Review

Vascular proteomics: Linking proteomic and metabolomic changes

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Cardiovascular diseases constitute the largest of death in the Western world. Various stressors, including elevated blood pressure, smoking, diabetes, and hypercholesterolemia directly or indirectly damage the vessel wall, eventually inducing arterial stiffness (arteriosclerosis) and lipid accumulation (atherosclerosis). However, the molecular mechanisms of atheroma formation are not yet fully clarified. While many investigators have used proteomic techniques to study cardiac diseases, vascular proteomics is still in its infancy. The present review highlights studies, in which proteomics has been successfully applied to study protein alterations in the vasculature. Furthermore, we will summarize our recent progress in combining proteomic and metabolomic techniques to reveal protein and metabolite alterations in the cardiovascular system: two-dimensional (2-D) gel electrophoresis proved to be highly complementary to nuclear magnetic resonance (NMR) spectroscopy, in that post-translational modifications of the most abundant enzymes were displayed on 2-D gels while NMR spectroscopy revealed changes in the corresponding metabolites. Importantly, the simultaneous assessment of protein and metabolite changes translated purely descriptive proteomic and metabolomic profiles into a functional context and provided important insights into pathophysiological mechanisms that would not have been obtained by other techniques.

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Abbreviations: EC, endothelial cell; **SMC**, smooth muscle cell

1 Introduction

Revealing the secrets of the genome [1–3] provided the basis for a better understanding of cellular and molecular mechanisms. However, as neither the genomic sequence nor the transcriptional profile can be directly correlated with protein expression [4], the importance of measuring protein levels has become increasingly clear. Proteins are involved in virtually every cellular function. Thus, most activities performed by a cell will be reflected in their proteome. The promise of proteomics is to perform large-scale studies of gene expression at the protein level,

which could lead to the discovery of novel proteins, novel markers of diseases, novel pathophysiological mechanisms and, last but not least, novel targets for drug development, providing a strong impetus for investment in these new technologies [5]. Without doubts, proteomics will redefine biomedical research in the postgenomic era, including research on cardiovascular diseases [6–10].

2 The pathophysiology of atherosclerosis

Cardiovascular diseases, such as heart attacks and stroke, account for over 50% of all deaths in industrialized countries, clearly outnumbering deaths attributed to malignant or infectious diseases [11, 12]. The underlying pathological process is a thickening of large arteries, called atherosclerosis [13–15]. Frequently affected vessels include the aorta as well as coronary, carotid, cerebral, and iliofemoral arteries. The strikingly nonrandom, geometrically defined localization of atherosclerotic lesions strongly suggests an influence of hemodynamic forces on the underlying pathologic mechanism [16]. This is supported by the fact, that veins do not develop atherosclerosis unless they are grafted from their low-pressure environment into the arterial circulation [16, 17]. Early atherosclerotic lesions may already occur in childhood, but overt clinical symptoms are generally absent until middle or old age. Although lesion development is clearly age-dependant [18, 19], atherosclerosis is not simply an inevitable degenerative consequence of aging, but rather a form of chronic inflammation [13].

The arterial wall is composed of three layers: the intima, the innermost layer lined with endothelial cells (ECs) on the luminal side; the media, consisting of several layers of smooth muscle cells (SMCs) and the adventitia, which is mainly formed of connective tissue (Fig. 1A). The endothelium is supposed to form a selective barrier between blood and tissue, however, life-long exposure to stress stimuli, *e.g.*, elevated levels of serum cholesterol, hypertension, diabetes mellitus, smoking, infectious agents, and obviously combinations of these or as yet unrecognized factors, cause endothelial injury, an initial event that precipitates the atherosclerotic process [13, 20]. The first manifestations of endothelial injury include increased trapping of lipoproteins in the arterial intima and the appearance of leukocyte adhesion molecules on the endothelial surface, triggering monocyte infiltration and initiating a chronic inflammatory process in the vessel wall [15].

While lipoproteins, such as low-density lipoproteins (LDLs), are protected from oxidation in the plasma compartment, they undergo extensive oxidation and enzymatic modifications in the arterial wall [14]. These modifications alter receptor-binding, *i.e.*, oxidized LDL cannot bind to classical LDL receptors, but is taken up by so-called scavenger receptors, that, in contrast to LDL receptors, are not downregulated by intracellular cholesterol and therefore greatly enhance cellular cholesterol flux [21]. As monocytes infiltrate the arterial intima, they become macrophages, express scavenger receptors and phagocytose-oxidized LDL until they become lipid-filled foam cells, a key event in atherogenesis [14]. Hence, the first recognizable lesion of atherosclerosis, the so-called 'fatty streak', constitutes an aggregation of lipid rich-macrophages within the intima [22]. Although the recruitment of monocytes to the intima may initially serve a protective function by removing subendothelial lipid accumulations that drive the atherosclerotic process, monocyte-derived macrophages and attracted lymphocytes will release cytokines advancing lesion progression further.

