Microarray analysis reveals pivotal divergent mRNA expression profiles early in the development of either compensated ventricular hypertrophy or heart failure

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Buermans, Henk P. J., Everaldo M. Redout, Anja E. Schiel, René J. P. Musters, Marian Zuidwijk, Paul P. Eijk, Cornelis van Hardeveld, Soemini Kasanmoentalib, Frans C. Visser, Bauke Ylstra, and Warner S. Simonides. Microarray analysis reveals pivotal divergent mRNA expression profiles early in the development of either compensated ventricular hypertrophy or heart failure. Physiol Genomics 21: 314–323, 2005. First published February 22, 2005; doi:10.1152/physiolgenomics.00185.2004.—Myocardial right ventricular (RV) hypertrophy due to pulmonary hypertension is aimed at normalizing ventricular wall stress. Depending on the degree of pressure overload, RV hypertrophy may progress to a state of impaired contractile function and heart failure, but this cannot be discerned during the early stages of ventricular remodeling. We tested whether critical differences in gene expression profiles exist between ventricles before the ultimate development of either a compensated or decompensated hypertrophic phenotype. Both phenotypes were selectively induced in Wistar rats by a single subcutaneous injection of either a low or a high dose of the pyrrolizidine alkaloid monocrotaline (MCT). Spotted oligonucleotide microarrays were used to investigate pressure-dependent cardiac gene expression profiles at 2 wk after the MCT injections, between control rats and rats that would ultimately develop either compensated or decompensated hypertrophy. Clustering of significantly regulated genes revealed specific expression profiles for each group, although the degree of hypertrophy was still similar in both. The ventricles destined to progress to failure showed activation of pro-apoptotic pathways, particularly related to mitochondria, whereas the group developing compensated hypertrophy showed blocked pro-death effector signaling via p38-MAPK, through upregulation of MAPK phosphatase-1. In summary, we show that, already at an early time point, pivotal differences in gene expression exist between ventricles that will ultimately develop either a compensated or a decompensated phenotype, depending on the degree of pressure overload. These data reveal genes that may provide markers for the early prediction of clinical outcome as well as potential targets for early intervention.

compensated hypertrophy; early time point; mitogen-activated protein kinase signaling; apoptosis

MYOCARDIAL VENTRICULAR HYPERTROPHY is a major risk factor that is associated with a higher incidence of clinical events, morbidity, and mortality from cardiovascular disease (32), generally leading to heart failure. Right ventricular (RV) hypertrophy is a general adaptive mechanism of the heart to increased workload, resulting from, i.e., chronic pulmonary hypertension, valvular disease, or left ventricular (LV) dysfunction. RV hypertrophy is aimed at normalizing ventricular wall stress and, as in the case of LV overload, depending on the degree or duration of the pressure overload, RV hypertrophy may progress from a compensated state into a pathological state with progressive myocyte apoptosis and compromised cardiac function, ultimately leading to congestive heart failure (6). Multiple signal transduction pathways are known to be involved in the remodeling of the heart (15), and hypertrophy and failure are characterized by varying degrees by changes in extracellular matrix composition, energy metabolism, contraction, adrenergic signaling, and calcium handling (5). Which of these changes is critical in the ultimate transition to heart failure is a matter of debate. More importantly, it remains unclear which signaling pathway or combination of signaling pathways mediate the development of pathological hypertrophy and whether the pathological phenotype is induced early during overload as a function of the degree of mechanical overload, or only as a secondary response when compensation is insufficient (15, 35).

To address this question, we used the established monocrotaline (MCT) model of chronic pulmonary hypertension and subsequent RV hypertrophy to determine gene transcription profiles early in the development of compensated and decompensated hypertrophy (8). MCT is a pyrrolizidine alkaloid, and its bioactive metabolite, which has a short half-life, selectively injures the vascular endothelium of the lung and induces pulmonary vasculitis and an increase in vascular resistance and pulmonary arterial pressure (49). With the MCT doses typically used in rats, the RV hypertrophy progresses to failure and death around day 28 in most animals, but up to 50% of the animals may develop compensated RV hypertrophy with no signs of failure (1, 2, 4, 40, 47, 50). Detailed analyses of the development of heart failure in this model have shown that critical changes in contractile function and expression of proteins involved in β-adrenergic signaling and calcium handling do not become apparent until after 3 wk (28, 31). Furthermore, pressure-dependent activation of pro-apoptotic pathways in the hypertrophied RV is suggested to underlie the transition to failure around this time (10). However, comparative analyses of the compensated and heart failure groups are few and are limited to late stages when the separate groups can be identified (1, 4, 40, 47). Following earlier work in our laboratory (29, 45, 47), we have now refined the MCT model to allow the selective...
induction of either compensated or decompensated RV hypertrophy by using a low or a high dose of MCT, respectively. Animals developing the pathological, decompensated phenotype die of congestive heart failure (CHF) 4 wk after MCT injection, whereas animals in the low-dose group developed a stable compensated hypertrophic phenotype (HYP) showing no signs of failure. Because the outcome of the hypertrophic process is known for both groups, early stages in the development of compensated and decompensated hypertrophy can be investigated with this model before phenotypic differences become apparent.

