Subtractive hybridization for differential gene expression in mechanically unloaded rat heart

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Subtractive hybridization for differential gene expression in mechanically unloaded rat heart. Am J Physiol Heart Circ Physiol 291: H2714–H2722, 2006. First published June 9, 2006; doi:10.1152/ajpheart.00445.2005.—The objective of this study was to identify differentially expressed genes in the mechanically unloaded rat heart by suppression subtractive hybridization. In male Wistar-Kyoto rats, mechanical unloading was achieved by infrarenal heterotopic heart transplantation. Differentially expressed genes were investigated systematically by suppression subtractive hybridization. Selected targets were validated by Northern blot analysis, real-time RT-PCR, and immunoblot analysis. Maximal ADP-stimulated oxygen consumption (state 3) was measured in isolated mitochondria. Transplantation caused atrophy (heart-to-body weight ratio: 1.6 ± 0.1 vs. 2.4 ± 0.1, P < 0.001). We selected 1,880 clones from the subtractive hybridization procedure (940 forward and 940 reverse runs assessing up- or downregulation). The first screen verified 465 forward and 140 reverse clones, and the second screen verified 67 forward and 30 reverse clones. On sequencing of 24 forward and 23 reverse clones, 9 forward and 14 reverse homologies to known genes were found. Specifically, we identified reduced mRNA expression of complex I (−49%, P < 0.05) and complex II (−61%, P < 0.001) of the respiratory chain. Significant reductions were also observed on the respiratory chain protein level: −42% for complex I (P < 0.01), −57% for complex II (P < 0.05), and −65% for complex IV (P < 0.05). Consistent with changes in gene and protein expression, state 3 respiration was significantly decreased in isolated mitochondria of atrophied hearts, with glutamate and succinate as substrates: 85 ± 27 vs. 224 ± 32 natoms O·min−1·mg−1 with glutamate (P < 0.01) and 59 ± 18 vs. 154 ± 30 natoms O·min−1·mg−1 with succinate (P < 0.05). Subtractive hybridization indicates major changes in overall gene expression by mechanical unloading and specifically identified downregulation of respiratory chain genes. This observation is functionally relevant and provides a mechanism for the regulation of respiratory capacity in response to chronic mechanical unloading.

suppression subtractive hybridization; respiratory chain gene; mitochondria

Myocardial atrophy has not been intensely investigated, presumably because it is not usually occurring in living organisms. The advent of left ventricular assist devices (LVADs) has changed this situation. LVADs are clinically used in patients with terminal heart failure as a bridge to transplantation or even destination (24). Studies have shown that, during this waiting time, unloading of failing hearts has beneficial effects on contractile function (8, 12). Implantation of LVADs has also been shown to increase patient survival until transplantation compared with drug therapy alone (24). In a few patients, cardiac function improved to an extent that transplantation was not necessary and the assist device could be explanted (18, 20).

These observations generated a surge of interest in the mechanisms underlying unloading (4, 8, 12, 13). For assessment of the mechanisms underlying recovery of failing hearts during unloading, an understanding of the physiological response to mechanical unloading is imperative. Infrarenal heterotopic heart transplantation has been found to be a convenient animal model for investigation of these mechanisms (9, 22). These hearts show a 20–40% reduction of heart weight within 1 wk of unloading (6, 16). We used this model previously to demonstrate changes in isoform gene expression resembling a fetal genotype (6, 10). We now use suppression subtractive hybridization to systematically identify differentially expressed genes (7, 14).

Suppression subtractive hybridization is a widely used powerful and highly effective technique for screening for differentially regulated genes (27, 28, 30, 31). With this technique, mRNA is transcribed into cDNA, enriched, and amplified by suppression PCR (7, 14). After suppression subtractive hybridization, PCR products reflecting differentially expressed genes can be separated by cloning and screened for verification of differential expression (7, 27). The verified clones can be sequenced and compared with known gene sequences (7, 27). Finally, the results should be validated by Northern blot analysis or PCR (27, 28).

In this study, we have used subtractive hybridization to identify 23 genes that are differentially expressed in the heterotopic transplanted rat heart. Specifically, we identified a significant reduction in mRNA expression of respiratory chain genes that was also apparent on the protein level. These changes were accompanied by decreased respiratory capacity of isolated mitochondria. We suggest that, in response to a chronic decrease in workload, the heart regulates its respiratory capacity mainly via changes in respiratory chain gene expression.