Importantly, local cytokine production stimulates SMC proliferation [11]. Normally, arterial SMCs are contractile and not very responsive to growth factors. The atherogenic environment, however, leads to a prominent structural reorganization in SMCs with loss of myofilaments and formation of an extensive endoplasmatic reticulum and a large Golgi complex termed synthetic phenotype [23]. Numerous contractile proteins gradually disappear, the SMCs lose their ability to contract, increase protein secretion and become more responsive to growth factors, which, in turn, stimulates medial SMC hypertrophy and/or intimal hyperplasia contributing to further enlargement of the growing lesion.

Two types of plaque growth characterize disease progression [18, 19]: The first resembles a slow and continuous plaque extension due to progressive accumulation of mononuclear cells and proliferation of SMCs. It relies on cumulative exposure of vascular risk factors and is often associated with extensive vascular remodelling, a compensatory enlargement of the artery at the site of the lesion. The second main type of plaque growth is characterized by an occasional prominent increase in lesion size with atherothrombosis being the main underlying pathomechanism. As lipid-loaded foam cells finally undergo cell death, they release their lipid content and form a necrotic core within the atherosclerotic plaque [15, 24] (Fig. 1B). If this necrotic core disrupts the endothelial lining it causes clot formation (thrombosis) [25], which may result in complete occlusion of the vessel and acute clinical symptoms: reduced blood flow leads to a reduced oxygen supply; tissues become ischemic and rapidly undergo necrosis, especially if their oxygen demand is high like in hearts and brains. Hence, plaque composition rather than the degree of arterial stenosis predicts the likelihood of rupture: large, fibrotic lesions can be relatively stable. In contrast, plaque rupture frequently occurs in relatively minor but lipid rich lesions [12, 26]. Although

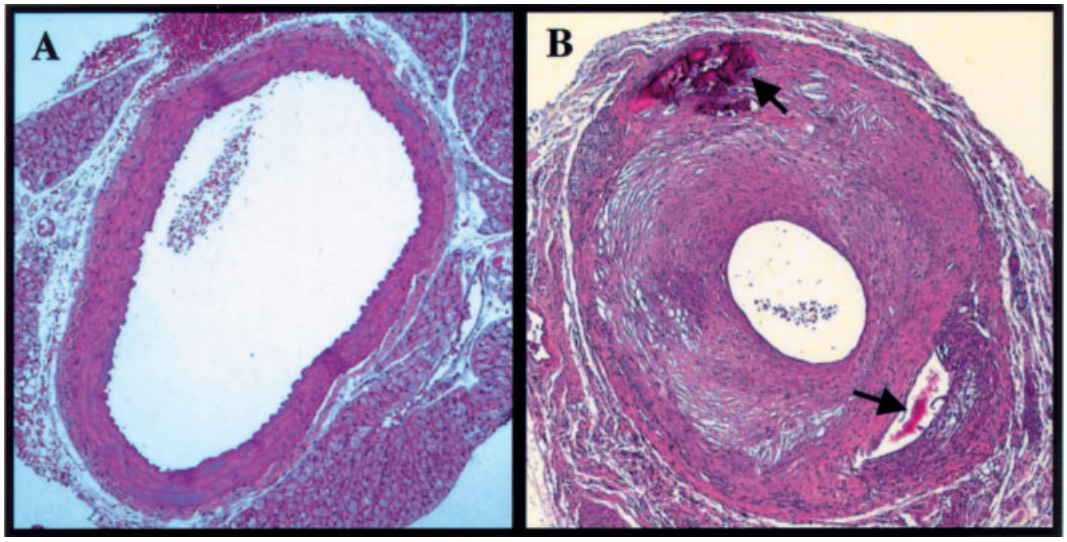

Figure 1. (A) Histological sections of normal artery stained with hematoxylin-eosin from wild-type mice and (B) atherosclerotic lesions from ApoE-deficient mice. Due to their elevated cholesterol levels, ApoE-deficient mice spontaneously display the entire spectrum of lesions observed during atherogenesis in humans. Arrows indicate necrotic core, magnification $\times$ 100.

the precise causes of atherosclerosis and plaque rupture are not yet fully clarified, there is strong evidence that inflammatory processes not only contribute to the initiation and progression of atherosclerotic lesions, but also determine plaque stability [27].

3 Vascular proteomics

In vascular research, proteomics is still in its infancy and the proteome of normal and pathological vessels is largely unknown. The main obstacle for applying proteomic analysis to vascular pathology is the heterogeneous cellular composition of atherosclerotic plaques [22]. Proteomic profiles of normal vessels are dominated by proteins of vascular SMCs, but will also contain proteins derived from ECs and other cell types including fibroblasts, myofibroblasts and dendritic cells. Atherosclerosis, however, is a chronic inflammatory disease and the chronic inflammatory process is generated by an interaction between infiltrating T-lymphocytes and monocyte-derived macrophages [13]. The latter are present in large numbers and will mask proteins derived from normal vessel wall components. Furthermore, cell viability within atherosclerotic lesions is severely compromised as evidenced by the formation of large necrotic cores. Therefore, differential display experiments might reflect the heterogeneous cell composition of advanced lesions compared to normal vessels without necessarily contributing to a better understanding of vessel pathology.

