Alterations in oxidative phosphorylation complex proteins in the hearts of transgenic mice that overexpress the p38 MAP kinase activator, MAP kinase kinase 6

Jason A. Wall, Jing Wei, Mimi Ly, Peter Belmont, Joshua J. Martindale, Diem Tran, Jun Sun, Wenzhong J. Chen, Wen Yu, Paul Oeller, Steve Briggs, Asa B. Gustafsson, Roberta A. Gottlieb, and Christopher C. Glembotski

1San Diego State University Heart Institute and The Department of Biology, San Diego State University, San Diego; 2Diversa Corporation, San Diego; 3Division of Biological Sciences, University of California-San Diego, La Jolla; and 4Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California

Submitted 13 December 2005; accepted in final form 10 May 2006

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: C. C. Glembotski, The SDSU Heart Institute and the Dept. of Biology, San Diego State Univ., San Diego, CA 92182 (cglembotski@sciencs.sdsu.edu).


Ischemia-reperfusion; mitochondrial complex proteins; mitogen-activated protein kinase (MAPK), showed that I/R injury is reduced in the hearts of transgenic mice that overexpress the p38 MAPK activator MKK6. This protection may be fostered by changes in the levels of many proteins not currently known to be regulated by p38. To examine this possibility, the authors employed the multidimensional protein identification technology MudPTP to characterize changes in levels of proteins in MKK6 transgenic mouse hearts, focusing on proteins in mitochondria, which play key roles in mediating I/R injury in the heart. Of the 386 mitochondrial proteins identified, the levels of 58 were decreased, while only 2 were increased in the MKK6 transgenic mouse hearts. Among those that were decreased were 21 mitochondrial oxidative phosphorylation complex proteins, which was unexpected because p38 is not known to mediate such decreases. Immunoblotting verified that proteins in each of the five oxidative phosphorylation complexes were reduced in MKK6 mouse hearts. On assessing functional consequences of these reductions, the authors found that MKK6 mouse heart mitochondria exhibited 50% lower oxidative respiration and I/R-mediated reactive oxygen species (ROS) generation, both of which are predicted consequences of decreased oxidative phosphorylation complex proteins. Thus the cardioprotection observed in MKK6 transgenic mouse hearts may be partly due to decreased electron transport, which is potentially beneficial, because damaging ROS are known to be generated by mitochondrial complexes I and III during reoxygenation.

The myocardium can be stressed by chronic increases in blood pressure, changes in neurohumoral substances, and ischemia followed by reperfusion (I/R). Numerous signaling pathways, including the mitogen-activated protein kinase (MAPK), are activated in stressed cardiac myocytes; in some cases, these signals foster protection, while in others they mediate damage (5, 22, 24, 42, 46). All three members of the MAPK family are activated during most myocardial stresses; however, the roles played by each in contributing to protection or damage are not entirely clear. For example, p38 MAPK has been reported to have both protective and damaging effects in the myocardium; this conundrum is addressed in recent reviews (1, 24, 31–33) and will, therefore, not be discussed in detail here. However, such apparently conflicting findings raise the possibility that p38 might serve either protective or damaging roles, depending on conditions, such as the cellular and temporal context and differential activation of p38 isoforms.

To begin to examine potential roles for p38 in the myocardium, the authors determined the effects of overexpressing an upstream activator of p38, MAPK kinase 6 (MKK6). They found that overexpression of MKK6 protected cultured cardiac myocytes against various types of stresses in a p38-dependent manner (54, 55). This protective effect has since been demonstrated by other laboratories, as well (28, 47). In genetically modified mice harboring an α-myosin heavy chain-driven MKK6 transgene, the authors found that p38 was activated, while neither JNK nor ERK was activated; moreover, ventricular morphology and function were similar to nontransgenic mouse hearts, and the transgenic mice exhibited no overt signs of heart dysfunction or early mortality. However, when exposed to I/R, either ex vivo or in vivo, the MKK6 transgenic mouse hearts exhibited significantly reduced tissue damage and better retention of contractile function (25).

We hypothesized that overexpression of MKK6 might lead to changes in the levels and/or phosphorylation states of p38 and p38-regulated proteins in ways that might contribute to the observed protection. Consistent with this hypothesis were several studies demonstrating that MKK6 transgenic mouse hearts showed increased expression of protective, known p38-regulated proteins (10, 25). However, it is also possible that many other proteins that are not currently known to be p38 regulated may be altered in the transgenic mouse hearts and that these alterations may contribute to the observed protection. In the present study, we addressed this possibility by using a proteomics approach that employed three-dimensional liquid chromatography coupled to tandem mass spectrometry (3D-LC-MS/MS) (49) to assess the relative expression levels of...
proteins in extracts prepared from MKK6 transgenic and nontransgenic mouse heart. This report focuses on proteins in mitochondria because they play a critical role in mediating the damaging effects of I/R. We observed the downregulation of numerous mitochondrial oxidative phosphorylation complex proteins, which are major sites of the generation of the reactive oxygen species (ROS) that mediate I/R injury in the heart. We also found that the MKK6 transgenic mouse hearts exhibited reduced I/R-mediated ROS generation, which may contribute to the protective phenotype in this line. These findings underscore the dynamic nature of the cardiac proteome and demonstrate how functional proteomics studies can provide important insight into the molecular mechanisms underlying the effects of I/R in the heart.

MATERIALS AND METHODS

Animals. Approximately 100 adult mice (Mus musculus, 6–8 mo of age, and 25 neonatal rats (Rattus norvegicus), were used in this study. All procedures involving animals were in accordance with institutional guidelines. The animal protocol used in this study was reviewed and approved by the San Diego State University Institutional Animal Care and Use Committee.

