Myoglobin-deficient mice activate a distinct cardiac gene expression program in response to isoproterenol-induced hypertrophy

Andrei Molojavyi,1* Antje Lindecke,2* Annika Raupach,1 Sarah Moellendorf,1 Karl Köhrer,2 and Axel Gödecke1

1Institut für Herz- und Kreislauffysiologie, and 2Biologisch-Medizinisches Forschungszentrum, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany

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Molojavyi A, Lindecke A, Raupach A, Moellendorf S, Köhrer K, Gödecke A. Myoglobin-deficient mice activate a distinct cardiac gene expression program in response to isoproterenol-induced hypertrophy. Physiol Genomics 41: 137–145, 2010. First published February 9, 2010; doi:10.1152/physiolgenomics.90297.2008.—Myoglobin knockout mice (myo−/−) adapt to the loss of myoglobin by the activation of a variety of compensatory mechanisms acting on the structural and functional level. To analyze to what extent myo−/− mice would tolerate cardiac stress we used the model of chronic isoproterenol application to induce cardiac hypertrophy in myo−/− mice and wild-type (WT) controls. After 14 days of isoproterenol infusion cardiac hypertrophy in WT and myo−/− mice reached a similar level. WT mice developed lung edema and left ventricular dilatation suggesting the development of heart failure. In contrast, myo−/− mice displayed conserved cardiac function and no signs of left ventricular dilatation. Analysis of the cardiac gene expression profiles using 40K mouse oligonucleotide arrays showed that isoproterenol affected the expression of 180 genes in WT but only 92 genes of myo−/− hearts. Only 40 of these genes were regulated in WT as well as in myo−/− hearts. In WT hearts a pronounced induction of genes of the extracellular matrix occurred suggesting a higher level of cardiac remodeling. myo−/− hearts showed altered transcription of genes involved in carbon metabolism, inhibition of apoptosis and muscular repair. Interestingly, a subset of genes that was altered in myo−/− mice already under basal conditions was differentially expressed in WT hearts under isoproterenol treatment. In summary, our data show a high capacity of myoglobin-deficient mice to adapt to catecholamine induced cardiac stress which is associated with activation of a distinct cardiac gene expression program.

heart; microarray; conductance manometry

THE DEVELOPMENT OF CARDIAC hypertrophy appears to represent a stereotypic response to reduce wall stress posed on the heart either by extrinsic factors such as elevated pre- and after-load or by intrinsic factors such as mutations in genes of the contractile apparatus or signaling cascades. It is now well known that cardiac hypertrophy may develop as a physiologic hypertrophy in response to endurance exercise or during pregnancy (5). This type of hypertrophy is associated with conserved or even enhanced contractile function and appears to be reversible upon cessation of the hypertrophy-inducing stimulus. In contrast, a pathologic hypertrophy may develop in response to chronic pressure overload or sustained neurohumoral stimulation. Characteristics of this form of hypertrophy are a decreased contractile function and a progression to left ventricular dilatation and heart failure (4). It is generally assumed that the heart has got two major gene expression programs, a fetal and an adult one. Reactivation of the fetal gene expression program is a hallmark of the development of cardiac hypertrophy. Among the induced changes upregulation of β-myosin heavy chain, skeletal muscle actin, as well as ventricular expression of ANP and BNP are characteristic features. However, the clear discrimination of different forms of hypertrophy precludes that a simple switchback of cardiac gene expression can explain the development of hypertrophy. More differentiated alterations are required to explain the differences in outcome.