In the present study, we used spotted oligonucleotide microarrays representing 4,803 known genes to analyze ventricular gene expression of control, HYP, and CHF animals early at the onset of myocardial ventricular hypertrophy, i.e., 2 wk after MCT administration. Analysis of the expression profiles revealed quantitative but also qualitative differences in gene expression in the hypertrophying RV that may be crucial for the ultimate development of either the compensated or the decompensated state.

METHODS

Animals. Animals were treated according to national guidelines and with the permission of the Institutional Animal Care and Use Committee of the Vrije University Medical Center. Male Wistar Rats, weighing 180–200 g (Harlan, Zeist, The Netherlands) were housed individually (250 cm²/animal) and received food and water ad libitum.

A total of 58 animals were randomly assigned to three groups. All animals received a single subcutaneous injection with either saline [control group (CON), n = 18], 30 mg MCT/kg body wt (HYP, n = 20), or 80 mg MCT/kg body wt (CHF, n = 20). Two weeks after treatment, 12 animals from each group were randomly picked and killed with a halothane overdose, and hearts and lungs were excised. A total of 2 ml of blood were taken and stored for subsequent measurement of plasma thyroid hormone level (T₃) by specific RIA as previously described (22). Hearts were rinsed by perfusion, and the LVs, RVS, and septum were separated. All tissues were weighed and snap frozen in liquid nitrogen and stored at −80°C. At 4 wk after treatment, the remaining animals were killed and blood samples, hearts, and lungs were collected.

Total RNA isolation. Total RNA isolation from the left and right ventricles from each animal was performed, using the TRIZol (Invitrogen) method according to the manufacturer’s protocol. Total RNA was quantified by A₂₆₀ measurement, and the A₂₆₀/A₂₈₀ ratio was used to check for possible contaminations. Sample integrity was also checked on a Agilent 2100 bioanalyzer. A common reference pool was constructed by pooling equal amounts of RNA from all samples used in the microarray experiments. Samples were dissolved in 100% formamide and stored at −80°C before use. RNA from six LVs, RVS, and septum were separated. All tissues were weighed and snap frozen in liquid nitrogen and stored at −80°C. At 4 wk after treatment, the remaining animals were killed and blood samples, hearts, and lungs were collected.

Total RNA isolation. Total RNA isolation from the left and right ventricles from each animal was performed, using the TRIZol (Invitrogen) method according to the manufacturer’s protocol. Total RNA was quantified by A₂₆₀ measurement, and the A₂₆₀/A₂₈₀ ratio was used to check for possible contaminations. Sample integrity was also checked on a Agilent 2100 bioanalyzer. A common reference pool was constructed by pooling equal amounts of RNA from all samples used in the microarray experiments. Samples were dissolved in 100% formamide and stored at −80°C before use. RNA from six LVs, RVS, and septum were separated. All tissues were weighed and snap frozen in liquid nitrogen and stored at −80°C. At 4 wk after treatment, the remaining animals were killed and blood samples, hearts, and lungs were collected.

Quantitative real-time PCR. Gene-specific primers (Invitrogen) were designed with Primer Express v.2.0 to generate amplicons with a length of 75–125 bp spanning exon-exon junctions. Primer pairs were checked for a linear response over a large cDNA input range and for nonspecific products with dissociation curves and 2% agarose gel electrophoresis. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as an internal control to normalize gene expression.

A total of 5 μg total RNA were used to generate cDNA strands in a 20-μl reaction volume, using the Cloned AMV First Strand Synthesis Kit (Invitrogen). An equivalent of 25 ng of total RNA was subsequently used in the amplification with 50 nM gene-specific primers and 4 μl of CYBR green master mix (Applied Biosystems) in a total volume of 8 μl using standard cycle parameters on an Applied Biosystems model 7700.

Western blotting. RV and LV tissue was homogenized in modified one-dimensional buffer [15% glycerol, 1% SDS, 0.0625 M Tris-HCl, pH 6.8, 500 μg/ml DTT (MP Biochemicals), 500 μg/ml Pefabloc SC (Roche), and 5 μl/ml phosphatase inhibitor cocktail-1 (Sigma)]. Samples were then sonicated for 30 min, incubated at 80°C for 5 min, and centrifuged for 20 min at 14,000 rpm. The protein samples were immunoblotted for phospho-p38 and MAPK phosphatase-1 (MKP-1; Santa Cruz Biotechnology).

Western blotting reagents were purchased from Amersham International. Equal amounts of protein (50 μg, bicinchoninic acid assay) were separated by electrophoresis (10% SDS-PAGE) and transferred to nitrocellulose membranes. Protein transfer was confirmed by Ponceau staining of the blot. Membranes were blocked with 5% nonfat milk in TBS-T (Tris-buffered saline plus 1% Tween) for 1 h at room temperature. Phosphospecific p38 and MKP-1 antibodies were separately incubated in TBS-T containing 5% nonfat milk (Bio-Rad) overnight. After membranes were washed with TBS-T, they were incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase for 1 h at room temperature. Bands were visualized by enhanced chemiluminescence and quantified using a FujiFilm LAS 3000 laser densitometer.

