Proteomic analyses are being increasingly used to identify protein changes accompanying changes in cellular function. An advantage of this approach is that it is largely unbiased by prior assumptions on the importance of each protein in the process under investigation. Here we have evaluated the protein changes that accompany the enlargement, or hypertrophy, of cardiomyocytes in culture. We have taken the additional step of comparing the changes that accompany a concentric hypertrophic phenotype stimulated by endothelin-1 exposure and an eccentric hypertrophic phenotype stimulated by leukemic inhibitory factor exposure. Following separation of the protein extracts by two-dimensional gel electrophoresis and staining with colloidal Coomassie Brilliant Blue, we identified 15 protein spots representing 12 proteins that changed in response to endothelin-1. In comparison, 17 protein spots representing 17 proteins changed in response to leukemic inhibitory factor, and 35 protein spots representing 28 proteins did not change under these conditions. Importantly the well established marker of cardiac pathology, atrial natriuretic factor, was identified as a protein up-regulated by both endothelin-1 and leukemic inhibitory factor exposure. Following separation of the protein extracts by two-dimensional gel electrophoresis and staining with colloidal Coomassie Brilliant Blue, we identified 15 protein spots representing 12 proteins that changed in response to endothelin-1. In comparison, 17 protein spots representing 17 proteins changed in response to leukemic inhibitory factor, and 35 protein spots representing 28 proteins did not change under these conditions. Importantly the well established marker of cardiac pathology, atrial natriuretic factor, was identified as a protein up-regulated by both endothelin-1 and leukemic inhibitory factor exposure (2.4 ± 0.8- and 2.2 ± 0.3-fold, respectively). However, nine of the observed protein changes occurred for only endothelin-1, whereas 11 of the changes occurred only with leukemic inhibitory factor exposure. These two different stimuli are therefore able to elicit unique changes in the protein expression profile of cardiac myocytes. This is consistent with the differences in morphologies noted as well as the different signaling pathways utilized by these different stimuli. Molecular & Cellular Proteomics 4:651–661, 2005.

Cardiac hypertrophy is a compensatory response of the heart to increased hemodynamic load. This enables the normal maintenance of cardiac output through an increase in the size of the individual cardiomyocytes and an overall increase in cardiac mass (1). Two morphologically different forms of hypertrophy are produced in response to increased hemodynamic load. Pressure overload, as observed during hypertension and aortic stenosis, produces a “concentric” form of hypertrophy that is characterized by the parallel addition of new sarcomeres. This results in increased cardiomyocyte length, increased cardiomyocyte width, and increased ventricular wall thickness (2). In contrast, volume overload as induced by chronic aortic regurgitation and mitral regurgitation produces an “eccentric” form of hypertrophy. Volume overload-induced eccentric hypertrophy is characterized by the addition of new sarcomeres in series, resulting in an increase in individual cardiomyocyte length and ventricular dilatation (3).

Changes in cell morphology resembling those observed during increased pressure and volume load can be induced in vitro by stimulating cultured neonatal cardiomyocytes with various growth factors and cytokines (4). These cultures therefore provide a robust system in which the effects of various hypertrophic agents can be studied. G-protein-coupled receptor agonists including endothelin-1 (ET-1),1 phenylephrine, and angiotensin II induce a form of in vitro hypertrophy comparable to that observed during pressure overload with cardiomyocytes exhibiting increased diameter due to the addition of new sarcomeres in parallel (5–7). In contrast, cytokines such as leukemic inhibitory factor (LIF) and cardiotoxin-1 that belong to the interleukin-6 family signal through the transmembrane gp130 protein to induce a form of hypertrophy resembling that produced during volume overload. Here cardiomyocytes increase in length due to the addition of sarcomeres in series (4, 6).

The defining molecular characteristics of cardiac hypertrophy include the increased synthesis of protein, the reorganization of sarcomeric proteins, and the re-expression of fetal

1 The abbreviations used are: ET-1, endothelin-1; LIF, leukemic inhibitory factor; ANF, atrial natriuretic factor; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase; 2DE, two dimensional gel electrophoresis; MHC, myosin heavy chain; MLC, myosin light chain.
cardiac genes including atrial natriuretic factor (ANF) (8–10). These changes are mediated following the activation of signaling cascades that act on various transcription factors and ultimately regulate gene and protein expression (11). Although the molecular events leading to the development of cardiac hypertrophy have not been fully elucidated, it is clear that cardiac hypertrophy is not exclusively regulated by a single signaling pathway. Instead different pathways may be used by different hypertrophic stimuli (12). For example, several different signaling cascades are activated following stimulation with ET-1. These include the protein kinase C pathway (13), the phosphatidylinositol 3-kinase pathway (14), and the three mitogen-activated protein kinase pathways that include the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases, and the p38 mitogen-activated protein kinases (15). Some of these changes such as activation of the phosphatidylinositol 3-kinase pathway (16) and the ERK and p38 mitogen-activated protein kinases (6) also follow the exposure to LIF; however, the Janus kinase/signal transducer and activation of transcription pathway is rapidly activated following exposure of cardiomyocytes to LIF (17).

