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Circadian Proteomics and Its Unique Advantage for Discovery of Biomarkers of Heart Disease

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1. Introduction

Current statistics from the World Health Organization, Heart and Stroke Foundation of Canada, and the American Heart Association show that cardiovascular disease remains a leading cause of death (Heart and Stroke Foundation of Canada [HSFO], 2011; Roger et al., 2011; World Health Organization [WHO], 2011). Innovative and integrative approaches aimed at understanding and treating heart disease are needed. In this chapter we introduce a novel field of investigation called cardiovascular circadian proteomics. This approach is based on the application of high throughput proteomic technologies for discovery of molecular processes in cardiovascular tissues over the 24-hour day/night cycles. Time is a crucial but frequently overlooked factor affecting our physiology in health and disease. Circadian cardiovascular proteomics offers considerable promise to advance our understanding of heart disease (indeed disease in general), and opens new avenues for treatment of patients clinically.

2. The circadian system and its importance to cardiovascular physiology

Life on earth is subject to a 24-hour day/night (circadian or diurnal) cycle. Circadian systems have evolved to allow physiological and behavioural processes to be synchronous with this cycle – mammals are adapted to sleep either during the day or at night. Circadian clocks allow us to entrain to environmental cues and hence anticipate the differing physiologic and behavioural demands of daily events. In mammals, the system is organized as a hierarchy with multiple oscillators, as has been well reviewed (Hastings et al., 2003; Rajaratnam & Arendt, 2001; Reppert & Weaver, 2001, 2002). At the top is the hypothalamic suprachiasmatic nucleus (SCN), a brain region functioning as a master rhythmic regulator (Figure 1A). The SCN integrates light information received from the eyes to coordinate clocks throughout the body. There are additional circadian regulatory systems such as the food entrainable clock (Storch & Weitz, 2009) which we are just now beginning to understand, but are beyond the scope of this review. We observe the output of the entrained clocks as daily physiologic rhythms, many of which are crucial to the cardiovascular system, such as the cyclic variation in heart rate (HR) and blood pressure (BP). Daily HR and BP follow the diurnal variation of our autonomic nervous system and increase around wake-time to help sustain cardiac output, then decrease during the period of vagal dominance at night and in the early morning (Guo & Stein, 2003; Imai et al., 1990).

Circadian rhythms also underlie the timing of onset of adverse cardiovascular events. The incidence of myocardial infarction in humans peaks in the morning (~6:00A.M.-12:00 noon) (Cohen et al., 1997; Goldberg et al., 1990; Muller et al., 1985). A similar pattern is observed in the incidences of sudden cardiac death (Muller et al., 1989; Willich et al., 1987), ventricular tachyarrhythmia (Eksik et al., 2007; Tofler et al., 1995), and rupture of aortic aneurysms (Manfredini et al., 2004; Mehta et al., 2002; Sumiyoshi et al., 2002). Precursor risk factors such as vasomotor tone, platelet aggregability, and other factors involved in thrombosis or thrombolysis also exhibit daily rhythms (Andrews et al., 1996; Angleton et al., 1989; Decousus et al., 1985; Maemura et al., 2000; Otto et al., 2004). Recent studies link timing of onset of adverse cardiac events with a circadian clock mechanism, reviewed in (Durgan & Young, 2010; Martino & Sole, 2009; Sole & Martino, 2009).

Proteins involved in the molecular clock mechanism have been identified in the last 10+ years, and daily oscillations of this mechanism in peripheral tissues, including the myocardium, are believed to be primarily combination of self-sustaining cycling along with neural/hormonal cues from the SCN. It is illustrated in Figure 1B and described in many excellent reviews (Hastings et al., 2003; Reddy et al., 2005; Roenneberg & Merrow, 2005). Though we focus here on protein cycling, it is important to note that there are post-translational rhythms as well, such as phosphorylation. Though beyond the scope of this review the reader is directed to several examples (Akashi et al., 2002; Lowrey et al., 2000; Iitaka et al., 2005; Yin et al., 2006).

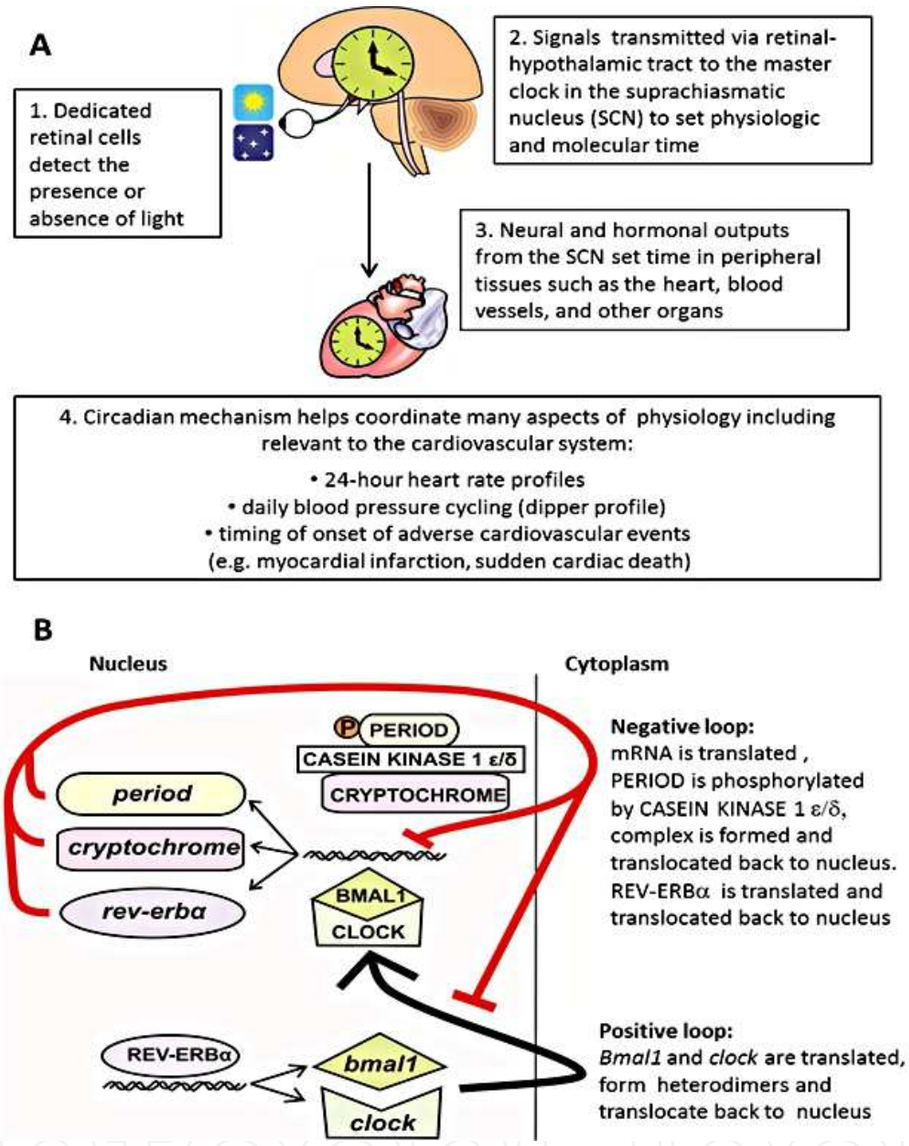
In the following sections, we describe how application of the circadian concepts, in combination with state-of-the-art proteomics, provides significant new opportunities for understanding disease physiology, for biomarker discovery, and helping patients clinically.

