Gene expression changes associated with fibronectin-induced cardiac myocyte hypertrophy

Hua Chen, Xueyin N. Huang, Alexandre F. R. Stewart, and Jorge L. Sepulveda. Gene expression changes associated with fibronectin-induced cardiac myocyte hypertrophy. *Physiol Genomics* 18: 273–283, 2004; 10.1152/physiolgenomics.00104.2004.—Fibronectin (FN) is an extracellular matrix protein that binds to integrin receptors and couples cardiac myocytes to the basal lamina. Cardiac FN expression is elevated in models of pressure overload, and FN causes cultured cardiac myocytes to hypertrophy by a mechanism that has not been characterized in detail. In this study, we analyzed the gene expression changes induced by FN in purified rat neonatal ventricular myocytes using the Affymetrix RAE230A microarray, to understand how FN affects gene expression in cardiac myocytes and to separate the effects contributed by cardiac nonmyocytes in vivo. Pathway analysis using z-score statistics and comparison with a mouse model of cardiac hypertrophy revealed several pathways stimulated by FN in cardiac myocytes. In addition to the known cardiac myocyte hypertrophy markers, FN significantly induced metabolic pathways including virtually all of the enzymes of cholesterol biosynthesis, fatty acid biosynthesis, and the mitochondrial electron transport chain. FN also increased the expression of genes coding for ribosomal proteins, translation factors, and the ubiquitin-proteasome pathway. Interestingly, cardiac myocytes plated on FN showed elevated expression of the fibrosis-promoting peptides connective tissue growth factor (CTGF), WNT1 inducible signaling pathway protein 2 (WISP2), and secreted acidic cysteine-rich glycoprotein (SPARC). Our data complement in vivo studies and reveal several novel genes and pathways stimulated by FN, pointing to cardiac myocyte-specific mechanisms that lead to development of the hypertrophic phenotype.

OUTSIDE-IN SIGNALING CHARACTERIZES the cellular effects mediated by interaction of cellular receptors with the extracellular matrix. In neonatal rat ventricular myocytes (NRVM), clustering of integrin receptors induced by fibronectin (FN) (39, 40) or RGD peptides (3) elicits a hypertrophic response, characterized by a 1.5- to 3-fold increase in cellular area and corresponding increases in global mRNA and protein synthesis, together with enhanced myofibrillogenesis and sarcomeric assembly. FN also induces increased formation of focal adhesions and costameres, which are sites of contact of extracellular matrix with integrins and associated cytoskeletal proteins associated with the Z-disks of the sarcomere (39). As in other models of hypertrophy, the hypertrophic response is accompanied by changes in gene expression, with increased expression of the natriuretic peptides atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) and fetal isoforms of sarcomeric proteins such as β-myosin heavy chain (βMHC) and α-skeletal actin (αSka).

The pathophysiological relevance of the FN-induced, integrin-mediated pathway is highlighted by the observation of increased deposition of FN in the extracellular matrix in animal models of cardiac hypertrophy and in patients with cardiac failure (5, 20, 22, 29, 42, 45). The increased synthesis of FN is in part mediated by stimulation of cardiac fibroblasts by angiotensin II, produced in response to mechanical overload (11, 20, 26, 52). The extracellular matrix remodeling associated with interstitial fibrosis can lead to contractile and diastolic dysfunction and consequent heart failure. In contrast to the development of fibrosis in the hypertrophic heart, the gene expression effects of increased FN deposition and consequent integrin activation have been less appreciated. That integrin-mediated pathways are critical for the response to hypertrophic stimuli is suggested by the observations that inhibition of outside-in integrin signaling blocks induction of the hypertrophic phenotype caused by phenylephrine (51, 56) or by mechanical stretch (1, 33).

In this study, we examined the changes in gene expression induced by FN outside-in signaling in NRVM using high-density oligonucleotide microarrays. The results obtained provide a framework to analyze in a comprehensive manner the transcriptional response to integrin activation and suggest novel pathways activated by FN in hypertrophic cardiac myocytes.

MATERIALS AND METHODS

**Isolation and culture of NRVMs.** Ventricular myocytes were isolated from 1- to 2-day-old newborn Sprague-Dawley rats according to an established protocol (14) and in compliance with the “Guiding Principles for the Care and Use of Laboratory Animals.” In brief, ventricles were dissected from 1 or 2 litters of about 10 pups each and incubated with Hanks’ balanced salt solution with 20 mM HEPES, pH 7.4, containing 2.0 mg/ml of trypsin and 20 μg/ml DNase I until complete cell dissociation. The cells were then washed, resuspended in minimal Eagle’s medium (MEM) with 5% bovine calf serum, and preplated in regular tissue culture dishes for 1 h to allow for attachment of nonmyocytes. After the preplating period, nonattached myocytes were removed by gentle pipetting and were plated in tissue culture dishes that were either untreated or coated with 0.5 μg/cm² of FN (Sigma catalog number F-1141; e.g., 1 ml of 10 μg/ml solution on a 6-cm diameter plate) or with 100 μg/cm² of gelatin, for 3 to 4 h. Cells were plated at a density of 500 cells/mm² and cultured for 24 h in MEM containing 5% bovine calf serum and 0.1 mM bromodeoxyuridine at 37°C in 1% CO₂. Using this protocol, we routinely achieve a purity of 90–95% myocytes, as assessed by sarcomeric α-actinin immunofluorescence. In some experiments, 1 μg/ml of anti-β1-integrin (anti-CD29, clone Ha2/5, no azide/no endotoxin; Pharmingen, San Diego, CA) or rabbit IgG were incorporated at all times in the culture medium. After 24 h of culture, the medium was replaced with MEM containing 10 μg/ml of transferrin, 10 μg/ml of...
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insulin, and 1 mg/ml of low-endotoxin bovine serum albumin (BSA). Insulin was omitted from the medium after 48 h of culture, and extracts were collected for RNA analysis after 72 h of culture.