Despite widespread investigation, it is not clear which signaling pathway(s) plays critical roles in mediating concentric hypertrophy and eccentric hypertrophy. Recent in vivo and in vitro evidence suggests that activation of the mitogen-activated protein kinase/ERK kinase (MEK1–ERK1/2) pathway is crucial in the development of concentric hypertrophy (15, 18) and that activation of the MEK5–ERK5 pathway mediates eccentric hypertrophy (19). These results imply that distinct signaling pathways regulate the development of concentric hypertrophy and eccentric hypertrophy. Consequently differential patterns of gene and protein expression could be associated with concentric hypertrophy and eccentric hypertrophy. In this study we have examined the protein expression profiles associated with concentric hypertrophy and eccentric hypertrophy. In this study we have examined the protein expression profiles associated with concentric hypertrophy and eccentric hypertrophy. In this study we have examined the protein expression profiles associated with concentric hypertrophy and eccentric hypertrophy.

Experimental Procedures

Isolation and Culture of Neonatal Cardiomyocytes—Cardiomyocytes from neonatal (1-day-old) Sprague-Dawley rats were isolated as described previously (20). Cells were resuspended in Dulbeccos modified Eagles medium:MEM (4:1) containing 10% (v/v) horse serum, 5% (v/v) heat-inactivated fetal bovine serum, 10 μM cytosine β-d-arabinofuranoside, 5 mM creatine, and 80 units/ml penicillin/streptomycin and preplated for 45 min to remove non-cardiomyocyte cells. For 2DE analyses cardiomyocytes were plated onto gelatin-coated 85-mm dishes (8 × 10⁶ cells in 6 ml) and cultured for 18–24 h before the medium was changed to serum-free Dulbeccos modified Eagles medium containing 5 mM creatine and 100 units/ml penicillin/streptomycin. The cardiomyocytes were cultured in serum-free medium for 24 h prior to treatment with either ET-1 (100 μM) or LIF (10 ng/ml) for 48 h.

Immunofluorescence—Immunofluorescence measurements were performed as described previously (6).

Experimental Design for 2DE—Primary cultures of neonatal cardiomyocytes were exposed to either control conditions, ET-1, or LIF in parallel culture. In addition, control and ET-1- and LIF-treated cultures from one experiment were simultaneously submitted to all steps of 2DE, including extraction, isoelectric focusing, SDS-PAGE, Coomassie Brilliant Blue staining, and quantitative analysis, so that variability between samples could be minimized.

Sample Preparation for 2DE—Medium was removed from the culture dishes, and cardiomyocytes were briefly rinsed (<10 s) twice with double deionized water before the addition of lysis buffer (300 μl) containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (v/v) IPG buffer pH 3–10 (Amersham Biosciences), 40 mM DTT, 2 μg/ml apro- tinin, 5 μg/ml leupeptin, 1 mM benzamidine, 10 μg/ml E64, and 1 mM EDTA. Samples were sonicated (5 × 1-s bursts) and left at room temperature for 2 h and then at 4 °C for 4 h before centrifugation (80,000 × g for 30 min at 4 °C). The supernatant was removed, assayed for protein concentration (Bio-Rad protein assay kit), and stored at −80 °C.

2DE—ReadyStrip TM IPG strips (pH 3–10, 18 cm, linear, Bio-Rad) were rehydrated overnight with 350 μl of sample (1 mg) that had been diluted into lysis buffer. First dimensional protein separation by isoelectric focusing was performed at 20 °C using an IPGphor™ isoelectric focusing system (Amersham Biosciences). The following isoelectric focusing protocol was used: a linear increase to 300 V over 2 h followed by a linear increase to 8000 V over 4 h after which the voltage was held at 8000 V until a total of 240,000 V-h had been reached. The total run time of 240,000 V-h was optimized with respect to protein spot migration and resolution. After isoelectric focusing the IPG strips were stored at −80 °C.

Immediately prior to SDS-PAGE, IPG strips were equilibrated for 25 min at 25 °C on an orbital shaker in equilibration buffer (6 M urea, 2% (w/v) SDS, 300 mM Tris/HCl (pH 8.8), 10% (v/v) glycerol, 2.5% (v/v) acrylamide, and 7.4 mM tri-n-butylphosphine). Following equili- bration, IPG strips were embedded in a 0.1% (w/v) agarose solution containing a trace amount of bromphenol blue on top of 1-mm thick 4% (v/v) stacking gels above 10% (v/v) polyacrylamide gels.

SDS-PAGE was performed in an Etan Daltix six electrophoresis system (Amersham Biosciences) at 2.5 watts/gel for 30 min followed by 25 mA/gel until the bromphenol blue front was 0.5 cm from the bottom of the gel. The separated proteins were visualized by Coomassie Brilliant Blue staining (1.8 M ammonium sulfate, 34% (v/v) methanol, 3.1% phosphoric acid, and 0.6% (w/v) Coomassie Brilliant Blue (G-250)) for 24 h. Stained gels were destained in deionized water for 16–18 h, scanned with an ImageMaster™ (Amersham Biosciences), and then soaked in a solution of 20% (v/v) ethanol and 5% (v/v) glycerol before being dried in a Hoefer® SE 1200 Easy Breeze™ air gel-drying system (Amersham Biosciences) according to the manu- facturer’s instructions.