METHODS

Animals. Male Wistar-Kyoto rats (275–300 g body wt; Charles River, Sulzfeld, Germany) were fed ad libitum and kept at 21°C with a 12-h:12-h light-dark cycle. The use of animals and the experimental

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PCR products were transferred on a membrane by spot blotting and 1% agarose gel electrophoresis. For “reverse Northern blot analysis” (1 min at 72°C, and 10 min at 72°C).

DNA inserts (3 min at 94°C, 30 cycles for 30 s at 94°C, 30 s at 50°C, 1 min at 99°C), and colony PCR was performed to amplify plasmid onies were transferred to 96-well PCR plates and lysed by heating (10 min at 98°C) and 1.5 min at 98°C and rehybridized (8 h at 68°C, 1st hybridization) with excess driver molecules (incubation overnight at 68°C; 2nd hybridization). Hybridized double-strand cDNA molecules, which are characterized by different adaptors bound to the 3’ and 5’ end at each strand, were amplified by two serial PCRs with two different primer pairs annealing to the adaptors (PCR1 consisted of 27 cycles for 30 s at 94°C, 30 s at 66°C, and 1.5 min at 72°C; PCR2 consisted of 10 cycles for 30 s at 94°C, 30 s at 68°C, and 1.5 min at 72°C). Rehybridized cDNA molecules with only one or no adaptor at the end were not amplified, and amplification of DNA molecules with equal adaptors at the 3’ and 5’ ends will be suppressed by preferentially building secondary structures, rather than binding PCR primers.

Cloning. cDNA PCR products were incubated with DNA ligase and linear plasmids for ligation of the cDNAs to the 3’ end. Ligated plasmids (2 μl) were incubated with competent bacteria for 30 min at 4°C and heated for 30 s at 42°C. Bacterial medium (250 μl) was added, and the mixture was incubated for 30 min at 37°C. Bacteria were plated on ampicillin-containing agar plates for single colony growth. Only bacteria with plasmids were ampicillin resistant and able to grow. To differentiate bacteria with plasmids with or without ligated PCR products, colonies were stained (blue/white) with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Single colonies were transferred to 96-well PCR plates and lysed by heating (10 min at 99°C), and colony PCR was performed to amplify plasmid DNA inserts (3 min at 94°C, 30 cycles for 30 s at 94°C, 30 s at 50°C, 1 min at 72°C, and 1 min at 72°C).

Gel electrophoresis and blotting. PCR products were verified by 1% agarose gel electrophoresis. For “reverse Northern blot analysis” PCR products were transferred on a membrane by spot blotting and blotting of separated PCR products on a high-density gel. For spot blotting, 30 μl of each PCR product were diluted by addition of 120 μl of H2O2, and 60 μl of each diluted PCR product were transferred to a well of a 96-well plate of a vacuum blotting apparatus for transfer on a nylon membrane. PCR products were separated by high-density gel electrophoresis on a 2% agarose gel (45 mM Tris base, 45 mM boric acid, 1.25 mM EDTA, and ethidium bromide, pH 7.5) using 5× sample buffer in a 1× final concentration (5× = 15% Ficoll, 50 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene-cyanole in 45 mM Tris base-45 mM boric acid-1.25 mM EDTA). The gel was denatured (twice for 10 min each in 1.5 M NaCl and 0.5 M NaOH) and neutralized (twice for 10 min each in 1.5 M NaCl and 0.5 M Tris base, pH 7.0), and PCR products were transferred on a nylon membrane overnight using 20× saline-sodium citrate (SSC) buffer (3 M NaCl, 300 mM sodium citrate, pH 7.0) and fixed on the membrane at 80°C for 2 h.
Screening. For effective reduction of false-positive results, two screenings were performed: a spot-blot screen followed by a high-density gel screen. Only markedly differentially expressed genes on spot-blot membranes were separated on a gel for a second screen. Blotted PCR products (spot-blot or high-density gel) of single clones were hybridized with radioactively labeled probes from total tissue RNA from unloaded or control hearts. Probes were synthesized using the Smart PCR CDNA synthesis kit (Clontech) as described by the manufacturer and labeled using the Megaprimre DNA-labeling system (Amersham Pharmacia Biotech). The membranes were prehybridized with denatured salmon sperm in hybridization buffer (2.5% dextran sulfate, 2X SSC buffer, 0.1% SDS, 0.1% sodium pyrophosphate, 2 mM EDTA, 0.2% Ficoll, 0.1% polyvinylpyrrolidone, and 0.2% BSA) for 1–4 h. For hybridization with the labeled cDNA probes, fresh hybridization buffer was used, and the probes were incubated overnight using denatured labeled probes (5 min, 95°C). Finally, membranes were washed twice in decreasing salt concentrations (2X SSC and 0.5% SDS followed by 0.1X SSC and 0.1% SDS) for 15 min. Differentially expressed genes as identified by the two screenings were sequenced and tested for homologies to known gene sequences in a gene data bank (BCM Search Launcher: Nucleic Acid Sequence Searches). For Northern blot analysis, the cDNAs were used as a probe for hybridization with blotted and fixed total tissue RNA in two different unloaded hearts to confirm actual differential mRNA expression, as described by von Stein et al. (27). Figure 2 shows an overview of suppression subtractive hybridization and subsequent screening and analysis of differentially expressed genes.