Microarray printing, probe generation, hybridization, and washing. The Compugen Rat OligoLibrary was purchased from Sigma-Aldrich. Oligos were resuspended to a concentration of 20 μM in a 150 mM sodium phosphate buffer, pH 8.5, and printed on Motorola CodeLink-activated slides with an Amersham Pharmacia Biotech Spotter. Features were spotted as duplicates on the microarray, with pairs divided over two different regions.

cDNA probes were generated from 100 μg of total RNA, with an oligo(dT) [(dT)20-VN] primer (Invitrogen) and SuperScript II Reverse Transcriptase (Invitrogen), with incorporation of aminomethyl-dUTP. Probes were indirectly labeled with Fluorolink Cy3 (sample) or Cy5 (reference pool) monofunctional dyes.

Hybridization protocol was adapted from Snijders et al. (41) with minor modifications. In brief, Cy3- and Cy5-labeled probes were overnight at 4°C. After membranes were washed with TBS-T, they were incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase for 1 h at room temperature. Band intensities were quantified using a FujiFilm LAS 3000 laser densitometer.
software. Array images were processed with Imagene v.5.1. Flagged features were excluded from further analysis. Mean Cy3 and Cy5 intensities were background corrected. Because there was only a very small location effect, the duplicate signals for each gene were averaged. The mean Cy3 and Cy5 channel intensities were balanced, and the Cy3-to-Cy5 log2 ratios were standardized to a Z-score with the fitted value as mean (μ) and the mean absolute distance as standard deviation (σ). Genes were considered to be suitable for further analysis if, for all three RV groups, genes were present in at least three of the six arrays per group. For the LV analysis, each gene had to be present in all six LV arrays to be accepted. These data have been deposited in the Gene Expression Omnibus (11) as series GSE1652.

Significance analysis. Clustering of the significantly regulated genes was performed on the basis of their significant differences, as determined in the three pairwise comparisons. If there was a significant difference between the CON and HYP group, it was denoted with a “1,” and if there was no difference observed, a “0.” This was also done for CON vs. CHF and HYP vs. CHF, respectively. These 1 and 0 notations were then combined as x-or-r, representing the CON vs. HYP, CON vs. CHF, and HYP vs. CHF comparisons, respectively. With three pairwise comparisons, there are a total of seven combinations possible. A gene with no significant difference between CON and HYP (0), a significant difference between CON and CHF (1), and a difference between HYP vs. CHF (1) would then be assigned to cluster 0-1-1. In the remainder of this article, these clusters will be referred to as clusters I–VII (see Table 3).

Note that this is a method that clusters only on the basis of significant differences between groups. It does not take the direction of change into account, i.e., up- and downregulated genes can be assigned to the same cluster.

Initially, differential expression between the CON, HYP, and CHF groups was assessed with the significance analysis of microarrays program, SAM (43). However, in contrast to 33 upregulated genes, only 4 were found that were downregulated (data not shown). This imbalance in the direction of regulation led to a search for alternative analysis methods for our dataset.

According to the theorem of Stouffer-Hemelrijk (42), the sum of $n$ standardized log ratios has a normal distribution with a mean of zero and a standard deviation of $\sqrt{n}$. So its quotient $Z = \frac{\sum z_i}{\sqrt{n}}$ has a standard normal distribution with a mean of zero and a standard deviation of 1. If

$$z_i(z_1, z_2, \ldots, z_n) \sim N(0,1)$$

then its sum

$$\Sigma z_i = z_1 + z_2 + \ldots + z_n \sim N(0, \sqrt{n})$$

and

$$\text{mean} Z_{\text{CON,HYP or CHF}} = \Sigma z_i/\sqrt{n} \sim N(0,1)$$

The difference between two pairwise conditions was tested on significance by Z-score in the following way

$$Z\text{-score} = (Z_2 - Z_1)/\sqrt{2} \sim N(0,1)$$

For each group, the mean $Z$ was calculated. Gene expression was considered to be significantly different if the absolute Z-score value was >2, which corresponds with a $P$ value < 0.025.

Statistical analysis for real-time PCR and hypertrophic parameters. Relevant parameters were evaluated for significant differences between groups with a one-way ANOVA, using the SPSS v.9.0 statistical package with the Bonferroni correction for multiple testing. Differences with a $P$ value <0.05 were considered significant.

RESULTS

MCT-induced development of compensated RV hypertrophy or heart failure. In previous studies, the use of a single dose of MCT of 40–60 mg/kg typically resulted in a mixed population of animals, developing either compensated hypertrophy or progressing to heart failure (1, 2, 4, 40, 47, 50). We found that administration of a high dose of MCT (80 mg/kg) induced RV hypertrophy that in all cases progressed to CHF. In contrast, 30 mg MCT/kg induced exclusively HYP. Figure 1 shows average growth curves of animals in both groups over a period of 4 wk. MCT administration resulted in a slight reduction of the rate of growth, but animals in the HYP group did not cease to grow, whereas the CHF group started losing weight around day 20. Apart from the progressive weight loss, all animals in this group showed secondary signs of right-sided CHF, including pleural effusion and ascites at the time of death at 4 wk. No such signs were present in any of the animals in the HYP group. In a parallel study, four HYP animals were killed at 12 wk, and none of these animals showed signs of heart failure (not shown).