Quantitative Analysis of Differential Protein Expression—Scanned gel images were analyzed using ImageMaster® 2D software (Amersham Biosciences). In brief, for an individual experiment each protein spot was manually matched between control and ET-1- and LIF-treated gels and assigned a number (spots 1–62). The volume of each protein spot was then manually determined using the “grow peaks” function, and the background surrounding each protein spot was manually subtracted using the “mode of non-spot” function. The volume of each protein spot was normalized by dividing by the total gel volume. Normalization diminished the inherent experimental variability associated with 2DE that is caused by factors such as different protein loading between gels. Differentially expressed cardiomyocyte
proteins were defined as those proteins that consistently increased or decreased in abundance in three or more independent experiments. Therefore only differences that were found to be present in all of the analyzed gel pairs were considered (21). Each independent experiment involved culturing cardiomyocytes from three or more groups of neonatal hearts. Within each experiment, protein expression of cardiomyocytes treated with either ET-1 or LIF was compared with an untreated control. Protein samples from separate experiments were not pooled for 2DE analysis as this would have obscured the biological variability of the experimental system. The variability in spot intensity between 2DE gels was assessed by calculating the coefficient of variation for protein spots from control gels and was calculated as being 56 and 74% for protein spots that were changed and unchanged during hypertrophy, respectively.

In-gel Trypsin Digestion of Cardiomyocyte Proteins—Protein spots were manually excised from the gels and cut into 1-mm² pieces with a scalpel. Gel pieces were then destained by rinsing three times in 25 mM ammonium bicarbonate in 50% (v/v) acetonitrile (45 min for each rinse) before being dried at 50 °C for 20 min. Gel pieces were then subjected to in-gel digestion for 16 h at 37 °C in 30 μl of digestion solution (12.5 μg/ml trypsin in 25 mM ammonium bicarbonate). Peptides were extracted from gel pieces using vigorous shaking at room temperature using a modification to the methods of Wilm and colleagues (22). Briefly 30 μl of 25 mM ammonium bicarbonate was added to the digestion solution mixture for 15 min before the addition of 30 μl of 100% (v/v) formic acid for another 15 min. Following removal of the supernatant, the extraction procedure was repeated two more times with 5% (v/v) formic acid in 100% (v/v) acetonitrile. The supernatants were pooled, and the sample was dried in a Speed-Vac. Extracted peptides were dissolved in 20 μl of 2% (v/v) formic acid.

Protein Identification by LC ESI-MS/MS—LC ESI-MS/MS analyses were performed using an Agilent 1100 Series HPLC system (Agilent Technologies) coupled to a Q-STAR Pulsar mass spectrometer (Applied Biosystems). Tryptic peptides were loaded onto a Zorba® SB-C18 column (15-cm length, 0.5-mm diameter, 5-μm bead size; Agilent Technologies) and separated with a linear gradient of 2% (v/v) acetonitrile, 0.1% (v/v) formic acid to 80% (v/v) acetonitrile, 0.1% (v/v) formic acid over a period of 30 min. Analyses of MS/MS data were performed by searching against the National Center for Biotechnology (NCBI) non-redundant data base using the Mascot search program (Matrix Science, London, UK; www.matrixsciences.com) to identify proteins of interest.

**RESULTS**

**Confirmation of Concentric and Eccentric Cardiomyocyte Hypertrophy**—Cardiomyocyte hypertrophy induced by either ET-1 or LIF was characterized by an increase in cell size when cells were viewed by phase-contrast microscopy (Fig. 1A). When these cells were fixed and subjected to staining for ANF and actin, an increased number of cells displayed perinuclear ANF staining and an increased level of actin organization within internal sarcomeric structures (Fig. 1B and Table I). In addition to these changes, cell area was found to increase by ~50–160% (Table I). ET-1 stimulated a concentric form of hypertrophy with an increased length and width of cardio-
Protein Expression during Cardiac Hypertrophy

myocytes and the parallel addition of sarcomeric units, whereas LIF induced an eccentric form of hypertrophy with cardiomyocytes displaying a marked increase in length (Fig. 1). These results confirmed that, under our culture conditions, ET-1 and LIF stimulate morphologically distinct forms of cardiomyocyte hypertrophy.

Protein Separation and LC ESI-MS/MS Identification—Despite being a relatively insensitive dye, Coomassie Brilliant Blue has a wide linear range (23) and therefore broad quantitative capacity, and it is for this reason that Coomassie Brilliant Blue was used to detect protein spots in our study. When extracts of cardiomyocytes were resolved by 2DE and then stained with Coomassie Brilliant Blue, more than 100 protein spots were visualized over the pI range of 3–10 and the molecular mass range of 14.3 to >220 kDa (Fig. 2). Of these, 62 protein spots were clearly and reproducibly resolved when multiple independent samples from control, ET-1-treated, and LIF-treated cardiomyocytes were subjected to analysis (Fig. 2). We used LC ESI-MS/MS to identify each protein by peptide fragmentation because we found that peptide mass fingerprinting could not unambiguously identify many of the proteins in our samples.2 On Fig. 2 we have indicated both the spot number and identity of each protein of our cardiomyocyte extracts.

The 62 protein spots were then analyzed for differential

2 T. M. Casey, P. G. Arthur, and M. A. Bogoyevitch, unpublished observations.

Fig. 2. 2DE of neonatal rat cardiomyocyte proteins. Solubilized proteins (1 mg) were separated by isoelectric focusing on ReadyStrip IPG strips (18 cm, pH 3–10) followed by SDS-PAGE on 10% polyacrylamide gels. Arrows indicate protein spots that changed in abundance during ET-1- or LIF-induced hypertrophy. Lines indicate proteins spots that did not change in abundance during either ET-1- or LIF-induced hypertrophy. SOD, superoxide dismutase.