MKK6 transgenic mice. The transgenic mice used in this study have been described previously (25). All experimental animals were F3 generation or later and were 5–6 mo of age.

Myofibril-depleted cardiac extracts. Myofibril-depleted extracts containing mostly subsarcolemmal mitochondria and cytosolic proteins were prepared as previously described (25).

Reduction, alkylation, and digestion. To control for animal-to-animal variability, myofibril-depleted cardiac extracts were prepared from 10 pooled transgenic and 10 pooled nontransgenic mouse hearts. In preparation for reduction, alkylation, and digestion, proteins in myofibril-depleted cardiac extracts were quantitatively precipitated with ProteoExtract Protein Precipitation Kit (cat. no. 539180, Calbiochem, San Diego, CA) and then resuspended in TNE [50 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA]. RapiGest SF Reagent (cat. no. 186001860; Waters) was added to 2 mg of protein in TNE for a final concentration of 1% RapiGest. Samples were heated and then deglycosylated (cat. no. 362280; Calbiochem) per the manufacturer’s protocol. Proteins were then reduced and carboxymethylated with 1 mM TCEP (cat. no. 20490; Pierce Chemical) and 0.5 mg/ml iodoacetamide (cat. no. 11149–5g; Sigma Chemical), respectively. Proteins were then digested once with Lys-C (cat. no. 11047825001; Roche) at weight ratio 1:200 (Lys-C:proteins) and twice with trypsin (cat. no. 11047841001; Roche) at weight ratio 1:100 (trypsin:protein). Samples were examined by gel electrophoresis and silver staining to ensure complete digestion. Samples were then treated with 50 mM HCl to degrade the RapiGest and then brought to a pH of 3.0 with NH4OH. The samples were centrifuged, and the supernatants were removed; the pellets were then resuspended in 70% 2-propanol.

LC-MS/MS. Aliquots of the soluble and insoluble fractions from the nontransgenic and transgenic mouse heart extracts, amounting to 650 μg of protein per aliquot, were fractionated by three-dimensional HPLC (reversed phase; size exclusion; reversed phase) followed by online MS/MS analysis, as previously described (49), with the exception of the following changes in the reversed-phase HPLC elution gradients. For the soluble fractions, the five reverse-phase (RP) gradients used were 0–8% B, 8–20% B, 20–35% B, 35–80% B, and 80–100% B (B = 80% acetonitrile, 19.8% H2O, 0.2% formic acid), each of which was followed by salt steps of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, and 1,470 mM ammonium acetate. For the insoluble fractions, the 5 RP gradients used were 0–25% B, 25–40% B, 40–80% B, and 80–100% B, each of which was followed by salt steps of 0, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, and 2,000 mM ammonium acetate. Eluted peptides were analyzed directly on an LTQ mass spectrometer equipped with a nanospray source (Thermo Finnigan, San Jose, CA). The LTQ mass spectrometer was set to divide the full MS scan into four smaller sections covering a total range of 400–1,800 mass-to-charge ratio. Each of the smaller MS scans was followed by 5 MS/MS scans of the most intense ions from the preceding MS scan. The raw data were then extracted and searched using the SEQUEST program, as previously described (49). By using previously described criteria (48, 51), the results were filtered to obtain the peptide matches and protein identifications. To estimate the frequency of incorrectly matched peptides, the database contained both the forward and reversed mouse sequences. By using this approach, the false hit rate is ~1.3%.

Statistical assessment of MS data. The goal of the proteomics portion of this study was to assess differential expression of proteins in the hearts of transgenic vs. nontransgenic mice but not to assess biological variability between individual animals within a line. Accordingly, we prepared two extracts, one consisting of 10 pooled transgenic mouse hearts and the other consisting of 10 pooled nontransgenic mouse hearts, and two complete proteomics analyses were performed on each extract. This approach has been validated as a method for assessing differential expression when evaluating biological variation is not the goal (23). Proteins that were not identified by at least one spectrum in each of the four runs were removed from further consideration. To account for slight run-to-run differences in MS detection sensitivity, the numbers of spectra observed for a given protein in runs 1 and 2 were normalized to the total spectra observed in each run. The remaining normalized data were log2-transformed and assessed for significant differences between transgenic and nontransgenic by using the local-pooled-error (LPE) test (21). This approach provides an estimate of the statistical confidence of the technical replication, not of the biological variation within each extract.

Bioinformatics. The accession ID for each protein, as provided from the SEQUEST search, was used in the web-based Clone/Gene ID Converter, http://idconverter.bioinf.cio.es/IDconverter.php to determine gene name, locuslink, and GenBank ID. GOminer was then used (56) to generate a Gene Ontology (GO) association for each gene by using the May 2005 GO database build, and all searches were limited to Mus musculus.

Immunoblots. Mitochondrial samples (0.5–1 μg protein per lane) were loaded onto a Bio-Rad 4–12% gel (cat. no. 345–0135; Bio-Rad, Hercules, CA), submitted to SDS-PAGE, and then transferred to polyvinylidene difluoride membranes. Blots were probed for components of the mitochondrial respiratory chain complexes by using monoclonal antibodies specific for each oxidative phosphorylation complex (cat. nos. MS105, MS203, MS304, MS407, and MS507 for complexes I-V, respectively, and as described in more detail in the legend to Fig. 1), or with OXPHOS monoclonal antibody cocktail (cat. no. MS601, Mitosciences, Eugene OR), which cross-reacts with the 20-kDa subunit of complex I, the 30-kDa subunit of complex II, the 50-kDa core2 protein of complex III, cytochrome-c oxidase II of complex IV, and ATP synthase F1α of complex V. For normalization purposes, blots were probed with an antibody to α-actinin (cat. no. A7811 Sigma, St. Louis, MO). Blots were then developed using Amershams ECLplus and a Typhoon. Gels were quantified with ImageQuant.