Table 1 summarizes organ and body weights of animals killed at 4 or 2 wk. The latter group was used in the microarray analysis described below. The significantly greater increase in lung weight in the CHF vs. the HYP group is indicative of the more extensive proliferative pulmonary vasculitis induced by the high dose of MCT (30). The increase is not related to edema, since dry-to-wet weight ratios of lung tissue did not differ between the groups at any time (not shown). Both groups developed a significant degree of RV hypertrophy in response to the increased pulmonary resistance, as evidenced by a 20% increase in the RV-to-LV + septum weight ratio already at 2 wk. The degree of hypertrophy was significantly greater in the CHF group (135%) compared with the HYP group (95%) after 4 wk. In line with the development of severe heart failure, plasma T3 levels were significantly reduced at 4 wk in the CHF group only. In an initial analysis of RV gene expression in this model, we determined mRNA levels of sarcoplasmic-endoplasmic reticulum Ca2+-ATPase (SERCA2a) and atrial natriuretic factor (ANF) as representative hallmark genes regulated in hypertrophy (Table 2). As expected, changes in the levels of SERCA2a and ANF message correlated with the severity of the hypertrophy. The data showed a progressive increase in ANF mRNA at 2 and 4 wk, which reached significance only in the CHF group. The levels of SERCA2a mRNA were reduced at 4 wk, and significantly more so in CHF compared with HYP.
animals. At 2 wk, however, no change was detectable in either group relative to CON.

Taken together, these data indicate that the low and high doses of MCT induce different degrees of pulmonary hypertension and ensuing RV hypertrophy, which in the latter case progresses to failure. A large-scale analysis of ventricular gene expression was next performed in the CON, HYP, and CHF groups at 2 wk to assess early differences in gene expression.

Microarray analysis. For the RV microarrays, 3,010 unique genes of the total 4,803 (62%) passed the required criteria for further analysis. All three RV groups were pairwise analyzed by a Z-test. All Z-test scores with an absolute value > 2 (Z-score > 2.0) were considered to be significant, which resulted in a total of 179 unique genes. Overall, there were more genes upregulated than downregulated in the RV, as can be seen in Table 3.

An analysis of the LV myocardium resulted in 10 differentially expressed genes, 4 of which were also regulated in the RV, although with different expression profiles between the three groups. We considered these LV changes of minor importance in our model.

All further analyses were performed on the 179 RV genes only. A list of all differentially regulated LV and RV genes is presented in Supplemental Table S1 (available at the Physiological Genomics web site).

Gene cluster classification. To classify the identified genes, they were clustered on the basis of their significant differences in expression between the three RV groups. This led to seven clusters (Table 3), of which four contain genes with a significant difference in expression between the RV HYP and RV CHF groups, i.e., clusters I, IV, V, and VI. Expression plots of all seven clusters are shown in Fig. 2, A–G. Genes in these separate clusters may be involved in hypertrophic processes that determine the specific development of the compensated and decompensated phenotypes.

Cluster I (Fig. 2A) contains genes with an expression profile that correlates with the degree of pressure overload, i.e., there is a significantly higher expression in the CHF than the HYP group, both of which are also significantly different from CON levels. Genes like these are likely to be associated with general hypertrophic remodeling processes that appear to be load dependent. Two of these genes, α-skeletal actin (S1) and myosin binding protein C (13), have both been shown to be involved in myocardial hypertrophy. Genes in cluster II (Fig. 2B) show an expression profile that is highly similar to that of cluster I, even though they only display significant differences in the CON vs. CHF comparisons. However, these genes still appear to display a load-dependent gene expression profile. Some downregulated genes in this cluster are cytosolic phospholipase A2, selenoprotein W, and dihydropryridine-sensitive calcium channel-α1, whereas β-catenin (Cantnb), cycdin D1, and α-tubulin were upregulated in CHF relative to CON. Similar to cluster I, cluster III (Fig. 2C) is composed of genes that appear to be associated with a general hypertrophy transcription program; however, their expression level is not sensitive to the difference in load between the HYP and CHF groups. Carmitine palmitoyltransferase-1B (Cpt1b) was downregulated, whereas α-smooth muscle actin was upregulated in this cluster. Cluster IV (Fig. 2D) contains genes that are regulated only in the CHF group. These genes may ultimately be associated with the development of a decompensated phenotype, since they are not differentially expressed in the HYP group at this point in time. In this cluster, several mitochondria-related genes were significantly regulated, i.e., the mitochondrial transcription factor A (mtTFA) was downregulated in contrast to the upregulation of the mitochondrial voltage-dependent anion channel-1 (VDAC1) and mitochondrial adenosine nucleotide translocator-1 (ANT1) genes.

