**Association of DJ-1/PTEN/AKT- and ASK1/p38-mediated cell signalling with ischaemic cardiomyopathy**

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**Aims**

Dilated cardiomyopathies from chronic ischaemia (ISCM) or idiopathic (IDCM) pathological mechanisms are accompanied by similar clinical symptoms but may differ in protein expression, cell metabolism, and signalling processes at the cellular level. Using a combination of proteomic and metabolomic profiling, we sought to decipher the relationships between the metabolism and cellular signalling pathways in human heart tissues collected from patients with ISCM, IDCM, and those without heart disease and dilation.

**Methods and results**

The comparative analysis suggested a decrease in glycolysis, Krebs cycle, and malate–aspartate shuttle activities in both types of cardiomyopathies and an increase in ketone body oxidation only in ISCM. Chronic ischaemic injury was associated with increased DJ-1 and decreased phosphatase and tensin homolog (PTEN) protein expression. The reduced PTEN expression was accompanied by increased phosphorylation of cell-protective AKT. Phosphorylation at T845 of apoptosis signal-regulating kinase 1 and p38 mitogen-activated protein kinase proteins, with no change in the phosphorylation of extracellular signal-regulated kinases, was also observed. The downregulation of peptidyl–prolyl cis/trans isomerase and NF-κB essential modulator potentially inhibits NF-κB-initiated processes.

**Conclusion**

The present study characterized differences in the molecular mechanisms, metabolism, and pathological cell signalling associated with ISCM and IDCM, which may provide novel targets for intervention at the cellular level.

**Keywords**

IDCM • ISCM • Cardiomyopathy • Metabolomics • Proteomics • Cell signalling

**1. Introduction**

Heart failure is the leading cause of cardiovascular morbidity and mortality worldwide. It affects ~5 million people in the USA alone with one in five people being at risk.¹² Heart failure often results from dilated cardiomyopathy (DCM), a condition characterized by left-ventricular dilatation, systolic and often diastolic dysfunction, reduced cardiac output, and significantly increased risk of sudden cardiac death.³⁴

Biomechanical stress including pressure or volume overload, tissue damage such as myocardial ischaemia, inflammation or infiltration, and genetic pre-conditions can initiate dilatation of the left ventricle and development of DCM.⁵ The molecular and genetic mechanisms of this multi-fatorial disease remain uncertain and involve multiple complex mechanistic interactions.¹⁵ Over the past few years, gene microarrays and proteomics technologies have been used widely to study various phases and aetiologies of cardiomyopathies as well as to identify biomarkers of cardiovascular diseases.⁶⁷ Several studies evaluating human DCM have found a consistent pattern of altered expression of genes involved in oxidative stress, apoptosis induction, cardiac structure, Ca²⁺ homeostasis, and energy metabolism.⁸⁹ Although ischaemic (ISCM) and idiopathic dilated cardiomyopathies (IDCM) have similar clinical features despite their different aetiologies, they differ in response to therapy and outcome.¹⁰–¹² Several clinical studies suggested that patients with ISCM have a higher degree of endothelial dysfunction when compared with patients with IDCM.¹³ A key role in the development of endothelial dysfunction and pathophysiology of myocardial dysfunction has been attributed to reactive

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oxygen species (ROS).\textsuperscript{14,15} It has been hypothesized that the myocardial energetic perturbations are a result of neurohumoral activation, increased free fatty acid metabolism, decreased glucose metabolism, and in some cases increased insulin resistance.\textsuperscript{14,15} Finally, myocardial ATP and phosphocreatine are depleted, creatine kinase activity reduced, and the efficiency of mechanical work diminished.\textsuperscript{16,17}

However, while the metabolic consequences of heart failure are well studied, and while it is well established that inadequate oxygen supply and the resulting ischaemic injury are the hallmark of ISCM, the molecular mechanisms leading to IDCM are not completely understood. It is reasonable to expect that understanding and comparing the cellular mechanisms causing ISCM and IDCM and their progression will provide a tool for earlier detection and intervention to improve outcomes. In addition, this may also lead to the identification of novel molecular therapeutic targets.

In this study, we employed a proteo-metabolomic strategy to identify differences in protein expression, cell metabolism, and cell signalling pathways in human cardiac tissue samples from patients with ISCM, IDCM, and normal hearts.