Immunofluorescence and image analysis. Cardiac myocytes were cultured in 8-well glass chamber slides (Lab-Tek II; Nalge Nunc, Naperville, IL) coated with gelatin or FN, as described above. After 4 days of culture, the cells were washed with phosphate-buffered saline (PBS), fixed in PBS containing 2% paraformaldehyde for 20 min, and permeabilized with pH 7.4 PBS containing 0.1% Triton X-100 for 20 min. Nonspecific binding was blocked by incubation for 1 h at room temperature. Myocytes were then double labeled for visual-0.1% Tween 20 (PBS-T) and 5% normal donkey serum. The following primary antibodies were diluted in PBS-T with 5% serum and incubated for 1 h at room temperature: 1) rabbit anti-ANP (Peninsula Laboratories, Belmont, CA) and 2) mouse monoclonal anti-sarcromeric α-actinin (Sigma, St. Louis, MO). The slides were then briefly rinsed in PBS-T and washed three times for 5 min in PBS-T, 0.3%, or 0.5% Tween 20 (PBS-T) and 5% normal donkey serum. After 24 h at room temperature, the slides were washed three times for 5 min with PBS-T and were mounted in 50% glycerol in PBS containing 1 µg/ml bis-benzimide (Hoechst 33258) nuclear stain. The cells were visualized in a Zeiss Axioplan 2 confocal imaging system or an Olympus BX40 epifluorescence microscope. Images were acquired with a SPOT camera, and image analysis was performed with Photoshop (Adobe Systems, San Jose, CA). To validate double or triple labeling experiments, appropriate negative controls omitting one of the secondary or primary antibodies were performed to rule out unwanted cross-reactivity between the various primary and secondary antibodies and to rule out optical leaching between the different filter combinations. For cell surface area measurements, several pictures were taken from representative fields using Nomarski interference optics on culture dishes or α-actinin immunofluorescence on fixed cells. At least 200 myocytes for each data point were analyzed with the NIH IMAGE software.

RNA purification and real-time RT-PCR. Cells were cultured as described above, and RNA was purified using TRIzol (Invitrogen, Carlsbad, CA), according to manufacturer instructions. RNA was precipitated from the TRIzol reagent with isopropanol and resuspended at 0.5–1.0 µg/ml using RNase Secure solution (Ambion, Austin, TX). RNA was treated with DNase I (DNA-free, Ambion) before reverse transcription. For reverse transcription, 60 ng of RNA were mixed with 5 µM random hexamers, 1 mM each dNTP, 7.5 mM MgCl₂, 40 U RNasin (Promega, Madison, WI), 1X PCR buffer II (Applied Biosystems, Foster City, CA) and 250 U of SuperScript II reverse transcriptase (Invitrogen). The reaction was incubated at 25°C for 10 min, 48°C for 45 min, and 95°C for 5 min, then cooled to 4°C. For SYBR Green quantitative real-time PCR, 2 µl of reverse transcription reaction was mixed with 400 nM each specific primer (see Table 2) and 1X SYBR PCR Master Mix (Applied Biosystems). The reaction was incubated in a model 7100 thermocycler (Applied Biosystems) for 45 cycles consisting of denaturation at 95°C for 15 s and annealing/extension at 58–60°C for 1 min. The critical cycle (Ct) was determined by the ABI Prism 7000 SDS software, and quantification of relative mRNA levels was performed by the software using a calibration curve obtained by serial dilutions of a standard RNA preparation from rat heart.

Target RNA preparation and microarray hybridization. Target RNA was extracted using TRIzol, as described above, and further purified by the RNeasy mini kit (Qiagen, Valencia, CA). Double-stranded cDNA was synthesized from total RNA (SuperScript II system; Invitrogen). An in vitro transcription reaction was then performed to obtain biotin-labeled cRNA from the double-stranded cDNA (Enzo BioArray High Yield RNA Transcript Labeling kit; Enzo Diagnostics, Farmingdale, NY). The cRNA was fragmented before hybridization, then mixed in a hybridization mixture containing probe array controls, BSA, and herring sperm DNA. A cleanup procedure was performed on the hybridization cocktail using an RNeasy spin column (Qiagen), after which it was applied to the Affymetrix RA230A probe array. Hybridization was allowed to continue for 6 h at 45°C, after which the arrays were washed and stained. Each probe array was scanned twice. The computer workstation automatically overlaid the two scanned images and averaged the intensities of each probe cell for greatest assay sensitivity.