In contrast to cluster IV, cluster V (Fig. 2E) represents those genes that are either up- or downregulated in the HYP group compared with both the CON and CHF groups. These genes may ultimately be associated with the development of compensated hypertrophy, since they are not differentially expressed in CHF. This cluster showed the upregulation of a protein tyrosine phosphatase (GenBank accession no. U02553). A locus-link query on this gene showed that it encodes a dual-specificity phosphatase known as MKP-1. MKP-1 is a predominantly nuclear protein that recognizes and deactivates MAPKs, such as p38-MAPK, by dephosphorylating the threonine and tyrosine residues with the T-X-Y se-

Table 1. Body weight, tissue wet weight, and plasma T3 level at 2 and 4 wk after MCT treatments

<table>
<thead>
<tr>
<th></th>
<th>CON 2 Wk</th>
<th>CON 4 Wk</th>
<th>HYP 2 Wk</th>
<th>HYP 4 Wk</th>
<th>CHF 2 Wk</th>
<th>CHF 4 Wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>284±4</td>
<td>258±5*</td>
<td>244±4*</td>
<td>352±14</td>
<td>332±10</td>
<td>250±6†</td>
</tr>
<tr>
<td>Heart, mg</td>
<td>924±28</td>
<td>835±31</td>
<td>812±28*</td>
<td>980±27</td>
<td>1090±30</td>
<td>1007±38</td>
</tr>
<tr>
<td>Heart/body wt, mg/g</td>
<td>3.25±0.08</td>
<td>3.23±0.08</td>
<td>3.32±0.07</td>
<td>2.80±0.09</td>
<td>3.29±0.11*</td>
<td>4.03±0.09†</td>
</tr>
<tr>
<td>RV, mg</td>
<td>162±8</td>
<td>170±9</td>
<td>167±9</td>
<td>167±11</td>
<td>304±23*</td>
<td>334±11†</td>
</tr>
<tr>
<td>RV/body wt, mg/g</td>
<td>0.57±0.02</td>
<td>0.65±0.03</td>
<td>0.68±0.03*</td>
<td>0.47±0.02</td>
<td>0.93±0.09*</td>
<td>1.33±0.02†</td>
</tr>
<tr>
<td>Lung, g</td>
<td>1.48±0.03</td>
<td>1.54±0.03</td>
<td>1.75±0.05†</td>
<td>1.33±0.11</td>
<td>1.65±0.04*</td>
<td>2.24±0.09†</td>
</tr>
<tr>
<td>Lung/body wt, mg/g</td>
<td>5.12±0.05</td>
<td>5.74±0.08*</td>
<td>6.99±0.10†</td>
<td>3.72±0.15</td>
<td>4.96±0.19*</td>
<td>9.00±0.42‡</td>
</tr>
<tr>
<td>Plasma T3, nmol/l</td>
<td>1.10±0.05</td>
<td>1.20±0.05</td>
<td>1.03±0.05</td>
<td>0.98±0.06</td>
<td>1.00±0.05</td>
<td>0.33±0.06*</td>
</tr>
</tbody>
</table>

Animals were killed 2 or 4 wk after receiving either saline (control; CON), 30 mg monocrotaline (MCT/kg (compensated hypertrophic phenotype; HYP), or 80 mg MCT/kg (congestive heart failure; CHF). Mean values ± SE of body wt, plasma thyroid hormone (T3) level, and tissue wet wt of whole heart, right ventricle (RV), and lung are given, as well as tissue-body wt ratios. RV/LV+S, wet wt ratio of RV over the combined wt of left ventricle (LV) and septum (S). Data were analyzed as described in METHODS; n = 12 for all parameters at 2 wk, except for lung wt (n = 6), and n = 8 for all parameters at 4 wk, except for the CON group (n = 6). *P < 0.05 relative to CON. †P < 0.05 relative to HYP.

1The Supplemental Material for this article (Supplemental Table S1) is available online at http://physiolgenomics.physiology.org/cgi/content/full/00185.2004/DC1.
quencing, MAPK signaling and in particular the activation of p38-MAPK have been implicated in the development of decompensated hypertrophy and heart failure. Consequently, the upregulation of MKP-1 could be critical in adaptive remodeling in the HYP group (see below and DISCUSSION).

Assigning a pathophysiological role to the last two clusters is more difficult. Cluster VI (Fig. 2F) contains genes that are either up- or downregulated in the CHF group relative to the HYP group only, with no significant differences relative to CON. Some of these genes show comparable expression profiles to cluster IV, whereas others show resemblance to genes in cluster V. However, because these genes do show a significant difference between the HYP and CHF group, they may still contribute to the distinct process leading to either compensated hypertrophy or heart failure. A nuclear receptor coactivator, transcriptional intermediary factor-2, was found to be significantly downregulated in CHF relative to HYP, whereas the endothelin receptor B and the calcitonin receptor-like receptor were upregulated in the CHF group.

Finally, cluster VII (Fig. 2G) contains genes that are only regulated in the HYP group relative to the CON group but not significantly different from CHF. This cluster showed a decrease in expression of γ-butyrobetaine hydroxylase (BBH) and platelet-derived growth factor B chain precursor. Thrombospondin-4 and the plasma protein α1-fetoprotein were upregulated in this cluster.