2. Methods

2.1 Tissue collection and selection
Protein expression was assessed in left-ventricular free wall samples from the human heart. The tissue was obtained from heart transplant recipients (ISCM \((n = 5)\) and IDCM \((n = 5)\) with left-ventricular dilation) or from transplant donors whose hearts were not utilized for transplantation (left ventricles with no dilation and normal systolic function \((n = 5)\), control samples) (see Supplementary material online, Table S1). After removal from the chest, the hearts were processed immediately on ice and tissue samples were put into liquid nitrogen and stored long-term in a \(-80^\circ C\) freezer.

The subjects were all male and age-matched (see Supplementary material online, Table S1). The majority of the individuals with ISCM and IDCM were on angiotensin-converting enzyme inhibitors, diuretics, and beta-blocker medications (see Supplementary material online). The majority of the individuals with ISCM and IDCM had left-ventricular dilation \((n = 5)\) with left-ventricular dilation and normal systolic function \((n = 5)\), control samples (see Supplementary material online, Table S1). The study was approved by the Colorado Multi-Institutional Review Board (COMIRB, Denver, CO) and conforms to the principles as outlined in the Declaration of Helsinki for the use of human tissue or subjects.

2.2 Tissue extraction for proteomics and western blot analysis
The frozen heart tissue samples were ground to a fine powder using a mortar and pestle and solubilized in lysis buffer containing protease and phosphatase inhibitors (Roche Diagnostics, Pleasanton, CA). The extracts were kept frozen at \(-80^\circ C\) for all subsequent analyses. The protein concentrations were determined using the Bradford protein assay kit (BioRad, Hercules, CA).

2.3 Proteomics analysis
A detailed description of the proteomics and mass spectrometry methods is available in the Supplementary material online, Appendix.

2.4 Western blot analysis
For western blots, tissue extracts were loaded onto Biorad Bis-Tris Criterion gels (various percentages). Proteins were separated using a Biorad Criterion electrophoresis system operating at 120 V and then transferred from the gel to an Immobillon-P membrane (200 mA, Millipore, Billerica, MA). Membranes were incubated with the primary antibody at 4°C overnight, after blocking with 5% milk or 2% BSA in PBS-Tween buffer. Antibodies used in this study included (mAb: monoclonal; pAb: polyclonal antibody; hosts: R: rabbit; M: mouse): DJ-1 (pAb), PTEN (mAb: phosphatase and tensin homolog), PIN1 (pAb: peptidyl-prolyl cis/trans isomerase, NIMA-interacting), ASK1 (apoptosis signal-regulating kinase 1) unmodified and phosphorylated at Ser967, Thr845, or Ser83 (all pAb), Akt unmodified and phosphorylated at Ser473 (both mAb), p38 and p42/44 MAP kinases unmodified (both pAb) and phosphorylated at Thr180/Tyr182 and Thr202/Tyr204, respectively (both mAb) (source of all antibodies above: rabbit; Cell Signalling Technology, Danvers, MA); CLIC1 (pAb, R; chloride intracellular channel protein 1), PRDX2 (mAb, R; peroxiredoxin 2), PRDX5 (mAb, R; peroxiredoxin 5), GSTM2 (pAb, R; glutathione S-transferase Mu 2), AST2 (pAb, R; aspartate aminotransferase), MDH1 (mAb, M; mitochondrial malate dehydrogenase 1), ALDH2 (mAb, R; mitochondrial aldehyde dehydrogenase 2), ACAT1 (mAb, M; acetyl-CoA acetyltransferase 1), SCOT1 (pAb, R; succinyl-CoA:3-oxoadipate-CoA transferase 1), NF-kB essential modulator (NEMO) (pAb, R; NF-kB-B essential modulator) (all Abcam, Cambridge, MA); actin skeletal muscle, HSP60 (heat shock protein 60) (both mAb, M; Lab Vision, Fremont, CA); OGDH (pAb, R; oxoglutarate dehydrogenase) (Santa Cruz Biotechnology, Santa Cruz, CA); and β-actin, actin cardiac muscle, vinculin (all mAb, M; Sigma-Aldrich, St Louis, MO). After membranes were washed three times, the secondary antibody (goat-anti rabbit and horse anti-mouse antibodies conjugated to horseradish peroxidase, Cell Signalling, Danvers, MA) was added. Membranes were subsequently treated with Pierce SuperSignal West Pico Solution (Pierce, Rockford, IL) following the manufacturer's instructions. A UVP system (Bioimaging Systems, Upland, CA) was used to detect the horseradish peroxidase reaction on the membrane. ImageJ software (NIH, Bethesda, MD) was used for quantitation. Densitometry data were normalized based on β-actin, which was shown not to change in pilot western blot experiments when compared with other housekeeping proteins including COX IV and GAPDH.

