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Proteomics and Metabolomics Combined in Cardiovascular Research

Manuel Mayr*, Basetti Madhu, and Qingbo Xu

Proteomics and metabolomics offer a nonbiased suite of tools to address pathophysiologic mechanisms from various levels by integrating signal transduction, cellular metabolism, and phenotype analysis. To link alterations of cellular proteins to metabolism and function, we have recently combined proteomic and metabolomic techniques. Examples, including genetic manipulation, ischemic preconditioning, atherosclerosis, and stem cell differentiation, are discussed to illustrate how the combination of these updated technologies may advance our understanding of cardiovascular biology. (Trends Cardiovasc Med 2007;17:43–48) © *2007, Elsevier Inc.*

Among the four big "-omics"—genomics, transcriptomics, proteomics, and metabolomics—there is an inverse correlation between the complexity of the biology we are trying to address and the maturity of the technology currently available. The importance of measuring proteins has become increasingly clear as mRNA transcripts cannot be directly correlated to protein expression (Gygi et al. 1999) and posttranslational modifications are known to be instrumental in many human diseases.

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• Proteomics

Recent progress in protein technology enables us to perform large-scale studies of gene expression at the protein level and characterize posttranslational modifications, i.e., by difference in gel electrophoresis (DIGE) (Unlu et al. 1997). Similar to microarrays, the DIGE approach uses sample multiplexing by prelabelling with different fluorescent probes before separation by two-dimensional (2-D) gel electrophoresis (Figure 1). Importantly, all gels in a DIGE experiment are normalized to the same internal standard pool, allowing reliable detection of differences as low as 10% in protein expression. Furthermore, different protein isoforms and posttranslational modifications are easily visualized on 2-D gels as a shift in molecular weight or isoelectric point. The possibility to isolate modified proteins from their native counterparts and to perform accurate quantitative comparisons within a large number of biologic replicates makes 2-D gel electrophoresis still a mainstream workhorse in proteomics (Gorg et al. 2004).

At the center of any proteomic approach, however, is the mass spectrometer (MS) (Pandey and Mann 2000). Proteins of interest are routinely subject to tryptic digestion to generate smaller peptides. Tryptic peptide mixtures are subsequently separated by liquid chromatography (LC) on a reverse-phase column before ionization and introduction into the mass analyzer. The latest tandem mass spectrometers (MS–MS) combine high mass accuracy with faster scan speeds and attomol on-column sensitivity. Moreover, complementary activation methods such as electron transfer dissociation preferentially fragment larger peptides with higher charge state and preserve posttranslational modifications such as phosphorylation and glycosylation, which are routinely lost during collision-induced dissociation. These recent advances in mass spectrometry, i.e., switching between electron transfer dissociation and collision-induced dissociation, may empower researchers to interrogate an entirely new subset of the proteome because proteins can undergo hundreds of different posttranslational modifications, but only few are routinely investigated in mainstream research.

For quantification, mass spectrometry–based approaches mainly rely on isotopic labeling methods (Washburn et al. 2001; Blagoev et al. 2003), which are well suited to detect accurate differences in pairwise comparisons or few biologic replicates. However, it remains a challenge to perform comparisons across a large number of clinical samples. Attempts are undertaken to perform label-free quantitation, but complex LC is required. Otherwise, quantitative differences in label-free LC–MS runs are influenced by coeluting peptides as highly abundant ions

