blood, the mechanisms by which diurnal protein abundance is regulated in blood is not known. One possible explanation is that the hepatic mRNA encoding these proteins is also rhythmic and thus regulates protein abundance through transcriptional/translational control. We investigated this by a comparison with a database for circadian gene expression (http://expression.gnf.org/cgi-bin/circadian/index.cgi). This is a well-documented, publicly available database based on Affymetrix microarray profiles of liver mRNA from C57Bl/6 mice maintained under circadian conditions. Notably, none of the proteins identified here exhibited rhythmic mRNA expression in the liver. These observations suggest that measuring BTOD in blood as a diagnostic tool is best served by individual protein profiles and not by extrapolation from liver mRNA data. To date, there is only one other study comparing protein versus mRNA cycling, in this case for soluble liver proteins versus the respective transcripts (32). They found no association between almost 50% of 49 proteins and their complementary genes even within the liver itself, thus providing further support for dissociation between cycling proteome and transcriptome. Furthermore, analogous dissociations between protein and gene have been reported in cancer disease models (19). We further constructed the shortest path interactions between the circadian clock mechanism and the blood proteins, using the knowledge-based database Ingenuity Pathways. This revealed distinct interactions via a single intermediary molecule, predominantly cytokines, and nuclear factors. The resulting interactome shown in Fig. 4 illustrates putative mechanisms for regulating protein abundance in blood. Thus, taken together, we conclude that tissue-specific expression demonstrates the feasibility of molecular timetables and that rhythmic genes are useful for time indicating in a tissue-specific manner. We now demonstrate cycling in blood and, further, the necessity of identifying specific protein biomarkers in blood for measuring body time.

There are several additional practical considerations to consider in further developing this approach for clinical diagnostics. The first is how many markers are required for an accurate test of BTOD. Given the design of ELISA-based protein diagnostics, cassettes containing antibodies to specific protein markers, it seems likely that fewer markers will be required than with the tissue arrays, although the exact number still requires empirical testing. A second consideration is that protein expressions in blood are strongly influenced by feeding. Thus it will be necessary to exclude rhythmic protein/peptides that reflect food intake and not BTOD. Once human markers are identified, it would seem prudent to at least test fasting
overnight versus regular meal intake, since this corresponds well with current clinical practices for blood sampling and could be a useful incorporation into the test design. Finally, an incorporation of application specific markers could increase the specificity of test design. For example, the largest trial testing the timing of therapy (chronotherapy) in cancer randomized 564 colon cancer patients to standard therapy with 5-fluorouracil (5-FU) and oxaliplatin versus a chronomodulated infusion of the same drugs with peak oxaliplatin delivery at 4:00 PM and 5-FU at 4:00 AM (17). The analysis of survival predictors showed that sex difference was the single most important factor. In men, the risk of death was decreased by 25% with chronotherapy versus standard drug treatment with median survival times of 21.4 and 18.3 mo, respectively. In women, the risk of death with chronotherapy was increased by 38% compared with standard therapy with median survival times of 16.3 and 19.1 mo. This study shows that sex differences in a therapeutic index are amplified when a drug is given consistently at a certain time of day, uncovering a significant difference in the circadian biology of men and women. Thus, the concept of BTOD may therefore be extended not only to determine the best time to give the drug but also to help differentiate between the best times for men and women. This might require adding to the profile some proteins specific to the therapy in question. For 5-FU-based therapy, this could, for example, include some of the important rhythmic enzymes for its metabolic pathways (46).

In summary, this study demonstrates changes in protein abundance in blood over a 24-h time period. SELDI is used for the creation of a fingerprint profile across the diurnal cycle. An MS/MS approach characterizes specific proteins cycling in blood. Importantly, we found little correlation between the cycling blood proteome and the corresponding circadian transcriptome (in hepatic tissue of origin). The practical application is to measure BTOD for molecular diagnostics and applications for clinical medicine. Overall, this allows us to effectively translate a technology mostly confined to the laboratory into something that may be applied clinically and diagnostically in medicine. Ultimately, the design of protein chips with specific markers will allow for rapid and effective characterization of body time; for example, as a simple ELISA-based cassette that can be used in routine or point-of-care medicine. One of the most practical applications is chronotherapy, which requires easily accessible markers of body time to optimize the timing of drug treatments. Additional applications might include managing rhythm disorders, such as occurs in jet lag, shift work, and sleep disturbance; all of these significantly impact on our productivity and health. In conclusion, there is tremendous potential in monitoring diurnal protein abundance in blood for the detection, treatment, prognosis, and prevention of disease and for the promotion of general good health.

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