3. Discovery of the circadian heart proteome

Our laboratory is focussed on discovering the circadian heart proteome both in normal tissue and in disease. Initial studies are done using murine models of cardiovascular disease, and later are translated clinically. Here we describe the circadian proteome in normal C57Bl/6 mouse heart, and in our well-established murine model of heart disease termed pressure-overload induced cardiac hypertrophy by **T**ransverse **A**ortic **C**onstriction (TAC). To induce heart disease, eight week old male mice were entrained to a 12:12 light (L): dark (D) cycle and administered TAC surgery where a ligature was placed distal to the third bifurcation of aorta (Figure 2A). In sham operated animals the surgical procedure was identical, but the ligature was not tightened. (Figure 2A). For proteomic studies, heart tissues were collected one week later (as the heart remodels), at six time-points 4 hours apart over the 24-hour L:D cycle (Figure 2B). The proteome was analyzed by two-dimensional difference in gel electrophoresis (2D-DIGE) and mass spectrometry (MS). Figure 2B illustrates the experimental workflow design. The technical details are described below.

3.1 Protein purification and labelling

The cytoplasmic soluble proteome was purified from either TAC or sham left ventricular heart tissue. Cardiac tissue was immersed in 600 µl ice-cold cell lysis buffer (10 mM Tris pH 8, 8 M Urea, 4% w/v 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and protease inhibitors). The lysis buffer helps to solubilize, denature, and



A) Light activates dedicated retinal receptors and signals the hypothalamic suprachiasmatic nucleus (SCN). The SCN is a master body clock, and orchestrates physiologic and molecular rhythms in peripheral organs including the heart. Rhythms relevant to the heart include the daily cycling of heart rate and blood pressure, also timing of onset of acute cardiac events such as myocardial infarction.
B) The molecular clock mechanism is dependent upon oscillating levels of proteins that interact via a 24-hour autoregulatory feedback. On the positive arm BMAL1 and CLOCK combine as heterodimers and bind to E-box elements upstream in the coding regions of other core clock elements, PERIOD and CRYPTOCHROME. These are phosphorylated by CASEIN KINASE 1 EPSILON (or DELTA), form heterodimers, then translocate to the nucleus. There, they bind to the same E-box elements thus negatively regulating their own expression. The positive loop also initiates production of RETINOIC ACID-RELATED ORPHAN NUCLEAR RECEPTOR ALPHA resulting in its inhibition, and completion of the 24-hour cycle. CAPITAL=Proteins; *italic*=mRNA.

Fig. 1. The Circadian System.

disaggregate proteins. Cell disruption was carried out using a Potter-Elvehjem tissue grinder. Following centrifugation the supernatant was collected and proteins were quantified by Bradford assay.

Prior to protein separation, 50 μg of each sample was labelled with CyDyes (Cy3/Cy5), and an internal control was pooled from both lysates and labelled with Cy2 (Figure 2B). To account for any bias due to preferential binding of CyDyes, a dye swap approach was also done in a separate experiment, so that the samples were alternatively labelled with the reciprocal dye. CyDyes form a covalent bond between their NHS ester reactive group and epsilon amino group of protein's lysine residues. The labelling reaction does not cause a significant change in isolectric point because lysine carries an intrinsic +1 charge at neutral or acidic pH, which is replaced by CyDye's +1 charge. CyDyes were added so that there was a stoichiometric excess of proteins and thus only 1-5% of lysines were labelled. The reaction was carried out for 30 minutes in the dark, and then quenched with 10 mM lysine.

3.2 First dimension isoelectric focussing

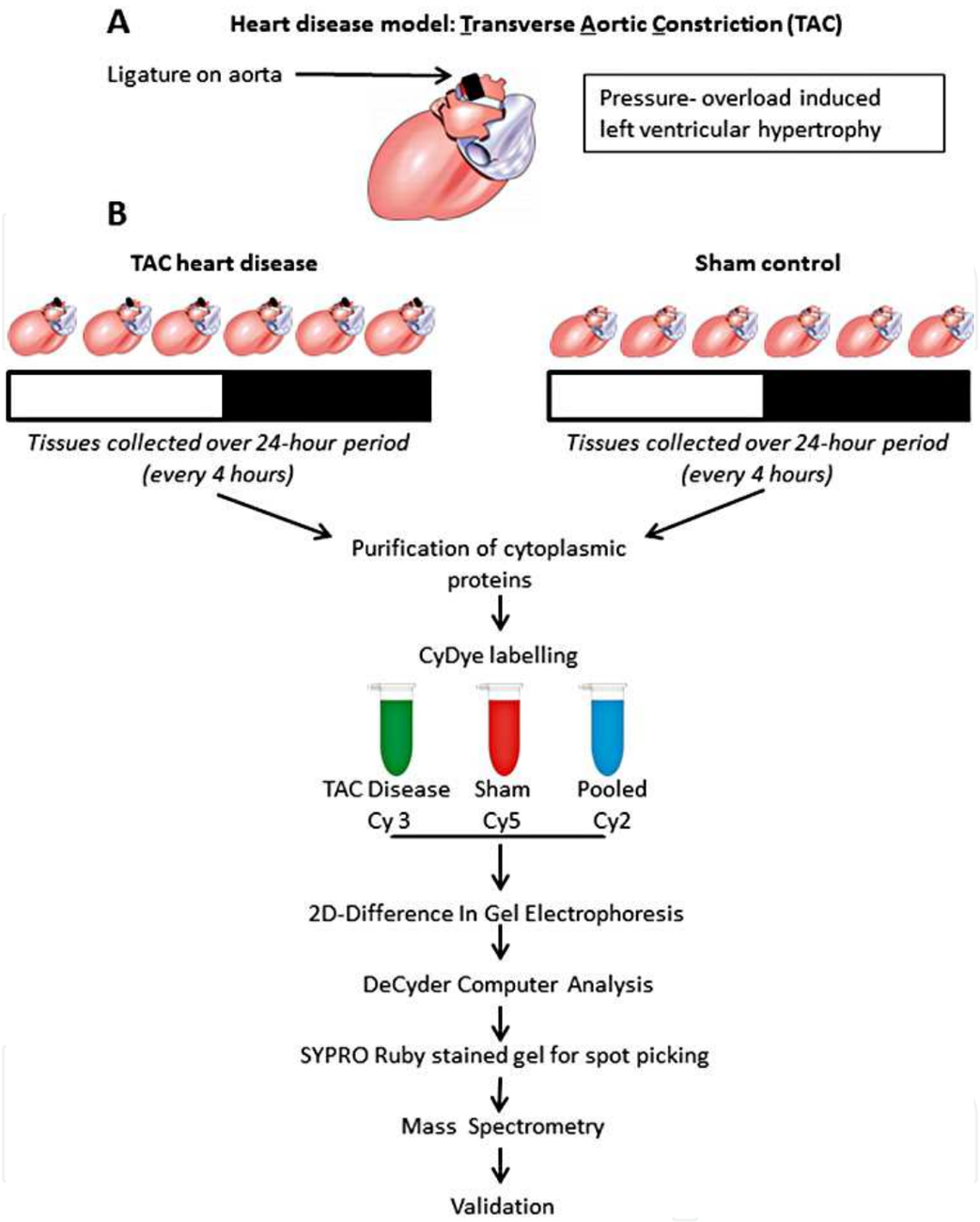
Proteins were separated in the first dimension by isoelectric focusing (IEF) based on our standard operating protocol (Hobson et al., 2007). In the experiment shown here (Figure 3) we used nonlinear 13 cm immobilized pH gradient (IPG) strips pH 3 – 10 (GE Healthcare). The CyDye labelled samples were combined and mixed with rehydration buffer (8 M urea, 2% w/v CHAPS, Dithiothreitol (DTT), 0.5% v/v carrier pharmalytes). Then, samples in rehydration buffer were applied at the bottom of the IPG strip holder. The strip was put on top of the sample solution and covered with paraffin oil to prevent crystallization of urea, water loss and carbon dioxide dissolving at the alkaline end of the strip. IEF was done using an Ettan IPGphore unit. The initial step was active rehydration, allowing proteins to slowly enter the strip along its whole length under the influence of a small current (30 V for 10 h). The next series of steps were 500 V step and hold for 2 h, 1000 V gradient for 1 h, 8000 V gradient for 2.5 h, 8000 V step and hold for 16000 V h, 500 V step and hold for 2 h. The result was that proteins migrated to a position in accordance with their isoelectric point.