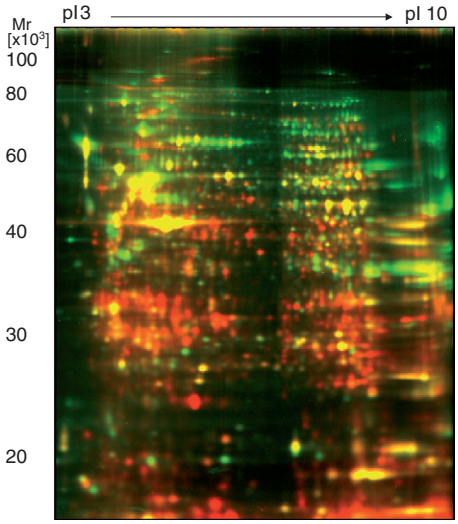

Figure 1. Difference in gel electrophoresis. Protein lysates of human umbilical vein endothelial cells and human peripheral blood mononuclear cells were labeled with Cy3 and Cy5 respectively and coseparated in large-format 2-D gels. Images were acquired on a fluorescence scanner. Differentially expressed proteins are highlighted in *green* (human umbilical vein endothelial cells) and *red* (peripheral blood mononuclear cells), respectively. Protein spots present in both samples appear in *yellow*. By labeling an internal standard pool consisting of an equal amount of both samples with Cy2 (not shown), the DIGE multiplexing approach allows accurate comparisons of protein expression even between very different cell lines.

suppress the ionization of less abundant ions. Undersampling of the mammalian proteome is another problem. This shortcoming in sensitivity becomes particularly apparent in proteomic analyses of body fluids. Plasma is the most complex proteome in the human body, and the concentrations of plasma proteins spans 10 orders of magnitude. Even if it were possible to remove 99.99% of albumin, ~5 μg/mL would remain, 1,000,000 times more than many proteins of low abundance, which are present in concentrations of <5 pg/mL (Anderson and Anderson 2002). Notably, depleting abundant plasma proteins may introduce quantification errors. For cells and tissues, subcellular fractionation is an effective method to increase proteome coverage (Huber et al. 2003), but important biologic information may be lost if only the organelle of interest is studied without considering associated changes in other cellular compartments.

- **Metabolomics**

Similar to proteomics, metabolomics is confronted with an overwhelming number of metabolites. Both MS- and nuclear magnetic resonance (NMR) spectroscopy-based metabolomic techniques have potential in becoming an integrated part of biologic studies along with proteomic techniques. MS-based methods offer better sensitivity but have the disadvantage of the ionizability of the metabolites. Moreover, no spectral LC–MS–MS libraries are available yet for metabolite identification, and accurate quantification is only achievable if authentic standards are spiked into the sample. In contrast, high-resolution NMR spectroscopy is able to satisfy all the criteria required for metabolomic studies, although NMR analysis of human plasma was only a weak predictor of coronary artery disease (Kirschenlohr et al. 2006).

Different NMR methods are available for measuring metabolites in vivo, unprocessed tissue, and tissue extracts: most of the in vivo magnetic resonance spectroscopy (MRS) studies in cardiovascular tissue have been done by phosphorus 31 (^{31}P) MRS to directly measure the high-energy metabolites involved in bioenergetics such as phosphocreatine, adenosine triphosphate (ATP), and inorganic phosphate. A recent review (Horn 2006) is a good source of information about the MRS applications in studying cardiac physiology. With respect to cardiac MRS, a major hurdle is the cardiac and breathing motions, which must be tagged to synchronized acquisition, but it is possible to acquire in vivo proton (^{1}H) MRS of cardiac tissues from volumes as small as 8 mm^3 (Schneider et al. 2004). The main limitations of in vivo MRS are the low sensitivity and poor resolution, which compromise quantitative accuracy. Concentrations of metabolites can only be estimated, with corresponding internal tissue water used as in vivo standard, or quantitative data must be reported as metabolite ratios. As