2.5 Tissue extraction for proton nuclear magnetic resonance (1H-NMR) spectroscopy
All tissue extractions were performed using a previously published perchloric acid extraction protocol, allowing for separation of water-soluble and lipid fractions.\textsuperscript{18} Lyophilized water-soluble tissue extracts were re-dissolved in 0.6 mL of deuterium oxide, centrifuged, and the supernatants neutralized to pH 7.2 to allow for precise chemical shift assignments. Lipid fractions were re-dissolved in 1 mL CD3OD/CDC13 mixture (1:2).

2.6 1H-NMR spectroscopy
High-resolution 1H-NMR experiments were performed using a Varian INOVA NMR 500 MHz spectrometer equipped with a 5 mm HCN PFG probe (Varian, Palo Alto, CA). For 1H-NMR analysis of water-soluble extracts, fully relaxed spectra with a standard water pre-saturation pulse program were recorded, whereas for analysis of lipids no pre-saturation pulse was used. Spectra were obtained at 12.0 p.p.m. spectral width (10 p.p.m. for lipids), 32K data arrays, and 64 scans with 90-degree pulses applied every 14.8 s. The quantification of metabolites was obtained using the trimethylsilyl propionic-2,2,3,3-d4 acid as an external standard and chemical shift reference (0 p.p.m.). The absolute concentrations of each metabolite were determined and normalized according to tissue wet weight, as previously described.\textsuperscript{18}

2.7 Statistical analysis
All numerical data were presented as mean ± standard deviation. Student's t-test or one-way analysis of variance (ANOVA) was used to determine differences among groups. Tukey's test was used as post-hoc test in combination with ANOVA to test for significances among groups. The
level of significance was set at \( P < 0.05 \) for all tests (SPSS version 19.0, IBM/SPSS, Chicago, IL).

3. Results

3.1 Oxidative stress response

The proteomic analysis identified the antioxidant enzymes peroxiredoxin 2, 5, and 6 as significantly changed between cardiomyopathy patients and controls. The directional changes were not uniform: while PRDX2 increased, PRDX6 decreased in both DCM groups. At the same time PRDX5 expression showed a decrease in the ISCM and no change in the IDCM group (see Supplementary material online, Table S2). Western blot analysis confirmed an increase of PRDX2 and a decrease of PRDX5 expression in ISCM samples compared with the control (Figure 1A and B). Neither PRDX2 nor PRDX5 expression in the patients with IDCM was significantly different from the controls (Figure 1A and B).

In addition to the peroxiredoxins, the glutathione-based oxidative stress response system was involved in DCM, namely GSTM2, the expression of which was more than two-fold lower in both DCM sample types than in the controls (see Supplementary material online, Table S2; Figure 1C). Heat shock proteins including HSP20, HSP27, and HSP60 were changed as well (see Supplementary material online, Table S2). In western blot analysis, HSP60 showed decreased expression in IDCM compared with ISCM samples (Figure 1D). In proteomic analysis, HSP60 and HSP27 were identified in multiple spots on the 2D-gels, suggesting involvement of post-translational modifications (PTMs) (see Supplementary material online, Table S2). We were able to identify one of the PTMs for HSP27: the isoform downregulated in ISCM tissues carried a phosphorylation site on the serine 82 residue (see Supplementary material online, Figure S1).

3.2 Metabolic pathways

3.2.1 Glucose metabolism

Glucose availability from glycogen seems normal or increased based on the normal (IDCM) or increased expression (ISCM) of glycogen phosphorylase (see Supplementary material online, Table S2). The glycolytical enzyme phosphoglycerate mutase showed significantly higher expression, but, in contrast, the expressions of triosephosphate isomerase and beta-enolase were markedly downregulated in ISCM and IDCM when compared with control samples (Supplementary material online, Table S2).

