Proteome Analysis of Secreted Proteins during Embryonal P19CLONE6 Cell Differentiation into Cardiac Myocytes Using 2-DE/MALDI-TOF

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Secreted proteins by cardiomyocytes, including paracrine factors and extracellular matrix proteins provide a means of communication in the heart between myocytes and non-myocytes. Heart disease encompasses a broad spectrum of pathological conditions and abnormal changes to the proteome can reflect these various disease states. In this project we set out to identify differentially secreted proteins that mediate cardiac myocyte differentiation using a cell model. The secretome of in vitro cardiac-like cells during differentiation of the multipotent embryonal carcinoma mouse cell line P19 clone 6, was subjected to 2-DE analysis. P19cl6 cells were differentiated in culture into beating cardiac myocytes by treatment with 1% dimethyl sulfoxide (DMSO). On day 10–12 of in vitro differentiation, RT-PCR analysis and immunohistochemical staining gave evidence that these cells were of cardiac origin as they expressed cardiac-specific markers: troponin T, beta myosin heavy chain and F-actin fibres were highly expressed in the treated cells. Serum-free supernatants from undifferentiated and differentiated cells were harvested, concentrated and desalted on Vivaspin columns at 5kDA MWCO. Secreted proteins were submitted to 2-DE coupled with MALDI-TOF peptide mass fingerprinting. Preliminary 2-DE results showed 17 proteins that were differentially expressed with five proteins only present in the control (not differentiated) cultures and 12 proteins only present in the differentiated beating cardiac myocyte cultures. Protein identification by peptide mass fingerprinting is currently under way. In conclusion, this study provides a tool for the characterization of the major proteins released by cardiac cells upon differentiation.

Proteomic Analysis of Membrane Microdomains Derived from Both Failing and Non-failing Human Hearts

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Eukaryotic cells plasma membranes are organized into microdomains of specialized function such as lipid rafts and caveolae, with a peculiar lipid composition being both highly enriched in cholesterol and glycosphingolipids. In addition to their role in regulating signal transduction, multiple functions have been proposed, such as anchorage of various receptors, trafficking of cholesterol, and regulation of permeability. However, an extensive and unbiased understanding of their protein composition in human heart, both in failing and non-failing conditions, is not yet available. Membrane microdomains were isolated from left ventricular tissue of both failing (n = "15") and non-failing (n = "15") human hearts by sucrose gradient ultracentrifugation. Protein composition and differential protein expression was explored by comparing large series of two-dimensional (2-D) maps and subsequent identification of spots by matrix-assisted laser desorption/ionization (MALDI) peptide mass fingerprinting and by LC/MS/MS analysis. Our data indicated that human heart membrane microdomains are enriched in chaperones, cytoskeletal-associated proteins, enzymes and protein involved in signal transduction pathway. In addition, differential protein expression profile revealed the presence of 30 proteins (chaperones, protein folding, cytoskeletal-associated proteins), that were specifically up- or down-regulated in human heart failure membrane microdomains (fold increase > 2, p < 0.05). In conclusion, the subproteomic analysis of human heart membrane microdomains allows the identification of multiple differentially expressed proteins, opening new perspectives for researcher to determine what role or roles they may play in any biological system or process such as human heart failure.
15.3
Characterization of Phosphorylated Proteins Associated with Ischemia/Reperfusion Injury in Rabbit Myocardium

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We present here a method for enriching and identifying phosphopeptides from within a complex mixture using micro-column titanium oxide chromatography and MALDI-TOF mass spectrometry, in association with phosphoprotein purification and phospho-fluorescence staining of two-dimensional electrophoresis gels generated from rabbit myocardial tissue subjected to brief ischemia/reperfusion injury (myocardial "stunning"). Brief ischemia followed by timely reperfusion (15 min ischemia/60 min reperfusion; 15I/60R) is unlikely to be sufficient for myocardial contractile dysfunction to be influenced by extreme changes in protein expression, but is more likely to result from subtle post-translational modifications to proteins representing key functional systems. We have utilized 2-DE gels and MS to begin characterizing protein modifications induced by brief ischemia/reperfusion (I/R) injury in the rabbit myocardium. Levels of the phosphorylated forms of two proteins, heat shock protein 27 kDa (HSP27) and alpha B-crystallin (ABC), increase greater than two-fold following 15I/60R. The addition of an oxygen free radical scavenger (N-mercapto-propionyl glycine; MPG) to the reperfusate (15I/60R/H11001 MPG) ameliorates contractile dysfunction and protects against the majority of protein changes seen in 15I/60R. This suggests that protein damage is mainly a result of reperfusion-associated injury. While the increase in ABC phosphorylation is inhibited by the presence of MPG, the OFR scavenger does not influence increased HSP27 phosphorylation, suggesting that the modification to HSP27 alone occurs during the ischemic period. These results were confirmed by tissue samples subjected to ischemia, but no reflow (15I/0R). This method will enable the rapid and simple identification of phosphoproteins and phosphorylation sites in proteomics experiments, and has allowed us to identify increased phosphorylation of HSP27 as a key marker of brief ischemic injury in the heart.