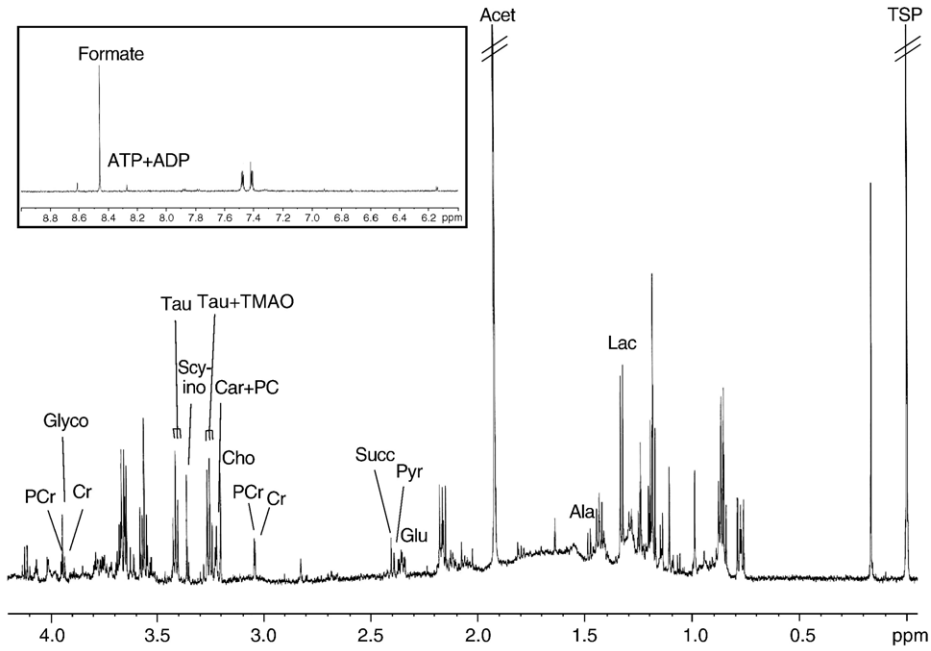

Figure 2. High-resolution proton NMR spectroscopy. Metabolites were extracted from a murine aorta derived from 18-month-old apoE$^{-/-}$ mice. Within the aliphatic region (−0.05 to 4.2 ppm) of the NMR spectra, resonances have been assigned to Lac, Ala, Pyr, Acet, Succ, Car, Cho, PC, Tau, Scy-ino, Glyco, TMAO, Glu, Cr, and PCr. ADP+ATP and formate are showing in the aromatic region of the spectra (6.0-9.0 ppm) (inset). Lac, lactate; Ala, alanine; Pyr, pyruvate; Acet, acetate; Succ, succinate; Car, carnitine; Cho, choline; PC, phosphocholine; Tau, taurine; Scy-ino, scylloinositol; Glyco, glycolic acid; TMAO, trimethylamino oxide; Glu, glutamate; Cr, creatine; PCr, phosphocreatine; ADP, adenosine diphosphate. Reprinted with permission from Mayr et al.: 2005. Proteomic and metabolomic analyses of atherosclerostic vessels from apolipoprotein E-deficient mice reveal alterations in inflammation, oxidative stress, and energy metabolism. Arterioscler Thromb Vasc Biol 25(10):2135–2142.

an alternative to in vivo MRS, tissue specimens can be rapidly excised and immediately frozen in liquid nitrogen. Subsequently, high-resolution ^{1}H NMR spectroscopy can be performed on tissue extracts (Figure 2). Although the ex vivo manipulation during the extraction procedure is a potential limitation, metabolite signals and resolution increase considerably compared to in vivo spectroscopy. Moreover, external standards allow accurate quantification, and spiking experiments can identify unassigned metabolites in the ^{1}H NMR profiles. Alternatively, high-resolution magic angle spinning ^{1}H NMR spectroscopy can be applied to unprocessed biologic tissues providing metabolite signals with a sensitivity and resolution comparable to high-resolution ^{1}H NMR, but quantification is not well established in this emerging technology.

- **Proteomics and Metabolomics Combined**

For reasons outlined above, ^{1}H NMR spectroscopy is our method of choice for combining proteomics and metabolomics. Notably, a reduction of a metabolite per se may be a consequence of either increased consumption or decreased production. Although enzymatic assays play an essential role in metabolic studies, these in vitro measurements are performed at optimal pH and in the presence of substrate plus required cofactors, which may not always be representative of the in vivo situation. Moreover, enzymatic activity may have been influenced by the extraction procedure and the disruption of the spatial organization. Because enzymatic activity is reflected in the expression and posttranslational modifications of enzymes, it can be interrogated by proteomic techniques. In this respect, 2-D gel electrophoresis offers a particular advantage by visualizing isoforms and posttranslational modifications of abundant enzymes as a shift in isoelectric point or molecular weight. The metabolic net effect of these changes can then be explored by use of metabolomic techniques. In combination, proteomics and metabolomics allow an integrated assessment of enzymes and their corresponding metabolites and provide a platform to interrogate tissue metabolism.