Genetically modified mice represent interesting models to study the influence of a specific mutation on the cardiac gene expression profile, because, depending on the capacity of the heart to compensate for the loss of a specific gene, major alterations of the cardiac gene expression profile under basal as well as stress conditions can be expected. Myoglobin knockout (myo−/−) mice have been studied extensively during the last years, and it has been demonstrated that the heart activates a large set of compensatory mechanisms to adapt to the loss of myoglobin’s oxygen transport and storage function (14). Indeed, despite the loss of myoglobin, knockout mice display no major functional limitations. They perform almost as well as their wild-type (WT) littermates and show a similar adaptation to chronic normobaric hypoxia (17, 22). An increased capillary density, elevated coronary flow, and a slightly enhanced hematocrit lead to steeper O2 gradients, which may increase O2 diffusion from erythrocytes to the mitochondria of cardiac myocytes (16). In addition, myo−/− hearts display an altered substrate selection. Whereas the heart of WT mice preferentially metabolizes fatty acids and less glucose, myo−/− mice reduce the use of fatty acids in favor of glucose for energy generation (8). Since the caloric equivalent is higher for glucose, this shift also may result in an oxygen-saving effect. In summary, all of these adaptive mechanisms appear to be sufficient to compensate for the loss of myoglobin.

In addition to oxygen-related functions, work in myoglobin knockout mice has underlined the importance of myoglobin for nitric oxide (NO) metabolism. Regulation of coronary flow and contractile function are more sensitive to endogenous NO in myo−/− mice (9). In addition, genetic and pharmacological inactivation of myoglobin in transgenic mice with massive overexpression of inducible NO synthase in the heart demonstrated that myoglobin plays an essential role in protection of the heart from NO-mediated stress (13). Both, ex vivo and in vivo 31P-NMR spectroscopy revealed that in the absence of myoglobin cardiac energetics were compromised because a drop in creatine phosphate was observed (7).
In the present report, we addressed the question to what extent myo−/− hearts might be able to adapt to elevated cardiac stress in vivo. As a stress model, WT and myo−/− mice were chronically treated with isoproterenol (ISO) to induce cardiac hypertrophy and heart failure. In this model, functional consequences of chronic ISO treatment as well as associated changes in the cardiac gene expression program were analyzed.

MATERIALS AND METHODS

Animals. WT (NMRI strain) and myo−/− mice littermates were used in these studies. Mice were kept under conventional conditions and had access to tap water and rodent chow diet ad libitum. All animal experiments were approved by the Bezirksregierung Düsseldorf and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996).

Anaesthesia and mini-pump implantation. For the implantation of osmotic minipumps mice were anesthetized with intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (2.5 mg/kg) diluted in phosphate-buffered saline (final volume 200 μl). An incision was made on the back of each animal between the shoulder blades, and a micro-osmotic pump (Alzet type 1002; Durect) containing ISO dissolved in saline solution (0.9% NaCl, 5 mM ascorbic acid) was inserted into the infraspinacular subcutaneous tissue. ISO was applied at a dose of 4 mg·kg⁻¹·day⁻¹.

For the assessment of functional parameters mice were anaesthetized with 1.5% isoflurane in a gas mixture of 30% oxygen in nitrogen with a flow of 50 ml/min. Mice were manually restrained, and anesthesia was quickly induced by placing their heads into a home-built nose cone. Mice were respiring spontaneously during the whole experiment.

Microcatheter measurements. After 2 wk of ISO application (4 mg·kg⁻¹·day⁻¹) left ventricular (LV) function was assessed in vivo in closed-chest anaesthetized mice. Anaesthetized mice were kept on a warming table at 37°C, and the right carotid artery and the right jugular vein were exposed. The Millar tip catheter was advanced through a small incision in the right carotid artery into the LV, and cardiac function was recorded for 15 min. Data were recorded and analyzed with dedicated software (EMKA, France).

Volumes (V) were calculated from conductance measurements according to the simplified equation $V = C(L - L_0)$. $L$ is the instantaneous conductance, $L_0$ is the parallel conductance of the surrounding tissues, especially of the LV myocardium. $L_0$ was determined for each heart at the end of the experiment by a calibration measurement using the hypertonic NaCl dilution method according to published procedures (11). In brief, the jugular vein was cannulated and a bolus of 10 μl hypertonic (15%) NaCl was injected. The NaCl solution transiently increased the conductance of blood and produced an offset of the volume signal, which was used to determine parallel conductance. $C$ was determined once for each specific catheter by measuring the conductivity of murine blood, which was filled into small cylinders of defined volumes. These data were used for linear regression analysis where $C$ defines the slope of the V/L relationship.