15.4
Differential Proteome Analysis of CRP-activated Platelets

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The activation of platelets by collagen is an essential event in prevention of bleeding. Glycoprotein VI (GPVI) is the major receptor responsible for intracellular signalling events and platelet activation by collagen. One of the major GPVI-selective agonists is collagen-related peptide (CRP). Based on our experience applying proteomics to the analysis of signalling cascades in platelets (Garcia A. et al., Blood 2004, 103, 2088–2095), we have undertaken a differential analysis of the human platelet proteome comparing basal and CRP-activated platelets.

Platelets were isolated from fresh blood and treated with vehicle (Basal) or 10 ug/mL CRP for 90 sec at 37 °C. Proteins were extracted from whole cell lysates and separated by two-dimensional gel electrophoresis (2-DE) using 4–7 and 6–11 pH gradients in the isoelectric focusing step. A detailed differential image analysis was carried out comparing gels from basal and CRP-stimulated platelets, allowing the detection of differentially regulated protein features. Those features were in-gel digested with trypsin and analysed by LC-MS/MS.

A total number of 117 differences were found in this analysis, 75 in 4–7 gels and 42 in 6–11. Most of the proteins identified corresponded to signalling proteins; some of them already known to be involved in platelet activation by CRP (e.g. Src, GADS, pleckstrin), and others not (e.g. RGS18, RKIP, ILK-1). Most of the differences identified are consistent with post-translational modifications (PTMs), mainly phosphorylation events. This study demonstrates that proteomics is a powerful tool to dissect signalling pathways in platelets, helping to build the basis for the development of therapeutic agents for thrombotic diseases.
15.5 Subcellular Organelle Analyses of Protein Signatures in Genetic Dilated Cardiomyopathy

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The objective of this study is to determine the effects of dilated cardiomyopathy (DCM) and heart failure on global patterns of protein abundance in mouse and human cardiac tissue. We have previously adapted a gel-free mass-spectrometry procedure for the investigation of biochemical adaptations in membrane proteins obtained from hyper- or hypocontractile hearts (Pan, 2004, PNAS, 101, 2241) and nuclear enriched proteins during early skeletal muscle differentiation (Kislinger, 2005, MCP, in press). We have expanded upon these initial studies which focused on restricted cellular compartments and now also isolate and investigate the cytosol, nuclei and mitochondria. Protein fractions isolated by differential centrifugation are subjected to proteolytic digestion with endoproteinase LysC and trypsin, precipitated, and applied to a capillary-scale liquid-chromatography column coupled to a tandem mass spectrometer. Spectra are compared under high statistical stringency parameters with known protein databases to identify proteins from each fraction. Previously, we reported that an inherited human DCM with refractory congestive heart failure was caused by a dominant R9C missense mutation in phospholamban, and we generated transgenic mice with this mutation (Schmitt, 2003, Science, 299, 1410). In this study, we compare age-matched wildtype mice to transgenic mice during the developmental progression of DCM. Significant biochemical and structural remodeling is observed involving more than 400 proteins. Proteins linked to major Gene Ontology (GO) terms associated with metabolism, intracellular signaling, and calcium ion binding are evident. We also determined consistent changes in expression levels of proteins previously shown to be affected in DCM including atrial natriuretic factor, annexin, and SERCA2. Experiments currently underway include the identification of protein signatures in a cardiac tissue explant from an R9C DCM patient vs. unaffected donor individuals, and their comparison to the R9C transgenic mice. In conclusion, we hope to understand the progression of DCM as well as identify novel biomarkers of early stage pathology in cardiac disease.