Real-time RT-PCR. Total cardiac muscle RNA was isolated from frozen tissue samples using the Qiagen RNeasy midi kit. Complementary DNA was synthesized using MultiScribe reverse transcriptase (Applied Biosystems). Real-time PCR was performed using the TaqMan fluorometric technique (ABI Prism, Perkin Elmer). Primers and probes were designed using Primer Express 1.3 software (Table 1). For each set of primers, basic local alignment search tool (BLAST) searches revealed that sequence homology was obtained only for the target gene.

Table 1. Primer and probe sequences used in quantitative PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH-DH subunit 2</td>
<td>AGGCTAACCCCTTGCTGCCCAAGAC</td>
<td>X14848</td>
</tr>
<tr>
<td>Reverse</td>
<td>AGGCGACAGATCACTGAGCAAA</td>
<td></td>
</tr>
<tr>
<td>SUC-DH subunit b</td>
<td>GGAGGCTGCATGATCCAGGAA</td>
<td>XM_216558</td>
</tr>
<tr>
<td>Reverse</td>
<td>TCTGCTAGGTCGGCCATCA</td>
<td></td>
</tr>
<tr>
<td>COX IV</td>
<td>CAATGGCAAGGATACTCCGTAA</td>
<td>X15029</td>
</tr>
<tr>
<td>Reverse</td>
<td>TTCAGGCCAGCGGATGAGAAAG</td>
<td></td>
</tr>
<tr>
<td>Histone 3B</td>
<td>CCAATTGTACCGCATCC</td>
<td>BC087725</td>
</tr>
<tr>
<td>Reverse</td>
<td>TTCAGCTGGACTGATGCTTGAAG</td>
<td></td>
</tr>
<tr>
<td>Probe</td>
<td>GCAGAGAAGGTTGAAGATACAG</td>
<td></td>
</tr>
</tbody>
</table>
| NADH-DH, NADH dehydrogenase; SUC-DH, succinate dehydrogenase; COX IV, cytochrome c oxidase subunit IV.

PCR to ensure specificity of amplification. Standard curves were measured using cDNA from control hearts, and results were calculated using the standard curve method (User Bulletin No. 2. Applied Biosystems). Target gene expression was normalized to histone 3B transcription as a housekeeping gene product, which was not different among the samples.

Immunoblot analysis. Rat hearts were excised, frozen in liquid nitrogen, reduced to powder in liquid nitrogen, and homogenized in a Potter-Elvehjem glass homogenizer containing homogenization buffer (10 mM NaCl, 25 mM NaF, 15 mM NaPO₄, 1 mM EDTA, 2 mM Na₃VO₄, 0.44 mM PMSF, 1% Triton X-100). One tablet of Complete Inhibitor Mix, and 20 mM Tris, pH 7.5). The homogenates were incubated on ice for 15 min and centrifuged at 600 g for 10 min. The resulting supernatant was solubilized in Laemmli buffer and run on a 10% acrylamide gel. Primary antibody incubations were performed in 3% nonfat dry milk in 0.1% Tween 20-containing TBS. The following primary antibodies were used: complex I (1:1,000 dilution; MitoScience), complex II (1:1,000 dilution; MitoScience), and complex IV (1:250 dilution; Invitrogen). Secondary antibodies (goat anti-mouse IgG) were diluted 1:40,000. Visualization was performed using an enhanced chemiluminescence system (Pierce). Subsequent Coomassie blue staining was used to assess protein loading.