2DE of neonatal rat cardiomyocyte proteins. Solubilized proteins (1 mg) were separated by isoelectric focusing on ReadyStrip IPG strips (18 cm, pH 3–10) followed by SDS-PAGE on 10% polyacrylamide gels. Arrows indicate protein spots that changed in abundance during ET-1- or LIF-induced hypertrophy. Lines indicate proteins spots that did not change in abundance during either ET-1- or LIF-induced hypertrophy. SOD, superoxide dismutase.
protein expression following the exposure of cardiomyocytes to either the concentric hypertrophic stimulus ET-1 or the eccentric hypertrophic stimulus LIF. The results of these analyses are shown in Table II for ET-1-treated cardiomyocytes and Table III for LIF-treated cardiomyocytes. We also include in Table IV an analysis of those protein spots not changing in response to either stimulus, thus providing a more complete proteomic map of cardiomyocytes.

Changes in Response to the Concentric Hypertrophic Stimulus ET-1—Fifteen protein spots representing 12 different proteins were present at different levels in ET-1-treated cardiomyocytes (Table II). Eight of these protein spots were present at higher levels in ET-1-treated cardiomyocytes, ranging from 1.5- to 3-fold increases. Most notable among these up-regulated proteins were the small heat shock protein pRM10 that was the most substantially decreased (2.6-fold lower). Although the function of this protein remains unreported, it is most similar in structure to a murine CC(A/T-rich)6GG (CARG)-binding factor-A (as annotated in the NCBI database [NCBI accession number BAA14181]) that acts as a transcriptional repressor (27).

Changes in Response to the Eccentric Hypertrophic Stimulus LIF—When a similar proteomic analysis was undertaken in LIF-treated cardiomyocytes, the abundances of 17 protein spots representing 17 different proteins were found to be changed (Table III). Thirteen of these protein spots were present at higher levels in LIF-treated cells, ranging from 1.3- to 4.7-fold increases. Most notable among these up-regulated proteins was mesoderm development candidate 2 (4.7-fold), a protein of unclassified function, but also up-regulated albeit to a lesser extent in response to ET-1 (2.8-fold; Table II). ANF was also up-regulated in response to LIF (2.2-fold), again consistent with its previous implications in cardiac pathology (26). Three protein spots were consistently down-regulated by 1.6–2.3-fold in response to LIF. Interestingly two of these (spots 2 and 23) contained proteins associated with the con-

| Spot no. | Accession no. | Protein identified | Peptides matched | Mascot score | Molecular mass (Da) | Functional class | -Fold change\(^d\) |
|----------|---------------|-------------------|-----------------|-------------|-------------------|-----------------|------------------|
| 7        | P19226        | 60-kDa heat shock protein, mitochondrial precursor (Hsp60) | 9              | 485         | 51,392            | Chaperone       | 1.7 ± 0.3 (3)    |
| 15       | AAD19638      | Nucleic acid binding factor pRM10 | 5              | 247         | 45,498            | Unclassified    | −2.6 ± 0.9 (4)   |
| 16\(^b\) | P19226        | 60-kDa heat shock protein, mitochondrial precursor (Hsp60) | 4              | 168         | 44,360            | Chaperone       | −1.3 ± 0.1 (3)   |
| 27       | P19226        | 60-kDa heat shock protein, mitochondrial precursor (Hsp60) | 9              | 461         | 33,806            | Chaperone       | −2.4 ± 1.3 (4)   |
| 28\(^a\) | AAA40891      | Clathrin light chain (LCB3) | 4              | 170         | 33,113            | Vesicular       | −1.2 ± 0.1 (3)   |
| 29       | XP_213755     | Wbscr1 | 5              | 192         | 32,508            | Protein processing | 1.5 ± 0.3 (3)   |
| 35\(^a\) | XP_218854     | Mesoderm development candidate 2 | 5              | 151         | 28,876            | Unclassified    | 2.8 ± 0.9 (3)    |
| 37       | NP_071985     | Peroxiredoxin 3 | 5              | 234         | 26,977            | Antioxidant     | 1.6 ± 0.2 (3)    |
| 38       | NP_476484     | Fertility protein SP22 | 7              | 357         | 25,061            | Unclassified    | −1.4 ± 0.2 (5)   |
| 41       | CAA42911      | αβ-crystallin (αβ-C) | 6              | 237         | 21,577            | Chaperone       | 1.9 ± 0.3 (5)    |
| 42       | CAA42911      | αβ-crystallin (αβ-C) | 6              | 245         | 20,941            | Chaperone       | 3.0 ± 0.8 (5)    |
| 47\(^a\) | AAA40736      | ANF | 2              | 81          | 14,157            | Signal transduction | 2.4 ± 0.8 (4)   |
| 49       | P08733        | Myosin regulatory light chain 2, ventricular/cardiac muscle isoform (MLC2) | 4              | 188         | 18,281            | Cytoskeletal     | −2.2 ± 0.9 (3)   |
| 50       | NP_058932     | Phosphatidylethanolamine-binding protein | 2              | 139         | 17,060            | Signal transduction | + (3)\(^c\) |
| 58       | XP_345562     | Similar to RIKEN cDNA 5730434103 gene | 3              | 132         | 20,137            | Unclassified    | −1.6 ± 0.2 (4)   |

\(^a\) Protein spots changed in parallel in both ET-1- and LIF-induced cardiac hypertrophy.

