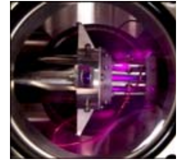
BOSTON UNIVERSITY SCHOOL OF MEDICINE
CARDIOVASCULAR PROTEOMICS CENTER

NHLBI PROTEOMICS



HIGHLIGHTS
PROGRESS REPORT
"Oxidative Protein Modifications in Cardiovascular Disease"

Reporting period: Feb. 1, 2007 to May 31, 2007

Progress for the trimester:

The projects making up the Cardiovascular Proteomics Center (CPC) are advancing on their specific aims and publishing regularly. The long list of publications of the previous trimester has been followed by a period of manuscript writing during the current trimester, according to the normal publication cycle. CPC investigators, project leaders and collaborators have submitted nearly 50 grant proposals, many proposing proteomics methodologies. Nine projects have been funded--most recently the five-year renewal of the "Genetic Modulation of Sickle Cell Disease" program, a major program of the Boston University Sickle Cell Center of Excellence, one of whose projects has a major proteomics specific aim. This project grew from a project 10 and Core laboratory collaboration. It is underway and aims to characterize protein changes and post-translational modifications of proteins as a function of sickle cell disease.

During the period of review we hosted the ninth Investigators Meeting of the NHLBI Proteomics Initiative April 18-19, 2007. A highlight of the meeting was its new open format. We debuted a tutorial workshop held on the morning prior to the investigators' meeting, open to staff members of NHLBI Proteomics Centers, and a new format for part of the investigators' meeting, open to the research community. The first day of the meeting was a closed session, featuring activities within the 10 Proteomics Centers and informal networking while the second day was a well-received research symposium.

At this point in the Proteomics Initiative, some location changes and turnover in personnel should be noted. Previously we reported that Joseph Loscalzo, M.D., Ph.D. (project leader of **projects 4 and 11**—see below), and Diane E. Handy, Ph.D. and Jane A. Leopold, M.D., researchers on these projects, moved to Brigham and Women's Hospital. The team remains committed to our proteomics research program as key personnel and, to that end, a subcontract is being established between Boston University and Brigham and Women's Hospital to support their continued involvement. **Projects 1 and 2** researchers, Drs. Takeshi Adachi and Ming Hui Zou, left Boston University, the first to return to Japan and the second, to join the University of Oklahoma faculty. The project leader, Dr. Richard Cohen, brought new researchers on board to partly replace the two 0.10-FTE co-investigator positions. John F. Keaney, M.D., **project 3** leader, moved to the University of Mass. Medical School in April 2007. His research associate at 0.10 FTE, Shane Thomas, Ph.D., had returned to Australia in 2004, without being replaced in the

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project. Dr. Keaney plans to continue collaborating with our proteomics center without a formal institutional arrangement. The research associate in **project 8**, Carsten Skurk, Ph.D., returned to Germany in 2004 after completing his postdoctoral fellowship. In partial fulfillment of the 0.10-FTE research associate budget, a student has received a stipend in project 8 since that time. Finally, in **project 12**, Ronglih Liao, Ph.D., a researcher at 0.03-FTE effort, moved to Brigham and Women's Hospital in 2005 with Dr. Loscalzo's group and terminated her involvement with the program. Douglas Sawyer, M.D., also a project 12 co-investigator at 0.03 FTE, left Boston University in 2006 for Vanderbilt University. As that project already has a sufficient number of investigators to complete the planned research, they have not been replaced.

As for new personnel, Joseph Zaia, Ph.D., will provide consultation on experimental setup, instruction, and assistance in data interpretation related to innovative protein analyses utilizing a newly-installed ThermoFisher LTQ-Orbitrap mass spectrometer within our proteomics center.