3.1 Proteomic analyses *in vivo*

Despite these limitations, the first attempts to analyze human atherosclerotic lesions by 2-DE were undertaken almost 20 years ago, when the absence of immobilized pH gradients still limited the reproducibility of 2-DE. Stastny *et al.* [28] compared the protein composition of human fibrofatty lesions with lesion-free segments of the human aortic intima. In those pioneering days of proteomic research, differences on gels had to be spotted by visual comparison rather than computer-assisted gel analysis. Thus, it is not surprising that only the most obvious changes were detected, namely that proteins originating from the plasma, *i.e.*, albumin, fibrinogen, immunoglobulin G, α-1 antitrypsin, transferrin, haptoglobin, apo A-I, and apo A-II, accumulated in diseased vessels [28]. In a later study, quantitation by laser densitometry confirmed a significant increase in the albumin/actin ratio in fatty streaks compared to normal arteries [29]. Similarly, Song *et al.* [30] found that plasma-derived proteins became more abundant in the arterial intima with aging and were related to intimal thickening. Overall, these findings probably reflect the compromised endothelial barrier function of diseased and aged vessels.

But even with recent improvements in 2-DE and the availability of sophisticated software packages for gel analysis, proteomic characterization of atherosclerotic lesions remains a challenge: recently, You *et al.* [31] analyzed coronary arteries from patients with coronary artery

disease and compared them with healthy controls (n = 10 and 7, respectively). 2-DE revealed a significant increase in ferritin light chain expression that was confirmed by Western blot analysis. The authors concluded that their results were consistent with the "iron" hypothesis, which proposes that iron storage contributes to atherosclerosis by modulating lipid oxidation. However, this study raises two questions: First, one would expect far more differences between healthy and diseased arteries indicating that despite a reasonable sample size differences were still masked by the biological variability between humans in general and between complex atherosclerotic lesions in particular and the experimental variability of proteomic analysis. Second, as mentioned above, the heterogeneous composition of atherosclerotic plaques complicates data interpretation. As mRNA levels of ferritin light chain were paradoxically decreased [31], the increase at the protein level might also be a consequence of monocyte infiltration in diseased arteries.

An interesting alternative approach to study atherosclerotic plaques with proteomic techniques was performed by Duran and Mas *et al.* [32]. They cultured normal arteries and carotid endarterectomy samples in protein-free medium and analyzed the supernatant by 2-DE. Proteins found to be released from plaques, but not from normal artery segments included proteins involved in reverse cholesterol transport (apolipoprotein B-100, apolipoprotein A-1), apoptosis (β-galactosidase-soluble lectin), protein degradation (ubiquitin carboxyl-terminal hydrolase 23), and antioxidants (Cu-Zn superoxide dismutase, peroxiredoxin 2). The more complicated the lesion, the higher was the number of proteins. Overall, the identified proteins fit well in the pathophysiological context of atherosclerosis. Nevertheless, it remains to be clarified whether these proteins are really "secreted" from the plaques as the authors suggested or whether passive diffusion into the cell culture medium is simply easier from plaques compared to normal arteries.

Another promising application of proteomics is to determine drug-effects on protein expression. Drug-eluting stents are highly successful in preventing restenosis [33–35]. One of the drugs used for stent coating is rapamycin (sirolimus), a well-established immunosuppressive drug, which inhibits SMC proliferation *via* mTor-signalling pathways [36, 37]. A proteomic comparison between the neointima of rapamycin and vehicle-treated vessels revealed that local rapamycin administration inhibited extracellular matrix synthesis after balloon injury [38]. This finding warrants further investigation as inhibition of extracellular matrix synthesis could compromise vessel stability. Although current evidence suggests that sirolimus-eluting stents are safe [39, 40], there is anecdotal evidence that drug-eluting stents might be associated with rare side-effects such as vessel dilatation or aneurysm formation [36]. Long-term follow-ups are clearly needed before a final assessment on the benefit of drug-eluting stents can be reached. Importantly, this example demonstrates that proteomics may reveal potential drug side-effects.

3.2 Proteomic analyses *in vitro*

To overcome the limitations of heterogeneity in cell composition, laser capture microdissection may be used to analyze specific cell types within tissues. However, collecting sufficient material for proteomic analysis is time-consuming and obtaining protein identification from limited amounts of tissues might be difficult. The most feasible alternative is to use cultured cells and to study proteomic changes in response to cardiovascular risk factors, such as high levels of glucose, cholesterol, and mechanical stress. As both SMCs and ECs play a dominant role in the development of atherosclerosis, they represent the predominant cell systems in studies addressing atherosclerosis.

3.2.1 Endothelial cells

Human umbilical cord endothelial cells (HUVECs) are the most popular *in vitro* model for human ECs. cDNA arrays were used to survey the transcriptional activity of about 11 000 genes in cultured HUVEC monolayers maintained under static (no flow) conditions and compared with two distinct fluid dynamic conditions – steady laminar flow or turbulent flow [41]. Mechanical forces are known to play a key role in the pathogenesis of atherosclerosis as spontaneous atherosclerosis develops at preferential sites along the vasculature. These regions experience specific hemodynamic conditions characterized by elevated stretch stress and shear stress with a cyclic reversal flow direction (named oscillatory or turbulent flow). This contrasts with plaque-free sections where the vessel is exposed to unidirectional (laminar) flow and comparably lower levels of stretch stress [16, 42]. Approximately 2500 genes showed detectable levels of expression in cultured endothelium and about 100 genes showed differential regulation in ECs exposed to laminar *versus* turbulent shear stress. Interestingly, laminar shear stress appeared to be a more potent stimulus than turbulent shear stress and more proteins were downregulated by shear stress than were upregulated. These data indicate that ECs can discriminate between distinct types of flow and that gene expression altered by mechanical stress may contribute to biomechanically induced "atheroprotective" and

"atheroprone" endothelial phenotypes. The entire data set can be found at a searchable database http://vessels.bwh.harvard.edu/papers/PNAS2001.