\(^b\) Protein spots that change in different directions in ET-1- or LIF-induced cardiac hypertrophy.

\(^c\) Spot not present on control gels.

\(^d\) Values in parentheses indicate the number of experiments in which protein spots were analyzed.
Protein Expression during Cardiac Hypertrophy

TABLE III
Identification of differentially expressed proteins during LIF-induced hypertrophy in cardiomyocytes

| Spot no. | Accession no. | Protein identified | Peptides matched | Mascot score | Molecular mass Da | pI | Functional class | -Fold change a |
|----------|---------------|--------------------|------------------|--------------|-------------------|----|-----------------|----------------|
| 2        | NP_036810     | Tropomyosin 4      | 9                | 470          | 65,012            | 28,492 | 4.8           | 4.7            | Cytoskeletal | -2.3 ± 0.2 (3) |
| 3        | AAB37701      | Tropomyosin 5      | 3                | 105          | 65,012            | 28,692 | 4.8           | 4.7            | Cytoskeletal | -1.4 ± 0.1 (4) |
| 6        | XP_218626     | Reticulocalbin 3   | 7                | 359          | 49,431            | 36,673 | 4.6           | 4.7            | Signal transduction | Chaperone | 1.4 ± 0.3 (3) |
| 7 a | P19226        | 60-kDa heat shock protein, mitochondrial precursor (Hsp60) | 9 | 485 | 51,404 | 60,917 | 6.0 | 5.9 | Chaperone | 1.6 ± 0.2 (3) |
| 16 b | P19226        | 60-kDa heat shock protein, mitochondrial precursor (Hsp60) | 4 | 168 | 44,874 | 60,917 | 7.0 | 5.9 | Chaperone | 1.9 ± 0.4 (5) |
| 22      | NP_071633     | Dimethylarginine dimethylaminohydrolase 1 | 4 | 158 | 32,508 | 31,406 | 6.3 | 5.8 | Chaperone | 1.9 ± 0.4 (5) |
| 23      | NP_058935     | Myosin heavy chain, polypeptide 6 (MHC6) | 9 | 490 | 31,841 | 223,370 | 7.0 | 5.6 | Chaperone | -2.2 ± 0.9 (3) |
| 28 a | AAA40891      | Clathrin light chain (LCB3) | 4 | 170 | 33,113 | 23,106 | 4.8 | 4.6 | Vesicular | 1.4 ± 0.2 (3) |
| 30      | P42930        | Heat shock 27-kDa protein (Hsp27) | 7 | 287 | 28,641 | 28,679 | 6.0 | 6.1 | Chaperone | -1.6 ± 0.2 (3) |
| 35 b | XP_218854     | Mesoderm development candidate 2 | 5 | 151 | 23,878 | 25,200 | 6.1 | 5.5 | Unclassified | 4.7 ± 2.0 (3) |
| 44      | 1NIWA         | Chain A, crystal structure of endothelial nitric-oxide synthase peptide bound to calmodulin | 6 | 283 | 18,071 | 16,515 | 3.5 | 4.1 | Unclassified | 1.5 ± 0.3 (5) |
| 46      | P08733        | Myosin regulatory light chain 2, ventricular/cardiac muscle isoform (MLC2) | 5 | 206 | 14,723 | 18,868 | 4.3 | 4.9 | Cyto skeletal | 1.4 ± 0.1 (4) |
| 47 a | AAA40736      | ANF | 2 | 81 | 14,157 | 12,528 | 4.0 | 6.8 | Signal transduction | 2.2 ± 0.3 (3) |
| 55      | NP_620242     | Heat shock 20-kDa protein (Hsp20) | 7 | 321 | 19,364 | 17,494 | 6.5 | 6.1 | Chaperone | 1.3 ± 1.0 (5) |
| 56      | XP_342967     | Heat shock protein, β-7 (cardiovascular heat shock protein)(vHsp) | 2 | 139 | 18,450 | 18,607 | 6.3 | 6.0 | Chaperone | 1.6 ± 0.1 (5) |
| 57      | AAA40996      | Cu,Zn-superoxide dismutase (Cu,Zn-SOD) (EC 1.15.1.1) | 6 | 391 | 16,710 | 15,700 | 6.3 | 5.9 | Antioxidant | 1.3 ± 0.1 (5) |
| 59      | NP_077325     | Adenylyl kinase 1 | 8 | 335 | 18,620 | 21,588 | 8.1 | 7.7 | Energy metabolism | 1.6 ± 0.2 (5) |
| 60      | P19804        | Nucleoside diphosphate kinase B | 6 | 237 | 17,906 | 17,272 | 7.5 | 6.9 | Signal transduction | 1.6 ± 0.3 (3) |

a Protein spots changed in parallel in both ET-1- and LIF-induced cardiac hypertrophy.

b Protein spots that change in different directions in ET-1- or LIF-induced cardiac hypertrophy.

c Values in parentheses indicate the number of experiments in which protein spots were analyzed.

The down-regulated protein spot contained Hsp27, a chaperone protein implicated in protection of myocytes isolated from adult hearts (25).