Because the potential of proteomics in cardiovascular research has been reviewed previously (Arrell et al. 2001; Macri and Rapundalo 2001; Loscalzo 2003; McGregor and Dunn 2003), we would like to focus on how the combination of proteomics and metabolomics may advance our understanding of cellular signaling and disease mechanisms, as illustrated briefly in four examples:

Combined with Genetic Manipulation

As a first proof of principle, we analyzed proteomic and metabolomic profiles of cultivated arterial smooth muscle cells (SMCs) after mutational ablation of the protein kinase C (PKC) δ gene (Mayr et al. 2004a). A single gene mutation can cause alterations of seemingly unrelated biochemical pathways, and it has been demonstrated previously that mitochondrial translocation of PKCδ is required for insulin stimulation of the pyruvate dehydrogenase complex in skeletal muscle (Caruso et al. 2001). Moreover, it was suggested that PKCδ activation increases O_2-derived free radical generation from mitochondria (Vanden Hoek et al. 1998; Wang et al. 2001). Similarly, PKCδ efficiency interfered with the glucose and energy metabolism of vascular SMCs and promoted an antioxidant state reflected by increased glutathione concentrations (Mayr et al. 2004a), which may be attributed to altered metabolic flux via alternative glucose pathways, as evidenced by our proteomic and metabolomic findings (Figure 3). Notably, the resistance to oxidative stress-induced cell death improved SMC survival in vein grafts, resulting in accelerated stenosis in a mouse model of vein graft disease (Leitges et al. 2001).