3.3 Second dimension electrophoresis

For the second dimension, the sample containing IPG strips were equilibrated with sodium dodecyl sulfate (SDS) running buffer (75 mM Tris (pH 8.8), 6 M Urea, 30% v/v Glycerol and 2% w/v SDS). DTT was added to reduce disulfide bonds, and iodoacetamide (IAA) to alkylate thiol groups what prevented disulfide bonds from reforming. After equilibration the IPG strip was applied directly on top of a large format 16 X 14 cm 12% acrylamide gel. Agarose was poured on top of the strip to prevent air from getting underneath and to hold strip in place. Molecular markers were applied 4 cm from the positive end of the strip. The proteins were then separated vertically according to their molecular weight by SDS-polyacrylamide gel electrophoresis (PAGE) on a DALT 6 electrophoresis unit at 20 ^{0}C, 6 W/ gel for 18 h.

3.4 Protein detection and bioinformatics analysis

After 2D-DIGE, the relative abundance of proteins from the TAC heart disease versus sham protein lysates was detected using a high resolution fluorescent scanner Typhoon 9410 (GE Healthcare). The excitation/emission wavelengths for the CyDyes were as follows: Cy2, 480 nm/530 nm (blue), Cy3, 540 nm/590 nm (green) and Cy5, 620 nm/680 nm (red). Images



A) Heart disease model. Pressure overload cardiac hypertrophy is induced by transverse aortic constriction (TAC). A ligature is placed distal to the third bifurcation of aorta. Sham animals undergo the same procedure except the ligature is not tightened. B) Two-dimensional difference in gel electrophoresis (2D-DIGE) and mass spectrometry (MS) approach to characterize the circadian cardiovascular proteome in health and disease. Heart tissue is collected from TAC disease and sham control animals at 6 timepoints over the 24-hour light/dark cycle. The cytoplasmic proteome from TAC vs. sham hearts is labelled with Cy3 and Cy5 dye, respectively. An internal control consists of pooled samples labelled with Cy2. After 2D-DIGE protein expression is analyzed with DeCyder. SYPRO Ruby gel for picking spots of interest is created. Proteins are excised and identified by MS. Results are validated by Western blot.

Fig. 2. Experimental design.

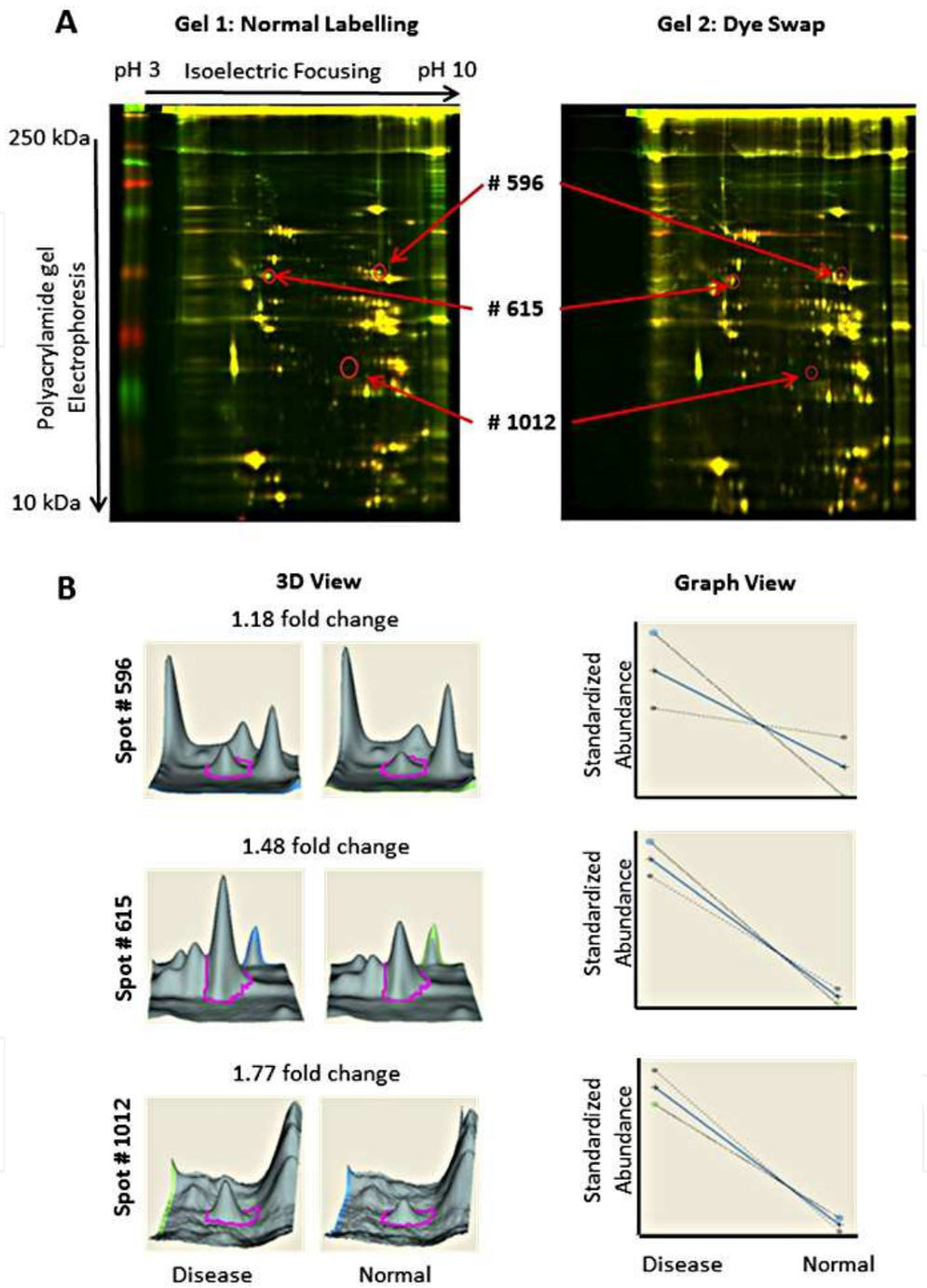
were visualized with Image Quant TL software. An overlay image of Cy3 and Cy5 scans is illustrated in Figure 3A. Proteins that had higher abundance in TAC heart disease vs. sham appeared as a red spot. Lower levels in TAC heart disease vs. sham appeared green. Equal amounts of protein in TAC heart disease vs. sham were yellow.

Statistical analysis and quantification of protein expression was achieved by DeCyder software (GE Healthcare). In the first step of DeCyder workflow, difference in gel analysis (DIA) used a codetection algorithm to detect and match differently labelled samples (Cy2, Cy3 and Cy5) within the same gel. It was necessary to define a specific area of interest within a gel and then manually confirm that the program detected all the spots within that area. Spot intensity corresponding to protein abundance was quantified after background subtraction and normalization. The second step termed biological variance analysis (BVA) simultaneously analyzed multiple DIGE gels by matching all the spots to a master gel, defined by user. Protein expression was compared between gels and statistically verified. Based on spot intensity, DeCyder constructed 3D views of relative protein abundance. The standardized volume of the peaks was used to calculate fold change.