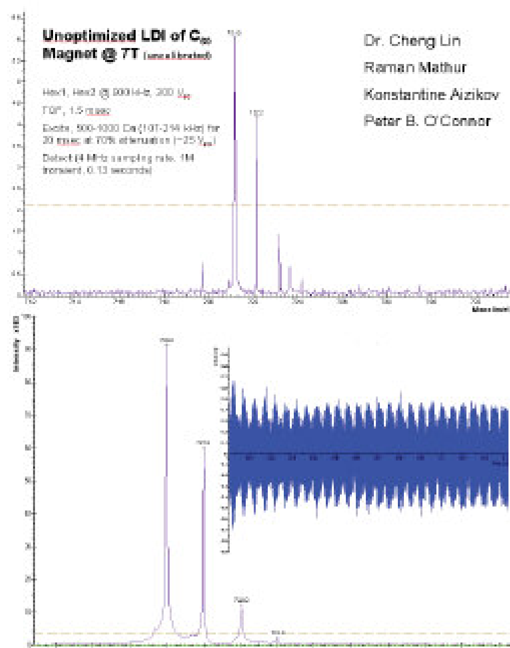


Figure 1. (top) The first signal from the Cryogenic FTMS instrument. (bottom) An optimized C_{60} signal from the Cryogenic FTMS. Inset shows the time domain signal.

Progress of individual CPC projects during the trimester is described below.

In the cryoFTMS laboratory (O'Connor), a major milestone occurred with achievement of first signal on the Cryogenic MALDI-FTMS prototype instrument being built in the laboratory. The instrument design was published in 2002 and funded by the NHLBI as a major instrument development project of the Proteomics Initiative. Cryogenic MALDI-FTMS (Matrix-Assisted Laser Desorption/Ionization—Fourier Transform Mass Spectrometry operating at very low temperatures) is a signal advance in FTICR (Fourier Transform Ion Cyclotron Resonance) mass spectrometry design. It involves close construction and integration of the FTICR with a modern cryogenic superconducting magnet design. The concept provides three major advantages. First, the magnet bore and FTICR cell chamber become very cold, which cryopumps the chamber and decreases the base pressure. Second, because of the cryopumping, the bore tube diameter can be much smaller, allowing high homogeneity and high magnetic fields to be generated at greatly reduced cost. Third, the cold surfaces can be used to cool a preamplifier for improved signal-to-noise ratio. In Figure 1, top, the signal is C_{60} laser desorbed, transferred down the two hexapoles, and detected in the ICR cell. Figure 1 (bottom) shows an optimized signal under current conditions. While many improvements still need to be made, and the amplifier now being used is at room temperature outside the vacuum, these figures demonstrate a functioning instrument. The researchers have achieved a critical threshold, a signal that can be used to better tune-in the instrument.

In the Core laboratory (McComb), researchers fully integrated new nano-HPLC equipment purchased from Waters Corp. for on-line LC-MS and LC-MS/MS studies of proteomics samples. They continued development of protocols for 2-dimensional abundant protein depletion using the Beckman Coulter HPLC system for processing of plasma samples from collaborator projects. The lab reports improved work flow protocols for automated 1D- and 2D-HPLC protein fractionation.

Protocols have been published and used in several projects and new ones are presented at research meetings. In addition, high-throughput methods for processing protein fractions from 1D- and 2D-HPLC using a novel 96-well array for sample processing continue to be investigated. Proteomics software programs developed with the help of two graduate students, Boston University Protein Identification Server (BUPID) and Boston University Database Search Shell (BUDSS), are being prepared for public release of the software code and both projects are being written for publication. Lastly, the high performance computer cluster for high-intensity bioinformatics processing, data capture, and storage, reported in the previous trimester, is now operational.

At the recent American Society for Mass Spectrometry annual meeting, June 3-7, 2007 in Indianapolis, FTMS and Core researchers presented 13 abstracts due to be submitted for publication as research articles over the next few months. An oral presentation by the Core laboratory director, "1D- and 2D-Protein Chromatography within the Proteomics Workflow for Enhanced Characterization of Protein Post-Translational Modifications," describes the means to rapidly fractionate proteins from complex samples and identify protein components. This workflow facilitates location and assignment of PTMs by integration of intact protein molecular weight information with peptide MS and MS/MS database searches. Using this methodology, intact proteins can easily be analyzed directly by MS, even at the low fmol level, with greatly improved protein identification.

In the Bioinformatics laboratory (Weng), researchers optimized BUPID for parallel computing. The software is now installed on a 16-way multi-processor server, with more comprehensive selection of protein sequence databases and faster computation time compared to the single-processor version.