Similarly, protein changes after exposure to oscillatory and laminar flow were analyzed in bovine aortic ECs by 2-DE [43]. Approximately 2500 spots were resolved within a pH gradient of 3–10, a figure that closely correlates to the number of expressed genes detected in the study mentioned above [41]. But in contrast to the microarray data, only a handful of proteins were found to be altered in response to mechanical stimuli, including a macrophage-capping protein (Cap G), a member of the gelsolin protein superfamily [44]. These findings highlight three major problems: First, whereas decreases in mRNA levels are likely to be accompanied by loss of protein expression, increased mRNA levels are not necessarily reflected in the proteome. Second protein separation techniques are less sensitive in detecting quantitative differences than microarrays. Third, the increased sensitivity of the microarray technology is associated with a high percentage of false-positive results and considerable experimental variation.

The first annotated 2-DE map of proteins expressed in HUVECs has been published recently [45] and is available on the web at http://www.huvec.com. Data were accumulated from separations conducted on 3–10, 4–7, and 5.5–6.7 pH gradients and displayed more than 1000 protein species. However, only 50 proteins within a very limited pH range (pH 4–7) have been identified so far and more proteins have to be identified to provide a representative overview about the most abundant proteins in this important cellular model. Then, such descriptive studies might become useful to all researchers working with these defined cell systems, although variations in running conditions between different laboratories and the use of different pH gradients for first dimension separation will always remain a limiting factor for the wider use of 2-DE maps.

3.2.2 Smooth muscle cells

Besides ECs, vascular SMCs are the principle cellular component of blood vessels. The most detailed map of SMC proteins currently available has been published by McGregor *et al.* [46]. Similar to the map for HUVECs, extracts of human saphenous vein medial SMCs were separated by 2-DE and about 130 proteins were identified. McGregor *et al.* also described early proteomic changes in venous SMCs exposed to hemodynamic stress [47]. Similar to ECs, alterations were observed for proteins of the gelsolin family. Additionally, actin filament remodelling was reflected in the proteome as dephosphorylation of heat shock protein 27 and decreased abundance of CapZ, two proteins responsible for capping the barbed end of actin filaments, highlighting mechanical stress-induced protein changes that favor the generation of contractile stress fibres [47]. A detailed map of arterial SMCs is not available yet, but we have identified 250 proteins in mouse aortic SMCs (Mayr *et al.*, unpublished data). This annotated map will be soon published on the web at http://www.vascular-proteomics.com.

So far, several functional studies have applied proteomic techniques to vascular SMCs: Patton *et al.* [48] identified differences in protein expression after subjecting serum-deprived rat aortic SMCs to growth stimuli. As described above, SMC proliferation is a key event in the pathogenesis of atherosclerosis. SMCs display two types of growth responses: atherogenesis involves a hyperplastic response, characterized by increased DNA and protein synthesis as well as cell division of intimal SMCs, while chronic hypertension predominantly causes SMC hypertrophy in the media, characterized by increased cell size and protein content without DNA synthesis or cell division, although this is subject to some controversy [49]. At least at the proteome level, hyperplastic (platelet-derived growth factor, 10% fetal calf serum) and supposedly hypertrophic agents (angiotensin II) were accompanied by similar changes in protein expression, including an increase in elongation factors and chaperones, such as heat shock protein 60, heat shock protein 70, and protein disulfide isomerase, suggesting that both types of growth require upregulation of the protein synthesis and folding machinery. These findings are consistent with *in vivo* data, reporting increased expression of heat shock protein 60 and 70 in atherosclerotic lesions [50, 51].

Cremona *et al.* [52] investigated aging-associated changes in vascular SMCs. By comparing protein expression in aortic SMCs cultured from newborn and aged rats, they identified a cellular retinol-binding protein, which was present in measurable amounts in cultured SMCs of old, but not young rats. Retinoids are implicated in vascular SMC differentiation and phenotypic modulation, which is a characteristic feature of atherosclerotic lesions.

Taurin *et al.* [53] analyzed subsets of soluble and membrane SMC proteins from control and ouabain-treated cells by 2-DE. Ouabain inhibits the cellular Na^+, K^+ pump and rescues SMCs from apoptosis in response to various stimuli including serum deprivation. Proteins induced after inhibition of the Na^+, K^+ pump included mortalin, a member of the heat shock protein 70 family. This protein inactivates the proapoptotic tumor suppressor gene, *p53,* providing a likely explanation for the protective effect of ouabain treatment against cell death. Importantly, there is strong evidence that *p53* is a crucial regulator of SMC proliferation [54] and apoptosis [55, 56] in atherosclerosis [57].