A comparison of the data presented in Table III with that in Table II indicates that only three protein spots were identified to change in a similar fashion when hypertrophy was induced in cardiomyocytes following exposure to ET-1 or LIF. These spots (indicated as Footnote a in Tables II and III) include one form of Hsp60 (spot 7) as well as mesoderm development candidate 2 (spot 35) and ANF (spot 47). In contrast, two spots changed in opposite ways in the ET-1- versus LIF-treated cells (indicated as Footnote b in Tables II and III). Specifically, one form of Hsp60 (spot 16) decreased in ET-1-treated myocytes but increased in LIF-treated myocytes, whereas clathrin light chain (spot 28) decreased in ET-1-treated myocytes but increased in LIF-treated myocytes. These results suggest that hypertrophic stimuli, as exemplified by ET-1 and LIF that are able to elicit different hypertrophic morphologies, also appear to result in changes in the levels of different subsets of proteins.

Proteins That Do Not Change in Abundance in Response to the Concentric Hypertrophic Stimulus ET-1 or the Eccentric Hypertrophic Stimulus LIF—Taking the results of Tables II and III together, 27 protein spots were reproducibly changed in response to ET-1 or LIF. An additional 35 protein spots representing 28 different proteins were reproducibly present but did not change in response to the hypertrophic stimuli, and these are summarized in Table IV. The major functional class represented by these proteins was cytoskeletal (9 of 28) with most of these proteins forming the sarcomeric structure of striated cardiac muscle. Other functional classes represented included those involved in energy and metabolism (6 of 28), chaperone functions in the cell (5 of 28), and protein processing and nucleic acid synthesis (5 of 28). Only one protein was of unclassified function, one protein has anti-

tractile apparatus (tropomyosins and myosin). The third down-regulated protein spot contained Hsp27, a chaperone protein implicated in protection of myocytes isolated from adult hearts (25).
TABLE IV
Identification of proteins showing unchanged expression during ET-1- and LIF-induced hypertrophy in cardiomyocytes

| Spot no. | Accession no. | Protein identified | Peptides matched | Mascot score | Molecular mass (Da) | pI | Functional class |
|----------|---------------|--------------------|-----------------|-------------|-------------------|----|-----------------|
| 1        | NP_033442     | Tropomyosin 2, β   | 8               | 337         | 74,473            | 4.9| Cytoskeletal    |
| 2        | P04692        | Tropomyosin 1 α chain | 14              | 589         | 74,473            | 4.9| Cytoskeletal    |
| 3        | AAB02288      | ATP synthase β subunit | 7               | 480         | 54,200            | 5.3| Energy metabolism |
| 4        | AAH61872      | Desmin            | 15              | 730         | 50,350            | 5.1| Cytoskeletal    |
| 8        | NP_037278     | Caldesmon 1       | 2               | 109         | 68,233            | 5.1| Cytoskeletal    |
| 9        | NP_037278     | Caldesmon 1       | 4               | 127         | 68,233            | 5.1| Cytoskeletal    |
| 10       | NP_620266     | Stress-induced phosphoprotein 1 | 6       | 175     | 63,679          | 6.8| Chaperone       |
| 11       | NP_620266     | Stress-induced phosphoprotein 1 | 5       | 136     | 63,386          | 6.8| Chaperone       |
| 12       | P11588        | Protein-disulfide isomerase A3 precursor (PDI) | 21   | 1165   | 58,076          | 6.3| Chaperone       |
| 13       | NP_036686     | Enolase 1, α      | 8               | 240         | 52,360            | 6.6| Energy metabolism |
| 14       | P19226        | 60-kDa heat shock protein, mitochondrial precursor (Hsp60) | 9    | 494    | 50,933          | 6.2| Chaperone       |
| 17       | XP_223190     | Heterogeneous nuclear ribonucleolar protein D-like protein | 4    | 144    | 40,271          | 6.6| Nucleic acid synthesis and processing |
| 18       | NP_036630     | Aldehyde reductase 1 (low K₈₆ aldose reductase) | 7    | 263    | 39,084          | 6.8| Other metabolic enzymes |
| 19       | XP_215692     | FATZ-related protein 2 | 6    | 201    | 35,481          | 6.8| Chaperone       |
| 20       | XP_217155     | Electron transfer flavoprotein a-subunit, mitochondrial precursor | 6    | 316    | 35,481         | 7.6| Energy metabolism |
| 21       | XP_217155     | Electron transfer flavoprotein a-subunit, mitochondrial precursor | 5    | 255    | 35,481          | 7.8| Energy metabolism |
| 24       | NP_446413     | Endoplasmic reticulum protein 29 | 8    | 405    | 31,260          | 6.8| Protein processing |
| 25       | NP_058936     | Myosin heavy chain, polypeptide 7 (MHC7) | 8    | 364    | 30,619          | 6.4| Cytoskeletal    |
| 26       | XP_343148     | Similar to RIKEN cDNA 1110005A23 | 5    | 241    | 33,806          | 6.9| Unclassified    |
| 31       | NP_446028     | Peroxiredoxin 6 | 7    | 414    | 28,054          | 6.1| Antioxidant     |
| 32       | P42930        | Heat shock 70-kDa protein (Hsp70) | 8    | 353    | 26,485          | 5.7| Chaperone       |
| 33       | NP_032857     | Prohibitin        | 8    | 422    | 23,550          | 4.7| DNA synthesis    |
| 34       | NP_036738     | Myosin light chain 3, alkali, cardiac ventricles (MLC3) | 12  | 734    | 23,334          | 5.3| Cytoskeletal    |
| 36       | NP_036738     | Myosin light chain 3, alkali, cardiac ventricles (MLC3) | 10  | 567    | 26,977          | 5.3| Cytoskeletal    |
| 39       | NP_062256     | ATP synthase subunit d | 9    | 377    | 23,334          | 6.3| Energy metabolism |
| 40       | NP_036738     | Myosin light chain 3, alkali, cardiac ventricles (MLC3) | 6    | 340    | 20,230          | 5.7| Cytoskeletal    |
| 43       | CAA42911      | αB-crystallin (αB–C) | 13  | 658    | 21,134          | 7.6| Chaperone       |
| 45       | XP_215116     | 60 S acidic ribosomal protein P2 | 5    | 274    | 16,255          | 4.1| Protein processing |
| 48       | PO8733        | Myosin regulatory light chain 2, ventricular/cardiac muscle isoform (MLC2) | 6  | 288    | 19,186          | 4.8| Cytoskeletal    |
| 51       | NP_058862     | Stathmin 1         | 6               | 254         | 18,450           | 5.9| Cytoskeletal    |
| 52       | NP_036738     | Myosin light chain 3, alkali, cardiac ventricles (MLC3) | 4    | 163    | 16,106          | 5.6| Cytoskeletal    |
| 53       | NP_036738     | Myosin light chain 3, alkali, cardiac ventricles (MLC3) | 5    | 179    | 14,321          | 5.8| Cytoskeletal    |
| 54       | NP_665726     | Cytochrome c oxidase, subunit Va | 4    | 186    | 14,157          | 5.1| Energy metabolism |
| 61       | PO388         | 40 S ribosomal protein S12 | 2    | 81     | 14,723          | 6.9| Protein processing |
| 62       | NP_032274     | Histidine triad nucleotide-binding protein | 2    | 139    | 31,117          | 6.7| Signal transduction |
Protein Expression during Cardiac Hypertrophy