Ischemic Preconditioning

Along with PKCε (Ping et al. 2002), PKCδ is the major PKC isoform implicated in

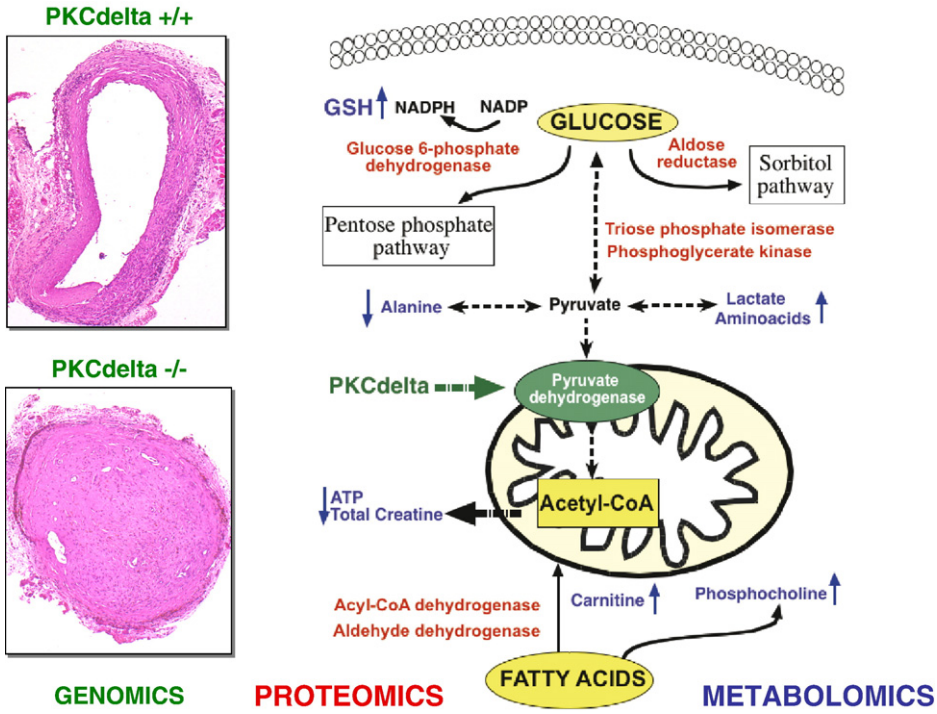

Figure 3. Schematic model of enzyme and metabolite changes in PKC$\delta^{-/-}$ SMCs. PKCδ is known to be involved in the regulation of the pyruvate dehydrogenase complex, which catalyzes the irreversible step from glycolysis to the citric acid cycle. Changes in enzymes and metabolites, as observed in our proteomic and metabolomic analysis, are highlighted in *red* and *blue*, respectively. Altered glucose metabolism was reflected in PKC$\delta^{-/-}$ SMCs as a drop in alanine, the transamination product of pyruvate, accumulation of lactate, decreased oxidation of certain amino acids, a diminished creatine pool, reduced ATP levels, increased fatty acid metabolism, and use of alternative glucose pathways such as the sorbitol and the pentose phosphate pathway. The latter serves to generate nicotinamide adenine dinucleotide phosphate, the cofactor required for GSH recycling, and is associated with elevated GSH levels, protecting PKC$\delta^{-/-}$ SMCs against oxidative stress-induced cell death contributing to accelerated vein graft stenosis. GSH, glutathione.

preconditioning-mediated cardioprotection (Inagaki et al. 2003). With the use of the combined proteomic and metabolomic approach, we compared the effects of ischemic preconditioning in PKC$\delta^{+/+}$ and PKC$\delta^{-/-}$ hearts (Mayr et al. 2004b, c). The response to ischemic preconditioning in wild-type hearts was characterized by a fragmentation of the pyruvate dehydrogenase complex, the link between glycolysis and aerobic respiration, and a down-regulation of cytosolic malate dehydrogenase, the enzyme responsible for transporting reducing equivalents from glycolysis into mitochondria. These enzymatic changes consistent with a "training" of cardiac metabolism towards anaerobic glycolysis were absent in hearts deficient for PKCδ. Moreover, cardiac lactate levels were reduced by ischemic preconditioning in PKC$\delta^{+/+}$ hearts, but remained higher in preconditioned PKC$\delta^{-/-}$ hearts. Even at baseline, loss of PKCδ altered the ratio of cardiac glucose to lipid metabolites in murine hearts but impaired metabolic adaptation exaggerated cardiac damage after ischemic preconditioning. A similar loss of preconditioning-induced cardioprotection has been previously observed in PKCε knockout mice (Gray et al. 2004). Thus, deficiency of either PKCε or PKCδ was sufficient to abrogate the cardioprotective effects of ischemic preconditioning, indicating that an individual PKC isoform may not be cardioprotective per se, but signaling networks associated with cardioprotection may require the presence of both PKC isoforms, and cardioprotective effects may be mediated by their combined orchestration of cardiac metabolism (Edmondson et al. 2002; Churchill et al. 2005).

Atherosclerosis

Atherosclerosis is widely viewed as an immune-inflammatory disease with hypercholesterolemia as the principal underlying stimulus for the chronic inflammatory response in the vasculature (Hansson et al. 2002). There is plenty of evidence suggesting that by modifying cholesterol levels, i.e., by statin treatment, one can also ameliorate the inflammation in the vasculature. However, because statins have a potent anti-inflammatory component in addition to their cholesterol-lowering effect, one cannot rule out that the inflammation in the vasculature is not exclusively related to cholesterol levels. Based on our proteomic and metabolomic analysis of aortas from apolipoprotein E (apoE)$^{-/-}$ mice (Mayr et al. 2005), we concluded that inefficient vascular glucose and energy metabolism coincides with increased oxidative stress in hyperlipidemic animals, as evidenced by oxidation of redox-sensitive proteins (Figure 4). Moreover, attenuation of lesion formation was associated with successful replenishment of the vascular energy pool and posttranslational modifications of cytosolic malic enzyme, which provides reducing equivalents for lipid synthesis and glutathione recycling. Supporting our findings are previous observations that insulin supplementation reduces lesion formation and oxidative stress in apoE$^{-/-}$ mice (Shamir et al. 2003), whereas overexpression of the uncoupling protein 1 promotes atherosclerosis by depleting energy stores and increasing superoxide production (Bernal-Mizrachi et al. 2005).