15.6 Proteomic Approaches to Investigate the Prevention from Oxidative Injury in Cultured Cardiomyocyte Cell Line by a Traditional Chinese Medicine, Shengmai San, as a Model of an Antioxidant-based Composite Formula

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The protective effect of Shengmai San (SMS) on oxidative damage in cultured rat cardiomyocyte H9c2 cell line was studied as a model of an antioxidant-based composite formula, which is usable for the treatment of oxidative stress-related complex disorders. This traditional Chinese herbal medicine consisting of Panax Ginseng, Ophiopogon Japonicus and Schisandra Chinensis, has been previously shown the effect to cure coronary heart disease and prevent cerebral oxidative injury. In our study, H9c2 cells were incubated with SMS for defined periods, followed with H2O2 treatment for 4 hours at 37 °C, then subjected to an MTT reduction and LDH cytotoxicity assay for determining the cell viability and toxicity. In addition, we evaluated the derivatization of protein carbonyls and applied DNP immunostaining, western blotting, redox proteomics with 2-D page and MALDITOF to characterize the pharmaceutical mechanism and protein profile changes. The results demonstrated that both protein carbonyl content and cell cytotoxicity increased in H9c2 cells after the H2O2 chase in a concentration and time-dependent manner. However, both H2O2-dependent carbonyl formation and cell cytotoxicity were obviously reduced in the cultured cells pretreated with SMS. At the same time, cell viability was also enhanced in the SMS-pretreated cells after the H2O2 abuse compared to the control samples as determined by the MTT assay. It is concluded that SMS may have high efficiency in scavenging potential hydroxyl radicals and result in the protective activity of SMS against the H2O2-mediated oxidative injury in cardiomyocytes.
Influence of a Cardiovascular Disease Lifestyle Intervention on the Plasma Proteome


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Cardiovascular disease (CVD) remains the leading cause of disability, death, and healthcare burden in the United States and other industrialized countries. The primary cardiovascular disorder responsible for the global CVD epidemic is atherosclerotic vascular disease. Although many patients with ischemic coronary disease receive surgical interventions in combination with lipid-lowering drug therapy as the main component of their treatment regime, an alternative noninvasive approach to CVD management involves intensive risk factor modification, including vigorous lowering of cholesterol through dietary changes, stress management, exercise, and group support.

In a prospective, nonrandomized, four-component lifestyle change program, our institute has clinically evaluated CVD risk factor response in over 250 patients, over the course of one year. Data collected on various anthropomorphic, biochemical, and psychometric endpoints has shown that patients make dramatic improvement in virtually all measured clinical parameters.

The purpose of this proteomic study is two fold: to explore low abundant protein changes, and to evaluate new approaches to isolating and identifying proteins. The 12 most abundant plasma proteins were depleted by antibody affinity chromatography, then low abundant fractions were pooled to increase protein concentrations. Following fractionation by 2D-LC, semi-quantitative differential protein analysis software detected multiple protein fractions that were differentially expressed in CVD patients during participation in the program. Fractions of interest were lyophilized, trypsinized, then analyzed using nanospray 2D-LC/MS/MS. Triplicate samples were run to ensure reproducibility. A scoring algorithm was employed to decrease false positive protein identifications. Via this approach 158 differentially expressed proteins were detected, which may lead to biomarkers that correlate with favorable changes in CVD risk factor profiles.

Relation between Different Apolipoprotein E Alleles and Paraoxonase-1 Activity in Early and Late Onset Coronary Artery Disease (CAD)

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The polymorphism of apolipoprotein E gene has functional effects on lipoprotein metabolism. The association of APOE allele frequencies with CAD remains unknown in developing countries. The other protein that has been associated with CAD is Paraoxonase 1 (PON 1). PON 1 is an enzyme that is associated with HDL and metabolizes organophosphates and may be antiatherogenic. To determine whether apoE polymorphism and PON 1 are associated with premature CAD, we examined the frequency of apo-e alleles among patients with early-onset CAD (n = 80; < 50 yr) vs. late-onset CAD (n = 80; > 65 yr) by molecular analysis. We also examined the activity of the PON 1 in both groups, and explored whether there was synergism between PON 1 activity and apoE alleles.

The mean of age in the early and late-onset groups were 44.9 ± 4.9 and 71.7 ± 2.5 years. The highest and lowest frequencies of the apoE alleles in the two groups belong to apo-e3 and apo-e4 respectively. The difference between apo-e2 apo-e3 apo-e4 alleles in early-onset to late-onset group, were significantly low, non significant and significantly high respectively. PON 1 activity was 402 ± 262 and 430 ± 205 (IU/ml) respectively. 35.0% of early-onset and 17.5% of late-onset group had the low level of PON 1. 86% of the early-onset CAD and all of the patients of late-onset CAD with apo-e3/e4 genotype, had low or moderate level of PON activity.