Tissue homogenization. Rats were anesthetized with an injection of pentobarbital sodium (50 mg/kg ip). Hearts were quickly excised and transferred to ice-cold cardioplegic solution containing 0.18 M KCl and 10 mM Tris·HCl, pH 7.4. The hearts were finely minced. One gram of tissue was added to 6 ml of KEA isolation medium (0.18 M KCl, 10 mM EDTA, and 0.5% BSA). The mixture was homogenized with an Ultra-Turrax T25 at 8,750 rpm for 20 s followed by one downpass in a Potter-Elvehjem glass homogenizer by a motor-driven Teflon pestle at 2,000 rpm. The pH was adjusted to 7.4. All steps were carried out at 4°C.

Isolation of mitochondria. Mitochondria were isolated by a modification of the procedure described by Tomec and Hoppel (26) in which 3 ml of whole heart homogenate were centrifuged at 750 g for 10 min. The pellet was treated with nagarse (5 mg/g wet wt) for 30 s and centrifuged at 7,000 g for 5 min. The resulting pellet was resuspended with KEA isolation medium and centrifuged at 700 g for 10 min. The pellet was resuspended and spun down three times at 7,000 g for 10 min. The last pellet was diluted with 500 μl of KEA isolation medium. All steps were carried out at 4°C. Protein concent-
tations were measured by the bicinchoninic acid protein assay (Bio-
Rad), with BSA as standard.

Citrate synthase activity. Citrate synthase (CS) serves as a mito-
chondrial marker enzyme (2). Total and free CS activities were
measured and used to calculate latent CS activity (CS\text{\text{total}} - CS\text{\text{free}}),
which is the fraction of total CS activity that is contained within the
mitochondria and not readily accessible by substrate. The CS ratio
(CSR) represents the relation of latent to free CS activity and serves
as an index of structural integrity of the mitochondrial preparation (2).
CS activity was determined at 25°C using a modification of the
procedure of Srere (25). Total CS activity was determined by preincu-
bation of samples with 2.5% Triton X-100, and free CS activity was
measured after preincubation in 0.9% NaCl.

Mitochondrial respiration. Oxygen consumption of isolated mito-
chondria was measured at 25°C with a Clark-type oxygen electrode
fitted to a 1-ml water-jacketed reaction chamber (1). Mitochondrial
preparations were added to respiration medium (210 mM mannitol, 70
mM sucrose, 5 mM KH$_2$PO$_4$, and 10 mM Tris-HCl, pH 7.4) to a final
concentration of 1 U CS/ml in the reaction chamber. With glutamate
or succinate as a substrate (10 mM), maximal oxygen consumption
(state 3 respiration) was stimulated by addition of 20 \mu l of 1 mM
ADP. State 4 respiration was measured as the rate of oxygen consump-
tion after total phosphorylation of added ADP. The respiratory
control ratio was calculated as the ratio of state 3 to state 4 respiration,
as defined by Chance and Williams (1). The ADP-to-O ratio was
calculated as described by Estabrook (11).

Statistical analysis. Values are means ± SE. Data were analyzed
using Student’s $t$-test. Differences among groups were considered
statistically significant at $P < 0.05$.

RESULTS

Effects of mechanical unloading on cardiac mass. Hearts
were explanted from donor rats, transplanted into the abdomen
of recipient rats (see METHODS), and excised for experiments
after 8 days of unloading. As shown in Table 2, heart weights
were significantly reduced after 8 days of unloading (recipient
rat), indicating significant heart atrophy. Hearts from nonop-
erated animals were used for comparison and are referred to as
controls.

Screening for differentially expressed genes. Screening for
differential expression of subtracted clones from the forward
run on spot-blot membranes is shown in Fig. 3. The subtracted
clones were blotted on two different membranes with equal
loading and distribution of samples. In an identical manner,
screening for differential expression of subtracted clones from
the reverse run was performed to reveal upregulated gene
expression.