In this study, and as shown in Figure 3B, we found three spots (# 596, 615, and 1012) that were upregulated in TAC heart disease compared to sham at our sleep-wake transition timepoint (ZT23). Spot # 596 had a 1.18 fold change, spot # 615 had a 1.48 fold change, and spot # 1012 had 1.77 fold change. To identify the proteins of interest, a pick gel was created containing 300 µg of unlabelled protein. Glass plates were treated with Bind-Silane (γ-methacryloxypropyltrimethoxysilane) so that the gel was covalently attached to the surface. After electrophoresis, proteins were fixed in 10% methanol and 7% acetic acid solution for 2 hours, then stained with SYPRO Ruby for 18 hours and destained in 40% methanol/10% acetic acid solution for 2 hours. The gel was scanned with a Typhoon 9410 scanner at 532 nm (670 BP 30 emission filter). Images were uploaded to DeCyder and matched to the previous DIGE gel. Unique coordinates for each spot were created using position markers. After manual confirmation, an Ettan Spot picker (GE Healthcare) was used to excise proteins from the gel.

3.5 Trypsin digestion and mass spectrometry

To determine the identification of the proteins in gel spots # 596, 615 and 1012, we used an In-Gel Tryptic Digestion Kit (Thermo Scientific) followed by mass spectrometry (MS). Trypsin is a serine protease that cleaves the peptide bonds at the carboxyl side of lysine and arginine amino acids. Following digestion, tryptic peptide fragments were separated by nano-LC which consisted of a trap column (300µm ID) and an analytical column (75 µm ID) packed with 5µm, 300Å Zorbax SB C18 beads. A linear binary gradient was used where solvent A was 98% H_2O:2% CH_3CN and 0.1% (v/v) formic acid and solvent B was 2% H_2O : 98% CH_3CN and 0.1% (v/v) formic acid. Peptides were eluted over a 2 - 95% solvent B gradient for 100 min at a rate of 300 nL/min. The eluant from the nano-LC was coupled to a hybrid triple/quadrupole linear ion trap mass spectrometer (QTRAP 4000, ABSciex) through a nano-spray ionization source equipped with a 15 µm ID emittor tip. Our preferred database for searching was Mascot (http://www.matrixscience.com). This database contains information on more than 10 million proteins, with inherent redundancies built in since peptides can correspond to more than one protein. Each of the spots generated a list of possible protein candidates, and Mascot score and E value helped identify the correct match. The molecular weight and pI of the identified protein was comparable to the expected molecular weight and pI of the corresponding spot from the 2-DE gel.



A) 2D-DIGE image. The gel on the left had proteins purified from TAC (heart disease) labelled with Cy3 and those from sham heart labelled with Cy5. Gel on the right shows alternatively labelled samples, where TAC proteins are labelled with Cy5 and sham with Cy3. Location of the proteins of interest is shown with arrows. B) DeCyder computer analysis of three identified protein spots # 596, 615 and 1012. Based on standardized abundance these protein spots increased in TAC vs. sham. Expression changes are shown by 3D and Graph views.

Fig. 3. Circadian Cardiovascular Proteomics.

3.6 Role of proteins identified in TAC heart disease

Our laboratory is interested in circadian cardiovascular proteomics to better understand molecular processes underlying heart disease and clinical treatments. The TAC upregulated spots 519, 615 and 1012 were identified by MS as Succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial (SCOT), Desmin (DESM), and PDZ and LIM domain protein 1 (PDLIM1) respectively. Three examples from ZT23 (one hour before lights on, murine sleep-time) were shown here; other proteins were identified from different times of day or night. Figure 4 shows a representative mass spectrum for one of the identified proteins, SCOT (spot #519), as well as the number and list of tryptic hits, Mascot score, E-value, and protein sequence. Functionally, SCOT is a mitochondrial matrix protein and is the key rate limiting enzyme for ketone body metabolism (Fukao et al., 2004; Orii et al., 2008). That it exhibits increased expression around sleep time suggests that it plays a role in changing cardiac energy sources. The second identified protein, DESMIN (spot # 615) is crucial for muscle structure and function, as reviewed in (Paulin & Li, 2004). The third protein, PDLIM1 (spot #1012) is a cytoskeletal protein involved in cardiac contractility and cardioprotection, as reviewed in (Arias-Loza et al., 2008; Johnsen et al., 2009).

3.7 Validation: Western blotting

Candidate circadian biomarkers identified by 2D-DIGE and MS are validated by Western blot protein expression analyses using an independent set of cytoplasmic soluble proteins from TAC and sham hearts. Proteins (20 μg) are separated according to their mass by 12% SDS-PAGE and transferred to polyvinylidene fluoride membrane (Bio-Rad) using a semi-dry transfer apparatus (Bio-Rad). Membranes are blocked for 2 hours at room temperature with 5% non-fat dry milk in TBS-T 0.05 % (20 mM Tris Base, 137mM NaCl, 0.05 % Tween 20, pH 7.6) and incubated overnight at 4 ^{0}C with primary antibodies against SCOT, DESM or PDLIM1. The antibodies are diluted according to the manufacturer's instructions. Anti-actin antibody (1:40000, Milipore) is used as a loading control. Immunoreactive protein bands are visualized with horseradish peroxidise-conjugated secondary antibodies (1:5000, Sigma) and ECLplus reagent (GE Healthcare). Blots are scanned using Storm 860 molecular imager (GE Healthcare) and protein expression is quantified by Image J software (NIH). Expression levels of SCOT, DESM and PDLIM1 in TAC samples at ZT23 are compared to sham, thus independently validating 2D-DIGE approach. Validating the candidate proteins/biomarkers in human samples will increase their significance and is key to translational applications.

4. Circadian proteomes in other body organs

Our time-of-day circadian approach led to discovery of SCOT, DESMIN, and PDLIM which were upregulated in TAC cardiac hypertrophy at sleep time. Other groups have also met with success investigating circadian proteomics in different tissues and clinical paradigms. A summary of identified circadian proteomes is shown in Table 1. The first identification of a circadian proteome was reported by Reddy and colleagues (Reddy et al., 2006), using a 2D-DIGE/MS approach to study murine liver. Mice were entrained to 12:12 L:D cycles, then placed in constant darkness (12:12 D:D) and sacrificed at 6 consecutive time-points 4 hours apart. It was observed that 135 (21%) out of 642 detected protein spots rhythmically cycled over the 24-hour period. Many of the newly identified circadian proteins were key rate limiting enzymes including ketohexokinase, succinate dehydrogenase 1, aldolase 2, enolase 1/aconitase 2, carbamoyl phosphate synthetase 1, CPS1, arginosuccinate synthetase 1, and arginase 1.