Our study suggests that there is a minor negative association between PON 1 activity and early-onset CAD, in the presence of the apo-e 4 allele. The association was positive with respect to apo-e 2 allele. Although PON activity was lower in early-onset group compared to the late-onset, the difference was not significant.
15.9
Proteomic Analysis of Vascular Cells during Left Ventricular Hypertrophy
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Left ventricular hypertrophy (LVH) is a potent, independent predictor of cardiovascular events, in which it dramatically increases the risk of stroke, coronary heart disease and heart failure. Whereas LVH alters the function of blood vessels, it also disrupts regulation of blood flow. We have previously shown for impairment of smooth muscle cell (SMC) function that properties of Ca\(^{2+}\)-activated K\(^+\) channels in coronary artery are altered, and so may account for reduced coronary reserve during LVH. We hypothesize that the alteration of cerebrovascular function underlie the cardiovascular event associated with LVH. Using two-dimensional gel electrophoresis and mass spectroscopy, we identified such candidate proteins. These results were confirmed by Western blot analysis. This change in protein expression during LVH correlates with the alteration of cerebral contractility. Our results provide novel target pathways for investigation that are important for cardiovascular event associated with LVH.

15.10
Mutation of Methyleneetetrahydrofolate Reductase Gene in CAD
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Objectives: The question of whether mild hyperhomocysteinemia is a risk factor for coronary artery disease (CAD) has long been debated and is still unclear.

Methods: Since the role of genetic susceptibility to coronary artery disease (CAD) seems to be quite important in young patients, we investigated methyleneetetrahydrofolate reductase (MTHFR) gene C677T polymorphisms in early-onset as the case group and late-onset as the control group, with angiographically proved CAD.

Results: allele frequencies for the “C” (wild-type) and “T” (mutant-type) alleles were 0.82 and 0.18 in early-onset cad patients and 0.71 and 0.30 in late-onset (controls), respectively. there was a significant difference in the distribution of mthfr alleles between the two groups. In the C/T genotype, serum homocysteine levels were significantly higher than C/C (p < 0.02) (14.5 +/- 0.9 vs. 24.7 +/- 5.4 umol/l). Serum total cholesterol and LDL cholesterol levels were significantly higher in C/C compared with C/T genotype. systolic and diastolic blood pressures in subjects with different cholesterol levels were significantly higher in C/C compared with C/T genotype.

Conclusion: mthfr c677t genotype was significantly related to hyperhomocysteinemia. In spite of the clear effect of the mthfr genotype on elevated homocysteine levels, the early-onset Iranian cad patients did not have a distinct profile of homocysteine or blood lipid levels.

15.11
Proteomic and Metabolomic Analysis of Atherosclerotic Aortas Derived from APOE\(^{-/-}\) MICE
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Background: Proteomics and metabolomics are emerging technologies to study molecular mechanisms of diseases. We applied these techniques to identify protein and metabolite changes in vessels of apoE\(^{-/-}\) mice on normal chow diet.

Methods and Results: Using two-dimensional gel electrophoresis and mass spectrometry, we identified about 100 protein species that were dynamically altered during various stages of atherogenesis. Immune activation, redox imbalance and impaired energy metabolism preceded lesion formation in apoE\(^{-/-}\) mice. Immunoglobulin deposition occurred prior to the accumulation of other serum proteins, implying an active trapping in the vessel wall. Oxidative stress in the vasculature was indicated by the oxidation status of 1-Cys peroxiredoxin and correlated to the extent of lesion formation in 12 month-old apoE\(^{-/-}\) mice. Nuclear magnetic resonance spectroscopy revealed diminished glucose metabolites and a marked depletion of the adenosine and creatine pool in vessels of young apoE\(^{-/-}\) mice. Attenuation of lesion formation was associated with alterations of NADPH generating malic enzyme, which provides reducing equivalents for lipid synthesis and glutathione recycling, increased utilisation of glucose and replenishment of the vascular energy pool, highlighting the intimate connection between oxidative stress and vascular energy metabolism.

Conclusion: Our study is the first proteomic and metabolic analysis of aortas from apoE\(^{-/-}\) mice and provides the most comprehensive dataset of protein and metabolite changes during atherogenesis published so far.