In Project 1 (Post-translational Modification of Vascular Proteins by Reactive Nitrogen Species, Cohen), further studies have been performed with sequence-specific nitrotyrosine antibodies towards MnSOD nitrotyrosine-34. Researchers observed intense staining of endothelial and smooth muscle cells in aorta from type 2 diabetic mice compared with normal mice. This observation indicates mitochondrial oxidant stress in the aorta of mice with type 2 diabetes. In addition, collaborative studies with Dr. Lee Ann MacMillan-Crow, University of Arkansas for Medical Sciences, were performed using the MnSOD nY-34 antibody. The studies demonstrated increased staining in kidneys of rats with both diabetic nephropathy and ischemia reperfusion. Coupled with our previous work, these studies indicate that MnSOD inactivation by tyrosine nitration is associated with metabolic disease.

In project 2 (Oxidation of Cardiovascular Protein Thiols, Cohen), the antibody developed to detect cysteine-674 sulfonic oxidation in SERCA proved to be specific in tests with various blocking peptides. The tests showed that the antibody staining of immunoblots and immunohistochemistry was prevented by the antigenic peptide, but not the scrambled antigenic peptide. In addition, researchers report that sufficient SERCA was purified from diabetic pig aorta to show that the key cysteine was significantly oxidized. These studies were carried out in collaboration with Dr. Christian Schoneich, University of Kansas, external advisor to the Cardiovascular Proteomics Center.

Project 3 (Post-translational Modification of eNOS by Hypochlorite, Keaney) investigators continued to acquire structural information on eNOS modification by HOCl. Initial success in generating reproducible eNOS modification has led to the stage in which they can interrogate spectra to arrive at structural solutions. Recently the project leader, Dr. Keaney, moved to University of Mass. Medical School and set up his laboratory where he will continue to lead project studies.

The working hypothesis of project 4 (Post-translational Modification of Vascular Proteins by Homocysteine, Handy and Loscalzo)—over-expression of glutathione peroxidase modulates intracellular oxidant levels resulting in alterations in cellular signaling and growth—is being tested. During the trimester researchers continued work to identify: 1) signaling targets that are altered in GPx-1 over-expressing cells (GPx-1 OE), and 2) the nature of the disulfide alterations found in GPx-1 OE. They have found that global disulfide formation is lower in GPx-1 OE and that epidermal growth factor receptor expression and signaling is reduced in GPx-1 OE. Some of these changes may be due to thiol modifications that could be the basis for the reduction in cell growth of these cells.

Results for **downstream signaling**: In GPx-1 OE cells, hydrogen peroxide or EGF-stimulated Akt and MAPK erk1/2 signaling is reduced however, p90RSK phosphorylation is increased. This activation occurs via MAPK kinase pathways as the MEK1/2 inhibitor inhibits this activation in both GPx1-OE and control cells. In some cell systems prolonged p90RSK phosphorylation has been associated with attenuation of cell growth. Results for **disulfide alterations**: Researchers have focused on 2-dimensional gels run first under non-reducing conditions and then reducing conditions in order to identify proteins that contain protein-protein disulfide bonds. Currently, they are developing methods to increase protein levels on these gels, such as fractionation and protein precipitation methods to concentrate samples. Results for **mitochondrial alterations** in GPx-1 OE: As a first measure, researchers analyzed mitochondrial potential using JC-1, a fluorescent dye that accumulates in mitochondria proportional to mitochondrial potential. The accumulation of JC-1 in mitochondria causes aggregate formation, shifting the fluorescence emission spectra from green to red. Researchers found that mitochondrial potential was significantly diminished in GPx-1 OE. Theoretically, potential measurements are independent of mitochondrial mass but researchers are undertaking additional studies to characterize mitochondrial content in their line of over-expressing cells. Using Mitotracker Green staining they have quantified mitochondrial mass and observed that the mitochondrial staining is reduced in GPx-1 OE.