Tissue	Methods	Proteins with circadian expression pattern	Function	Reference
Liver CD1 mice	Mice were entrained under 12:12 L:D and then transferred to 12:12 D:D (constant darkness). Liver tissue was collected every 4-h over 24-h starting at CT 0 (n = 3/ time point) 2D-DIGE MALDI-TOF MS LC MS/MS	Arginosuccinate synthetase 1 (ASS1)	Urea cycle	(Reddy et al., 2006)
		Carbamoyl phosphate synthetase 1 (CPS1)	Urea cycle	
		Arginase 1 (ARG1)		
		Ketohexokinase (KHK)	Fructose metabolism	
		Succinate dehydrogenase 1 (SDH1)		
		Aldolase 2 (ALDO2)	Glycolysis	
		Enolase 1 (ENO1)		
		Aconitase 2 (ACO2)	Kreb's cycle	
Pineal gland Wistar rats	Rats were entrained under 12:12 L:D. Pineal glands were collected at ZT6 (6 hours after lights on) and ZT18 (6 hours after lights off) (n = 8/ time point) 2D-PAGE MALDI-TOF MS	**Dark period (rodent wake time)**		(Moller, Sparre et al. 2007)
		Alpha enolase	Glycolysis pathway	
		Gamma enolase		
		Vimentin	Morphogenesis	
		Creatine kinase, B chain	Energy transduction	
		Guanine deaminase	Purine catabolism	
		Peptidyl-prolyl cis-trans isomerase A	Protein folding	
		Annexin A2 and A5	Ca^{2+}-regulated membrane-binding	
		Light period (rodent sleep time)		
		Malate Dehydrogenase	Krebs cycle	
		Citrate synthase		
		Triosephosphate Isomerise		
		Ubiquinolcytochrome c reductase core protein I	Mitochondrial electron transport	
		RNA recognition motif	Processing of pre-mRNAs	
		Chaperonin containing TCP1	Chaperone, signal transduction	
		ER protein ERp29 precursor(Erp31)		
		Transitional endoplasmic reticulum ATPase	Vesicle budding from the endoplasmic reticulum	
		Complement C3 Precursor	Activation of the complement system	
		Phosphoserine phosphatise	Biosynthesis of serine from carbohydrates	
		Contrapsin-like protease inhibitor 1 and 3 precursor	Protein metabolism	

Tissue	Methods	Proteins with circadian expression pattern	Function	Reference
Retina C57BL/6 mice	Mice were entrained under 12:12 L:D and transferred to 12:12 D:D. Retinal tissue was collected every 6-h over 24-h starting at CT 2 (n = 5/ time point) 2D-PAGE MALDI-TOF MS	N-ethylmaleimide-sensitive fusion protein	Vesicle transport	(Tsuji, Hirota et al. 2007)
		Charged Multivesicular body protein 4b		
		Reticulocalbin-2 precursor	Calcium binding	
		Calbindin D28	Photoreceptor adaptation, gating of photic input	
		Heterogeneous ribonucleoprotein A/B	RNA-binding	
		T-complex 1 subunit delta	Protein folding, phototransduction, morphological changes	
		Leukotriene A4 hydrolase	Metabolism	
		Proteasome subunit alpha type 1	Protein degradation	
SCN releasate Long-Evans/BluGill rats	Rats were entrained under 12:12 L:D and transferred to 12:12 D:D. Samples were taken every 4-h over 24-h starting at CT 0 LC MALDI-TOF MS LC MS/MS	Angiotensin I		(Hatcher et al., 2008)
		Arginine vasopressin[b]		
		Proenkephalin 219–229		
		Galanin		
		Neurokinin-B		
		Neurotensin		
		POMC, melanotropin α		
		PEN		
		Big LEN		
		Little SAAS		
		Somatostatin-14[b]		
		proSomatostatin 89-100		
		Substance P		
		Thymosin β-4		
Urine *Tau* mutant hamsters	Hamsters entrained under 14:10 L:D cycle. SDS-PAGE LC MS/MS	Cytochrome C (CYCS)	Apoptosis	(Martino et al., 2008)
Blood C57Bl/6	Mice entrained to 12:12 L:D cycle SELDI	Fingerprinting assay	Proof of concept that de-novo proteins cycle in the blood, and that the daily rhythmic variation changes in heart disease	Ref Martino same one as below
Blood C57BL/6 mice	Mice entrained under 12:12 L:D cycle. Samples collected every 4 hours, starting at ZT 23 (n = 3/ time point) SDS-PAGE LC MS/MS	Transthyretin	Transports thyroxin (T4) and retinol (vitamin A)	(Martino, Tata et al. 2007)
		Apolipoprotein A1 precursor	Lipid and cholesterol regulation	
		Apolipoprotein E precursor		
		Apolipoprotein J		
		Plasminogen	Plasmin formation	
		Complement C3 precursor	Activation of compliment system	

Tissue	Methods	Proteins with circadian expression pattern	Function	Reference
Blood metabolome C57BL/6 and CBA/N mice	Mice entrained under 12:12 L:D cycles, then transferred to either 12:12L:D or 12:12 D:D. Blood collected in fasting animals over 24-h starting at ZT 4 or CT 4 LC-MS CE-MS	Trimethylamine N-oxide		(Minami et al., 2009)
		Glutamine		
		2-Aminobutyrate		
		Cytidine		
		Sarcosine		
		Carnitine		
		Valine		
		Tryptophan		
		4-Guanidinobutyrate		
		Isoleucine		
		3-Methylhistidine		
		Leucine		
		Proline		
		Guanidoacetate		
		1-Methylnicotinamide		
		Citrulline		
		Creatinine		
		Glycine		
		Methionine sulfoxide		
		a-Aminoadipate		
		Methionine		
		Phenylalanine		
		N,N-Dimethylglycine		
		Thr 13C		
		Threonine		
		Ornithine		
		Hydroxyproline		
		Creatine		
		Corticosterone		
		lysophosphatidylcoline		

Table 1. Circadian proteomes.

The circadian proteome in the rat pineal gland was identified using 2D-PAGE, silver stain and tandem mass spectrometry (MS/MS) (Moller et al., 2007). In this study, rats were entrained to 12:12 L:D cycle. Pineal glands were collected at two timepoints: ZT06 which is 6 hours after lights on (light phase, rats asleep) and ZT18 which is 6 hours after lights off (dark phase, rats awake). A total of 1737 pineal gland proteins were detected, 35 showed greater abundance during sleep time and 25 during wake time. Proteins upregulated during wake time were involved in glycolysis (alpha- and gamma-enolase), morphogenesis (vimentin), energy transduction (Creatine kinase), purine catabolism (guanine deaminase), protein folding (peptidyl-prolyl cis–trans isomerase A), and Ca^{2+}-dependent membrane binding (annexin A2, annexin A5). Proteins with increased abundance during sleep mapped to the Krebs cycle (malate dehydrogenase, citrate synthase, triosephosphate isomerise), mitochondrial electron transport (ubiquinol-cytochrome c reductase core protein 1), RNA binding and processing (RNA recognition motif), protein folding (chaperone-containing TCP1, ER protein ERp29 precursor), cell transport (transitional ER ATPase), complement (C3 precursor), and metabolism (phosphoserine phosphotase, contrapsin-like protease inhibitor 1 and 3 precursors).

The circadian proteome of the mouse retina was characterized by 2D-PAGE and Coomassie Brilliant stain (Tsuji et al., 2007). C57Bl/6 mice were entrained at 12:12 L:D cycle, then placed in constant darkness (12:12 D:D) and sacrificed on the fourth day at 4 timepoints: CT2, CT8, CT14, and CT20. CT2 corresponded to subjective dawn and CT12 to subjective dusk. A total of 415 protein spots were detected and 11 exhibited circadian rhythms. The cycling proteins were important in vesicular transport (N-ethylmaleimide-sensitive fusion protein, charged multivesicular body protein 4b), calcium-binding (calbindin D28, reticulocalbin-2 precursor), RNA-binding (heterogeneous ribonucleoprotein A/B), protein folding (T-complex 1 delta), metabolism (leukotriene A4 hydrolase), and protein degradation (proteasome subunit alpha1).