Finally, Liao *et al.* [58] purified and identified proteins secreted from vascular SMCs in response to oxidative stress, using capillary chromatography and ESI-MS/MS. One of the secreted oxidative stress-induced factors was heat shock protein 90. Interestingly, depletion of heat shock protein 90 from the conditioned medium significantly inhibited ERK-signalling in response to oxidative stress implicating this heat shock protein as important mediator for the effects of reactive oxygen species on vascular function. The mechanisms, by which intracellular signalling proteins that lack signal peptide sequences, such as heat shock proteins, can be secreted in response to stress, is currently unknown, but the phenomenon has been widely reported for chaperones of the cyclophilin family [59]. Furthermore, there is strong evidence for cell surface expression of heat shock protein 60 in the vasculature [60, 61] and serum levels of circulating heat shock protein 60 are elevated in patients with atherosclerosis [62] and borderline hypertension [63]. Thus, chaperones in general and heat shock protein 60 in particular may represent important mediators in atherosclerosis as they alter cell-signalling [64] and inflammatory responses [65, 66].

4 Translating proteomic findings into biological function

The holistic approach taken by proteomics is clearly an advantage, in that it is less biased by experimental design, but it can also produce data that may not be interpretable. Displaying proteomic changes without translating them into a functional context is only of limited scientific value and fails to deliver on the true potential of proteomics. Ideally, a thorough analysis of the proteome is not only descriptive, but rather includes some measure of function. Protein expression profiling *per se* provides no information regarding their functional state. Hence, many manuscripts using exclusively proteomic techniques remain purely descriptive and data interpretation is often speculative. Instead, proteomics is more likely to succeed in mainstream research if used in combination with other established technologies ranging from biochemistry and molecular biology to analytical protein chemistry.

Proteomic technologies are rapidly evolving, but currently none of them is able to deliver absolute quantification of complex protein mixtures such as cell lysates and tissue extracts in a fully automated fashion. Based on our experience, 2-DE together with MS is still the most suitable technique, especially for combining proteomics with metabolomics. Although 2-DE is already 30 years old [67], it has yet to be supplanted for quantitative comparisons of complex proteins mixtures [9, 68]. It is true that 2-DE is biased towards long-lived, abundant proteins; however, this consideration enhances the possible significance of differentially expressed proteins, which therefore cannot be minor cellular components. It is also true that 2-DE does not reflect a true representation of hydrophobic, highly insoluble, very basic, very small, and very large proteins [69], but all of these limitations can, at least partially, be addressed [69]. Furthermore, recent improvements in 2-DE allow a more reliable quantification, *e.g.*, by using an internal standard in the difference in gel electrophoresis (DIGE) approach [70], or better proteomic coverage, *e.g.*, by running narrow-range IPG strips after sample prefractionation [71]. Importantly, by trying to overcome the weaknesses of 2-DE [72], one must not forget to exploit its major advantage, namely its ability to display post-translational modifications [68, 69], which are known to be instrumental in the development of many diseases.

The true strength of 2-D gel images is the visualization of post-translational modifications as a shift in p*I* and M_r of the resolved proteins [68]. This additional information is invaluable for the detection of changes in protein isoforms and proved to be essential for successfully combining proteomic and metabolomic techniques [73–75]: enzymatic activity is frequently regulated by post-translational modifications and not only changes in protein expression. Thus, by replacing 2-D gels with automation-friendly techniques, such as LC-MS/MS [72, 76], one may overcome certain limitations of 2-D gels, but crucial information on post-translational modifications is lost. In fact, protein derivatization techniques, such as isotope-coded-affinity tags (ICAT)-labelling [76], might cause post-translational modifications that were not the result of the original experimental conditions [9].

As differences observed on 2-D gels reflect either changes in post-translational modification or protein expression [68], theoretically each of these differences would have to be characterized further. But since hundreds of changes may be observed between two cell lines, this represents a time-consuming and therefore impractical task. Frequently, individual proteins are selected for further analysis, guided by the specific research objectives and the expertise of the scientist. Surprisingly, enzymatic changes tend to be ignored in this selection process despite the fact that enzymes are among the most abundant proteins resolved on 2-D gels. But translating enzymatic changes into a functional context is challenging as differences at a proteome level will not allow conclusions about enzymatic activity and multiple enzymatic changes might be present in a single sample. Thus, additional experimental steps are clearly needed for correct interpretation of enzymatic alterations at the proteome level.

5 Metabolomics

High-resolution NMR analysis has the potential to reveal the metabolic net effect of enzymatic changes observed at the protein level. NMR technologies allow a quantitative analysis of small metabolites in a given cell or tissue. They offer the advantage of obtaining information on metabolites without the need to have prior knowledge of the study systems and are able to satisfy all the criteria required for metabolomic studies [77]: robustness, reproducibility, the ability to identify unknown metabolites, and the potential to incorporate them into models of theoretical biochemical networks.