Procedure for selection and identification of differentially
expressed genes. Table 3 shows the numbers of clones used
from subtractive hybridization for the screening procedures
and the following identification and verification of differenti-
ally expressed genes. On the first screen, investigation of 940
arbitrarily selected single colonies of forward and reverse runs
on spot-blot membranes revealed markedly increased signals

Table 2. Body and heart weights of donor and recipient
rats, after 8 days of unloading

<table>
<thead>
<tr>
<th></th>
<th>Donor Rat</th>
<th>Recipient Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>297 ± 16</td>
<td>286 ± 15</td>
</tr>
<tr>
<td>Heart wt, mg</td>
<td>1.25 ± 0.05</td>
<td>0.48 ± 0.05*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *$P < 0.001$ vs. donor rat.

Fig. 3. Verification of differentially expressed subtracted clones by spot
blotting of clones from the forward run with radioactive markers and compar-
sion of signals on the X-ray screen. Subtracted clones were blotted on 2
different membranes (A and B), with equal loading and distribution of samples.
Signals of single clones after hybridization with labeled probes from atrophied
hearts (A) are compared with signals of single clones after hybridization with
labeled probes from control hearts (B). Arrows, clones demonstrating less
signal in atrophied than in control hearts, which indicates downregulation.

of 465 colonies of the forward run and 140 colonies of the
reverse run. On the second screen, cDNAs were separated by
gel electrophoresis before blotting to exclude contaminations
producing additional signals (not shown). The second screen
confirmed 67 of 236 tested clones of the forward run and 30 of
140 tested clones of the reverse run. Twenty-four clones of the
forward and 23 clones of the reverse run displaying marked
differences between atrophied and control cDNA were arbi-
trarily selected for sequencing and analyzed for homologies to
known genes in a gene data bank (BCM Search Launcher:
Nucleic Acid Sequence Searches). Sequence analysis revealed
homologies to known genes for 9 forward and 14 reverse
subtracted clones.

Identified and verified differentially expressed genes. Table
4 shows upregulated and downregulated genes of atrophied
hearts as identified by subtractive hybridization. Four genes of
the forward run and five genes of the reverse run were arbi-

With glutamate and succinate as substrate, state 3 respiration of mitochondria of atrophied and control hearts is shown in Fig. 3. Total CS activity was latent CS activity). Mitochondria of atrophied and control hearts (93.5–94% of the mean total CS activity) were isolated from hearts with glutamate (2.0 ± 0.4 vs. 2.0 ± 0.1 for atrophy vs. control, not significant) as substrate. ADP-to-O ratio, which is an indirect measure of coupling of oxygen consumption to ATP production, was significantly different in isolated mitochondria from atrophied hearts with glutamate (3.5 ± 0.7 vs. 6.2 ± 0.9 for atrophy vs. control, P < 0.05) but was not different when succinate was used as substrate. The ADP-to-O ratio, which is an indirect measure of coupling of oxygen consumption to ATP production, was not different in isolated mitochondria from atrophied hearts with glutamate (2.0 ± 0.4 vs. 2.0 ± 0.1 for atrophy vs. control, not significant) or succinate (1.4 ± 0.2 vs. 1.5 ± 0.1 for atrophy vs. control, not significant) as substrate.

Expression of respiratory chain genes in atrophied hearts. Using subtractive hybridization, we identified differences in the expression of respiratory chain genes. For further confirmation and accurate quantification, we investigated the mRNA expression of NADH dehydrogenase subunit 2 and cytochrome c oxidase subunit IV by RT-PCR. We found a 49% reduction of complex I mRNA content and a trend toward reduced expression of complex IV mRNA (−35%; Fig. 5). In addition, we investigated the expression of succinate dehydrogenase subunit b mRNA, which was reduced by 61% in atrophied hearts.

Expression of respiratory chain proteins in atrophied hearts. We also investigated protein expression of complexes I, II, and IV in atrophied hearts. As demonstrated in Fig. 6, protein levels of each of the complexes were significantly reduced in unloaded hearts: −42% for complex I, −57% for complex II, and −65% for complex IV.

Mitochondrial respiratory function of atrophied hearts and CS activity. To determine whether the identified changes in respiratory chain gene and protein expression are accompanied by functional changes, we isolated mitochondria and assessed their respiratory activity. We used CS activity to characterize the isolated mitochondria and their structural integrity. Table 5 shows total, free, and latent CS activity and CSR (i.e., ratio of latent to free CS) of whole heart homogenates and isolated mitochondria of atrophied and control hearts. Total CS activity was significantly decreased in homogenates and isolated mitochondria of atrophied hearts compared with controls. Mean values of CSR, an index of structural integrity, were 15.2−17.1, which indicate a high degree of structural integrity in isolated mitochondria of atrophied and control hearts (93.5–94% of the total CS activity was latent CS activity).