These data indicate that, early in the development of either compensated or decompensated RV hypertrophy, qualitative differences in addition to quantitative differences in mRNA expression already exist between both hypertrophic phenotypes.

p38-MAPK phosphorylation in HYP and CHF. As mentioned in the previous section, MKP-1-mediated deactivation of p38-MAPK might play a role in the differentiation between compensated and decompensated hypertrophy.

Therefore, we first assessed p38-MAPK activation in RV samples from CON, HYP, and CHF animals. Western blot analysis of phospho p38-MAPK levels showed marked activation in the CHF group only (Fig. 3A). This activation was restricted to the RV, since an analysis of the LV showed no difference in phospho p38-MAPK levels among the three groups (Fig. 3B). Next, we examined whether the absence of p38-MAPK activation in the HYP group was associated with increased MKP-1 expression, as suggested by the microarray expression data. Indeed, MKP-1 protein levels in the RV of the HYP group were clearly increased relative to CON levels, with considerably less expression in the CHF group (Fig. 3C).

Real-time PCR gene confirmations. From the significantly regulated genes, 12 were selected to be confirmed by real-time PCR. Nine of these showed expression profiles in agreement with the microarray data, although with occasional differences in the fold changes that were observed (Fig. 4). Three of the twelve genes showed no change in expression (monoamine oxidase A and calpain-3) or a decrease in expression where an increase was expected (SUP-data, not shown).

DISCUSSION

In this study, we used 65-mer spotted oligonucleotide microarrays representing 4,803 known rat genes to probe the ventricular myocardial transcriptome for differential expression of genes at an early stage of development of either compensated or decompensated RV hypertrophy. Although the degree of RV hypertrophy was similar in both MCT-treated groups 2 wk after injections, their gene expression profiles were strikingly different. Clustering revealed expression profiles that were uniquely associated with the development of either the compensated or decompensated hypertrophic phenotype. As we will discuss below, we found genes that were not previously associated with hypertrophy and heart failure as well as genes that are well known to be involved. Some of these may prove to be pivotal in the ultimate development of either compensated hypertrophy or failure, such as the genes involved in MAPK signaling and apoptosis pathways.

These divergent transcription profiles, caused by different degrees of pressure overload, were generated by using a novel adaptation of the MCT model for RV hypertrophy.
Model. The rat MCT model is widely used to study RV hypertrophy in response to a chronically elevated hemodynamic load, with most studies reporting progression of RV hypertrophy to failure and death in the majority of animals around day 28 after a single injection of 40–60 mg MCT/kg. However, with the use of these doses of MCT, up to 50% of the animals may develop stable compensated RV hypertrophy with no signs of failure (1, 2, 4, 40, 47, 50). The animals that do progress to failure show a significantly greater degree of RV hypertrophy and more pronounced changes in RV gene expression, reflecting the development of higher pulmonary arterial pressures (23, 33, 47). A single dose of MCT may thus induce a graded response, which is most likely related to differences between individual animals in rate of MCT metabolism or susceptibility to its bioactive metabolite. The MCT model could therefore provide a valuable tool to study the development of compensated hypertrophy or failure in response to pressure overload, but a comparison of early stages has not been possible, because both groups cannot be reliably distinguished until after 3 wk, when differences become apparent (28, 31).

In the present study, we show that a dose of 80 mg MCT/kg consistently induced RV hypertrophy progressing to CHF, whereas 30 mg MCT/kg induced only compensated RV hypertrophy (HYP). The values for body weight, plasma T3 levels, and index of RV hypertrophy, i.e., RV-to-LV + septum weight ratio, for the CHF group at 4 wk (Table 1) are identical to those reported earlier for the subgroup of animals that developed CHF after a dose of 40 mg MCT/kg (47). Furthermore, the parameters of the animals that developed compensated hypertrophy in that study are identical to those in the present HYP group (Table 1), with a significantly smaller degree of RV hypertrophy in the HYP relative to the CHF group (47). The reduced levels of plasma T3 at 4 wk in the CHF group only may serve as an indicator of the severity of heart failure. In our earlier study, we showed that the ultimate 60% drop in plasma T3 is characteristic of severe nonthyroidal illness and, although in part related to a diminished caloric intake, it is primarily the effect of the condition of heart failure (27). The more pronounced reduction of RV mRNA levels of SERCA2a in CHF vs. HYP at 4 wk (Table 2) also confirms our earlier observation and those made by others (28). At 2 wk we already find significant RV hypertrophy, in line with previous findings (33), but the degree of hypertrophy is the same for both groups. Nevertheless, lung weight was already significantly more increased in the CHF compared with the HYP group (Table 1), indicating more extensive vascular injury and proliferative vasculitis in this group (the weight increase was not associated with edema) (23, 33). A consequently higher hemodynamic load and RV wall stress in this group is indicated by the greater increase in ANF mRNA expression in RV from the CHF group (Table 2), which is in agreement with earlier studies (10, 26, 46). Furthermore, the absence of an effect on SERCA2a expression in either group at 2 wk supports the notion that essential changes in gene expression occur after this time (28, 31).