The circadian peptidome released from rat hypothalamic SCN was characterized using a gel-free approach (Hatcher et al., 2008). Long-Evans/BluGill rats were entrained to 12:12 L:D and then transferred to constant darkness. Animals were sacrificed during subjective day-time (CT 0-12) and brain slices containing SCN and optic nerve were prepared. SCN releasates were obtained from extracellular fluid with pipettes containing solid-phase extraction material or from the SCN itself with micrometer-sized beads with solid-phase extraction material. Samples of releasate were collected at the beginning and end of CT 0–4, 4–8, 8–12, 12–16, 16–20, and 20–24 intervals and analyzed with MALDI TOF MS. Identified peptides were independently verified with LC-MS/MS. Arginine vasopressin showed a robust circadian rhythm with a peak during sleep time. Other identified peptides were angiotensin I, arginine vasopressin^{b}, proenkephalin, galanin, neurokinin-B, neurotensin, melanotropin α, PEN, big LEN, little SAAS, somatostatin-14b, proSomatostatin, substance P and thymosin β-4. Interestingly, this study also detected previously unknown peptides, which prompted further investigation of their biological role.

5. The circadian proteome helps to understand disease and identify new biomarkers

Disturbances of the circadian rhythm, such as might occur in humans in shift work or sleep disorders, can affect many physiologic processes (e.g. circadian rhythms of heart rate, body temperature, sympathetic nervous activity, chemical, inflammatory, and metabolic processes). The proteome should characteristically change as well, revealing de novo biomarkers. We investigated this using a +/tau hamster model bearing a mutation in the core circadian clockwork protein casein kinase 1 epsilon (Martino et al., 2008). The circadian rhythm disruption in these animals etiologically caused heart and kidney disease. The animals exhibited profound proteinuria. To detect proteomic changes, urine samples collected from +/tau vs. controls were analyzed on 10-20% tricine gels stained with Coomassie dye. Bands were excised from the gel, trypsin digested, subjected to MS/MS on a LCQ DECA XP ion trap, and analyzed using Sequest. Protein identification was validated by Western blot. As shown in Table 1, a ~15 kDa protein band, appearing only in the urine of the +/tau animals, was identified by MS as cytochrome c, a biomarker of cellular apoptosis. Apoptosis was confirmed in the renal tissues of +/tau mutants by terminal uridine deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining. Thus by using circadian approaches, a novel biomarker of renal disease was discovered. This was a particularly exciting discovery as there are notably very few biomarkers of developing renal disease. For example, a usual biomarker for kidney disease is creatinine, however, this

represents just chemical waste generated from muscle metabolism and is only an indirect marker of renal function. Here we identified a direct indicator of early and ongoing tissue damage, by investigating circadian proteome expression. This marker is easily obtained by collecting urine samples, thus making it potentially ideal for point-of-care or routine diagnostics. Additional examples of circadian-based biomarkers, including many fundamental to human health and disease, are described below.

6. Circadian biomarkers in blood

Circadian proteomics can be used for discovery of de-novo blood biomarkers of heart disease. Established cardiovascular factors (e.g. hypertension, smoking, diabetes) do not fully explain the risk for heart disease, and there is substantial interest in the development of new biomarkers to identify persons at risk and who may be targeted for preventative measures. In those with heart disease, biomarkers are in demand to help track disease progression and aid physicians in better treating their patients. Although a number of strategies are currently used to identify biomarkers, there have been very few clinical advances, and thus new approaches such as circadian proteomics are warranted.

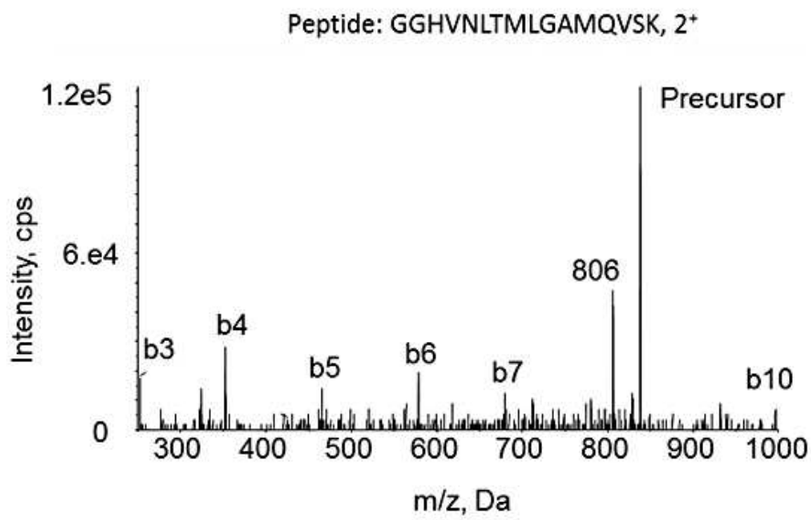
As an example, we recently investigated diurnal proteome cycling in murine blood (Martino et al., 2007). Blood was the preferred tissue for biomedical investigation because of its ease of accessibility and minimal invasiveness for sampling. As shown in Table 1, the first approach was proof of concept and used surface-enhanced laser desorption ionization (SELDI) MS. Only proteins retained on ion exchange solid phase chromatographic surfaces (or chips) were examined; substances with other biochemical properties remain open to future investigation. Expression profiles were collected over a wide mass range; those of lower molecular weight (1-10 kDa range) classically contained bioactive peptides, while those in the midrange (10-50 kDa) and larger (>50 kDa) reflected peptides/proteins involved in cell structural and functional processes. With SELDI MS we essentially created a fingerprint of blood protein expression over the 24-hour time, from which one could then quantify, statistically analyze and graph data to visualize daily protein rhythms.

Since one of the drawbacks of SELDI MS was a relative inability to further identify the fingerprint proteins, the diurnal blood proteome was also characterized following prefractionation on column chromatography with an ion exchange resin (Martino et al., 2007). As shown in Table 1, proteins comprising effluent, salt elution, and bead retentive fractions were visualized by SDS-PAGE and silver stain. Protein bands that exhibited cyclic variation over 24 hours were excised, trypsin digested, and then injected by electro spray ionization (ESI) into a LCQ DECA XP ion trap. Proteins were identified by comparison searching molecular mass against murine databases. Many of the identified blood proteins with diurnal expression linked to cycles in physiology were those released from liver, such as transthyretin, apolipoprotein A1 precursor, apolipoprotein E precursor, apolipoprotein J, plasminogen and complement C3 precursor. Ultimately, comparing daily rhythms in sera from healthy individuals vs. heart disease patients would allow for the creation of new biomarker profiles and discovery platforms.

Another study examining diurnal biomarkers in blood measured 24-hour profiles of small chemical substances (peptides, amino acids, hormones) (Minami et al., 2009). As shown in Table 1, blood was drawn every 4 hours over the 24-hour period from CBA mice maintained

SCOT: Succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial

A



B

Peptides

KGNAHKI
KLLSSGLRL
KCTLPLTGKQ
KGVFDVDKKN
RNFNLPMCKA
KDGSVAIASKPRE
KFYTDPVEAVKD
KYGDLANWMIPGKM
KGMGGAMDLVSSSKTKV
RMISSYVGENAEFERQ
RGGHVNLTMLGAMQVSKY
RQFLSGELEVELTPQGTLAERI
RAGGAGVPAFYTSTGYGTLVQEGGSPIKY

Total number of peptides sequenced: 24
Mascot score: 150
E-value: 4.0 ×10^{-21}

C

Protein sequence and peptide coverage

*MAAL***KLLSSGLRL***GASARSSRGALHKGCVCYFSV*
*STRHHT***KFYTDPVEAVKD***IPNGATLLVGGFGLC*
GIPENLIGALLKTGVKDLTAVSNNAGVDNFGLGLL
*LRSKQIK***RMISSYVGENAEFERQFLSGELEVEL**
TPQGTLAERIRAGGAGVPAFYTSTGYGTLVQ
EGGSPIKYNKDGSVAIASKPRE*VREFNGQHFIL*
*EEAITGDFALVKAWKADRAGNVIFRKSA***RNFNLP**
MCKA*AGTTVVEVEEIVDIGSFAPEDIHIPKIYVHRL*
IKGEKYEKRIERLSLRKEGDGKGKSGKPGGDVRERI
IKRAALEFEDGMYANLGIGIPLLASNFISPNMTVH
LQSENGVLGLGPYPLKDEADADLINAGKETVTVLP
*GASFFSSDESFAMI***RGGHVNLTMLGAMQVSK**
YGDLANWMIPGKMVKGMGGAMDLVSSSK
TKV*VVTMEHSA***KGNAHKI***MEKCTLPLTGKQCV*
*NRIITE***KGVFDVDKKN***GLTLIELWEGLTVDDIKKST*
GCDFAVSPNLMPMQQIST

A) Representative mass spectrum of the SCOT peptide: RGGHVNLTMLGAMQVSKY. B) A total of 24 peptides were identified, corresponding to 13 unique sequences. The Mascot score is 150, E-value is 4.0 ×10^{-21}. C) Identified peptides and their corresponding match to the SCOT protein sequence.