Echocardiography. The animals were imaged in the parasternal short-axis plane through the anterior chest using a Hewlett Packard Sonos 5500 Ultrasound System equipped with a 15 MHz linear-phased array transducer. Routinely, data were acquired with a frequency of 70–80 Hz resulting in 7–9 images per heart cycle. To optimize the penetration depth for cardiac imaging the finger of a latex glove was filled with acoustic coupling gel (Aquasonic 100, Parker, Orange, NJ) and then placed over the tip of the transducer. The mice were kept at 37°C throughout the whole experiment. For analysis of cardiac function and hypertrophy the systolic (PWDs) and diastolic (PWDd) posterior wall dimensions as well as systolic (LVd) and diastolic (LVId) left ventricular inner diameters were measured. End systolic (ESV) and end diastolic (EDV) volumes were calculated from LVd and LVId using the Teichholz formula (26). Ejection fraction was derived from LVd and LVId as $EF = (LVId - LVd)/LVId$.

Total RNA isolation and cDNA synthesis. Hearts were collected, briefly washed free of blood in saline solution, and cut with a scalpel blade transversally to remove the atria and the heart base including the valves. Total RNA was isolated form the remaining left and right ventricular tissue using TRIzol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instruction followed by an additional column-based cleanup step (RNasey; Qiagen, Hilden, Germany). Quantification of RNA was performed by $A_{260}$ measurement (NanoDrop; Kisker, Steinfurt, Germany). The integrity of total RNA was checked by capillary electrophoresis (2100 Bioanalyzer, Agilent Technologies, Palo Alto, CA). Labeled cDNA probes were synthesized from 20 μg of total RNA. Reverse transcription was carried out in a 30 μl reaction volume with 0.5 μg oligo-dT₁₄-primer, 10 mM DTT, 20 U RNaseOut, 200 μM each of dATP, dCTP, dGTP, 80 μM dUTP, 40 μM Cy3-dUTP or Cy5-dUTP (Amersham Pharmacia Biotech, Freiburg, Germany), 200 U of Superscript III (Invitrogen, Karlsruhe, Germany). The labeled cDNA was purified using QIAquick PCR purification columns (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Microarray analysis. To study the differences in gene expression between WT and myo−/− mice in response to ISO treatment, we used oligonucleotide based microarrays. The Mus musculus AROS Oligo Set V4.0 was obtained from Operon (Cologne, Germany). Oligonucleotides (70 mers) were dissolved in amino spotting buffer to a concentration of 20 μM (Genetix, Hampshire, UK) and spotted onto UltraGap slides (Corning, Corning, NY). After the printing process the oligonucleotides were UV cross-linked (630 mJ/cm²) to the slide surface. Prior to hybridization the slides were incubated in a pre-warmed BSA blocking solution containing 5% SSC, 0.1% SDS, and 0.1 mg/ml BSA at 42°C for 45 to 60 min. Subsequently, slides were rinsed twice in 0.1× SSC for 5 min and for 30 s in double-distilled water, both at room temperature. The slides were then dried in a nitrogen flow. Cy3-/Cy5-labeled cDNA pools were combined in hybridization buffer (final concentration 50% formamide, 5× SSC, 0.1% SDS). Hybridization was carried out in a humid chamber at 42°C for 16 h. After the hybridization step, unbound cDNA and hybridization buffer were removed by several washing steps (2 times 10 min 2× SSC, 0.1% SDS, 5 times 1 min 0.1× SSC, and 10 s 0.01× SSC).

Data acquisition and analysis. Fluorescence signals were visualized by a GenePix 4000B laser scanner (Axon). GenePix Pro software (v. 6.0) was used to calculate fluorescence intensities. Data sets were analyzed by two different methods to get a robust set of differentially expressed genes. On the one hand, we used the ratio-based statistical methods to detect differentially expressed genes. On the other hand, we used a fluorescence intensity-based method to detect differentially expressed genes. For that purpose, we adjusted the raw fluorescence data for each wavelength of each slide to a common value and filtered for quality criteria (signal to noise ratio > 3, flag count = 0). Differentially expressed genes of the compared conditions were determined by Student’s t-test. Genes with significantly different expression levels ($P < 0.05$) in both analyses were included in the analysis.