Proton (^{1}H) NMR has been used in a first 'proof-of-principle' demonstration of a practical metabolomic screening method in a eukaryotic genome [78], with the main objective of ultimately predicting the function of 'silent' genes in yeast. The deletion of silent genes causes no noticeable effect on metabolic fluxes or growth and yet these genes account for about 85% of the yeast genome. The effects of changes in enzyme activity on metabolite concentrations can be much larger than their effects on metabolic fluxes so examining metabolite concentrations is likely to be a good method for revealing the role of 'silent genes' [79]. So far, most metabolomic research has been performed on plants or microorganisms and relatively little has been published on metabolomics in humans or animals [80].

The role of each gene product in metabolism is the link between the genotype and the phenotype. A single gene mutation may cause alterations of metabolite levels of seemingly unrelated biochemical pathways and this is likely to happen when genes are constitutively overexpressed or knocked-out by targeted disruption of the endogenous gene. In order to help researchers to understand such systems, a comprehensive and quantitative analysis of proteins and metabolites is required, with experimentally robust and reproducible methodologies that aim to include all classes of compounds with high recovery.

Similarly to proteomics, NMR analysis will be restricted to the most abundant metabolites. But this limitation actually makes both techniques complementary: proteomics visualizes changes in the more abundant proteins, including many enzymes, and NMR analysis reveals differences in the more abundant metabolites. Therefore, NMR will provide crucial information for a functional interpretation of proteomic data, while proteomics contributes to a better understanding of NMR data by highlighting the enzymes or enzymatic pathways involved [81]. In combination, these methods have a great potential for exploring systems with changes in metabolic pathways following gene mutations.

6 Combining proteomics and metabolomics

To prove the feasibility of this concept, we have recently characterized proteomic and metabolomic changes in PKCdelta knockout (PKCδ -/-) hearts [73, 74]. Strikingly, most of the changes observed in PKCδ -/- hearts were related to energy metabolism (Table 1). Enzymes involved in glycolysis appeared to be decreased compared to wild-type controls, *e.g.*, glycerol-3-phosphate dehydrogenase (spot 1, -2.2 fold, p = 0.011), pyruvate kinase 3 (spot 2, -2.0 fold, p = 0.007), and lactate dehydrogenase 1 (spot 3, -3.5 fold, p = 0.001). In contrast, enzymes related to lipid metabolism were found to be markedly upregulated in PKCδ -/- hearts, *e.g.*, a three- and fivefold increase in acyl-CoA dehydrogenase (spot 6, p = 0.024 and 0.026, respectively) (Fig. 2A). NMR analysis of cardiac metabolites proved the functional relevance of the proteomic changes at a metabolic level (Fig. 2B): a pronounced decrease in the ratio of glycolytic endproducts (alanine + lactate) to end products of lipid metabolism (acetate) demonstrated an impairment of aerobic glucose metabolism and a compensatory increase in lipid utilization in PKCδ -/- hearts, which is in perfect agreement with the proteomic findings [73]. Strikingly, proteomic differences for glycolytic enzymes disappeared rapidly after exposure to brief episodes of ischemia [74] (Fig. 3A), indicating that they represented differences in post-translational modifications rather than protein expression. Moreover, these sudden changes in post-translational modifications observed at the protein level corresponded to changes in enzymatic activity, as the ratio of glucose to lipid metabolites became similar to wild-type controls (Fig. 3B). Thus, metabolomic data can substantiate the functional relevance of enzymatic changes observed at a proteomic level and 2-D gels appear to be particularly suited for these kind of analyses, as they have the potential of displaying post-translational modifications, which alter enzymatic activity and thereby cellular metabolite concentrations.

In addition, we performed the first proteomic and metabolomic analysis of cultivated vascular SMCs. By use of 2-DE, we identified more than 30 alterations in protein species between SMCs derived from PKCδ+/+ and PKCδ-/- mice [75]. Similar to our *in vivo* findings, proteomic changes involved enzymes related to glucose and lipid metabolism. Although vascular SMC metabolism differs profoundly from cardiomyocytes, in that glucose is the main source of energy for SMC, but not for cardiomyocytes, high-resolution NMR spectroscopy of PKCδ-/- SMCs indicated a similar impairment in aerobic glycolysis. Furthermore, our proteomic data suggested that PKCδ-/-SMCs may have a compensatory increase in

Table 1. Differentially expressed enzymes in protein profiles of PKCδ +/+ and PKCδ -/- hearts

No.	Protein identity	NCBI Entry No.	Function	Calculated pI/ mass, Da ($\times 10^3$)
Glucose metabolism				
1	Glycerol-3-phosphate dehydrogenase	31981769	Glycolysis	6.2 / 81.4
2	Pyruvate kinase 3	20890302	Final step in glycolysis	6.7 / 57.9
3	Lactate dehydrogenase 1	13529599	Anaerobic glycolysis	8.2 / 34.5
4	RIKEN cDNA 261020716	13195670	Glucose/ribitol dehydrogenase	6.3 / 54.9
5	Mercaptopyruvate sulfurtransferase	20149758	Conversion of cysteine to pyruvate	6.1 / 33.0
Lipid metabolism				
6	Acyl-CoA dehydrogenase, short chain-specific	20841295	β-oxidation of short-chain fatty acids	8.9 / 44.9
7	Aldehyde dehydrogenase 4, family member A1	18848352	Oxidation of aliphatic and aromatic aldehydes	8.4 / 61.8
8	Peciprotein	12805053	Peroxisomal enoyl-CoA isomerase	8.1 / 39.4
9	Propionyl-CoA carboxylase, α-subunit	21450241	β-Oxidation of odd-chain fatty acids	7.0 / 79.9
10	3-Hydroxyacyl-CoA dehydrogenase type II	7949047	Short-chain/hydroxy-steroid dehydrogenase	8.8 / 27.4
11	Ke 6 protein	1103844	Alcohol/steroid dehydrogenase	6.1 / 26.7
12	Malic enzyme, supernatant	6678912	NADPH generation	7.2 / 63.9
TCA cycle				
13	Oxoglutarate dehydrogenase, lipoamide, E1 component	20853413	Citric acid cycle	6.4 / 116.4