Data analysis. GeneChip expression software (Affymetrix, Santa Clara, CA) was used to determine the absolute analysis metrics using the probe arrays hybridization measure after scanning the arrays, as directed by the Affymetrix GeneChip standard procedure. The average difference for each probe set is an average of the differences between the “perfect match” (PM) and control “mismatch” (MM) probe intensities and is directly related to the level of expression of the transcript. The GeneChip Detection Algorithm was used to calculate a detection p value using an empirical distribution function of probe intensities. The detection p value was used to determine the detection score [R = (PM – MM)/(PM + MM)] and the threshold Tau = 0.015, tested by a one-sided Wilcoxon signed rank test. Only those probe sets with detection P values <0.05 were called “present” and considered for further analysis in this study. The level of expression of each transcript (signal) was determined by GeneChip using a one-step Tukey biweight estimate. The data points for each gene of the FN or control arrays were normalized using a targeted normalization procedure in which the average signal from the 3′ GAPDH probe sets was defined as unchanged. The signals from all the experimental arrays were then multiplied by a normalization factor to obtain similar average intensity between arrays for the 3′ GAPDH probe sets. This normalization method was chosen because the global increase in gene expression associated with hypertrophy precludes global or even selected “housekeeping” gene normalization procedures. We selected GAPDH because it has been broadly used as an invariant control for mRNA expression in studies of cardiac myocyte hypertrophy, and in our experience GAPDH mRNA expression by Northern blot or real-time RT-PCR shows very little variation in cells plated in the presence or absence of FN and other hypertrophic agents (unpublished observations). The corrected signals after the normalization procedure were then used to determine the fold change of FN relative to control by dividing the signal of each probe set in the FN array by respective signal in the control array. Each experiment was performed in duplicate, and the fold change ratios from each replicate were averaged in the final analysis. The complete data set was deposited into the NCBI Gene Expression Omnibus (GEO) database with the accession number GSE1055.

Pathway analysis. Pathway analysis was performed using the GenMAPP software version 1.0 (Gladstone Institutes, UCSF, San Francisco, CA) (12), downloadable from http://www.genmapp.org. This software uses an identifier for each probe set to display the expression level of each gene in a pathway. We used the Swiss-Prot identifiers for each gene based on the annotation data provided by Affymetrix, as well as a table relating rat GenBank identifiers to Swiss-Prot numbers obtained with the Dragon program (http://pevsnerlab.kennedykrieger.org/annotate.htm). When Swiss-Prot identifiers were not available, the GenBank identifier was used and replaced in the respective gene slot in the pathway. The annotation for each probe set of the RA230A array was verified by downloading the latest annotation file from Affymetrix. In addition, we performed BLAST analysis using the probe target sequence described by Affymetrix for all the probe sets with significant levels of expression (present) where there were discrepancies between various probe sets.
corresponding to the same transcript or where the annotation was “transcribed sequences.” When genes in a pathway were not automatically found in the array with the GenMAPP software, we used NCBI BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/) and BLAT alignments to the UCSC rat genome browser (http://genome.ucsc.edu) (27) to identify sequences homologous to the probes in the array. Using these strategies, we assigned a specific gene (with the corresponding HUGO Gene Nomenclature Committee approved gene symbol) to 7,250 RAE230A probe sets corresponding to 5,664 different genes, compared with the original 5,193 probe sets corresponding to 4,662 different genes from the Affymetrix annotation files. Moreover, in several instances, the original annotation was corrected based on the results of BLAST analysis.

All the pathways in the GenMAPP database were analyzed to determine whether a significant number of genes in each pathway were affected by FN vs. control (15). We examined the frequency of genes fulfilling a certain criterion (such as increase ≥1.5) in each group or pathway and compared with null hypothesis expected frequency for that group or pathway based on the total number of genes examined on the array. The z-score was derived by dividing the difference between the observed number of genes meeting the criterion in a specific group or pathway and the expected number of genes based on the total number of genes in the array meeting the criterion and standardizing by dividing by the standard deviation of the observed number of genes under the hypergeometric distribution. The equation used was:

\[
z = \frac{(r - \pi N)}{\sqrt{n R(N - R)(N - 1)}}
\]

where \(N\) = the total number of genes measured in the array, \(R\) = the total number of genes meeting the criterion, \(n\) = the total number of genes in a specific group or pathway, and \(r\) = the number of genes meeting the criterion in a specific group or pathway.

A positive z-score indicates that more genes than expected fulfilled the criterion in a certain group or pathway; therefore, that group or pathway is likely to be affected by FN.

RESULTS

Induction of hypertrophy by FN. Cardiac myocytes plated on FN displayed several features of hypertrophy (Fig. 1). First, there was a significant increase in average cell area, both in live, unstained myocytes (Fig. 1, A–C) as well as in fixed cells immunostained with anti-sarcomeric α-actinin antibody (Fig. 1, compare E to G). Second, there was a marked increased in sarcomeric organization and thickness (Fig. 1, D and E). Third, there was a significant increase in the expression of ANP and BNP, as measured by the percentage of actinin-positive cardiac myocytes expressing ANP (Fig. 1, H–J).
parallel increase in the mRNA levels of ANP and BNP relative to GAPDH, as measured by quantitative real-time RT-PCR (Fig. 1) and Northern blot (not shown). To demonstrate that this increase in ANP and BNP expression was due to FN outside-in signaling through the integrin receptors, cells were plated and cultured in the presence of anti-β1-integrin (CD29) antibody at all times before RNA extraction. Normal mouse IgG at the same concentrations was used as control. The results (Fig. 1) show that β1-integrin binding to FN is required for induction of the hypertrophic phenotype.