Measurements of respiration of isolated mouse heart mitochondria. Isolation of mouse heart mitochondria and measurement of respiration were performed as described previously (36), with the following modifications. Briefly, mitochondria enriched in subsarcolemmal mitochondria were isolated from mouse hearts by brief Polytron homogenization in ice-cold MDE buffer (200 mM mannitol, 70 mM sucrose, 2 mM EGTA, 5 mM MOPS, pH 7.4) followed by two quick strokes of a loose fit Potter-Elvehjem tissue grinder. Mitochondria were collected by centrifugation at 3,000 g following two centrifugations of the homogenate at 600 g. All work was performed on wet ice at 0°C. Oxygen consumption was measured at 30°C with a Clark-type oxygen...
**METHODS.**

Described in **MATERIALS AND METHODS.** A human mitochondrial extract was (18-kDa subunit IV), and complex V (55-kDa subunit ATP synthase), as described in **MATERIALS AND METHODS.** A human mitochondrial extract was used as a control (H) to identify subunits in each complex. M = nontransgenic mouse heart mitochondrial extract. The migration positions of molecular mass markers ranging from 20 to 75 kDa are shown on each blot. B: Decreased mitochondrial complex proteins: cytochrome complex immunoblots. Mitochondria were isolated from NTG and TG mouse hearts \((n = 4)\), separated by SDS-PAGE, and blots were probed with a mixture of the antibodies described in A. Actinin was used as a loading control. C: Immunoblot quantification. The immunoblots shown in B were quantified as described in **MATERIALS AND METHODS.**

**Superoxide production in mouse hearts.** Superoxide production was assessed in hearts following ex vivo I/R via the conversion of dihydroethidium (DHE) to ethidium, as previously described (36). Briefly, frozen hearts were slightly thawed, sliced into 1-mm sections, and incubated with 2 µM DHE for 15 min in the dark. Images of sections were acquired using an ultraviolet transilluminator and captured by using a Kodak DC120 digital camera with Kodak Digital Science 1D software. Images were analyzed with Adobe Photoshop 7.0, and the percentage of cells exhibiting conversion of DHE (superoxide production) was quantified as the ratio of fluorescent pixels to the total heart area.

**Mitochondrial swelling assay.** Mitochondria were isolated as previously described (25) and resuspended in swelling buffer \((10 \text{ mM Tris pH 7.4, 120 mM KCl, 20 mM MOPS, 5 mM KH}_2\text{PO}_4)\) to a protein concentration of 0.25 µg/µL. Aliquots of 50 µg were used per well in a 96-well plate to which 250 µM Ca\(^{2+}\) was added to induce mitochondrial swelling. In some samples, 15 µM cyclosporine A was added to verify that swelling was due to mitochondrial permeability transition pore opening. The absorbance was assessed on a Molecular Devices Versamax plate reader at 520 nm for 30 min after Ca\(^{2+}\) addition. Samples were assessed for statistical significance by using a one-way ANOVA with a Student-Newman-Keuls post hoc analysis.

**Superoxide production in cultured cardiac myocytes.** Neonatal rat ventricular cardiac myocytes (NRVCs) were isolated and cultured as previously described (27). Cells were then infected by using adenovirus (AdV)-mediated gene transfer of either control, wild-type MKK6 (MKK6wt), or constitutively active MKK6 (MKK6E) constructs, as previously described (18); all strains of AdV used encode a cytomegalovirus-driven green fluorescent protein (GFP) gene, which allowed for the positive identification of infected cells. After 48 h in serum-free medium, cells were subjected to hypoxia \((-0.3\% \text{ O}_2\) for 10 h) in a glucose- and serum-free medium and then to re-oxygenation \((21\% \text{ O}_2\) for 21 h) in glucose-containing, serum-free medium, which are conditions that simulate ischemia and reperfusion (sl/IR), as it occurs in vivo (27). Cells were then stained with JC-1 (cat. no. T-3168, from Invitrogen-Molecular Probes, Eugene, OR) at a concentration of 1 µg/ml of media for 30 min, after which they were washed three times with serum-free medium and then resuspended in minimal media. After 22 h of reoxygenation, green and red images of the cells were captured and analyzed by using Adobe Photoshop. Cells were then scored for those that were infected (GFP positive) and those that retained JC-1 staining. Statistical analysis was performed by using a one-way ANOVA with a Student-Newman-Keuls post hoc analysis.

**Superoxide production in cultured cardiac myocytes.** Neonatal cardiac myocytes were subjected to simulated ischemia by incubating cells in ischemic buffer (in mM: 125 NaCl, 8 KCl, 2.5 NaHCO\(_3\), 2.5 MgSO\(_4\), 1.2 CaCl\(_2\), 6.25 NaHCO\(_3\), 100 2-deoxyglucose, 5 Na-lactate, 200 µM oligomycin, and the maximal respiration rate was measured after uncoupling the mitochondria with 2 µM FCCP. Data were analyzed by using Student’s t-test.
RESULTS

Myofibril-depleted mouse ventricular proteome. Because of their relatively high abundance in the heart, it was determined that myofibril-related proteins would reduce our ability to detect lower abundance proteins. Accordingly, because they were not the focus of this study, we used a previously described fractionation procedure (25) to prepare myofibril-depleted extracts. We then carried out LC-MS/MS analyses of extracts prepared from nontransgenic and MKK6 transgenic mouse hearts, as described in MATERIALS AND METHODS. The proteins identified using SEQUEST were sorted into GO categories on the basis of their functions and subcellular locations (56), as described in MATERIALS AND METHODS. In this study, we focused on proteins in the mitochondria because they are known to play a critical role in mediating the effects of I/R.