alternative glucose pathways as evidenced by the marked upregulation of aldose reductase and glucose-6-phosphate dehydrogenase, the rate-limiting enzymes of the sorbitol and the pentose phosphate pathway, respectively. The latter was associated with a corresponding rise in cellular glutathione (GSH) levels. GSH is a tripeptide with a free sulfhydryl group, and of paramount importance in maintaining the reducing intracellular environment [82, 83]. NADPH provides the reducing equivalents for regeneration of GSH, and NADPH levels, in turn, are regenerated through the action of glucose-6-phosphate dehydrogenase. Cells with reduced levels of glucose 6-phosphate dehydrogenase activity have decreased GSH levels and are known to be more sensitive to oxidative stress [83]. Consequently, increased GSH protected PKCδ-/- SMCs against oxidative stress-induced cell death. These results provide a mechanistic explanation for our previous *in vivo* finding that vein grafts derived from PKCδ-deficient mice showed a marked increase in lesion formation compared to wild-type controls, as resistance to apoptosis contributed to SMC accumulation [84].

Taken together, enzymatic and metabolic changes were remarkably consistent in PKCδ-/- mice: similar alterations were found in different cell types, namely SMCs and cardiomyocytes, under *in vivo* as well as *in vitro* conditions. Thus, proteomic and metabolomic techniques can be applied to tissues as well as cell cultures, and cautious conclusions about the *in vivo* situation may be justified on the basis of *in vitro* findings.

7 Perspectives

The past several years have seen an increase in the number of proteomic studies in the cardiovasculature, with the overwhelming majority of work being carried out with cardiac tissue as extensively reviewed elsewhere [7–10, 85]. Vascular proteomics is still in its infancy, although a few studies have already used proteomic techniques to clarify pathogenetic mechanisms of atherosclerosis. We demonstrate that 2-DE is the preferred choice for a combined proteomic and metabolomic analysis, as they display post-translational modifications reflecting enzymatic activity. In view of the rapid progress in proteomics, we expect that proteomic techniques will be more widely applied to vascular research for clarifying alterations of proteins and their functions related to cell-signalling, differentiation, migration, apoptosis, and proliferation. These findings may lead to a new strategy for prevention and treatment of cardiovascular diseases such as atherosclerosis-induced heart disease and stroke.

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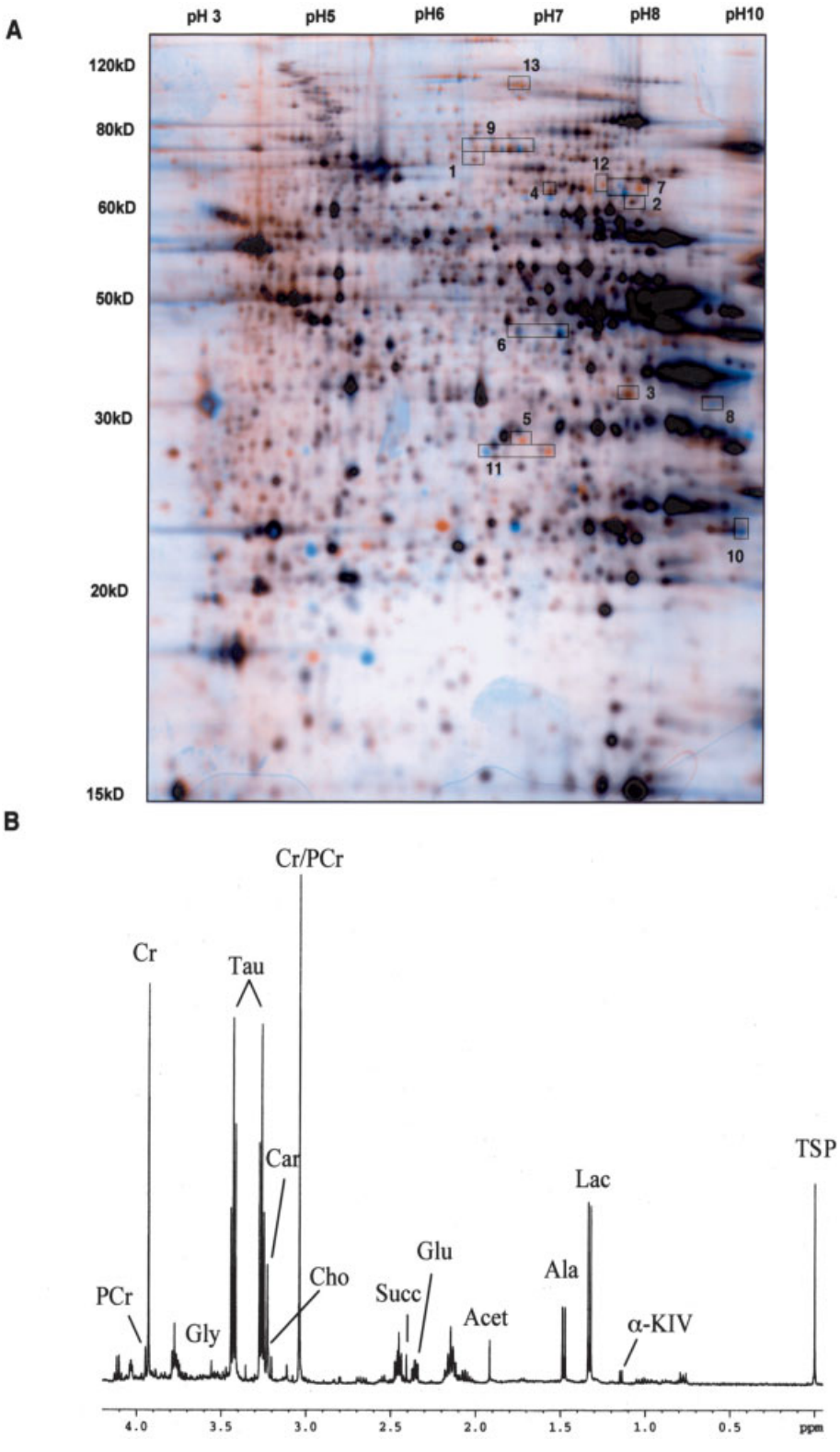