**Microarray analysis of FN-induced gene expression changes.** We analyzed global gene expression changes in cardiac myocytes plated in FN-coated plates compared with uncoated plates using Affymetrix RAE230A microarrays. Each RAE230A array contains a total of 15,875 probe sets, excluding the control sets, which represent 14,046 unique UniGene identifiers (3,142 of UniGene identifiers are represented by more than one probe set). A total of 7,250 probe sets, representing 6,630 unique UniGene transcripts, were called present by the GeneChip detection algorithm in either the replicate FN or control arrays. Only these were considered for further analysis. Surprisingly, despite an obvious change in phenotype with significant increases in cell area, sarcomeric assembly, and ANP/BNP expression, very few genes were markedly altered by FN. For example, only 296 unique transcripts were induced more than 2-fold, and only 20 were induced more than 3-fold. Fourteen of the transcripts induced more than 3-fold were expressed at very low levels (signal <200) and most likely represent false-positive signals. The only transcripts induced more than 3-fold with high levels of expression (signal >500) were ANP (4.4-fold), cytochrome P-450 subfamily 51 (4.0-fold), and component of oligomeric Golgi complex 1 (3.2-fold, Table 1). Similarly, of 242 genes downregulated more than 2-fold (to <0.5 of control level), only 2 unique transcripts were repressed more than 3-fold and had initial expression levels greater than 500 (Table 1).

We performed real-time RT-PCR in independent samples to verify the accuracy and validity of our array results (Table 2). In general, the results of RT-PCR and microarray quantification were in agreement in the direction, if not the exact magnitude, of change. All the genes tested were upregulated in both assays. Moreover, we used anti-CD29 antibody to block FN outside-in signaling through β1-integrins and measured Cyp51 induction (Fig. 2). The results confirm that most if not all of the induction of Cyp51 by FN is due to outside-in signaling involving β1-integrin.

**Group and pathway analysis.** Table 3 shows the z-scores calculated for selected gene groups and pathways. The z-score was obtained using the formula described in MATERIALS AND METHODS.
METHODS, by comparing the proportion of genes increased by 1.5 or more by FN, among all unique genes with a present call (1,170/6,630 = 18%), vs. the proportion of genes increased ≥1.5-fold among all present unique genes that belong to a specific group or pathway. The z-score values greater than 2.0 are generally considered significant. Note that the z-score depends on the total number of genes examined; therefore, it is higher in larger groups. We validated this statistical approach by determining the z-score for irrelevant groups such as genes symbols starting with a specific letter and obtained z-scores below 2.0.

Table 3 shows that general biosynthesis pathways related to hypertrophy were significantly upregulated by FN, but there is specificity in which components of the general assembly machinery were upregulated at the mRNA level. For example, not all genes involved in translation were upregulated (z = 1.1), but ribosomal proteins and genes involved in ribosomal metabolism were significantly upregulated (z = 9.0–12.2). Similarly, mRNAs for most transcription factors and genes involved in transcription were not induced (z = 0.7), but RNA polymerase-related genes were (z = 3.8). In addition to assembly pathways, the proteolytic ubiquitin-proteasome machinery was mildly upregulated (z = 2.6–3.4).

Among the cytoskeleton-associated genes, tubulins and tubulin-associated genes were significantly upregulated (z = 3.9), whereas actin-related genes as a group were not (z = 1.2). However, individual genes, such as skeletal-α-actin (Acta1), were significantly induced. Myosins and tropomyosins are a heterogeneous group with highly expressed sarcomeric-specific genes and a large variety of nonsarcomeric myosins. Among the highly expressed myosins and tropomyosins (normalized levels >200), a significantly larger proportion of genes was upregulated (z = 2.8). These include tropomyosins 1, 2, and 3 (Tpm1–3), βMHC (Myh7), cardiac myosin-binding protein C (Mybp3), cardiac myosin light chains (Myl2, Myl3, Myl4), and smooth-muscle myosin light chain (Myl6).

Three metabolic pathways achieved high z-scores: cholesterol biosynthesis (z = 8.9), fatty acid biosynthesis (z = 4.2), and the mitochondrial respiratory chain (z = 6.4). Again, the z-scores are specific for these pathways, and they are much reduced when the groups are widened. For example, considering all genes involved in fatty acid metabolism reduces the z-score from 4.2 to 2.5, suggesting that our statistical analysis correctly identifies significantly coregulated genes specific to the anabolic pathway.

Comparison with an aortic banding mouse model. Using the LocusLink gene symbol field as a link between the rat and mouse annotation databases and the Resourcerer program for matching Affymetrix GeneChips (http://pga.tigr.org/tigr-scripts/magic/r1.pl), we compared our results to those of aortic-banded mice, published by the Harvard Cardiovascular Genomics group (8, 19) (Table 4 and Supplemental Table S1, Physiol Genomics • VOL 18 • www.physiolgenomics.org

Table 3. Pathway analysis of genes affected by FN in NRVM

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<tr>
<td>Name</td>
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<tr>
<td>Unique genes, present</td>
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</table>

Pathway analysis and z-score calculation was performed by comparing the frequency of genes upregulated by FN in selected groups vs. the overall frequency of upregulation in the entire universe of unique genes expressed as described in MATERIALS AND METHODS. Groups were defined by MAPP pathway software, or by searching the RAE230A array annotation database. "FN/C ≥ 1.5" represents the number of genes in the group that were induced by FN by a factor of 1.5 or greater. "Present" means that the genes were called “present” by GeneChip software detection algorithm in at least one array.
Table 4. Genes upregulated in in vivo hypertrophy but not in purified NRVM