State 3 respiration of whole heart homogenates and isolated mitochondria of atrophied and control hearts is shown in Fig. 7. With glutamate and succinate as substrate, state 3 respiration was significantly decreased in homogenates of atrophied hearts. Maximal oxygen consumption was also markedly reduced in isolated mitochondria of atrophied hearts with both substrates. This reduction could be a consequence of the reduced CS activity that we observed in these hearts. We therefore related state 3 respiration to CS activity. State 3 respiration was still markedly reduced in isolated mitochondria of atrophied hearts. The respiratory control ratio, which is the ratio of state 3 to state 4 respiration, was significantly reduced in isolated mitochondria of atrophied hearts when glutamate was used as substrate (3.5 ± 0.7 vs. 6.2 ± 0.9 for atrophy vs. control, P < 0.05) but was not different when succinate was used as substrate.

Mechanical unloading of the heart results in changes of gene expression (5). We previously demonstrated a switch in iso-

### Table 3. Clones selected for verification by two screens, identification of homologies to known genes, and verification by Northern blot analysis

<table>
<thead>
<tr>
<th>Subtracted Clones</th>
<th>Forward run</th>
<th>Reverse run</th>
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<tbody>
<tr>
<td>Clones 1st screen</td>
<td></td>
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</tr>
<tr>
<td>No. selected</td>
<td>940</td>
<td>940</td>
</tr>
<tr>
<td>No. positive</td>
<td>465</td>
<td>140</td>
</tr>
<tr>
<td>Clones 2nd screen</td>
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<td></td>
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<td>140</td>
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<tr>
<td>No. positive</td>
<td>67</td>
<td>30</td>
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<tr>
<td>No. sequenced</td>
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<td>23</td>
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</table>

### Table 4. Genes identified by subtractive hybridization as differentially expressed in unloaded rat hearts

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
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<tbody>
<tr>
<td><strong>Decreased expression</strong></td>
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<tr>
<td>NADH-DH subunit 2*</td>
<td>NC_001665</td>
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<tr>
<td>COX subunit 1*</td>
<td>NC_001665</td>
</tr>
<tr>
<td>Acetyl-CoA synthetase*</td>
<td>NM_019811</td>
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<td>Myoglobin*</td>
<td>NM_021588</td>
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<tr>
<td>Hb β-chain complex</td>
<td>NM_033234</td>
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<tr>
<td>Voltage-dependent anion channel 3</td>
<td>NM_031355</td>
</tr>
<tr>
<td>Myomoglobin</td>
<td>NM_022382</td>
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<tr>
<td>Cis-golgβ p28</td>
<td>NM_053584</td>
</tr>
<tr>
<td>PAC clone RP4-691F11</td>
<td>AC004859</td>
</tr>
<tr>
<td>Guanine nucleotide-releasing protein (ms4)</td>
<td>NM_001007678</td>
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<tr>
<td><strong>Increased expression</strong></td>
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<tr>
<td>HSP70*</td>
<td>NM_024351</td>
</tr>
<tr>
<td>MHC class I heavy chain*</td>
<td>NM_001008839</td>
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<td>MHC class II α-chain*</td>
<td>NM_011008047</td>
</tr>
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<td>β-Microglobulin*</td>
<td>NM_012512</td>
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<tr>
<td>Lysozyme*</td>
<td>NM_012771</td>
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<tr>
<td>Ubiquitin C</td>
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<tr>
<td>α-Tubulin 2</td>
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<td>Ribosomal protein S4</td>
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<td>Eefl1</td>
<td>NM_175838</td>
</tr>
<tr>
<td>STAT1</td>
<td>NM_032612</td>
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HSP70, 70-kDa heat shock proteins; MHC, major histocompatibility complex; Eef1a1, eukaryotic translation elongation factor 1α; STAT1, signal transducer and activator of transcription 1. * Genes confirmed by Northern blot analysis (Fig. 4).
form gene expression of several proteins of unloaded hearts, leading to the hypothesis of reactivation of a fetal gene expression pattern (6). Other reports showed downregulation of mRNA expression of several different genes, including mRNA for main regulators, enzymes, and proteins of myocardial substrate metabolism [e.g., peroxisome proliferator-activated receptor-α (PPARα) and uncoupling proteins-2 and -3] (32).