15.12
Integrated Proteomic and Metabolomic Analysis Reveals Metabolic Remodelling in Human Persistent Atrial Fibrillation
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Background: Atrial fibrillation (AF) leads to several different forms of remodelling processes within the atria, which contribute to its self-perpetuating nature. The exact mechanisms underlying atrial remodelling are as yet not fully understood.

Methods and Results: To assess the remodeling processes in persistent AF, we analyzed the protein and metabolite changes in human atria, which were fibrillating or in sinus rhythm, by using 2D gel electrophoresis and nuclear magnetic resonance spectroscopy (NMR). Firstly, structural remodelling of the cytoskeleton was evident on 2D gels as fragmentation of microtubules and alterations in desmin and small heat shock proteins. Secondly, oxidative stress during AF resulted in the depletion of certain antioxidants. Western Blot analyses confirmed lower levels of antioxidants such as peroxiredoxin 1 and catalase in AF. Thirdly, several enzymes involved in glucose, fatty acid and energy metabolism were differentially expressed in our proteomic study. NMR analysis showed a marked rise in beta-hydroxybutyrate, suggesting increased ketone body utilisation. The cellular energy pool (ADP+ATP pool, creatine) was maintained and phosphocreatine was elevated during persistent AF.

Conclusions: This study is the first to utilise a combination of proteomic and metabolomic techniques on human myocardial tissue to demonstrate that persistent AF represents a hypermetabolic state with increased ketone body oxidation, lactate accumulation and a rise in phosphocreatine. This “metabolic remodelling” may be a key element contributing to the self-perpetuating nature of this resilient arrhythmia.
Biochemical and Proteomic Characterization of the Murine Cardiac 26S Proteasome

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The 26S proteasome system is the major intracellular quality control system that targets misfolded proteins for refolding or degradation. By maintaining proper intracellular protein homeostasis, a well organized and well regulated 26S proteasome system is essential to the viability of eukaryotic cells. Characterization of the proteasome in murine heart is an important step towards understanding the role of the proteasome system in cardiac pathophysiology. The 26S proteasome is composed of a 20S core complex and one or two 19S regulatory complexes. Biochemical studies showed that the proteolytic activities as well as the amount of 20S proteasome in the heart are significantly less than those of the 20S in the kidney, liver and lung. Purification and characterization of the murine cardiac 26S proteasome identified the expression of at least 37 distinct subunits in this vital cardiac organelle. Western immunoblotting confirms the expression of key subunits in the isolated cardiac cells. Among the subunits detected in the cytosolic fraction of the normal cardiac tissue are the three inducible 20S subunits, β1i, β2i, and β5i. Importantly, an alternative splicing isoform of a 19S subunit was found together with its dominant isoform. The co-expression of the three inducible 20S isoforms as well as that of the alternatively spliced subunits in the 19S proteasome suggest a complex level of regulation on this protein degradation machinery in the heart. In addition, LC/MS/MS characterized that a subset of these 37 subunits were N-terminally myristoylated (e.g., RPT3); separately, a subset of these subunits were N-terminally myristoylated. After undergoing ischemia/reperfusion the amount of 26S proteasome present in the heart (determined by western blotting of both 19S and 20S subunits) showed no significant change compared to the sham heart. However, the β1 and β5 proteolytic activities, but not the β2 activity, of the 20S proteasome were selectively reduced in the ischemic heart, indicating regulation of the cardiac 26S proteasome system by ischemia/reperfusion injury.

Phosphorylation Is a Major Regulator of the Proteolytic Activity of Cardiac 26S Proteasomes