oxidant properties, and one is considered to act in signal transduction.

**DISCUSSION**

Apart from the initial proteomic studies of heart failure using human and animal models (28–30), very few recent studies have evaluated a global proteome of the cardiomyocyte. Instead, as outlined by Vondriska and Ping (31), many have now chosen to define the cardiomyocyte as an integration of multiple functional subproteomes, each organized to perform a unique function. For example, subproteomes such as the cardiomyocyte mitochondria have been assessed (32) as have signaling modules including protein kinase C (33) and phosphoproteomes following β-adrenergic stimulation (34). In those cases where a global proteome has been assessed, only a relatively small number of protein changes have been noted, and an even smaller number of proteins actually have been identified. For example when right atrial tissue from patients with dilated cardiomyopathy have been compared, 25 statistically significant changes were noted, and only 12 of these proteins have been identified (Refs. 35 and 36, and for a review, see Ref. 37). Furthermore, despite the widespread acceptance of the neonatal rat cultured cardiomyocyte system as an in vitro model to study the events of cardiac hypertrophy (10, 38, 39), there is a surprising paucity of studies evaluating global protein changes in these cells. In three studies, long term adrenergic stimulation has been evaluated (40–42), but only in the latter of these were proteins identified. Proteins present at higher levels included myosin light chain 1 and myosin light chain 2 atrial isoforms, chaperonin cofactor a, nucleoside diphosphate kinase a, and Hsp27, whereas mitochondrial matrix protein p1 and NADH:ubiquinone oxidoreductase 75-kDa subunit were decreased.

Our study therefore is the first to present a global proteomic analysis of cardiomyocytes following exposure to two hypertrophic agents, ET-1 and LIF, highlighting both the similarities in their responses but also the significant differences in the protein profile. Several aspects of the analysis and interpretation of the proteomic data as well as the implications of the data in understanding the hypertrophic process are discussed in the following sections.

**Analysis and Interpretation of Proteomic Data**—2DE followed by mass spectrometry has proven to be most successful in the analysis and identification of abundantly and moderately expressed proteins (43). For accurate comparisons of protein levels, 2DE requires that each individual protein spot highlighted via protein staining procedures contains only a single protein and furthermore that all proteins are resolved as single rather than multiple protein spots.

In our study, LC ESI-MS/MS analyses has revealed that one differentially expressed protein spot (spot 2) was comprised of two distinct proteins, tropomyosin 4 and tropomyosin 5. This co-migration was consistent with the similarity in both molecular mass and pI values for tropomyosin 4 and tropomyosin 5. We therefore could not determine whether either or both of these proteins were down-regulated in response to LIF. Our interpretation of LC ESI-MS/MS analyses was further complicated by the appearance of several proteins in multiple protein spots on 2DE gels with only 4 of the 62 spots being represented by distinct proteins. Differentially expressed proteins represented by more than one protein spot included Hsp60 (present in protein spots 7, 14, 16, and 27), Hsp27 (present in protein spots 30 and 32), MLC2 (present in protein spots 46, 48, and 49) and α-B-crystallin (present in protein spots 41, 42, and 43). We therefore could not determine whether changes in the total abundance of any of these proteins had accompanied the hypertrophic events in the presence of ET-1 or LIF. In previous studies of cardiac proteomics, MLC2 has been identified as a group of protein spots (42, 44, 45) as has Hsp27 (46). In the case of Hsp27, previous studies have also indicated that some spots increased in dilated cardiomyopathy, whereas others decreased possibly as a result of enhanced degradation in heart failure (47).