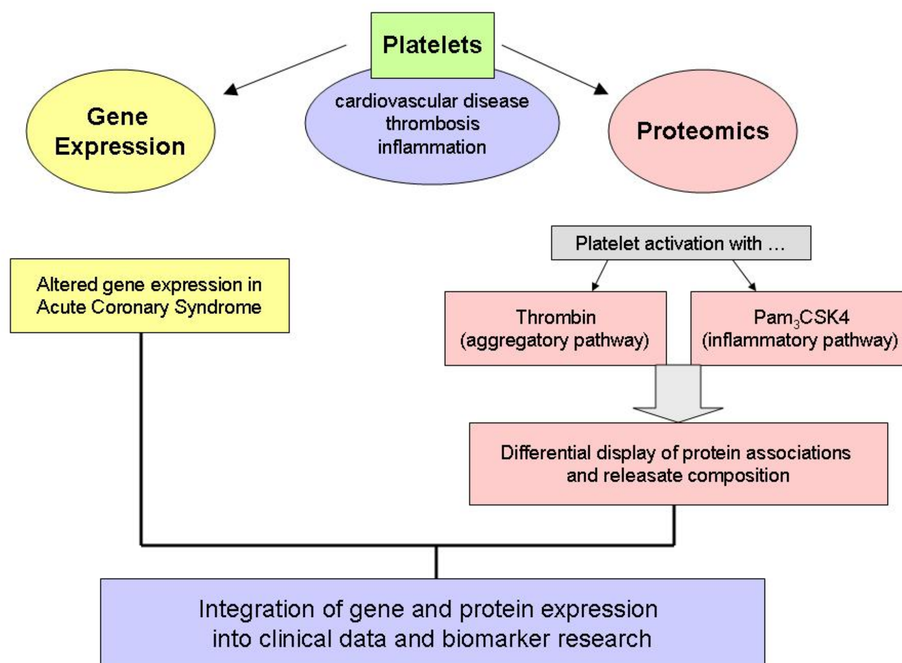
Underpinning project 5 (Circulating Surrogate Target Cells of Oxidant Stress, Freedman) studies is a hypothesis that activation of platelets by thrombotic or immune pathways leads to differential regulation of protein interactions and/or granule release. In addition to hemostasis, platelets mediate inflammation and clearance of bacteria from the bloodstream. As with platelet-platelet interactions, platelet-bacteria interactions involve release of granular content and cytoskeletal rearrangements in the platelet. It is not known if the activation of platelets by thrombotic or immune pathways leads to differential regulation of protein interactions and/or granule release. Researchers summarize the work on **FXIIIA-associated proteins**:

- Identification and confirmation of five proteins that specifically interact with FXIIIA in resting as well as activated platelets: thrombospondin, gelsolin, FAK, fibrinogen b and myosin.
- Gelsolin and FAK are novel FXIIIA-associated proteins not previously reported to interact with FXIIIA.
- In activated platelets, the intensity of the association with FXIIIA changes depending upon the type of stimulation and was found to be reduced notably in Pam3CSK4-activated platelets as compared to thrombin-activated platelets.
- Factor XIIIA is a good example of the activation of platelets with thrombin vs. Pam3CSK4 leading to differential protein associations.

As for their work on **platelet releasates:**

- The platelet releasate showed dramatic differences after 15 min of thrombin vs. Pam3CSK4 activation (about 80 proteins were differentially displayed).

- In addition to FXIIIA, several other proteins released by the platelets, such as gelsolin and fibrinogen β, expressed a differential pattern of release.



The investigators conclude that that the stimulation of platelets with thrombin (aggregatory pathway) vs. Pam3CSK4 (inflammatory pathway) leads to differences in protein associations as well as to differences in the releasate composition. They are continuing their work to obtain more evidence of this novel finding. Ultimately they wish to integrate gene and protein expression profiles into clinical data and biomarker research, as illustrated on the chart.

In project 6 (Reverse Engineering of Protein Networks, Collins), a two-stage method to identify transcription factor regulators (TF) from the expression profile of their targets (stage one), and from a second set of microRNA mediators involved in the regulation of TFs (stage two), has been employed. This process allows investigators to tunnel the information from the expression profile of individual genes to higher level regulators. The method has been applied to expression profiles of a breast cancer study with the goal to identify mediators of recurrent and non-recurrent breast cancer and to identify genes associated with disease progression. Many TFs were identified as differential regulators between two patient populations, one with estrogen receptors and non-recurrent tumors and the other with ER but without recurrent tumors: ARNT, ATF5, ESR1, GATA3, HOXD3, JUN, LEF1, MYCN, NFYA, NKX3-1, RARA, RARB, REL, RFX5, SMAD3, SPI1, STAT2, STAT5B, STAT6, TCF3, TFAP2C, TP73L.