It cannot be ruled out that the dose of 80 mg/kg has additional effects, compared with the low dose of MCT, that are not related to the degree of pulmonary vasculitis and subsequent RV hypertrophy. However, with respect to ventricular gene expression, such additional systemic effects of MCT...
appear to be marginal, given that only 10 LV genes were differentially regulated in the analysis of all groups. Taken together, the data indicate that our current MCT protocol predictably induces different degrees of pulmonary hypertension, leading to either compensated or decompensated RV hypertrophy and failure.

**Expression profile clusters.** Clustering the regulated RV genes on the basis of their significant differences among the three groups classified them to the seven distinct groups of genes (Fig. 2, A–G). For the majority of the genes, when either up- or downregulated in the HYP relative to the CON group, the expression in the CHF group showed a greater change in the same direction, i.e., clusters I, II, and III. Genes in these clusters appear to be load sensitive and are associated with general hypertrophic remodeling in both compensated and decompensated hypertrophy.

There were two clusters of genes that were uniquely associated with either the compensated hypertrophic phenotype (cluster V) or decompensated phenotype (cluster IV). This indicates that heart failure is not merely a result of quantitative gene expression differences, but that there is a certain group of genes the expression of which is associated with the development of either maladaptive ventricular remodeling or adaptive hypertrophy (see below). Our data indicate that, although the compensated and decompensated myocardium do have a lot of regulated genes in common, their expression profiles are in fact strikingly different.

**Altered gene expression in HYP and CHF.** In addition to several well-known genes involved in hypertrophic remodeling, e.g., α-smooth muscle actin, α-skeletal actin, and α-tubulin (5), several other genes emerged as well. In pressure overload-induced cardiac hypertrophy and heart failure, myocardial energy utilization is switched from fatty acids to glucose as a substrate. In line with this, Cpt1b and BBH were downregulated in clusters III and VII. Cpt1b is situated in the outer mitochondrial membrane, where it is involved in the transport of long-chain fatty acyl-CoAs from the cytoplasm into the mitochondria, thereby regulating mitochondrial β-oxidation rates (9). BBH catalyzes the formation of L-carnitine from γ-butyrobetaine and is essential for the transport of activated fatty acids across the mitochondrial membrane (17).

Although hypoxia-inducible factor (HIF)1α was not significantly regulated in our analysis, a more hypoxia-responsive gene, HIF3a (21), was found to be upregulated in cluster III. This indicates some degree of cellular hypoxia in both HYP and CHF. We did not find a change in HIF3a gene expression in the LV myocardium, thereby excluding systemic hypoxia as a cause for the expression of this gene in the RV.
In cluster IV we found the metabotropic glutamate receptor-1 (mGLUR1) to be specifically downregulated in the CHF group. mGLUR1 has been shown to be expressed in the central nervous system, where it is involved in preventing neuronal apoptosis via Homer-phosphatidylinositol 3-kinase (PI3-kinase) enhancer-large (PIKE-L) complex interaction, leading to PI3-kinase activation (39). A pathophysiological role for mGLUR1 in the heart has not been described, although cardiac mGLUR1 expression has been reported (19). Downregulation of this gene in the heart in CHF could impair anti-apoptotic effects, which might be crucial in the development of the decompensated phenotype. However, more research will be needed to confirm a role for mGLUR1 in heart failure.

Interestingly, the growth hormone (GH) receptor was upregulated in HYP (cluster VII). Although no other groups showed a significant difference in expression, the HYP vs. CHF comparison, with a [Z-score] of 1.95, was almost significantly different. The expression profile of this gene shows a striking similarity to cluster V, the HYP-specific cluster. Recently, González-Juanatey et al. (20) showed that GH attenuated apoptosis in cardiomyocytes via calcineurin signaling. In addition to this, they showed a decrease in p38-MAPK phosphorylation level after GH treatment. As will be discussed below, p38-MAPK signaling in relation to apoptosis may play a pivotal role in the development of the HYP and CHF phenotypes.

Putative mediators in compensated hypertrophy or heart failure. It is impossible within the limits of this paper to present a full description of the possible functional significance of regulated genes. However, we did find several genes that may play a crucial role in the development of either compensated or decompensated ventricular hypertrophy.

Our data show the HYP-specific upregulation of MKP-1 in cluster V. MKP-1 recognizes and deactivates MAPKs with higher preference for p38-MAPK and c-Jun NH2-terminal kinases (JNK1/2) than for extracellular signal-related kinases (ERK1/2). Evidence is accumulating that MKP-1 is involved in a negative feedback loop mechanism suppressing MAPK signaling activity. MAPK pathway activation ultimately leads to the phosphorylation and activation of one of three end effectors, i.e., p38-MAPK, JNK1/2, or ERK1/2, all of which can activate a wide array of transcription factors, thereby contributing to altered gene expression. MAPK signaling has repeatedly been shown to be of major importance in ventricular remodeling due to pressure overload (37). Recently, it has been shown that p38-MAPK acts as a pro-death effector and that attenuation of p38-MAPK phosphorylation was associated with the upregulation of anti-apoptotic bcl-2, both in vitro and in vivo (24). In a study of MCT-induced RV hypertrophy, an increase in bcl-2 expression was observed in an early stage of hypertrophy, whereas it was markedly downregulated in the transition to heart failure, where it was associated with severe apoptosis (10). Transient MKP-1 overexpression reduced myocardial hypertrophy-associated gene expression, as evidenced by attenuated ANF, β-myosin heavy chain, and α-skeletal muscle actin promoter activity after phenylephrine stimulation of isolated rat neonatal ventricular cardiomyocytes (16). MKP-1 transgenic mice also showed, in addition to blocked activation of p38-MAPK, JNK1/2, and ERK1/2 after acute catecholamine stimulation, that expression of MKP-1 attenuated hypertrophy, in response to both pressure overload and prolonged isoproterenol infusion (3).