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<th>Symbol</th>
<th>Title</th>
<th>Avg FN</th>
<th>Avg FN/C</th>
<th>Base</th>
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<th>4 h</th>
<th>24 h</th>
<th>48 h</th>
<th>1 wk</th>
<th>8 wk</th>
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<td>0.7</td>
</tr>
<tr>
<td>Hspca</td>
<td>Heat shock 90-kDa protein 1, alpha</td>
<td>2,996</td>
<td>0.8</td>
<td>1,624</td>
<td>1.7</td>
<td>2.4</td>
<td>2.5</td>
<td>1.4</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Mlf</td>
<td>Macrophage migration inhibitory factor</td>
<td>517</td>
<td>1.2</td>
<td>215</td>
<td>0.6</td>
<td>1.2</td>
<td>1.6</td>
<td>1.9</td>
<td>3.6</td>
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<tr>
<td>Mlp</td>
<td>MARCKS-like protein</td>
<td>1,049</td>
<td>0.9</td>
<td>320</td>
<td>1.1</td>
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<td>2.4</td>
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<td>1.9</td>
<td>1.2</td>
</tr>
<tr>
<td>M22</td>
<td>Metallothionein-II (MT-II)</td>
<td>1,521</td>
<td>0.7</td>
<td>3,463</td>
<td>2.4</td>
<td>2.0</td>
<td>4.4</td>
<td>1.9</td>
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<tr>
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<td>367</td>
<td>0.8</td>
<td>789</td>
<td>1.5</td>
<td>1.8</td>
<td>2.5</td>
<td>1.6</td>
<td>1.3</td>
<td>1.2</td>
</tr>
<tr>
<td>Timp1</td>
<td>Tissue inhibitor of metallocproteinase 1</td>
<td>654</td>
<td>1.1</td>
<td>1,468</td>
<td>0.7</td>
<td>0.9</td>
<td>3.8</td>
<td>9.4</td>
<td>2.5</td>
<td>1.0</td>
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</table>

Selected genes that were upregulated in an aortic banding mouse model of cardiac hypertrophy (8, 19) but were not induced in NRVM stimulated with FN are shown. "Avg FN" indicates average level of expression after normalization in FN-treated NRVM. "Avg FN/C" indicates average ratio of expression in FN-treated vs. control animals. "Base" indicates average level of baseline expression in sham-operated animals. Numbers represent the ratio of gene expression in aortic-banded vs. sham-operated animals. Values ≥1.5 are in bold font.

available at the Physiological Genomics web site).1 In this mouse model of cardiac hypertrophy, gene expression arrays were done on RNA obtained from hearts at 1 h, 4 h, 24 h, 48 h, 1 wk, and 8 wk postaortic banding and compared with sham-treated control animal (8, 19). We were able to match 4,304 Bgn, 5,779 Col1a, 614 Empl, 797 Fbn1, 3,337 Fn1, 206 Hsp1a, 2,996 Hspca, 517 Mlf, 1,049 Mlp, 1,521 M22, 367 Oazn, 654 Timp1 by phosphorylation (7) and showed unchanged mRNA levels of these translation factors in cardiac hypertrophy. Interestingly, elongation factor eEF2 is regulated posttranscriptionally.

On the other hand, we identified 124 genes significantly upregulated both in the primary NRVM culture model and in vivo (Supplemental Table S1). These include genes involved in apoptosis and cell cycle (8), calcium handling (9), metabolism (20), signal transduction (14), transcription (8), translation (19), and associated with the cytoskeleton or the sarcomeres (20). Many of the genes representing the pathways identified on Table 3 were also induced in vivo (Supplemental Table S1).

**Protein synthesis.** Ribosomal proteins represented the group affected the most by FN, in part because of their large number, resulting in the highest z-score (Table 3). Since many of the ribosomal proteins were also induced in the mouse aortic banding model (Supplemental Table S1), these data suggest that ribosomal protein abundance increases in hypertrophic cardiac myocytes. In addition, we measured significant increases in the mRNA abundance of subunits of translation factors eIF2, eIF3, and eEF1 and in aminoacyl-tRNA synthetases Vars2, Cars, and Sars (Supplemental Table S1). To our knowledge, this is the first report suggesting the involvement of these translation factors in cardiac hypertrophy. Interestingly, elongation factor eEF2 is regulated posttranscriptionally by phosphorylation (7) and showed unchanged mRNA levels in our experiments.

**Protein degradation.** We have identified the proteolytic ubiquitin-proteasome pathway as a target for upregulation by FN (Table 3). The z-scores for ubiquitin and proteasome-related genes were 3.4 and 2.6, respectively. These relatively low z-scores reflect the fact that several ubiquitin and proteasome-related genes were not upregulated, or only slightly.