Figure 2. Heart protein extracts were separated on a pH 3–10 nonlinear IPG strip, followed by a 12% SDS polyacrylamide gel. Spots were detected by silver staining. (A) Direct overlay of average gels from PKCδ +/+ and -/- hearts. Each average gel was created from four single gels (total $n = 8$) using the Proteomweaver software (Definiens). Differentially expressed spots are highlighted in color (orange and blue for PKCδ +/+ and PKCδ -/- hearts, respectively). Boxed areas highlight differences in post-translational modification/expression of enzymes. The enzymes were identified by MS and are listed in Table 1. ^{1}H NMR spectra of PKCδ +/+ hearts were obtained using a Bruker 500 Mhz spectrometer (B). Resonances have been assigned to α-ketoisovalerate (α-KIV), lactate (Lac), alanine (Ala), acetate (Acet), glutamate (Glu), succinate (Succ), creatine (Cr), phosphocreatine (PCr), choline (Cho), carnitine (Car), taurine (Tau), and glycine (Gly). Sodium 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate (TSP) was added to the samples for chemical shift calibration and quantification.

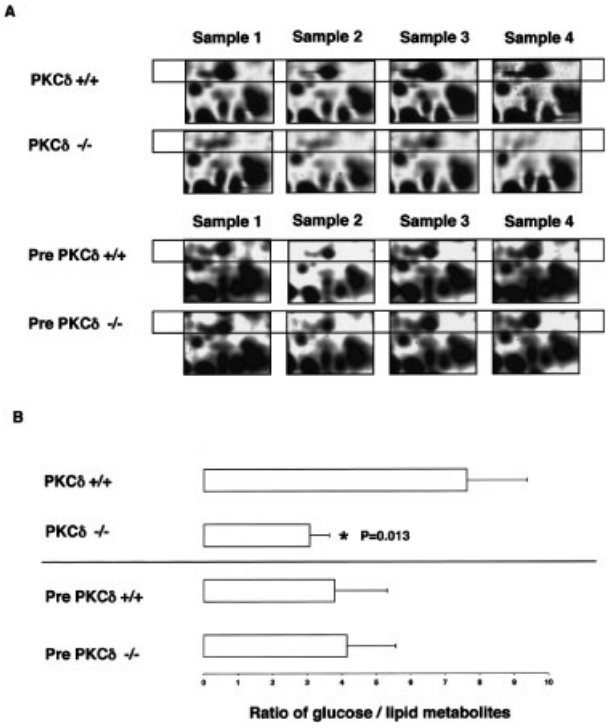

Figure 3. (A) Expression of lactate dehydrogenase 1 in PKCδ +/+ and PKCδ -/- hearts before and after ischemic preconditioning. Paradoxically, brief episodes of ischemia-reperfusion protect heart tissue from a subsequent prolonged ischemia by delaying cardiomyocyte death. This phenomenon is called ischemic preconditioning. Note the obvious difference in the protein pattern between PKCδ +/+ and PKCδ -/- hearts under normoxic conditions, which disappears rapidly in response to ischemic preconditioning (Pre). (B) Similarly, a significant difference in the ratio of glucose/lipid metabolites, as obtained from NMR spectra using the following calculation (alanine + lactate) / (acetate), is only observed in normoxic, but not in preconditioned hearts. Thus, 2-D gels visualize post-translational modifications, which are related to enzymatic activity and reflected in metabolite levels.

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