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The cardiac 26S proteasome is an organelle composed of a collection of multiprotein complexes, serving as the essential protein degradation system in the heart. We investigated whether phosphorylation of the proteasome has a functional role in regulating the proteolytic activity in the heart. Studies using electrophoresis combined with liquid chromatography tandem mass spectrometry revealed distinct types of post-translational modifications on a subset of the proteasome subunits including phosphorylation, N-terminal myristoylation, and N-terminal acetylation. Purified murine cardiac 20S proteasomes were separated by two-dimensional gel electrophoresis (2DE) and immunoblotted with anti-Ser, anti-Thr and anti-Tyr antibodies. These experiments demonstrated distinct phosphorylation 2DE patterns, documenting serine, or threonine, or tyrosine phosphorylation of a subset of cardiac proteasome subunits. These experiments also confirmed the phosphorylation of two subunits previously reported in non cardiac proteasomes (α6 and α7) as well as the phosphorylation on two subunits that are characteristics of the cardiac proteasomes subunits (α1 and β3). Results obtained from the 2DE analysis with Pro-Q dye (a fluorescent dye which specifically binds phosphorylated proteins) further confirm the phosphorylation of these proteasome subunits. We next examined the effect of phosphorylation on the proteolytic activity of distinct 20S proteasomes subunits. Dephosphorylation of the 20S proteasome (confirmed by Pro-Q staining of the 20S subunits) with Calf intestinal alkaline phosphatase (CIAP) resulted in no change in the proteolytic activity of the β1 proteasome subunit; in contrast, dephosphorylation induced a 3 fold increase in the proteolytic activity of the β2 proteasome subunit, and a more than 10 fold increase in the proteolytic activity of the β5 proteasome subunit, when compared to the phosphorylated proteasome. These data provide the first direct evidence demonstrating regulation of the proteolytic activity in a proteasome subunit specific fashion, documenting phosphorylation as a critical regulatory mechanism to modulate this vital organelle in the heart.
Proteomic Analysis of Intact Mitochondrial Membrane Protein Complexes Containing ANT and VDAC; Implications for Mitochondrial Permeability Transition in Murine Heart

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Pathologic cellular stresses, such as cardiac ischemia/reperfusion, can induce a defect known as mitochondrial permeability transition (MPT). MPT involves permeabilization of the inner membrane, mitochondrial swelling, and cytochrome c release and can lead to cell death. Two protein families, the inner membrane adenine nucleotide translocase (ANT) and outer membrane voltage dependent anion channel (VDAC), have garnered significant attention in the setting of MPT because of their abilities to function as non-selective channels. Despite recent studies that implicate prevention of MPT as a potential cardioprotective intervention, the molecular basis of this phenomenon in the heart remains unclear. To analyze intact MPT pore complexes, Percoll gradient-purified mitochondria were treated with the covalent cross-linker disuccinimidyl suberate (1:50, DSS:Protein) to fix proteins, membranes disrupted with 1% dodecylmaltoside to release the fused complexes, and the complexes purified by sequential immunoprecipitation for ANT and VDAC. Following SDS-PAGE separation, proteins were identified by LC/MS/MS analysis of tryptic peptides and database mining. We observed multiple intact complexes containing purported members of the MPT pore, including the central pore-forming units, ANT and VDAC, as well as heretofore unrecognized members, such as the potassium channel beta 1 subunit and ATP synthase H+ transporter. Immunoblotting (IB) detected enhanced levels of creatine kinase and ATP synthase beta subunit in specific supramolecular complexes following treatment with the cross-linker, illustrating the use of this approach to immobilize native complexes. To examine the relevance of these intact complexes to the phenomenon of MPT, we quantitatively analyzed (via IB) specific complexes containing ANT and VDAC in the normal heart and in hearts from genetically-cardioprotected mice (PKCε transgenic mice). The data indicate decreased formation of ANT-VDAC complexes in the protected heart, supporting the notion that decreased assembly of MPT complexes may promote cell survival. These studies represent, to our knowledge, the first proteomic characterization of the MPT pore subproteome in the heart and provide a framework of distinct native MPT pore complexes.

Identification of Brain Ischemia-induced Changes in Protein Profiles by Quantitative Proteomics

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This study is to provide a proteomic view of brain ischemia-induced changes in protein expression. The proteomes of control and ischemic rat brains were analyzed with a quantitative proteomic approach, using a newly developed LC-MS/MS system, the so-called Protein Expression System that does not require sample labeling of any kind for quantitation. This system utilizes reproducible chromatography and high mass precision to track peptides in repeated LC-MS/MS analyses of the sample with alternating high and low energy. Soluble fractions of brain homogenates were digested with trypsin and analyzed. Quantitative differences between proteomes of control and ischemic brains were determined by comparing chromatographic peak areas of each identified peptide. To date, over five hundred proteins have been identified and quantified. Among the identified proteins, more than one hundred proteins showed quantitative differences between control and ischemic brains. The majority of the ischemia-induced changes were an up-regulation; this trend of change of expression agrees well with published genomic studies on ischemic rodent brains. Western blot analyses of selected proteins confirmed our proteomic data. Furthermore, results of informatic analyses of proteins that showed a change after brain ischemia revealed that proteins in the secretory pathway (secretory proteins and their receptors), as well as proteins involved in neurotransmission and ion transportation were particularly responsive to brain ischemia. Following the leads from this study, the kinetics of ischemia-induced changes in protein expression is being further investigated.