The appearance of proteins as multiple protein spots in our present study could reflect the presence of similar isoforms of these proteins, or alternatively these different forms may arise from post-translational modifications such as phosphorylation, glycosylation, or proteolytic cleavage. In support of the possibility that proteolytic cleavage contributes to some of the different protein spots identified, spots 23 and 25 were also found to contain fragments of MHC6 and MHC7, respectively. These protein fragments were indicated by the differences between experimental and theoretical molecular mass values (MHC6 experimental molecular mass = 31,841 Da, theoretical molecular mass = 222,370 Da; MHC7 experimental molecular mass = 30,619 Da, theoretical molecular mass = 222,945 Da). Because the detected peptides only covered amino acids in the C-terminal regions of MHC6 and MHC7, this suggests the proteolytic cleavage and loss of the N terminus of each protein. Our results are also in agreement with previous studies that have demonstrated that MHC from heart and skeletal muscle resolves as fragments during 2DE (45, 48).

Apart from these differences in molecular mass noted for MHC6 and MHC7, experimental molecular mass and pI values generally compared well to corresponding theoretical values because molecular mass differences did not exceed 10,000 Da, and pI values did not differ by more than 1.0 pH unit. However, there were several notable exceptions. Six proteins were found to have higher than expected experimental molecular mass values. These included reticulocalbin 3 (spot 6), nucleic acid binding factor pRM10 (spot 15), clathrin light chain (spot 28), RIKEN cDNA 1110005A23 (spot 26), and histidine triad nucleotide-binding protein (spot 62). Similarly the experimental pI values of nucleic acid binding factor pRM10 (spot 15) and ANF (spot 47) were lower than the expected pI values. Post-translational modifications such as phosphorylation and glycosylation may account for the observed differences between experimental and theoretical mo-
distinct patterns of protein expression during ET-1-induced hypertrophy and LIF-induced hypertrophy of cardiomyocytes—The results of a number of studies have suggested that a “fetal gene program” underpins the hypertrophic responses of the heart (8–10). This suggests that there is a conserved program of gene expression and thus a conserved series of protein expression changes that accompanies cardiomyocyte hypertrophy. However, the application of global analyses such as proteomics together with microarray analyses has the power to test this hypothesis. In the only direct comparison of cardiac hypertrophy as a result of four different interventions, namely overexpression of protein kinase C-e activation peptide, overexpression of calsequestrin, overexpression of calcineurin, and overexpression of Gaq, only ANF was commonly regulated (51). In our present study, LC ESI-MS/MS analyses of the 62 protein spots results revealed that 9 and 11 proteins were differentially expressed during ET-1-induced and LIF-induced hypertrophy, respectively. Wbscr1, mesoderm development candidate 2, peroxiredoxin 3, and ANF were increased during ET-1-induced hypertrophy, whereas nucleic acid binding factor pRM10, clathrin light chain, fertility protein SP22, and RIKEN cDNA 573043I03 were decreased. LIF-induced hypertrophy was associated with increases in reticulocalbin 3, dimethylarginine dimethylaminohydrolase 1, clathrin light chain, mesoderm development candidate 2, calmodulin, ANF, Hsp20, cvHsp, Cu,Zn-superoxide dismutase, adenylate kinase 1, and nucleoside diphosphate kinase 1. Only two proteins, ANF and mesoderm development candidate, were co-regulated during concentric and eccentric hypertrophy. The abundance of one form of Hsp60 (spot 7) was increased during both ET-1- and LIF-induced hypertrophy; however, we were unable to determine whether the total abundance of this protein was increased during both forms of hypertrophy because Hsp60 was represented by multiple protein spots. Clathrin light chain was differentially expressed during both forms of hypertrophy but not co-regulated because its abundance was decreased during concentric hypertrophy and increased during eccentric hypertrophy.

Interestingly several proteins that changed in abundance during ET-1- or LIF-induced cardiac hypertrophy in vitro, MLC2, Hsp60, Hsp27, and αB-crystallin, have also been shown to change in human/animal models of heart disease (Refs. 28, 44, 47, and 52–54; and for a review, see Ref. 55). That more similarities in protein expression were not found is not surprising given the differences in factors such as duration of the hypertrophic process (long term in human/animal models of heart disease versus short term in our in vitro study).

Taken together, at least in this in vitro model of cardiac hypertrophy, our findings suggest that concentric hypertrophy and eccentric hypertrophy are associated with distinct patterns of protein expression. These results are consistent with the different signaling pathways that regulate concentric hypertrophy and eccentric hypertrophy (15, 18, 19). Furthermore neither concentric hypertrophy nor eccentric hypertrophy was associated with a specific change in a distinct functional group of proteins. Instead differentially expressed proteins were from a wide range of functional classes and included proteins involved in signal transduction, protein folding (chaperones), endocytosis (vesicular proteins), antioxidant defense, maintenance/alteration of cellular structure (cytoskeletal proteins), hydrolase activity, and energy metabolism. The hypertrophic response, even in this simple in vitro system, is therefore accompanied by a complex series of protein changes. It remains to be evaluated which of these changes are essential for the hypertrophic response or which may contribute to the known cardioprotective actions of ET-1 or LIF (56, 57). Newly emerging technologies of RNA interference will likely help evaluate these outstanding questions.

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