Applying the method to lung epithelium data from a smoking cessation study, researchers identified six TFs as differential regulators: CEBPD, ESR2, HOXC11, HOXD3, PAX6, RUNX1.

After performing enrichment on the differential TF regulators in the breast tissue and lung epithelium samples, researchers observed 52 (breast) and five (lung) miRNAs as being significantly enriched and plan further experimental follow-up to determine the functional significance of the findings and to characterize the regulatory influence.

This systems biology approach to transcriptional regulatory networks has afforded the research team an expanded look at transcriptional regulation on genome-wide scale, in studies of the function and regulation of the Lon protease, a weakly specific stress-condition protease whose multifaceted cellular role is only partially understood. Researchers hypothesize that, in addition to Lon's known

role as a protease, Lon could also perform the role of a transcriptional modulator, either directly or indirectly, but likely in a DNA-localized fashion. They designed and performed a series of ChIP-qPCR experiments, in which an over-expressed and tagged Lon protease was cross-linked to DNA, precipitated, and purified. Early results indicate that Lon-bound genomic DNA is strongly enriched in regions of predicted transcriptional activity of the Lon protease, supporting the hypothesis of Lon's dual role. More evidence for this striking example of two-level regulation is being sought.

In project 8 (Circulating Endothelial Progenitor Cells, Walsh), researchers continued analysis of transcript expression in skeletal muscle of transgenic mice and continued a collaboration with project 2 to analyze insulin signaling and ROS-stress in this system.

In project 11, (Post-translational Oxidative Modification of Vascular Proteins in Key Antioxidant Deficiency States, Leopold and Loscalzo), researchers performed characterization studies in vascular smooth muscle cells treated with aldosterone in which they demonstrated increased oxidant stress, and decreased soluble guanylyl cyclase activity and cGMP production, as compared to vehicle-treated control cells. Researchers successfully purified alpha- and beta-subunits of guanylyl cyclase protein from bovine aortic vascular smooth muscle cells using a modified antibody immuno-precipitation protocol. Protein isolated from cells was treated with aldosterone or vehicle as a control. Three cysteine residues on the beta-subunit at the active site that are likely candidates for oxidative modification were identified. The researchers tested siRNA to decrease G6PD expression in smooth muscle cells, optimized transfection, and demonstrated approx. 70% in G6PD expression. They characterized G6PD-deficient smooth cells exposed to aldosterone and found elevated levels of oxidant stress and decreased soluble guanylyl cyclase activity and cGMP production as compared to vascular smooth muscle cells with normal G6PD activity exposed to aldosterone. They also report similar results obtained for soluble guanylyl cyclase activity in cells exposed to aldosterone.

Project 12 (Antioxidant Deficiency and the Heart, Colucci) reports progress in two areas: **Hydrogen peroxide-induced contractile dysfunction is mediated through oxidation of SERCA on cysteine-674**. H_2O_2 induces thiol oxidative post-translational modifications of the calcium handling protein sarco/endoplasmic reticulum calcium ATPase (SERCA), leading to a reduction of its activity and contractile dysfunction. Investigators tested whether cysteine-674 of SERCA is critical for H_2O_2-induced contractile dysfunction in adult rat ventricular myocytes (ARVM). **Results:** After 14 min of H_2O_2 exposure, cell shortening was reduced in WT-SERCA overexpressing ARVM (-43±21%) but not in C674S-SERCA overexpressing ARVM (+49%±31%, $p<0.05$ vs WT-SERCA, n=21-24/group). Compared with WT-SERCA ARVM, expression of C674S-SERCA attenuated the H_2O_2-induced reduction in calcium transient amplitude (-51±11% vs -87±6%, $p<0.05$) as well as 50% calcium reuptake time (46±3ms vs 60±3ms, $p<0.05$ C674S-SERCA vs WT-SERCA), reflecting a decrease in SERCA activity in WT-SERCA but not in C674S-SERCA ARVM. H_2O_2 decreased BIAM-labeling in ARVM overexpressing WT-SERCA by 40%±7% ($p<0.01$,n=3) but failed to decrease BIAM-labeling in ARVM overexpressing C674S-SERCA. These findings suggest that oxidation on Cys674 of SERCA is responsible for H_2O_2-induced reduction in its activity leading to contractile dysfunction.