Moreover, it is tempting to speculate that such metabolic disturbances in the vasculature also stimulate local secretion of inflammatory cytokines. In skeletal muscle, e.g., interleukin 6 (IL-6) has been implicated as an "exercise factor" that acts in a paracrine manner on neighboring muscle cells and is triggered by a depletion of glycogen stores within muscle fibers to increase glucose supply. Notably, the rise of IL-6 during prolonged exercise can become as high as in sepsis. Similarly, mechanical stretch is a potent inducer of IL-6 in vascular SMCs (Zampetaki et al. 2005). Thus, besides their important role in regulating immune-inflammatory responses in atherosclerosis, the pronounced metabolic actions of cytokines such as IL-6 may aim to maintain glucose homeostasis in the SMC compartment and contribute to the general adaptation of the vasculature to stress conditions.

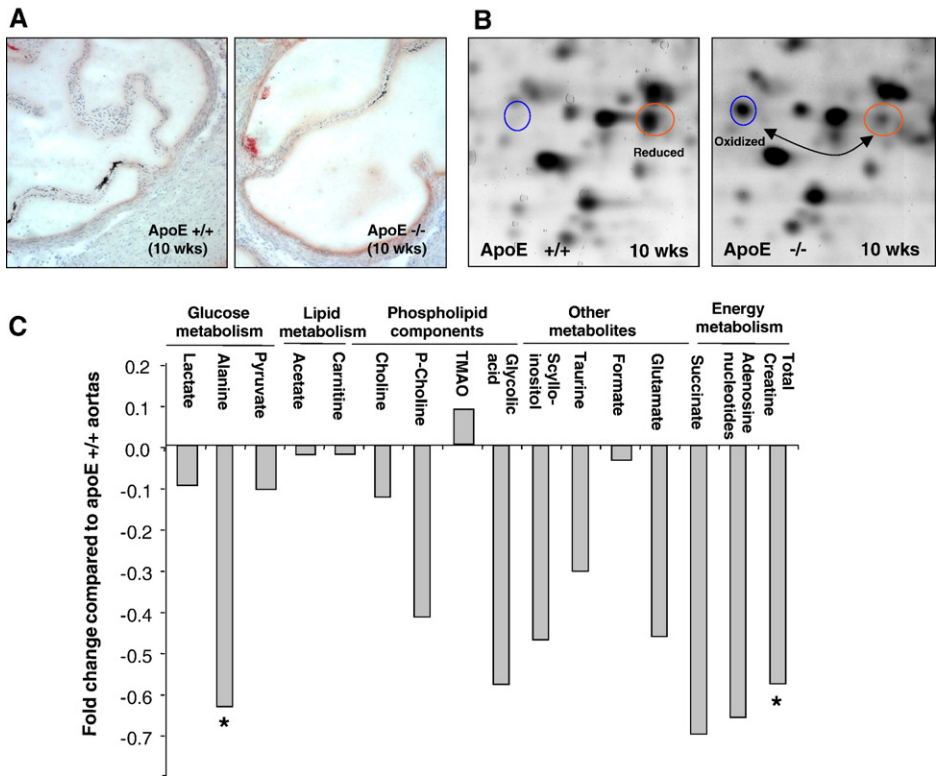

Figure 4. Oxidative stress and energetic impairment in apoE$^{-/-}$ aortas. (A) shows representative photographs of oil red O-stained sections from aortic roots, indicating early lesions (red color) of 10-week-old apoE$^{-/-}$ mice. (B) highlights quantitative changes in expression of the oxidized and reduced form of 1-Cys peroxiredoxin (peroxiredoxin 6). The spot pair corresponding to the oxidized and reduced isoform is marked with an arrow. The histogram in (C) summarizes the relative changes of metabolites in aortas derived from 10-week-old apoE$^{-/-}$ mice compared to wild-type controls (C). Note that a reduction of energy metabolites coincides with increased oxidation of redox-sensitive proteins during early stages of atherogenesis.