1The Supplementary Material for this article (Supplemental Table S1) is available online at http://physiolgenomics.physiology.org/cgi/content/full/00104.2004/DC1.
upregulated, by FN. Two of those genes (Uchl1, Usp18) were also upregulated in vivo (Supplemental Table S1). Uchl1 codes for a carboxy-terminal ubiquitin esterase involved in removing ubiquitin from small adducts, whereas Usp18 is a member of the ubiquitin-specific proteases that disassemble polyubiquitin chains into free monomeric ubiquitin. Additional genes induced by FN and involved in the ubiquitin-proteasome pathway included ubiquitin proteases (Usp1, Usp2, Usp4, Usp10, Usp15, Usp39, Usp49), ubiquitin precursors (FAU, Uba52, Ubb, Ubc), members of the E2-ligase complex (Ube2d2, Ube2j1, Ube2v1, Ube2v2), E3-ligase and cofactors (Sugt1, Rbx1, Rnf7, Spop, Fbx16, Fbx07, Fbxo22, Fbxw5, Tceb2, Cop1), and components of proteasome 19S (Psmc2, Psmc3, Psmc5, Psmd1, Psmd9–11, Psmd13) and 20S (Psma7, Psmb3–6, Psmb8, Psmb10) subunits (results not shown).

Metabolic pathways. Virtually all of the expressed cholesterol biosynthesis genes present in the RAE230A array were significantly induced by FN (Fig. 3). A rate-limiting enzyme, the P-450 cytochrome CYP51, responsible for the sterol 14α-demethylase reaction, is coded by one of the genes with the highest level of induction by FN (Table 1). Several genes in the cholesterol biosynthesis pathway (Cyp51a1, Dhcr7, Hmgcs1, and Sgcs) were also induced by aortic banding (Supplemental Table S1). We confirmed the induction of several of the cholesterol biosynthesis genes by RT-PCR (Table 2).

Changes in fatty acid metabolism have been observed in cardiac hypertrophy. These usually involve a decrease in fatty acid β-oxidation and reversion to the fetal phenotype of glucose utilization as a major source of energy. We did not observe any major changes in glucose metabolism or fatty acid oxidation gene expression, although we did see a minor decrease in Cpt1b (0.8-fold), the muscle isoform of carnitine palmitoyltransferase and rate-limiting enzyme for transporting long-chain fatty acid into the mitochondria, as well as a decrease in long-chain fatty acid-CoA ligases Fac12 (0.8-fold) and Fac13 (0.6-fold) and fatty acid transporter CD36 (0.7-fold). In addition, we observed a mild increase in the insulin-insensitive glucose transporter Slc2a1 (GLUT1, 1.4-fold), whereas the insulin-regulated transporter Slc2a4 (GLUT4) was undetectable. These results are consistent with a shift from exogenous fatty acid oxidation to glucose metabolism for energy production. In addition to a decrease in fatty acid oxidation, our data suggest a potential increase in fatty acid synthesis. We detected significant amounts of fatty acid synthase (Fasn) mRNA (normalized levels = 1.028) in induced NRVM, and induction by FN was 2.4-fold. Several other genes in the fatty acid biosynthesis/elongation/desaturation pathway were also present at high levels and induced by FN (z = 4.2, Table 3 and Supplemental Table S1).

Fig. 3. Diagram of cholesterol biosynthesis pathway gene expression changes induced by FN in cardiac myocytes. The diagram was adapted from files available at the GenMAPP website (http://www.genmapp.org), originally created by Michael Lieberman and Manny Ramirez. The diagram was modified to match the RAE230A probe sets and several new genes and interactions were introduced. Genes induced ≥1.5-fold are shown in orange, genes induced ≥2.0-fold are shown in red, and genes with expression changes between 0.7 and 1.5 are shown in yellow. Genes in gray were not called “present” by the GeneChip software, whereas genes in white are not represented by any probe sets in the RAE230A array. The red arrows represent genes known to be induced by the transcription factor SREBP1, whereas the broken red arrow shows the gene (mevalonate kinase) affected by SREBP2 but not SREBP1.
Induction of cholesterol and fatty acid biosynthesis in other cell types has been linked to activation of the transcription factors SREBP1 and SREBP2. Microarray analysis of mouse liver mRNA expression in SREBP1a and SREBP2 transgenic mice and SREBP cleavage-activating protein (SCAP) knockout mice revealed 33 genes that are targets of both SREBP1a and SREBP2. 18 genes activated by SREBP1a but not SREBP2, and 10 genes that are targets of SREBP2 only (23). When we applied the pathway analysis to the 38 SREBP1a target genes represented in the RAE230A array and expressed in NRVM, 32 were induced ≥1.5-fold by FN (Table 3). These genes include all of the 17 cholesterol biosynthesis pathway genes in Fig. 3, plus the LDL receptor, involved in cholesterol uptake, INSIG1, a repressor of SREBP induced by negative feedback when adequate sterol levels are present in the cell (23), SREBP1 itself, 9 fatty-acid synthesis genes (Me1, Acacb, Acac, Fasn, Acat2, Elovl6, Faci5, Fads1, Fads2), and 3 other genes (Gstt3, Mac30, Tkt).

Respiratory chain. Induction by FN of several electron transporters and other genes involved in the mitochondrial respiratory chain was observed in our experiments (z = 6.4, Table 3). These include genes in the NADH-ubiquinone oxidoreductase complex I (15 of 25 expressed genes were induced), ubiquinol cytochrome c reductase complex III (7 of 11 induced), cytochrome c oxidase complex IV (10 of 16 induced), and ATP synthase complex V (6 of 16 induced). Complex IV includes Cox17, a protein essential for the assembly of functional cytochrome c oxidase (CCO) and for delivery of copper ions to the mitochondrion, which was induced 1.9-fold by FN. Interestingly, other genes involved in regulation in copper transport and metabolism were also induced by FN, including Slc31a1, a copper transporter, and Atox1, a copper chaperone (Supplemental Table S1).