We now set out to systematically identify differentially expressed genes after mechanical unloading of the heart by suppression subtractive hybridization. This method allows a highly effective and simultaneous identification of downregulated and upregulated genes by normalization during the subtraction process (i.e., forward or reverse subtraction) (27, 28). A two-step screening substantially limited a possible isolation of false-positive clones (27). A total of 1,880 clones were tested for differential gene expression. Of the selected genes for analysis, we found 10 to be downregulated and 14 to be upregulated in the mechanically unloaded rat heart. We verified 9 of these 24 genes by Northern blot analysis, and all 9 genes could be confirmed to be differentially expressed.

A disadvantage of the procedure is that the homologies of the differentially expressed sequences to known genes never Fig. 4. Representative Northern blot analyses of selected genes that were found to be differentially expressed in unloaded hearts by subtractive hybridization (see Table 4). A, atrophy; C, control. Top blots: target gene expression. Bottom blots: 28S rRNA expression as a loading control. MHC, major histocompatibility complex.

Fig. 5. Real-time RT-PCR determination of mRNA expression of NADH dehydrogenase (NADH-DH) subunit 2, succinate dehydrogenase (SUC-DH) subunit b and cytochrome c oxidase IV (COX IV) of control and atrophied hearts (n = 4). *P < 0.05; ***P < 0.001 vs. control.
reach 100% because of species differences. Another shortcoming is the relatively laborious method used to select and verify clones through the screening process. This drawback has been overcome recently by the development of microarray analysis, which is now able to screen for differentially expressed genes in the entire genome (21). However, despite major advances in this technology in recent years, the difficulties derived from the myriad of potential sources of random and systematic measurement error in the microarray process and from the small number of samples relative to the large number of variables (probes) complicate data analysis and interpretation and can jeopardize the validity of microarray findings (19, 21). Our approach appears highly reliable, because we could verify all genes by Northern blot analysis identified as differentially expressed by suppression subtractive hybridization. The results support the notion that suppression subtractive hybridization with two-step screening is a powerful procedure for effective screening for differentially expressed genes.

We have demonstrated reduced mRNA expression of respiratory chain genes. In addition, we found reduced respiratory chain protein levels. To test the functional relevance of these changes, we determined state 3 respiration of isolated mitochondria from unloaded hearts. Indeed, we found that state 3 respiration of isolated mitochondria of unloaded hearts was significantly decreased with the NADH-linked substrate glutamate and the FADH-linked substrate succinate. State 3 is a measure of maximal respiratory activity of mitochondria under conditions of substrate saturation and stimulation by ADP. Under these conditions, the respiratory chain enzymes are thought to perform maximal catalytic activity. If this is true, changes in state 3 respiration are determined by changes in respiratory chain protein levels. Indeed, we found that reduced state 3 respiration is accompanied by reduced protein expression. Although we have not determined the catalytic activity of single respiratory chain enzymes, we suggest that the down-regulation of these enzymes may be responsible for the reduced state 3 respiration of isolated mitochondria of unloaded hearts.

CS is a mitochondrial marker enzyme and, therefore, may reflect the amount of mitochondrial material in a mitochondrial

Table 5. Total, free, and latent CS activity and CS ratio of whole heart homogenate and isolated mitochondria of control and unloaded hearts