We observed spectra for 386 mitochondrial proteins. However, for 161 of the 386, fewer than one spectrum was observed in at least one of the four analyses; because of this low spectral count, these proteins were not retained for statistical analyses. Thus the remaining 225 mitochondrial proteins were assessed for statistical differences between the two mouse lines, by using a procedure similar to a previously published method (7), which validated the use of LC-MS/MS spectral data to compare the levels of a protein in control vs. treated samples. Using this method, we estimated that there was no statistical difference in the levels of 165 of the 225 mitochondrial proteins in nontransgenic and transgenic mouse hearts (see Table 1). However, 60 of the 225 mitochondrial proteins were estimated to be present at significantly different levels, with 58 proteins being decreased and 2 exhibiting increases in the transgenic mouse hearts (see Table 2). We were surprised to find 21 proteins involved in oxidative phosphorylation among the 58 proteins that were decreased (see Table 2, rows 1–21). Alterations in the levels of certain oxidative phosphorylation complex proteins have been shown to reduce the assembly of the complexes (45). Accordingly, immunoblots were carried out by using monoclonal antibodies directed against portions of each of the mitochondrial oxidative phosphorylation complexes. Control immunoblots demonstrated that subunits from each of the five mitochondrial complexes in nontransgenic mouse heart mitochondria cross-reacted with the appropriate antibodies and migrated to the same positions as the corresponding subunits in a commercially available control human cell mitochondrial preparation (Fig. 1A). When blots were probed with a mixture of all five antibodies, transgenic mouse heart mitochondria exhibited decreases of 20–40% in the levels of components in complexes I, II, III, IV, and V (Fig. 1, B and C). These findings support the proteomics results, providing additional data using different methods that mitochondrial oxidative phosphorylation complex levels are decreased in the transgenic mouse hearts.

Mitochondrial respiration, ROS, and mitochondrial membrane potential. To further validate the proteomics and immunoblot results, we examined potential physiological consequences of decrease in mitochondrial oxidative phosphorylation complex proteins. We hypothesized that transgenic mouse heart mitochondria would display decreased respiration rates. Accordingly, electron transfer capacities of isolated mitochondria were assessed, as previously described (36); representative tracings are shown in Fig. 2A. Compared with nontransgenic mice, respiration rates of mitochondria isolated from transgenic mice were impaired when substrates for complexes I (palmitoyl carnitine), II (succinate), or IV (TMPD/ascorbate) were used. We found that state 3, state 4 (not shown), and FCCP-uncoupled, maximal respiration were reduced by 30–50% in the MKK6 transgenic mitochondria (Fig. 2, B and C). These results provide a functional validation of the observed changes in the mitochondrial complex proteome in MKK6 transgenic mouse hearts.

On reperfusion, ROS are generated by complexes I and III (17, 41, 44). Because the MKK6 transgenic mice exhibited decreases in several complex I and III proteins, we hypothesized that, compared with nontransgenic, they would also generate less ROS. Accordingly, free radical generation was measured in isolated perfused mouse hearts from both mouse lines. After 2 min of reperfusion, we found that compared with transgenic, the nontransgenic mouse hearts exhibited a significant increase in free radical generation, as measured by dityrosine formation (Fig. 3A). The generation of superoxide in mouse hearts after 30 min of ischemia and 15 min of reperfusion was measured by conversion of dihydroethidium (DHE) to ethidium. We found that, compared with transgenic mice, the nontransgenic mouse hearts subjected to I/R exhibited a significant increase in DHE conversion (Fig. 3B). Taken together, these results suggest that during I/R, MKK6 transgenic mouse heart mitochondria generate less ROS than nontransgenic mouse hearts.

ROS are among several factors that increase during reperfusion that can stimulate the opening of the mitochondrial permeability transition pore (MPTP) (16). Because the transgenic mouse heart mitochondria exhibited lower rates of ROS generation, we hypothesized that they might also exhibit reduced MPTP activation. Accordingly, mitochondria were isolated from the hearts of both mouse lines, and Ca\(^{2+}\)-induced MPTP opening was assessed by measuring mitochondrial swelling (2). On treatment with Ca\(^{2+}\), we observed a more rapid decrease in absorbance of the nontransgenic mouse heart mitochondria than transgenic, suggesting that compared with nontransgenic, the MKK6 transgenic mice exhibit reduced Ca\(^{2+}\)-stimulated MPTP activation.

Effects of MKK6 overexpression in primary cardiac myocytes. We next evaluated whether MKK6 overexpression in isolated cardiac myocytes affected mitochondrial function. Accordingly, primary neonatal rat ventricular myocytes were infected with a recombinant adenoviral strains that encode either MKK6wt or MKK6E (18). The cells were then exposed to sI/R, as previously described (27). The status of the mitochondrial membrane potential, which is an estimate of the electrochemical gradient across the inner mitochondrial membrane, and thus MPTP opening, was assessed with the fluores-
cent dye JC-1, as previously described (8, 11). After sI/R, there was a reduction in the number of cells able to retain the mitochondrial membrane potential in cultures infected with the control AdV, while cells infected with AdV-MKK6wt or AdV-MKK6E exhibited very little change in mitochondrial membrane potential (Fig. 4A). The generation of superoxide was also examined in the cultured cardiac myocytes, where we found significantly lower rates of DHE conversion in AdV-MKK6wt- and AdV-MKK6E-infected cultures compared with those infected with the control AdV (Fig. 4B). Although the neonatal cardiac myocytes do not necessarily represent all aspects of the adult myocyte phenotype, these results indicate that in cultured cardiac myocytes, overexpression of either MKK6wt or MKK6E confers some of the same characteristics observed in MKK6 transgenic mouse hearts, including reduced ROS generation and retention of the mitochondrial membrane potential.