Our observation that MKP-1 is specifically upregulated in the HYP group suggests blocked pro-death signaling mediated via p38-MAPK dephosphorylation, which is not present in the CHF group. Western analysis indeed confirmed the upregulation of MKP-1 in the RVs of the HYP group, which consequently showed no p38-MAPK activation, in contrast to the RVs of the CHF group.

Subsequent increases in bcl-2 levels in the HYP group will then exert their anti-apoptotic effects in the compensated hypertrophic myocardium only. High levels of bcl-2 were indeed found at an early stage of hypertrophy by Escarnt-Laubriet et al. (10), whereas it was markedly downregulated in the subsequent transition to heart failure. This decrease in bcl-2 levels coincided with a stimulation of cyclin D1 expression, a pro-apoptotic mediator, leading to extensive myocardial apoptosis. Indeed, our array data show a significant increase in cyclin D1 expression in the CHF group relative to CON (cluster II), which was confirmed by real-time PCR.

In addition to the above-mentioned alterations in the expression of key pro-apoptotic and pro-survival signaling molecules, we found that the genes encoding the two major components of
the mitochondrial permeability transition pore complex (PTPC), i.e., the mitochondrial ANT1 and the mitochondrial VDAC1, were significantly overexpressed in CHF but not in HYP. This is particularly relevant in light of the potential role of p38 and JNK in the mitochondrial death pathway (36, 38). However, Western blot analysis did not yet indicate altered expression of either VDAC1 or ANT1 at this point in ventricular remodeling (data not shown). This could be explained by a time lag between mRNA and actual protein expression, which is supported by the observation that apoptosis is not yet detected at day 14 in the MCT model (10).

Mitochondrial involvement was also suggested by the significantly reduced expression of the mitochondrial transcription factor A (mtTFA) in CHF but not in HYP. mtTFA controls mitochondrial replication and transcription (12, 34). Mitochondrial synthesis and function require an estimated 1,000 polypeptides, 37 of which are encoded by mitochondrial (mt)DNA, the rest by nuclear (n)DNA (14). This suggests that reduced mitochondrial biogenesis via mtTFA downregulation and associated mitochondrial dysfunction may play a role in the development of pathological hypertrophy. Reduced mtTFA levels were also found at end-point heart failure in a study of aortic stenosis-induced LV hypertrophy 6 mo after surgery (18). In fact, mitochondrial dysfunction enhances oxidative stress, which may directly trigger mitochondrial membrane permeabilization and subsequent apoptosis by opening of the PTPC (7, 48).

The changes described above (see also Fig. 5 for a schematic summary) indicate that there is a fundamental shift in the balance of pro-apoptotic vs. anti-apoptotic signaling in the two different hypertrophic phenotypes studied. Already early in myocardial remodeling, hearts that will eventually develop heart failure appear to be sensitized to apoptosis, in contrast to compensated hearts, which seem to have become more resistant. Similar changes have recently been described by Kang et al. (25), both in vivo and in vitro.

The concept that there are signaling pathways specifically linked to either compensated or decompensated hypertrophy has been gaining more acceptance over the last couple of years (for a recent review, see Ref. 35). However, the time point at which these discriminating signals are activated, i.e., the suggested transition of hypertrophy to heart failure, is unknown. Our observation that discriminating pathways have already been invoked very early in myocardial remodeling, and not after compensation has failed, challenges the paradigm that there is in fact such a transition.

Clinical relevance. Primary pulmonary hypertension is a disease that usually has a rapid course. Patients become symptomatic with nonspecific complaints in about 2 yr. On presentation, patients may show rapid development of right failure [New York Heart Association (NYHA) classes III and IV], some with only moderately increased pressures, whereas a subgroup of patients showing pressures up to systemic levels remains NYHA class I, indicating successful compensatory RV hypertrophy. The ability to make a reliable early prognosis concerning the progression of the disease state will be invaluable to clinicians. The use of diagnostic expression microarrays may therefore be ideal to distinguish patients at risk early after clinical presentation. However, much work remains to be done before such diagnostic arrays are proven to be reliable and highly reproducible (44). Our data indicate that such early differences can be detected in an animal model for RV hypertrophy secondary to pulmonary hypertension. These data may provide target genes for phenotypic screening early in the development of heart failure and suggest potential targets for early clinical intervention.

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