Cytoskeletal and sarcomeric proteins. Our statistical analysis of pathways affected by FN in cardiac myocytes uncovered significant changes in genes involved in microtubule assembly and sarcomeric proteins (Table 3). FN induced α-tubulin genes Tubα1 and Tubα4, β-tubulin genes Tubb3, Tubb2, and Tubb5, as well as microtubule-associated proteins Mtap1α and Mtap4 (Supplemental Table S1) and tubulin- and microtubule-maintenance chaperones, including T-complex subunits 1, 3, 4, 5, and 7 and tubulin cofactors A, B, D, and E (not shown).

In contrast to microtubule proteins, actins and actin polymerization genes as a group were not significantly altered by FN (z = 1.2). However, several genes playing important roles in actin polymerization were upregulated, including Actn1, Cfl1, Cnn2, Coro1c, Cyr61, Enah, Fbln2, and Tagln, drebrin 1, thymosin β4x, filamin C, and Arp2/3 complex members (Supplemental Table S1). Interestingly, melusin, a β1-integrin binding protein linking mechanical stretch and cytoskeletal dynamics in cardiac myocytes (6), was induced 2.1-fold by FN. These results suggest that changes in actin polymerization and cytoskeleton dynamics may result from FN-induced cardiac hypertrophy.

Genes coding for sarcomeric proteins were also significantly induced by FN (Table 3 and Supplemental Table S1). These include the fetal isoforms αSKA (Acta1) and cardiac BMHC (Myh7), well known to be upregulated in cardiac hypertrophy, as well as myosin light chains, cardiac myosin-binding protein C, troponin C and T, and tropomyosin genes (Supplemental Table S1). The tropomyosin genes are regulated by alternative splicing in a tissue-specific manner (57), and the RAEC230A array probe sets can distinguish some of the alternatively spliced isoforms. For example, probe set 1370287_a_at recognizes the smooth muscle-specific exon 9d and showed an expression level of 1.157 and induction of 1.3-fold by FN, whereas probe set 1368724_a_at recognizes the striated muscle-specific exon 9b and showed an expression level of 16,414 and induction of 2.1-fold by FN in cardiac myocytes. Our results suggest that transcriptional upregulation of these sarcomeric genes beyond the general increase in transcription accompanying hypertrophy underlie not only increased sarcomeric assembly, but also actual shifts in the isoform composition of the sarcomeres.

DISCUSSION

In this study we have confirmed that FN induces a hypertrophic phenotype in NRVM characterized by increased cellular area, enhanced myofibrillogenesis, and sarcomeric assembly, together with gene expression changes, including expression of fetal genes such as ANP, BNP, αSKA, and βMHC. Moreover, we reported for the first time the results of global gene expression analysis in hypertrophic primary cardiac myocytes using high-density oligonucleotide microarrays. Our data uncovered several pathways induced by FN and provide a framework for comparing whole heart gene expression changes associated with in vivo cardiac hypertrophy with expression changes intrinsic to cardiac myocytes. The statistic pathway analysis we used allowed us to distinguish noise from truly induced genes, even when the induction was as small as 1.5-fold. Our data, together with other studies in culture and in vivo, confirm that hypertrophy involves global changes in biosynthesis pathways, including increased transcription, translation, and synthesis of cholesterol and fatty acids, which are consistent with the significant increases in cell size and complexity characteristic of hypertrophy. In addition, hypertrophy is accompanied by induction of specific genes, such as those involved in the sarcomeric assembly and the natriuretic peptides, which are upregulated to levels beyond the global increase in transcription and translation.

One important feature of hypertrophic cardiac myocytes is increased protein synthesis, achieved in part by an increase in translational capacity. The increase in translational capacity is supported by our observation of FN-induced expression of ribosomal protein genes, resulting in the highest z-score of the pathways examined (Table 3). It is known that hypertrophy and integrin activation increases the translational efficiency of ribosomal protein mRNAs (3, 54). Therefore, it is likely that the abundance of ribosomal proteins increases during hypertrophy due to increased transcription and increased translation. Together with increased transcription of the 45S rRNA precursor (2, 36), the resulting increased translational capacity allows for enhanced protein synthesis required for hypertrophic growth.

In addition to the increase in translational capacity, hypertrophic cardiac myocytes are characterized by increased translational efficiency. The rate-limiting step of regulation of translation efficiency involves the regulation of the activity of translation initiation factor eIF4E, which modulates the formation of the eIF4F complex and affects the affinity of mRNA for the ribosome. We did not detect a change in mRNA abundance.
levels of eIF2 and eIF3, which are involved in recruitment of eIF4E, but this protein is mostly regulated by phosphorylation and association with 4E-binding proteins in cardiac myocytes (55). However, we did measure increases in mRNA levels of eIF2 and eIF3, which are involved in recruitment of capped mRNAs to the ribosome, eEF1 subunits α1, β, γ, and δ, which bind aminoacyl-tRNA complexes (7), and valine, serine, and cysteine-tRNA synthetases, suggesting that synthesis of certain aminoacyl-tRNA and their recruitment to the ribosome are limiting in hypertrophic cardiac myocytes.