Stem Cell Differentiation into Vascular Cells

Stem cell research holds great promise for regenerative medicine and tissue engineering and provides exciting new avenues in the treatment of cardiovascular diseases. By now, numerous studies have demonstrated that vascular progenitor cells, including endothelial and smooth muscle progenitors, are present in circulating blood and have the capacity to differentiate into SMCs and endothelial cells (Asahara et al. 1997; Simper et al. 2002), thereby contributing to vascular repair, remodeling, and atherosclerotic lesion formation (Hillebrands et al. 2001; Hu et al. 2002; Tanaka et al. 2003). Since progenitor cells are already used in clinical trials, the lack of specific cell markers is currently one of the most pressing problems in stem cell research (Hristov and Weber 2004; Xu 2006). Importantly, Yin et al. (2006) have recently shown that SMCs derived from embryonic stem cells can express a panel of smooth muscle markers, but their proteome remains very different from aortic SMCs indicating that the same marker proteins used for characterizing mature cell populations may not necessarily be sufficient to classify stem-cell-derived cells. Instead, the ultimate phenotype of a cell is reflected in their instantaneous proteomic profile, and proteomics may offer an opportunity to progress towards a molecular classification of stem-cell-derived cells, based on comparative analysis of protein expression patterns with other vascular cell types (Figure 1). Moreover, proteomics could help to identify therapeutic markers in stem cell therapy and drug targets for promoting stem cell differentiation.

• Conclusion

To understand complex biologic systems, detailed examination of the properties of their constituent parts is essential but insufficient. The quest for single signaling pathways in multifactorial disease processes has the illusion of simplicity but requires higher levels of integration. In our opinion, the combination of proteomic and metabolomic techniques provides an opportunity to bridge the gap between molecular and systems biology, which will be indispensable for addressing the multiple facets of cardiovascular diseases.

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Basic Mechanisms of Oxidative Stress and Reactive Oxygen Species in Cardiovascular Injury

Christopher A. Papaharalambus and Kathy K. Griendling*

The development of vascular disease has its origins in an initial insult to the vessel wall by biological or mechanical factors. The disruption of homeostatic mechanisms leads to alteration of the original architecture of the vessel and its biological responsiveness, contributing to acute or chronic diseases such as stroke, hypertension, and atherosclerosis. Endothelial dysfunction, macrophage infiltration of the vessel wall, and proliferation and migration of smooth muscle cells all involve different types of reactive oxygen species produced by various vessel wall components. Although basic science and animal research have clearly established the role of reactive oxygen species in the progression of vascular disease, the failure of clinical trials with antioxidant compounds has underscored the need for better antioxidant therapies and a more thorough understanding of the role of reactive oxygen species in cardiovascular physiology and pathology. (Trends Cardiovasc Med 2007;17:48–54) © *2007, Elsevier Inc.*

The fundamental processes underlying the development of vascular diseases such as atherosclerosis, restenosis, and hypertensive vascular remodeling have their origins in an initial insult to the vessel wall. Such an insult may arise from mechanical disruption that occurs during percutaneous coronary angioplasty or at regions of oscillatory shear stress, or it can result from biological causes, such as hypercholesterolemia, excess free radicals, diabetes, increased concentrations of plasma homocysteine, or infectious agents. Injury promotes disruption of the homeostatic mechanisms of the endothelial protective barrier, changing its vasoreactivity, increasing its adhesiveness to leukocytes, and altering its permeability. This endothelial dysfunction also leads to the formation of vasoactive molecules, which in turn induce inflammatory genes, inactivate nitric oxide ($NO^{\cdot}$) and its protective functions, and activate matrix metalloproteinases (MMPs), leading to extracellular matrix remodeling and increased smooth muscle cell (SMC) growth, migration, and proliferation. Each of these processes contributes to thickening of the vessel wall or the formation of lesions that can lead to hypertension, acute myocardial infarction, and stroke.

• Reactive Oxygen Species and Vascular Injury

In the past decade, a staggering amount of evidence implicates a common denominator, reactive oxygen species (ROS),

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