Fig. 4. MS based identification of Succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial (SCOT).

under L:D or D:D conditions and LC-MS analysis was performed that led to the detection of 176 negative and 142 positive ion peaks. Importantly, in transgenic murine model of clock disruption (Cry1-/- and Cry2-/-), expression of these metabolites was altered, suggesting that they were controlled by the clock mechanism. The authors demonstrated that their metabolite timetable could be used to accurately determine body time in mice with different genetic background, age, sex and feeding regime. The overall purpose of doing this was to create maps of body time, which could be applied clinically to optimize understanding of disease or determine the best times for administering drugs and therapies.

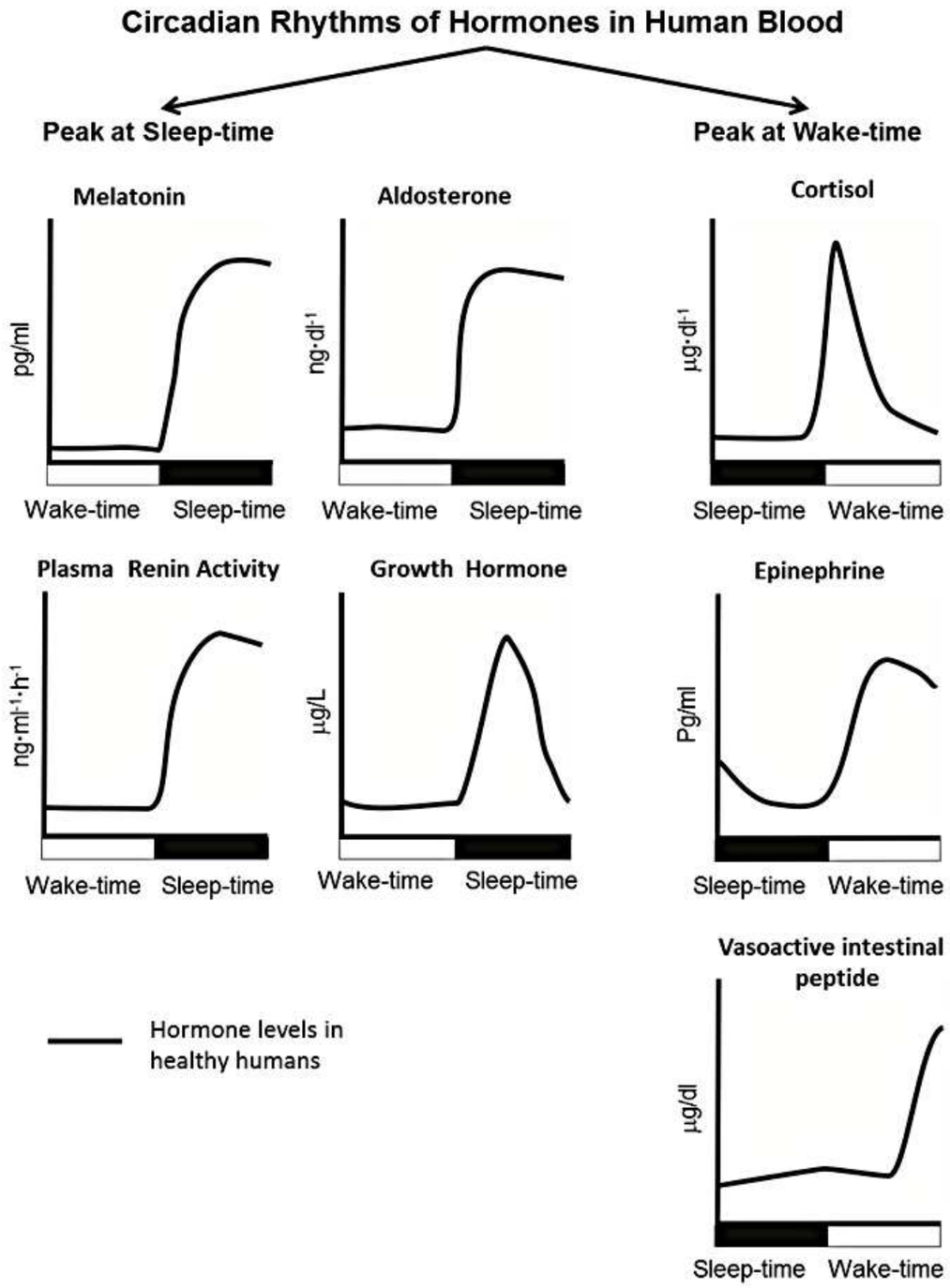
7. Circadian rhythms in neuro/hormone biomarkers

Long before protein cycling was even discovered, it was already known that some key neuroendocrine hormones important to the heart had daily rhythmic patterns of expression. Cycling of many of these hormones drives the protein rhythms we observe in normal heart and other tissues, and the changes that occur in disease. Further details are described below and in Figure 5 and Table 2.

7.1 Sleep-time hormones

The first example is melatonin, and its expression in humans is elevated in the dark but suppressed by light (thus it is sometimes called the "hormone of darkness") (Brzezinski, 1997). It has a cardioprotective effect through antioxidant and anti-inflammatory activity, reviewed by (Tengattini et al., 2008). It is a useful circadian biomarker of heart disease. Nocturnal melatonin levels were low in patients with acute myocardial infarction (MI) vs. healthy controls within the first 24 hours after hospital admission (Dominguez-Rodriguez et al., 2002). Also, patients with ST-segment MI who developed adverse events during follow-up had significantly lower nocturnal melatonin levels than patients without the events (Dominguez-Rodriguez et al., 2006). In another study it was revealed that nocturnal melatonin levels were reduced in patients with cardiac syndrome X vs. controls (Altun et al., 2002). Second, the renin-angiotensin-aldosterone system (RAAS) also exhibits circadian rhythm. Aldosterone produced by the adrenal cortex regulates Na^+ and K^+ homeostasis. Aldosterone plasma levels peak during sleep time (11:00 P.M. – 7:00 A.M.) (Charloux et al., 1999). As a biomarker of heart disease, it has been reported that while normal subjects showed an 81% decrease during the wake period, there was only a 40% decrease in low renin hypertensive patients (Grim et al., 1974). Similarly, plasma renin activity (PRA) cycles in healthy subjects with peak activity during sleep (Charloux et al., 1999). Patients with hypertensive heart disease exhibited a greater increase in PRA during sleep-time as compared to normotensive patients (Tuck et al., 1985).