**DISCUSSION**

In the present study we found that 21 mitochondrial oxidative phosphorylation complex proteins, 24 proteins involved in fatty acid oxidation (FAO), and 2 proteins of the pyruvate dehydrogenase complex were reduced in MKK6 transgenic mouse hearts (Fig. 5). We also found that several proteins involved in apoptosis, mitochondrial biogenesis, and ROS metabolism were decreased (Table 2). Because of the I/R-protective phenotype exhibited by MKK6 transgenic mouse hearts and the central role played by certain oxidative phosphorylation proteins in generating the ROS that can mediate I/R damage, we focused our analyses in this study on the oxidative phosphorylation proteins.

We observed decreased levels of each of the five mitochondrial complexes in the MKK6 transgenic mouse heart mitochondria when examined by immunoblotting (Fig. 1) and decreases in complexes I, III, and V in the proteomics analysis (Table 2 and Fig. 5). Complex I, which is also known as NADH ubiquinone oxidoreductase, or NADH dehydrogenase, is comprised of 46 subunits, and at 950 kDa, it is the largest of the respiratory chain components (6). Complex I catalyzes electron entry into electron transport from NADH that is derived from the TCA cycle and fatty acid oxidation (Fig. 5). In the MKK6 mouse hearts, we observed 11 proteins that were decreased, two of which were Ndufs1 and 4. Decreases in the levels of Ndufs4 and Ndufs1 have been found to reduce the levels of the holocomplex I and to result in reduced electron transport and ROS generation on hypoxia or reoxygenation (20). This is consistent with our findings that complex I-mediated oxygen utilization was reduced (Fig. 2) and that the generation of ROS upon reperfusion of hearts or reoxygenation of cultured cardiac myocytes was reduced (Figs. 3 and 4). Moreover, Ndufs1 and Ndufs2 are the core subunits that are critical for the function of complex I.

---

Fig. 2. Respiration studies of mitochondria isolated from MAPK kinase 6 (MKK6) TG and NTG mouse hearts. A: representative O2 electrode tracings obtained with NTG and TG mouse heart mitochondria are shown. Scale indicates nA O2 consumed per unit time. Each tracing represents the data obtained from mitochondria pooled from 2 mouse hearts. Inflections represent changes in respiration rates observed on addition of the compounds shown. B: state 3 respiration rates. The respiration rates were determined from tracings similar to those shown in A after the addition of the substrates shown, which allows the estimation of electron transport in complexes I, II, and IV. State 3 respiration is the rate obtained after the addition of 2 μM ADP. Data are shown as the means of n = 5 independent analyses of mitochondria from 10 TG and 10 NTG mouse hearts SD. *NTG different from TG, P ≤ 0.05, as determined by using Student’s t-test. C: maximal respiration rates. The respiration rates were determined as described in B. Maximal respiration is the rate obtained after the addition of 2 μM FCCP. Data are shown as means (n = 5 independent analyses of mitochondria from 10 TG and 10 NTG mouse hearts) ± SD. *NTG different from TG, P ≤ 0.05, as determined by using Student’s t-test.
essential for electron transfer from NADH to ubiquinone and for the generation of the protonmotive force (6). Because we observed reductions in both Nudufs1 and 2, we would expect that proton pumping would also be impaired in MKK6 mouse heart mitochondria. Although we did not examine pyridine nucleotide levels, given the central roles metabolic roles of NADH in carrying electrons from the TCA cycle and FAO to complex I (Fig. 5), reduction of complex I in the transgenic mouse hearts would be expected to decrease the rates of NADH oxidation in the MKK6 mouse mitochondria.

Complex II, also known as succinate-ubiquinone oxidoreductase, or succinate dehydrogenase, is composed of four subunits and participates in both electron transport and the TCA cycle. Although we did not observe reductions in any complex II proteins that reached statistical significance in the proteomics analysis, we detected reduced levels of subunit b of succinate dehydrogenase (Sdhb) by immunoblotting (Fig. 1). Although few studies have examined the effects of decreased complex II, as expected, this complex has been shown to be rate limiting for succinate oxidation (4), consistent with our observations that the MKK6 transgenic mouse heart mitochondria exhibit reduced respiration when succinate is provided as a carbon source (Fig. 2).

Fig. 3. Analyses of free radical generation and mitochondrial swelling. A: dityrosine formation. Hearts from NTG or TG mice were perfused ex vivo for either 27 min (control, n = 5 from each line) or for 25 min of global ischemia followed by 2 min of reperfusion (I/R; n = 5 from each line). Free radical release was estimated by measuring dityrosine generation, as described in MATERIALS AND METHODS. Results are means ± SE. *Different from value shown, P ≤ 0.05, as determined using Student's t-test. B: superoxide production. Hearts from NTG or TG mice (n = 3 from each line) were perfused ex vivo for either 45 min (control) or for 30 min of global ischemia, followed by 15 min of reperfusion (I/R). Hearts were then analyzed for superoxide production by dihydroethidium (DHE) conversion, as described in MATERIALS AND METHODS. Results are means ± SE. *Different from value shown, P ≤ 0.05, as determined using Student's t-test. C: mitochondrial swelling. Mitochondria were isolated from NTG and TG mouse hearts, and the rate of swelling after addition of 250 μM Ca was determined as described in MATERIALS AND METHODS. Ca-stimulated mitochondrial swelling was inhibited by cyclosporine A (not shown), confirming that this measurement represented an estimation of mitochondrial permeability transition pore opening. Results are means (n = 5 hearts from each line) ± SE. *Different from values shown, P ≤ 0.05, as determined by using Student's t-test.