With regard to the Krebs cycle, we observed an increased expression of OGDH in ISCM only (see Supplementary material online, Table S2, Figure 2A). A marked decrease of fumarate hydratase in DCM samples suggests a decreased production of malate, an important metabolite of the malate–aspartate shuttle (see Supplementary material online, Table S2). Furthermore, downregulation of the...
‘shuttle’ enzymes MDH1 and AST2 was found, pointing towards the disturbance of intra-mitochondrial NADH production and electron transfer in DCM heart tissues (see Supplementary material online, Table S2, Figure 2B and C).

3.2.2 Fatty acid metabolism and utilization of ketone bodies
In addition to the altered glycolysis and Krebs cycle, DCM was also associated with redistribution of fatty acid metabolism, especially under ischaemic conditions. Here, the ischaemic injury induced an increased expression of OXCT1 and ACAT1 enzymes. Their levels in ISCM samples were significantly higher than in IDCM and controls (see Supplementary material online, Table S1, Figure 2D and E). At the same time, the expression of mitochondrial ALDH2 was significantly lower in ISCM samples compared with IDCM and controls (see Supplementary material online, Table S2, Figure 2F).

3.3 Cytoskeleton reorganization
Proteins involved herein included the cardiac and skeletal isoforms of actin, desmin, ezrin, myosin, cristallin chains, and vinculin. Using western blot analysis, both actin isoforms showed a higher expression in the ISCM than in the IDCM or control samples (see Supplementary material online, Table S2, Figure 3A and B). On the other hand, titin and vinculin were downregulated in both DCM tissues (see Figure 2A). This suggests a reorganization of the cytoskeleton in DCM heart tissues.

![Figure 2](https://academic.oup.com/cardiovascres/article-abstract/97/1/66/372196/1) Western blot analysis of proteins involved in the regulation of metabolic pathways. (A) Krebs-cycle enzyme OGDH was upregulated in ISCM samples. (B) MDH1 was downregulated in both ISCM and IDCM samples. (C) AST2 was downregulated in ISCM samples compared with controls. (D) Fatty acid metabolism and ketone body utilization regulators OXCT1 and (E) ACAT1 were upregulated in ISCM samples. (F) ALDH2 was upregulated in IDCM samples. Densitometry data were normalized based on the amount of β-actin. Data are presented as means ± standard deviations (n = 4; *P < 0.05; **P < 0.01; ***P < 0.001).
Supplementary material online, Table S2 and 3C). In comparison to the controls, expression of desmin as well as tubulin was upregulated in samples from DCM patients (see Supplementary material online, Table S2). A differential regulation of the nuclear transporter protein CLIC1 was also detected. The expression of this protein was significantly decreased in IDCIM when compared with ISCM and controls (see Supplementary material online, Table S2 and Figure 3D).

3.4 Cellular signalling pathways

3.4.1 DJ-1/PTEN signalling
Dj-1 expression was significantly higher in ISCM samples than in IDCIM and controls (see Supplementary material online, Table S1 and Figure 4A). Dj-1 interacts and negatively regulates PTEN, which was found downregulated only in ISCM (Figure 4B).

3.4.2 MAPK signalling
Owing to interactions between Dj-1/PTEN and MAPK signalling, we investigated the impact of the downregulation of PTEN on MAPK proteins. Indeed, a change in the phosphorylation of the p38 protein was identified. p38 was upregulated in ISCM, but downregulated in IDCIM samples when compared with the controls (Figure 4C). At the same time, no change in ERK1/2 (p42/44) protein activity was noted (Figure 4D). On the basis of this differential regulation of MAP kinases, we assessed the pathways upstream of p38 MAPK and investigated the involvement of ASK1. Our analysis showed that the increased phosphorylation at T845 was associated with an increase in phospho-p38 in ISCM heart tissues (Figure 4E). The ratio of inactive S83 phospho-ASK1 (dephosphorylated form is active) was decreased in both sets of DCM samples (Figure 4F), while the inactive S967 phospho-ASK1 was present to a larger extent in IDCIM samples (Figure 4G).