Finally, growth hormone secretion by the anterior pituitary gland is pulsatile and has a circadian rhythm with the highest peak occurring at midnight (Surya et al., 2006). Growth hormone is involved in regulation of cardiac metabolism and contractility, reviewed by (Volterrani et al., 2000). In some heart failure patients (New York Heart Association classes I-III) there is a loss of circadian rhythm and overall reduction of growth hormone secretion (Duncan et al., 2003).



Left; Melatonin, aldosterone, plasma renin activity, growth hormone peak during sleep time. Right; cortisol, epinephrine, vasoactive intestinal peptide peak during wake time. X-axis: white bar = light period, black bar = dark. Rhythms illustrated here are based on the references listed in Table 2.

Fig. 5. Dirunal cycling of hormones in blood.

Hormone	Selective function(s) relevant to the cardiovascular system	Hormone Cycling		References
		In healthy humans	Altered cycling in specified cardiovascular disease	* Used in Figure 5 § Heart disease # Additional references
Melatonin	Antioxidant, circadian entrainment	Nocturnal peak in blood (in the dark) Secretion is suppressed by light and activated in the dark	Nocturnal decrease observed within the first 24h period in patients with myocardial infarct (MI) Low levels of melatonin correlate with adverse events during follow up in ST-segment MI patients Nocturnal decrease in patients with cardiac syndrome X vs. healthy controls	* (Brzezinski, 1997) # (Claustrat et al., 1986) # (Follenius et al., 1995) § (Dominguez-Rodriguez et al., 2006) § (Dominguez-Rodriguez et al., 2002) § (Altun et al., 2002)
Aldosterone	Regulates Na^+/K^+ homeostasis, blood pressure	Peak serum levels during sleep time	Does not decrease normally during the day in low renin hypertensive patients vs. healthy controls	* (Charloux et al., 1999) # (Katz et al., 1975) # (Lightman et al., 1981) § (Grim et al., 1974)
Plasma Renin Activity	Regulates blood pressure	Significantly higher plasma activity during sleep time	Significantly upregulated only at night in some patients with essential hypertension	* (Charloux et al., 1999) # (Katz et al., 1975) § (Tuck et al., 1985)
Growth Hormone	Affects metabolism and contractility	Nocturnal plasma peak Exhibits pulsatile expression	Overall decrease and loss of circadian rhythm in some patients with chronic heart failure (NY Heart Association classes I-III)	* (Surya et al., 2006) # (Takahashi et al., 1968) # (Hartman et al., 1991) § (Duncan et al., 2003)

Hormone	Selective function(s) relevant to the cardiovascular system	Hormone Cycling		References
		In healthy humans	Altered cycling in specified cardiovascular disease	* Used in Figure 5 § Heart disease # Additional references
Cortisol	Regulates blood sugar levels, increases blood pressure	Serum levels peak around wake-time	Peaks at 4:00 A.M. in some low renin hypertensive patients	* (Charloux et al., 1999) * (Lightman et al., 1981) # (Lockinger et al., 2004) # (Cugini et al., 1991) § (Grim et al., 1974)
Epinephrine	Increases heart rate and blood pressure Mediates sympathetic response	Plasma levels peak during wake time	Nocturnal increase in some patients with obstructive sleep apnea vs. healthy controls	* (Linsell et al., 1985) # (Scheer et al., 2009) # (Akerstedt & Froberg, 1979) § (Alonso-Fernandez et al., 2005)
Vasoactive intestinal peptide	Regulates vasodilation, heart rate, force of contraction	Plasma peak before sleep (20:00), trough at night (0:00)	Circadian rhythm is lost in patients with orthotopic heart transplant	* (Cugini et al., 1991) # (Cugini et al., 1992) § (Cugini et al., 1993)

Table 2. Circadian rhythms in neuro/hormones in humans

7.2 Wake time hormones

Cortisol exhibits diurnal variation with serum levels peaking early in the morning around wake time and troughing late in the evening (Charloux et al., 1999; Lightman et al., 1981). It may also be a useful biomarker or mediator of disease as plasma cortisol peaks earlier at 4:00 A.M. in some low renin hypertensive patients, as compared to 8:00 A.M. in normal controls (Grim et al., 1974). Epinephrine stimulates sympathetic activity including in the cardiovascular system. It exhibits a robust endogenous circadian rhythm that peaks during wake time (Linsell et al., 1985), and this expression changes in obstructive sleep apnea heart failure patients, in which there is increased nocturnal sympathetic tone (Alonso-Fernandez et al., 2005; Bradley & Floras, 2009). Lastly, vasoactive intestinal peptide affects vasodilation, heart rate and force of contraction, reviewed by (Henning & Sawmiller, 2001). It has a circadian rhythm with a peak before sleep time (20:00) and a trough later at night (0:00) (Cugini et al., 1991). The rhythm of this peptide is lost in patients with orthotopic heart transplant (Cugini et al., 1993).

8. Clinical translation

Discovery of the circadian cardiovascular proteome and it's endogenous drivers provides a new understanding of cardiovascular health and disease, where time is a new paradigm of functional significance. Proteomic biomarkers can be easily implemented with ELISA-type point-of-care diagnostic platforms that could be routinely applied in physician offices, or even potentially in the consumer's home. One of the practical applications of cardiovascular circadian proteomics is chronotherapy, which requires easily accessible markers of body time for optimizing the timing of drug treatments. For example, we recently demonstrated that the efficacy of treatment with the angiotensin converting enzyme inhibitor (ACEi) captopril exhibits a diurnal pattern, consistent with the diurnal variation in ACE expression (Martino et al., 2011). ACEi are common medications given to cardiovascular patients with hypertension, after a myocardial infarction or with heart failure. We found that drug administration at sleep-time improved heart function, but wake-time did not differ from placebo. This approach can be used in other diseases such as cancer (Hrushesky & Bjarnason, 1993; Innominato et al., 2010) and neuroendocrine disorders (Chung et al., 2011) as well.

9. Conclusion

Circadian cardiovascular proteomics is an important new area of research that provides an excellent opportunity to elucidate molecular processes that underlie our health and disease across the 24-hour light/dark period. At this early stage, temporal analysis of the proteome in cardiovascular tissues (i.e. heart or blood) of experimental animal models reveals remarkable 24-hour variations in protein abundances. Diurnal protein profiles differ remarkably between health and disease. Characterization of these proteins is the key to understanding normal body physiology as well as providing new diagnostic capabilities, and new approaches to treatment by aiding in the design of personalized therapeutics.

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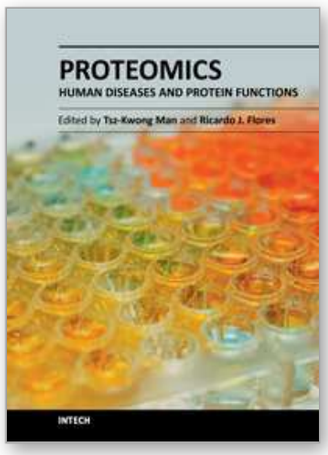

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Biomedical research has entered a new era of characterizing a disease or a protein on a global scale. In the post-genomic era, Proteomics now plays an increasingly important role in dissecting molecular functions of proteins and discovering biomarkers in human diseases. Mass spectrometry, two-dimensional gel electrophoresis, and high-density antibody and protein arrays are some of the most commonly used methods in the Proteomics field. This book covers four important and diverse areas of current proteomic research: Proteomic Discovery of Disease Biomarkers, Proteomic Analysis of Protein Functions, Proteomic Approaches to Dissecting Disease Processes, and Organelles and Secretome Proteomics. We believe that clinicians, students and laboratory researchers who are interested in Proteomics and its applications in the biomedical field will find this book useful and enlightening. The use of proteomic methods in studying proteins in various human diseases has become an essential part of biomedical research.

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