Fig. 4. Effect of wild-type MKK6 (MKK6wt) or constitutively active MKK6 (MKK6E) overexpression in cultured cardiac myocytes. Primary neonatal rat ventricular myocytes were infected with adenovirus (AdV)-control (Con), AdV-MKK6wt, or AdV-MKK6E and then treated with simulated ischemia/reperfusion (sI/R) and analyzed for mitochondrial membrane potential (A) or for superoxide formation (B) as described in MATERIALS AND METHODS. Results are means ± SE. *P < 0.05, **P < 0.01, different from control as determined by using Student's t-test.
Complex III, also known as ubiquinol-cytochrome c oxidoreductase, is composed of 11 subunits and catalyzes electron transfer from reduced ubiquinone to cytochrome c. The proteomics analysis indicated that six complex III subunits were reduced in the MKK6 mouse hearts. Two of the complex III subunits that were reduced were Uqcrcl and Uqcrcl2, which are also known as ubiquinol cytochrome c oxidoreductase core proteins 1 and 2. Uqcrcl and Uqcrcl2 mediate the formation of the complex between cytochromes c and c1, and they are believed to be the primary site of ROS generation by reverse electron transport during hypoxia as well as reoxygenation (15). This is consistent with our findings of increased ROS generation in the MKK6 mouse hearts and cultured cells on reoxygenation. The cytochrome c1 (cyc1) subunit of complex III accepts electrons from Uqcrfs1, which is also known as Rieske iron-sulfur polypeptide I, and transfers them to soluble cytochrome c (37, 38); cyc1 and Uqcrfs1 were both reduced in the MKK6 mouse hearts (Table 2), which is likely to cause reduced electron flow-coupled proton translocation and thus decrease the overall activity of complex III (50).

Complex IV, also known as cytochrome c oxidase, is composed of 13 different subunits; cytochrome c shuttles electrons from complex III to complex IV. Although we did not observe any reduction in cytochrome c or any complex IV proteins in the proteomics analysis, the immunoblot showed a reduction of subunit IV of complex IV (Fig. 1), suggesting that electron flow from complex III to IV would be impaired in the transgenic mouse hearts examined in this study.

We also observed reduced levels of Atp5l and Atp5h of complex V, both of which are subunits of the transmembrane F0, proton transport mediating portion of F1F0 ATP synthase (Fig. 5) (13). Moreover, there was a reduction in the levels of the Atp5d and Atp5o of complex V, which are subunits of the F1 complex of ATP synthase that constitutes the catalytic site of the enzyme. Given the reductions in the levels of these four important ATP synthase subunits, we would expect decreased proton transport, as well as ATP generation in the MKK6 transgenic mouse heart mitochondria. We have yet to directly measure ATP generation, and while we have not observed any overt reduction of contractile performance in the MKK6 transgenic mouse hearts, they do exhibit a slightly lower resting heart rate, as well as marginally reduced fractional shortening (25), which could be clues that contractility is compromised. Nonetheless, the MKK6 transgenic mice, which do not display altered life spans, do not exhibit any overt effects of reduced oxidative respiration, nor are the hearts abnormal in terms of gross or microscopic histology or nonexercised function. Future studies examining ATP generation in MKK6 transgenic mouse hearts, as well as the effects of exercise on cardiac function and performance, will be required to address possible detrimental effects of the reduced oxidative respiration.

It is of interest to consider how MKK6-mediated p38 activation might cause a reduction in oxidative phosphorylation complex protein expression. The expression of most oxidative phosphorylation complex proteins is regulated by nuclear factors (NRF) 1 and 2, as well as PGC-1α, all of which coordinate with peroxisome proliferator-activated receptor (PPAR)-α and PPAR-β to induce the transcription of many nuclear genes that encode mitochondrial proteins (14, 19). However, to the best of our knowledge, p38-mediated decreases in the levels of oxidative phosphorylation complex proteins have not been observed. In fact, there is evidence to the contrary, suggesting that p38 actually increases the expression of oxidative phosphorylation protein levels we observed are not actually mediated through the PPARs and PGC-1α but instead involve other transcription factors. For example, p38 can phosphorylate PGC-1α, as well as p160 myb binding protein, a PGC-1α suppressor; these phosphorylation events increase PGC-1α-mediated coactivation of PPAR-α-dependent gene induction, which leads to increased levels of oxidative phosphorylation proteins (3, 12). Perhaps the decreases in oxidative phosphorylation protein levels we observed are not actually mediated through the PPARs and PGC-1α but instead involve other transcription factors. For example, Sp1 represses expression of certain oxidative phosphorylation protein genes (34). p38 has been shown to activate Sp1 in macrophages (52), vascular smooth muscle cells (26), and fibroblasts (9), suggesting a possible mechanism by which MKK6-activated p38 might depress oxidative phosphorylation protein levels in the transgenic mouse hearts examined in the present study. Addition-
ally, it may be possible that MKK6-mediated effects on oxidative phosphorylation protein levels could also be indirect and/or posttranscriptional.

The reduction of oxidative phosphorylation proteins in the transgenic mouse hearts is qualitatively similar to that observed in failing hearts, where there is a shift in mitochondrial substrate utilization from primarily fatty acid oxidation (FAO) in the healthy heart, to glucose oxidation in the diseased heart (35, 39, 40, 43). This metabolic shift has been considered by some to contribute to the failing heart phenotype; however, others believe that it may be an adaptive response that allows for preserved ATP generation during times when mitochondrial oxidative phosphorylation is reduced. Because this shift is commonly associated with a reduction in the levels of the proteins responsible for FAO, it is of interest that 26 of the 60 changed mitochondrial proteins in MKK6 transgenic mouse hearts are involved in fatty acid transport into the mitochondrion and FAO (see Table 2 and Fig. 5); 24 of those 26 FAO proteins.