3.4.3 AKT, PIN1, NEMO, and NF-κB signalling
We identified changes in the active form of AKT and its co-regulator PIN1 (see Supplementary material online, Table S1). The active form, S473 phospho-AKT, was increased in both types of DCM (Figure 5A). PIN1 was downregulated in ISCM samples only (see Supplementary material online, Table S1 and Figure 5B). With PIN1 being involved in the regulation of NF-κB signalling,19 we showed that NEMO was also downregulated only in ISCM samples (Figure 5C).

3.4.4 1H-NMR metabolomic analysis
1H-NMR analysis of water-soluble metabolites revealed lower concentrations of creatine in both sets of DCM samples when compared with the controls (see Supplementary material online, Table S3). The data also indicated a reduced conversion of choline into phosphocholine resulting in lower phosphatidylcholine concentrations (see Supplementary material online, Table S3).

Figure 3 Western blot analysis of proteins involved in the cytoskeletal reorganization of the myocardium. (A) Cardiac actin and (B) skeletal actin were significantly higher in ISCM samples. (C) Vinculin was significantly lower in both ISCM and IDCIM samples. (D) CLIC1 was significantly lower in IDCIM samples. Densitometry data were normalized based on the amount of β-actin. Data are presented as means ± standard deviations (n = 4; *P < 0.05; **P < 0.01; ***P < 0.001).
The concentrations of glycolytical lactate were higher in IDC and ISCM than in control heart tissues (see Supplementary material online, Table S3). The concentrations of Krebs cycle metabolites, citrate, and succinate were 69% ($P < 0.05, n = 5$) and 66% ($P < 0.01, n = 5$) of the controls in ISCM, while there was no corresponding difference between IDC tissue samples and controls (see Supplementary material online, Table S3). The concentrations of glucose in the IDC and ISCM samples were similar to those of control samples, whereas the concentration of acetate was significantly higher in ISCM samples (see Supplementary material online, Table S3).

4. Discussion

The present study is based on human cardiac tissue samples from age- and gender-matched patients with two types of DCM and a non-failing heart control group. This investigation was restricted to male subjects to eliminate the gender variability in this pilot study. Unlike previous plasma-based metabolomic analyses of myocardial ischaemia, our study directly investigated human cardiac tissues. We used a two-step strategy: a proteo-metabolomic approach to generate mechanistic hypotheses and western blotting to confirm and further elucidate changes in cell signalling pathways.

The differences between IDC and ISCM are summarized in Figure 6. Our results supported the current view of metabolic remodelling in the failing heart muscle (Figure 6A). We also went a step further and identified novel signalling mechanisms that are likely to contribute to the development of ISCM and IDC. We identified the processes responsible for cytoskeletal reorganization and characterized the shifts in energy-generating metabolic fluxes (Figure 6A). For the first time, we were able to show an involvement and differential regulation of DJ-1/PTEN/AKT-, ASK1/p38-, PIN-1, and NEMO-mediated pathways in ISCM and IDC tissue samples (Figure 6B).

4.1 Cardiac metabolism in DCM and the role of oxidative stress

Our findings point towards a redistribution of the metabolic fluxes within the injured heart, showing an increase in its glycolytic lactate production. This is in accordance with findings described in the literature.16,17,21–24 In both DCM groups, there was an increase in the expression of phosphoglucomutase suggesting enhanced glycogen metabolism into glucose-6-phosphate as a substrate of glycolysis. It can be speculated that the observed decrease of triosephosphate isomerase might increase the activity of glycerophosphate shuttle, which can be a source of intramitochondrial NADH. The increased lactate level can be explained by decreased activity of the Krebs cycle allowing metabolism of more pyruvate into lactate.

Decreased expression of fumarate hydratase and malate dehydrogenase in both DCM groups suggests a reduced Krebs-cycle activity, a finding supported by the observed decrease in the concentrations of Krebs cycle intermediates pyruvate and citrate. Decreased malate dehydrogenase level may result in decreased intramitochondrial NADH generation via the malate–aspartate shuttle. In this metabolic shift, the increased expression of oxoglutarate dehydrogenase in combination with the decreased expression of fumarate hydratase results in the increased production of succinyl-CoA, a key metabolite in ketone body utilization. As mentioned previously, human heart can utilize ketone bodies for energy production, a path clearly favoured under ischaemic conditions and high OXCT1 expression.25 With regard to the lipid metabolism, ACAT1 was increased in both DCM heart tissues and accompanied by increased contents of intracellular cholesterol.