To perform an exploratory pathway analysis the advanced pathway analysis tool within the GeneSpring GX 10 software package (Agilent Technologies, Waldbronn, Germany) was used to identify global molecular networks. The resulting networks are based on information available from curated databases implemented in the GeneSpring GX
Contractile parameters such as \( \frac{dP}{dt_{\text{max}}} \) and \( \frac{dP}{dt_{\text{min}}} \) and the heart rate of ISO-treated WT animals was elevated (Fig. 3). This effect was observed after 14 days of ISO stimulation. Compared with vehicle-treated animals, calculated in vivo by means of conductance manometry, these parameters were increased in myo-/- mice behaving differently in that LVIs and ESV decreased under ISO, whereas LVIDd and EDV were not significantly altered (Fig. 2). Fractional shortening increased in myo-/- hearts under ISO and was not altered in WT hearts.

In a separate experimental approach, cardiac function was analyzed in vivo by means of conductance manometry after 14 days of ISO stimulation. Compared with vehicle-treated animals, heart rate of ISO-treated WT animals was elevated (Fig. 3G). Contractile parameters such as \( \frac{dP}{dt_{\text{max}}} \) and \( \frac{dP}{dt_{\text{min}}} \) and the preload-independent parameter \( \frac{dP}{dt_{\text{max}}/iP} \) were also elevated (Fig. 3, A–C). No differences between WT and myo-/- mice in these parameters were detected, neither under basal nor under stimulated conditions. As in the echocardiographic measurements, the increase in these parameters most likely reflected the effect of ISO, which still persisted after 2 wk of treatment. Similar to our observations using echocardiography, ISO induced in WT mice enlargement of ESV and EDV (Fig. 3, E and F) that was accompanied by a decrease of EF by 30%. Representative recordings shown in Fig. 3H underline these findings and show that WT mice develop LV dilatation in response to ISO treatment as is indicated by the rightward shift of the pressure-volume (P-V) curve. In contrast, myo-/- mice showed no such alterations of the P-V loops after ISO treatment.

In a further set of experiments we addressed the question whether the observed differences in cardiac function went along with specific differences in the cardiac gene expression program. Therefore, we analyzed the cardiac gene expression profiles in a total of 32 hearts subdivided into four groups (8 WT vehicle, 8 WT ISO, 8 myo-/- vehicle, 8 myo-/- ISO). Cy3- and Cy5-labeled cDNA were hybridized to oligonucleotide arrays as outlined in Fig. 4A. This approach allowed the

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**Fig. 1.** Effect of isoproterenol (ISO) on hearts and lungs. A: cardiac hypertrophy is revealed by the normalized heart weights in sham and ISO-treated animals. B: lung edema in wild-type (WT) mice after ISO treatment as determined by lung dry/wet weight. Data represent means ± SD for \( n = 10 \) animals per group. * \( P < 0.05 \), ** \( P < 0.01 \) vs. basal conditions, ## \( P < 0.01 \) vs. myoglobin knockout (myo-/-) mice (ISO).

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**Fig. 3.** A: Representative recordings shown in Fig. 3H underline these findings and show that WT mice develop LV dilatation in response to ISO treatment as is indicated by the rightward shift of the pressure-volume (P-V) curve. In contrast, myo-/- mice showed no such alterations of the P-V loops after ISO treatment.

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**Table 1.** ISO treatment massively altered cardiac function in WT and myo-/- mice. The influence of ISO was evident by the increase in heart rate, which occurred in WT and myo-/- mice to a similar extent. In addition we found an increase in systolic and diastolic left ventricular posterior wall thickness (LVPWs; LVPWd) that is in line with the development of cardiac hypertrophy in response to chronic ISO treatment. Measurement of systolic and diastolic left ventricular inner diameter (LVIDs, LVIDd) revealed an increase in both values in WT hearts.