The 165 mitochondrial proteins that did not exhibit significant differences between transgenic (TG) and nontransgenic (NTG) mice [i.e., local pooled error (LPE) < 0.05] are shown. Nos. of spectra observed for each protein, normalized as described in MATERIALS AND METHODS, are shown for the two NTG analyses (columns 1 and 2) and the two TG analyses (columns 4 and 5). The proteins in this table were sorted by numbers of spectra observed in NTG run 1 (column 1). The only proteins shown are those for which at least 1 spectrum was observed in each of the 4 analyses. Column 7 shows the gene symbol, as found by Mouse Genome Informatics search at http://www.informatics.jax.org/.
Table 2. Significantly changed mitochondrial proteins

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>5</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NTG</td>
<td>NTG</td>
<td>TG</td>
<td>TG</td>
<td>p-value</td>
</tr>
<tr>
<td>1</td>
<td>57</td>
<td>62</td>
<td>26</td>
<td>28</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>2</td>
<td>138</td>
<td>139</td>
<td>252</td>
<td>261</td>
<td>0.0002</td>
</tr>
<tr>
<td>3</td>
<td>388</td>
<td>394</td>
<td>674</td>
<td>673</td>
<td>0.0001</td>
</tr>
<tr>
<td>4</td>
<td>421</td>
<td>432</td>
<td>825</td>
<td>820</td>
<td>0.0001</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>67</td>
<td>39</td>
<td>42</td>
<td>0.0001</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>7</td>
<td>2</td>
<td>2</td>
<td>0.0001</td>
</tr>
<tr>
<td>7</td>
<td>41</td>
<td>25</td>
<td>8</td>
<td>8</td>
<td>0.0001</td>
</tr>
<tr>
<td>8</td>
<td>96</td>
<td>67</td>
<td>55</td>
<td>55</td>
<td>0.0001</td>
</tr>
<tr>
<td>9</td>
<td>149</td>
<td>111</td>
<td>115</td>
<td>84</td>
<td>0.0001</td>
</tr>
<tr>
<td>10</td>
<td>133</td>
<td>127</td>
<td>175</td>
<td>111</td>
<td>0.0001</td>
</tr>
<tr>
<td>11</td>
<td>16</td>
<td>31</td>
<td>23</td>
<td>17</td>
<td>0.0001</td>
</tr>
<tr>
<td>12</td>
<td>511</td>
<td>514</td>
<td>414</td>
<td>392</td>
<td>0.0001</td>
</tr>
<tr>
<td>13</td>
<td>830</td>
<td>858</td>
<td>754</td>
<td>681</td>
<td>0.0001</td>
</tr>
<tr>
<td>14</td>
<td>41</td>
<td>38</td>
<td>34</td>
<td>18</td>
<td>0.0001</td>
</tr>
<tr>
<td>15</td>
<td>145</td>
<td>150</td>
<td>137</td>
<td>86</td>
<td>0.0001</td>
</tr>
<tr>
<td>16</td>
<td>32</td>
<td>34</td>
<td>30</td>
<td>12</td>
<td>0.0001</td>
</tr>
<tr>
<td>17</td>
<td>130</td>
<td>127</td>
<td>116</td>
<td>90</td>
<td>0.0001</td>
</tr>
<tr>
<td>18</td>
<td>23</td>
<td>25</td>
<td>22</td>
<td>12</td>
<td>0.0001</td>
</tr>
<tr>
<td>19</td>
<td>436</td>
<td>480</td>
<td>416</td>
<td>196</td>
<td>0.0001</td>
</tr>
<tr>
<td>20</td>
<td>907</td>
<td>870</td>
<td>786</td>
<td>429</td>
<td>0.0001</td>
</tr>
<tr>
<td>21</td>
<td>44</td>
<td>40</td>
<td>37</td>
<td>12</td>
<td>0.0001</td>
</tr>
<tr>
<td>22</td>
<td>30</td>
<td>32</td>
<td>30</td>
<td>16</td>
<td>0.0001</td>
</tr>
<tr>
<td>23</td>
<td>48</td>
<td>55</td>
<td>47</td>
<td>22</td>
<td>0.0001</td>
</tr>
<tr>
<td>24</td>
<td>73</td>
<td>81</td>
<td>69</td>
<td>43</td>
<td>0.0001</td>
</tr>
<tr>
<td>25</td>
<td>398</td>
<td>371</td>
<td>339</td>
<td>202</td>
<td>0.0001</td>
</tr>
<tr>
<td>26</td>
<td>215</td>
<td>218</td>
<td>193</td>
<td>178</td>
<td>0.0001</td>
</tr>
<tr>
<td>27</td>
<td>4</td>
<td>10</td>
<td>4</td>
<td>2</td>
<td>0.0001</td>
</tr>
<tr>
<td>28</td>
<td>153</td>
<td>158</td>
<td>139</td>
<td>103</td>
<td>0.0001</td>
</tr>
<tr>
<td>29</td>
<td>138</td>
<td>87</td>
<td>94</td>
<td>69</td>
<td>0.0001</td>
</tr>
<tr>
<td>30</td>
<td>83</td>
<td>76</td>
<td>54</td>
<td>42</td>
<td>0.0001</td>
</tr>
<tr>
<td>31</td>
<td>106</td>
<td>92</td>
<td>116</td>
<td>40</td>
<td>0.0001</td>
</tr>
<tr>
<td>32</td>
<td>48</td>
<td>37</td>
<td>19</td>
<td>26</td>
<td>0.0001</td>
</tr>
<tr>
<td>33</td>
<td>21</td>
<td>24</td>
<td>20</td>
<td>12</td>
<td>0.0001</td>
</tr>
<tr>
<td>34</td>
<td>174</td>
<td>142</td>
<td>137</td>
<td>102</td>
<td>0.0001</td>
</tr>
<tr>
<td>35</td>
<td>55</td>
<td>37</td>
<td>39</td>
<td>19</td>
<td>0.0001</td>
</tr>
<tr>
<td>36</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>5</td>
<td>0.0001</td>
</tr>
<tr>
<td>37</td>
<td>342</td>
<td>323</td>
<td>295</td>
<td>227</td>
<td>0.0001</td>
</tr>
<tr>
<td>38</td>
<td>44</td>
<td>40</td>
<td>39</td>
<td>18</td>
<td>0.0001</td>
</tr>
<tr>
<td>39</td>
<td>23</td>
<td>18</td>
<td>8</td>
<td>12</td>
<td>0.0001</td>
</tr>
<tr>
<td>40</td>
<td>105</td>
<td>88</td>
<td>59</td>
<td>42</td>
<td>0.0001</td>
</tr>
<tr>
<td>41</td>
<td>42</td>
<td>51</td>
<td>42</td>
<td>21</td>
<td>0.0001</td>
</tr>
<tr>
<td>42</td>
<td>121</td>
<td>92</td>
<td>85</td>
<td>70</td>
<td>0.0001</td>
</tr>
<tr>
<td>43</td>
<td>18</td>
<td>18</td>
<td>11</td>
<td>9</td>
<td>0.0001</td>
</tr>
<tr>
<td>44</td>
<td>37</td>
<td>37</td>
<td>33</td>
<td>16</td>
<td>0.0001</td>
</tr>
<tr>
<td>45</td>
<td>101</td>
<td>75</td>
<td>75</td>
<td>46</td>
<td>0.0001</td>
</tr>
<tr>
<td>46</td>
<td>419</td>
<td>468</td>
<td>400</td>
<td>360</td>
<td>0.0001</td>
</tr>
<tr>
<td>47</td>
<td>662</td>
<td>541</td>
<td>533</td>
<td>496</td>
<td>0.0001</td>
</tr>
<tr>
<td>48</td>
<td>6</td>
<td>21</td>
<td>14</td>
<td>4</td>
<td>0.0001</td>
</tr>
<tr>
<td>49</td>
<td>216</td>
<td>152</td>
<td>95</td>
<td>117</td>
<td>0.0001</td>
</tr>
<tr>
<td>50</td>
<td>27</td>
<td>31</td>
<td>26</td>
<td>37</td>
<td>0.0001</td>
</tr>
<tr>
<td>51</td>
<td>19</td>
<td>27</td>
<td>21</td>
<td>14</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