To compensate for changes in energy supply and demand, cardiomyocytes can activate the creatine kinase system, which produces ATP faster than other pathways.26 Intracellular concentration of creatine, as well as the expression of creatine kinase M, the enzyme responsible for conversion of phosphocreatine to creatine, was reduced in both types of DCM. This inadequate response of creatine utilizing pathways in DCM may result in decreased ATP restoration and availability in energy demanding situations.26,27

In order to identify the mechanisms underlying the discussed shifts in cell metabolism, we investigated what role the oxidative stress plays herein. Interestingly, antioxidant stress response seemed to have been differently regulated in IDC vs. ISCM tissues. On one side, a relative lack of protective ALDH2 activation28 in ISCM as opposed to IDCM samples was noted; on the other side, a differential regulation of peroxiredoxins 2 and 5 was observed.

While there was no change in the IDCM, the expression of PRDX2 was higher and the expression of PRDX5 was lower in ISCM samples when compared with controls. This observation supports the results of a recent study which showed that the failing myocardium responds to oxidative stress by decreasing the expression of PRDX5.29 Both peroxiredoxins have been shown to act as protective ROS scavengers.30,31 PRDX2 is a typical Cys-2 peroxiredoxin localized in the cytosol and nucleus,30 whereas PRDX5 is a unique atypical Cys-2 peroxiredoxin found in the cytosol, the mitochondria, peroxisomes, and the nucleus.29 Because of wide distribution of PRDX5, it could be speculated that this antioxidant molecule is attacked and degraded first by ROS then PRDX2; however, the preservation of PRDX2 expression, could also indicate involvement of other, oxidative stress-unrelated signalling pathways that could directly influence its level.
HSP27, in its unmodified and phosphorylated forms (at serine 82), was lower in ISCM when compared with IDCM and control samples. Increased levels of phospho-HSP27 have been hypothesized to be protective against vascular disease possibly by stabilizing the actin cytoskeleton within endothelial and/or smooth muscle cells.32

4.2 Cytoskeletal reorganization

In ISCM, but not IDCM, our results showed an increased expression of the skeletal and α-cardiac actin forms. Previous reports suggested a connection between increased expression of α-skeletal actin in the diseased human heart and increased myocyte stretch, increased wall stress, and pressure overload; however, the authors did not observe skeletal actin overexpression in IDCM.33 An increased expression of α-skeletal actin was observed in chronically worsening heart failure in association with decreased PTEN and increased AKT activity,34 which was also the case in our ISCM samples. The higher expression of tubulin and desmin in IDCM and ISCM than in the controls was in agreement with the literature.35

The chloride channel CLIC1 was another protein that was found expressed at higher levels in ISCM than in IDCM and control samples. CLIC1 contributes to the formation and stabilization of collaterals during vascular development36 and it is involved in the regulation of cellular redox status.37 On the basis of our results, it can be speculated that the differential expression of CLIC1 is a possible link responsible for the differential regulation of stress-response mechanisms and changes in cytoskeletal reorganization observed between ISCM and IDCM samples.

4.3 The effects of signalling pathways in the pathogenesis of ISCM and IDCM

DJ-1 signalling has gained substantial interest due to its involvement in the development of cancer and neurological diseases, and more importantly for the present study, its role in cellular stress response.38,39 Increased DJ-1 has been shown to provide protection against oxidative stress in stroke38 and protects the heart from ischaemia reperfusion injury.40 The present study is the first to show that ISCM itself is associated with increased levels of protective DJ-1.

DJ-1 negatively regulates the activity of PTEN, a major cell-cycle/ proliferation and glycolysis regulator.41 In ISCM samples, increased DJ-1 expression associated with reduced PTEN levels, which was accompanied by increasing AKT activity.41 A less prominent increase of
AKT activity was observed in IDCM samples without decreased PTEN expression (Figure 4B).