The robust increase in protein synthesis during cardiac myocyte hypertrophy is accompanied by moderate increases in protein degradation, resulting in higher protein turnover and net accumulation of proteins. The ubiquitin-proteasome pathway plays the major role in proteolytic degradation of misfolded or damaged proteins, turnover of short-lived proteins, and also of sarcomeric proteins (17). We have identified the ubiquitin-proteasome pathway in cardiac myocytes to synthesize and elongate fatty acids has been poorly studied, since the heart is thought incapable of cardiac myocytes to synthesize fatty acids (16, 21). In our NRVM cultures, we have detected labeled linoleic acid (34), and there is no satisfactory explanation for the heart (21). In our NRVM cultures, we have detected significant mRNA expression and induction by FN of fatty acid synthetase (Fasn) and other genes in the fatty acid biosynthesis pathway. Other studies have detected Fasn mRNA in the heart and reported regulation by starvation (28), glucocorticoids (48), and thyroid hormone (4). It is possible that in the perfused adult heart (16, 21) fatty acid synthesis is undetectable, but in cultured NRVM fatty acid synthesis plays a role in hypertrophic growth.

Since all of the cholesterol biosynthesis genes and nine fatty acid biosynthesis genes induced by FN are also known targets of transcription factor SREBP1 (23), our data suggest that SREBP1 may be co-coordinating the induction of these two pathways to maintain the proper balance of cholesterol and phospholipid-associated fatty acids in hypertrophic cardiac myocytes. We are currently testing the hypothesis that activation of SREBP1 during cardiac hypertrophy is responsible for the induction of transcription of cholesterol and fatty acid biosynthesis genes.

Induction of respiratory chain components has not been well studied in hypertrophic myocytes. ATP synthase activity is induced by increased contraction and inotropic agents in cardiac myocytes (13), and ATP synthase subunit c mRNA was induced by norepinephrine in the rat heart (32). In our experiments we observed induction of components I, III, IV, and V of the mitochondrial respiratory chain. The highest induction in the ATP synthase complex F1 was observed in subunit e (2.4-fold). Interestingly, regulation of subunit e by hypoxia at the pretranslational level has been previously observed in cardiac myocytes (31). It is possible that transcriptional induction of several limiting components of the respiratory chain is required to meet the increased energy demand of the hypertrophic myocytes.

Given the recently identified role of calcineurin in hypertrophy (37), it is interesting that FN did not induce the expression of any of the isoforms of calcineurin or the calcineurin/NFAT-target gene Dscr1, which can be induced by mechanical stretch or adrenergic stimulation (49, 53). On the other hand, FN induced calmodulin 1 (Supplemental Table S1), which activates calcineurin in response to elevated intracellular Ca^{2+} levels. FN also induced the calcineurin binding protein myoznin-2 (calsarcin-1), which tethers calcineurin to the Z-line of sarcomeres (18), calcium/calmodulin-dependent protein kinase I, which synergizes with calcineurin in promoting hypertrophy (43), and frequent homolog, which can substitute for calmodulin and regulate cardiac Ito K^+ channels (38). It is possible that FN induces a permissive state for calcineurin activation but is insufficient for the full induction of calcineurin, which may require increased intracellular Ca^{2+} levels induced by mechanical stretch or adrenergic agonists.

We have identified tubulins and microtubule-associated proteins, such as Mtap4, as targets of FN-induced upregulation. Microtubule density is increased in cardiac hypertrophy and may interfere with contractility and play a role in heart failure progression (25, 46, 47, 50). Mtap4 is a microtubule-stabilizing protein and is thought to be involved in microtubule densification during cardiac myocyte hypertrophy (46, 50). Interestingly, we also observed induction by FN of several chaperones implicated in tubulin and microtubule maintenance. These results suggest that both pretranslational and posttranslational mechanisms are involved in tubulin upregulation and microtubule stabilization in hypertrophic cardiac myocytes.

FN induced the connective tissue growth factor (CTGF) gene, coding for a cysteine-rich, glycosylated protein that regulates proliferation of fibroblast and other mesenchymal cells and induces secretion of extracellular matrix proteins. CTGF is induced by TGF-β in cardiac fibroblasts and myocytes and is thought to play a role in heart fibrosis (9). FN also upregulated WNT1 inducible signaling pathway protein 2 (WISP2), another member of the CTGF family, but its expression in the heart has not been previously reported. Another extracellular protein induced by FN, the secreted acidic cysteine-rich glycoprotein (osteonectin, SPARC), is abundant in remodeling tissues and in diseases associated with fibrosis. It was reported to be induced by β-adrenergic stimulation in the myocardium of adult rats (35) and in a transgenic mouse model of hypertrophy (24). It is tempting to speculate that secretion of the cysteine-rich proteins SPARC, CTGF, and WISP2 by cardiac myocytes may be responsible in part for the fibroblast proliferation and fibrosis associated with pathological hypertrophy and heart failure in vivo and may explain the increased mRNA levels of various collagen genes, FN, biglycan, and
other extracellular matrix genes, which were not induced by FN in purified NRVM but were upregulated in the mouse aortic banding model (Table 4).

In summary, our model of FN-induced hypertrophy together with our statistical analysis of gene expression patterns and comparison with the mouse aortic banding model revealed several pathway and gene expression changes not previously associated with cardiac hypertrophy. Our studies also appear to distinguish cardiac myocyte-specific from non-myocyte-dependent pathways. Further work is required to determine how critical these gene expression changes are for a complete cardiac hypertrophy phenotype and to evaluate how modulation of these changes may affect progression to heart failure.

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GRANTS

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REFERENCES


FIBRONECTIN-INDUCED CARDIOMYOCYTE HYPERTROPHY