The mitochondrial proteins that exhibited significant differences between TG and NTG mice (i.e., LPE ≤ 0.05) are shown, sorted by function. Nos. of spectra observed for each protein, normalized as described in MATERIALS AND METHODS, are shown for the two NTG analyses (columns 1 and 2) and the two TG analyses (columns 4 and 5). The only proteins shown are those for which at least 1 spectrum was observed in each of the 4 analyses. For reference purposes, the values obtained for cytochrome oxidase IV (Cox4), which did not exhibit any significant difference between NTG and TG samples (see row 30 in Table 1), are shown in row R at the end of the table. Columns 6 and 7 are the averages of columns 1 and 2, and of columns 4 and 5, respectively. Column 8 shows whether the value for this protein is increased (1) or decreased (2) in TG compared with NTG. Column 9 shows the gene symbol, and column 10 shows the gene name and, for the oxidative phosphorylation proteins, the complex to which they belong. Column 11 shows the function of each protein as follows: ox phos, oxidative phosphorylation; FAO, β-fatty acid oxidation; AA meta, amino acid metabolism; apop, apoptosis; biogen, mitochondrial biogenesis, e.g., protein import; channel, channel protein; glycol/TCA, glycolysis or tricarboxylic acid cycle; ROS meta, reactive oxygen species metabolism; ?, unknown function. All gene symbols and names can be found at Mouse Genome Informatics as http://www.informatics.jax.org/.
proteins were reduced in MKK6 mouse hearts. This suggests that the MKK6 transgenic mouse hearts may exhibit a metabolic switch similar to that observed in failing hearts. The implications of such a switch on myocardial response to I/R are not known. However, it is possible that a switch away from fatty acid to glucose metabolism may be an adaptive response that might allow for the preservation of ATP generation, even though oxidative phosphorylation is reduced.

In addition to the reduced oxidative phosphorylation and FAO proteins, we observed reductions in several proteins involved in apoptosis (prohibitin and programmed cell death 8), as well as the reduction of a mitochondrial channel protein (VDAC3). It is possible that reduced levels of these proteins could contribute to the protective phenotype exhibited by the MKK6 transgenic mouse hearts. However, reduction in the levels of Card1, a chaperone involved in mitochondrial bio genesis, and Sod2, which metabolizes mitochondrial matrix ROS, in the transgenic mouse hearts would not be expected to support a more protected phenotype.

Like other proteins, p38 functions in the context of complex networks; therefore, the functional impact of p38 stretches well beyond the scope of our current knowledge. The results of the present study emphasize the extent of what remains to be discovered about the widespread effects of MKK6/p38 activation on the cardiac proteome. As such, proteomic analyses have the potential to identify previously uncharacterized effectors of such pathways and, as demonstrated in the current study, they will contribute to physiologically important phenotypes.

REFERENCES


27. Novalija E, Kevin LG, Camara AK, Bosnjak ZJ, Kampine JP, and Stowe DF. Reactive oxygen species precede the epsilon isoform of protein

GRANTS

This work was supported by National Institutes of Health Grants HL-63975, NS/HL-25037, and HL-75753 to C. C. Glembotski and a predoctoral fellowship from the American Heart Association to J. J. Martindale.