Furthermore, it is known that mitogen-activated protein kinase (MAPK) signalling also plays an important role in the progression of heart failure, with the expression of phosphorylated p38 shown to be lower in IDCM than in the controls. Our results showed a significantly higher p38 activity in ISCM and a significantly lower p38 activity in the IDCM hearts when compared with controls. This suggests that different mechanisms are involved in the regulation of p38 kinase activity in IDCM and ISCM. In both, however, the activity of ERK1/2 (p42/44) MAPK proteins remained unchanged. This observation supports previously published data, which showed that in PTEN knock-out mice, the loss of PTEN signalling does not have an influence on the phosphorylation of the extracellular signal-regulated kinase ERK1 and ERK2 proteins, but that it does induce the phosphorylation of p38 MAPK.

Regulation of p38 activation partially relies on the activity of the unique MAPK kinase kinase ASK1. ASK1 is unique among the MAPKKK as it appears to directly regulate cell death through p38, JNK, and other effectors and at the same time it has been shown to function as a regulator of cardiac remodelling and hypertrophy. It is regulated by phosphorylation and dephosphorylation at four different amino acids; activation occurs through phosphorylation on threonine 845, or dephosphorylation of serine 83, serine 967, and serine 1034. In ISCM samples, a non-significant dephosphorylation of S967 of ASK1 were found (Figure 4G and E). At the same time,
there was a significant phosphorylation of S967 in IDCM samples (Figure 4G). It is described that calcinurin dephosphorylates S967 and activates ASK1 allowing the phosphorylation of T845 which is a negative feedback mechanism for the calcinurin/NFAT pathway.45 We did not investigate the level and activity of calcineurin in this study but we can speculate that normal or elevated activity of calcineurin in ISCM decreased the phosphorylation of S967 contributing to ASK1 and p38 activation. In IDCM, the level of calcineurin might be low allowing the phosphorylation of S967 of ASK1 above the level of the control samples, which inactivates ASK1 and might keep apoptosis at lower levels. In normal condition, ASK1, AKT, and HSP90 form a complex in which AKT phosphorylates S83 of ASK1. In cell stress, the complex has conformational changes, which decreases the phosphorylation of S83 and allows autophosphorylation of T845 of ASK1 inducing apoptosis.47 Cell stress can be expected in both DCM, which might manifest in significantly less phosphorylation of S83 of ASK1 compared with control (Figure 4F). Decreased phosphorylation of S83 and S967 was accompanied by significant phosphorylation of T845 of ASK1 in ISCM (Figure 4E). Decreased activity of protein phosphatase 5 (PP5), which dephosphorylates T845, cannot be excluded as a contributing factor in ISCM samples.48 There was no significant phosphorylation of T845 of ASK1 in IDCM samples (Figure 4E). This can be explained by the presence of phosphorylated S967 and normal activity of protein phosphatase 5 (PP5). The role of PP5 in different DCM samples needs to be further investigated. PIN1 and NEMO are both essential regulators of NF-κB,19,49 a major player in various pathophysiological processes in the human heart. Interestingly, they are differently regulated in ISCM vs. IDCM, with ISCM showing a downregulation of both PIN1 and NEMO. The knockdown of NEMO leading to a blockade of NF-κB activation has recently been shown to be partly responsible for the development of dilated cardiomyopathy and heart failure.50 The same publication also discussed the role of oxidative stress in NEMO-deficient cardiomyocytes as a critical pathological component that could be attenuated with antioxidants in vivo.50 Our results suggest that ISCM injury could highly benefit from an antioxidant treatment, since in ISCM several signalling pathways involved in the oxidative stress response are affected, some being protective and others attenuating the negative effects at the same time.

In summary, we performed an in-depth analysis of well-matched small cohorts of cardiac tissue samples from patients with normal heart, ISCM, or IDCM. Although the number of qualified samples was relatively small, based on the homogeneous and very consistent effects and differences observed in both types of cardiomyopathy when compared with the controls, it seems reasonable to conclude that the number of samples was sufficient to draw valid conclusions in this mechanistic study. In addition to future studies with larger number of samples, studies of gender and ethnicity differences are warranted.

We were able to show an involvement and differential regulation of Dj-1/PTEN/AKT-, ASK1/p38-, PIN-1, and NEMO-mediated pathways in ISCM and IDCM for the first time (Figure 6). An in-depth understanding of the molecular mechanisms including signal transduction pathways in ISCM and IDCM may provide novel targets for diagnostic strategies and treatment interventions at cellular levels.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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