Dilated cardiomyopathy and progressive myocardial failure in Gαq over-expressing mice are associated with oxidative post-translational modifications of RyR2 and SERCA2. Cardiac myocytes from Gαq mice have reduced contractile amplitude associated with decreased systolic and prolonged diastolic Ca^{2+} transients. Investigators have shown that cardiac-specific over-expression of the anti-oxidant, catalase, in Gαq mice prevented progression to failure but not the initial dilated

phenotype. Investigators tested whether the initial cardiomyopathic phenotype and the subsequent progression to myocardial failure are mediated by ROS-independent and ROS-dependent mechanisms, respectively. **Results:** Hearts were isolated from 8 or 20 wk-old wild-type (WT), Gαq-overexpressing (Gq) or Gαq-catalase (GqCat) overexpressing transgenic mice (n=3-6 in each group). SERCA and NCX protein expression remained unchanged in all groups at both ages. In contrast, RyR2 protein expression was reduced by 63%±18 at 8 wks and by 61%±28 at 20 wks in Gq mice compared with WT ($p<0.05$). Catalase over-expression did not prevent the decrease in RyR2 expression in Gq mice. RyR2 mRNA level (real-time RT-PCR) was reduced in 20 wk-old Gq and GqCat mice compared with WT mice (0.65±0.02 and 0.67±0.05, respectively, vs 1.12±0.14, $p<0.05$). At 8 weeks, no oxidative modifications (labeling with biotinylated iodoacetamide BIAM), were detected for RyR2, NCX, or SERCA2. At 20 weeks, BIAM labeling was unchanged for NCX and RyR2; however, SERCA2 BIAM labeling was reduced by 37±5% in Gq mice compared with WT mice ($p<0.001$). Catalase over-expression reduced SERCA oxidation (-17±8%, $p<0.05$ vs WT, $p>0.05$ vs Gq). To assess SERCA2 oxidation, heart sections were stained with anti-SERCA antibody raised against a peptide containing the sulfonylated Cys674 residue. Compared with WT, increased staining in Gq mice was observed, which was prevented by catalase over-expression. **Conclusion:** In Gαq mice, the initial cardiomyopathy is associated with downregulation of RyR2 mRNA expression that is ROS-independent. In contrast, the subsequent progressive myocardial failure is associated with increased oxidative stress and oxidative post-translational modification of SERCA2 on Cys674 that is mediated by hydrogen peroxide.

List of published papers and manuscripts in progress and those submitted for publication:

1. Kaur P and O'Connor PB. Quantitative determination of isotope ratios from experimental isotopic distributions. *Anal Chem* 2007; 79:1198-1204.
2. Odhiambo A, Perlman DH, Huang H, Costello CE, Farber HW, Steinberg MH, McComb ME, Klings ES. Identification of oxidative post-translational modification of serum albumin in patients with idiopathic pulmonary arterial hypertension and pulmonary hypertension of sickle cell anemia. *Rapid Commun Mass Spectrom* 2007; in press.
3. Perlman DH, Huang H, Dauly C, Costello CE, and McComb ME. Coupling of protein HPLC to MALDI-TOF MS using an on-target device for fraction collection, concentration, digestion, desalting, and matrix/analyte co-crystallization. *Anal Chem* 2007; 79: 2058-2066.
4. Saba JA, McComb ME, Potts DL, Costello CE, Amar S. Proteomic mapping of stimulus-specific signaling pathways involved in THP-1 cells exposed to *Porphyromonas gingivalis* or its purified components. *J Proteome Res* 2007; 2007: 6(6): 2211-21. Epub 2007 May 4.
5. Ying J, Tong XY, Pimental DR, Weisbrod RM, Trucillo MP, Adachi T, Cohen RA. Cysteine-674 of the sarco/endoplasmic reticulum calcium ATPase is required for the inhibition of cell migration by nitric oxide. *Arterioscler Thromb Vasc Biol* 2007; 27 (4):783-90. PMID: 17234728.
6. Zhao C and O'Connor PB. Removal of polyethylene glycols from protein samples using titanium dioxide. *Anal Biochem* 2007: doi:10.1016/j.ab.2007.03.024.