<table>
<thead>
<tr>
<th></th>
<th>Control Hearts</th>
<th>Unloaded Hearts</th>
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<tbody>
<tr>
<td><strong>Whole heart homogenate</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS activity, wet wt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>120±4</td>
<td>97±5</td>
</tr>
<tr>
<td>Latent</td>
<td>97±5</td>
<td>63±8†</td>
</tr>
<tr>
<td>Free</td>
<td>23±1</td>
<td>22±4</td>
</tr>
<tr>
<td>CS ratio</td>
<td>4.3±0.4</td>
<td>3.0±0.4*</td>
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<tbody>
<tr>
<td><strong>Isolated mitochondria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS activity, U/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>17.5±2.0</td>
<td>11.2±2.6*</td>
</tr>
<tr>
<td>Latent</td>
<td>16.5±1.8</td>
<td>10.5±2.4*</td>
</tr>
<tr>
<td>Free</td>
<td>1.0±0.2</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>CS ratio</td>
<td>17.1±2.5</td>
<td>15.2±1.2</td>
</tr>
<tr>
<td>Protein content, mg/ml</td>
<td>9.7±0.7</td>
<td>7.8±0.5*</td>
</tr>
<tr>
<td>CS total/protein, U/mg</td>
<td>1.8±0.1</td>
<td>1.4±0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 5). CS, citrate synthase. *P < 0.05; †P < 0.01 vs. control hearts.
preparation (2). We found that total CS activity is significantly reduced in homogenates and mitochondrial isolates of atrophied hearts. Although the results suggest that mitochondrial material is reduced in homogenates and isolated mitochondria of atrophied hearts, they do not allow us to distinguish whether the number of mitochondria is reduced and the individual mitochondrion is normal or whether all mitochondria contain less enzyme. We related CS activity to the protein content in isolated mitochondria (Table 5). The finding that the ratio was not different between the groups supports a reduction in mitochondrial number and/or volume, instead of a mitochondria-specific downregulation of CS activity. We did not perform electron microscopic studies, which may address this issue.

The reduction in CS activity may contribute to the decreased state 3 respiration. CS is a tricarboxylic acid (TCA) cycle enzyme, and both substrates used in this study are fed into the TCA cycle (glutamate) or directly converted by a TCA cycle enzyme (succinate). A reduction in TCA cycle flux may decrease state 3 respiration by limiting NADH- or FADH-linked electron transfer to the respiratory chain. To assess the contribution of the decrease in CS activity to the reduction in state 3 respiration, we related state 3 respiration to CS activity. Because state 3 respiration was still reduced by 50% with both substrates, this mechanism may be less important.

The reduction in respiratory chain protein expression in response to mechanical unloading is not reduced to a single enzyme but instead involves several complexes of the respiratory chain. Also, NADH- and FADH-linked respiratory activity were reduced in mitochondria of unloaded hearts to exactly the same extent. This might suggest an adaptation via a mechanism that is able to regulate components of the respiratory chain in a rather comprehensive way. In this respect, we identified a significant downregulation of PPARα coactivator-1α (PGC-1α; unpublished observations). PGC-1α is a transcriptional coactivator that is able to coactivate and/or induce the expression of nuclear respiratory factors, thereby increasing their activity and ability to increase gene expression (29). Nuclear respiratory factor recognition sites have been identified on several respiratory chain gene promoters (15). Therefore, it is tempting to speculate that the adaptive changes in respiratory chain gene and protein expression in response to a reduction in myocardial workload may involve the PGC-1α signaling cascade.

As mentioned above, we observed no change in the CS-to-protein ratio in mitochondria of unloaded hearts, despite reduced CS activity in these hearts, suggesting that mitochondrial number and/or volume may be reduced. This result is consistent with the reduced expression of PGC-1α in atrophied hearts. PGC-1α can activate the full program of mitochondrial biogenesis, and PGC-1α expression in the heart is sharply increased at the time of birth (17, 23, 29). Thus mechanical unloading may also lead to a reduction in mitochondrial biogenesis, potentially mediated via reduced expression of PGC-1α in these hearts.

Irrespective of the exact cause of the downregulation of respiratory capacity, the results may have practical relevance in the context of reloading an unloaded ventricle. It is conceivable that the sudden reloading of mechanically unloaded ventricles (as in mechanical failure of assist devices or reloading without a weaning period) cannot be met by appropriate increases in

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**Fig. 7.** State 3 respiration of whole heart homogenate (A) and isolated mitochondria (B) and related to citrate synthase activity in isolated mitochondria (C) of control and atrophied hearts with glutamate (solid bars) or succinate (hatched bars) as a substrate ($n = 5$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; all vs. control.
ATP production, because the respiratory gene machinery is not adequately expressed. Although we do not know whether the heart may manage such situations by increasing the coupling of ATP production to oxygen consumption (e.g., by reducing uncoupling protein activity), the unchanged ADP-to-O ratios in unloaded hearts in this study may argue against it. Further studies are necessary to address this issue as well as the impact of mechanical unloading on gene expression and function of failing hearts.

In conclusion, we have shown that subtractive hybridization indicates major changes in overall gene expression by mechanical unloading and specifically identifies downregulation of respiratory chain genes. We suggest that, in response to a chronic decrease in workload, the heart regulates its respiratory capacity mainly via changes in respiratory chain gene expression.

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