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**Data deposition.** The results of the microarray analyses were submitted to the National Center for Biotechnology Information’s Gene Expression Omnibus database and can be accessed under accession no. GS16246 and GS16243, respectively.
use of the ratio-based statistical analysis package Limma (23, 24). On the other hand, the fluorescence data were used for an analysis based on spot intensities (for details see MATERIALS AND METHODS). To minimize false positives and to obtain a robust set of differentially expressed genes we defined the intersection of genes identified by the ratio- and intensity-based analyses as the final set of differentially expressed genes for further analyses.

In line with the well-compensated phenotype of myoglobin-deficient mice we detected major differences in the basal cardiac gene expression profile between WT and myo−/− hearts (Suppl. Table S1). However, the surprising result was that chronic ISO treatment resulted in major differences in cardiac gene expression between WT and myo−/− hearts. As shown in Fig. 4B, intensity-based data analysis led to the detection of 280 differentially expressed transcripts in WT hearts after ISO treatment. A similar number of genes was found by means of the ratio-based analysis. However, when both lists of genes were compared a set of 180 genes was detected by both methods (see Suppl. Table S2). In myo−/− hearts ISO induced less changes in gene expression than in WT hearts. The total number of differentially expressed genes detected by the ratio- and intensity-based analysis was approximately only half of that found in WT hearts. Similar to the WT, the number of genes identified by both methods, namely 92 transcripts, represented 50% of the WT value (Suppl. Table S3). A comparison of these gene lists identified only 40 differentially expressed transcripts in WT as well as myo−/− hearts in response to ISO (Suppl. Table S4). Among these 40 genes we predominantly found genes that are associated with cardiac hypertrophy and remodeling, including ANP precursor NppA, collagens such as COL1A1, COL1A2, and extracellular matrix components including extracellular matrix protein 1 (ECM1) and fibronectin 1 (Fn1). One hundred forty genes in WT and 52 genes in myo−/− hearts were specifically altered in their expression depending on the genotype. In WT hearts additional components of the extracellular matrix were found to be upregulated (collagen 3A1, decorin, biglycan, lumican, etc.). In myo−/− hearts genes encoding proteins of carbohydrate and fatty acid metabolism were altered (e.g., phosphorylase kinase, pyruvate dehydrogenase kinase 4, enoylCoA hydratase, isovaleryl CoA hydratase). Besides these genes involved in metabolism, two genes encoding proteins with functions in attenuation of apoptosis (Pim1) and muscle repair (SNF1LK) are noteworthy candidates identified in ISO-treated myo−/− hearts.

To further analyze the gene expression data we performed a hierarchical cluster analysis based on the Treeview Algorithm (6). Therefore, a gene expression matrix containing all differentially expressed transcripts was created. This matrix was based on the normalized expression ratios of 16 two-color hybridizations according to Fig. 4A. As shown in Fig. 5, this cluster analysis led to major subclusters according to genotype and experimental condition. The comparison of WT and myo−/− hearts under basal conditions and ISO stress led to the identification of four distinct subclusters (Fig. 5, Ia/b, II, III, IVa,b) indicated by differently colored dendrograms (for gene lists see Supplementary Tables: Subcluster Ia/b, Suppl. Table S5; subcluster II, Suppl. Table S6; subcluster III, Suppl. Table S7; subcluster IVa/b, Suppl. Table S8). The green and red subclusters contained differentially expressed genes which were either upregulated (red subcluster /III) or downregulated (green subcluster /Ia) in WT and myo−/− hearts in response to ISO. In contrast, the blue (II) and purple (IVb) subclusters primarily contained transcripts which were differentially expressed in WT and myo−/− hearts under basal conditions and not affected by ISO. Interestingly, each of the subclusters I and IV contained an additional subgroup indicated by white and yellow boxes (Fig. 5). Intriguingly, the genes of subcluster IVa were upregulated in myo−/− hearts under basal conditions (yellow box, Suppl. Table S8) and also induced in WT hearts by ISO. Similarly, genes that were downregulated in myo−/− hearts under basal conditions (white box, subcluster Ia) were also downregulated in ISO-treated WT hearts. To verify the altered gene expression detected by our in-house manufactured oligonucleotide arrays, we analyzed several targets by real-time PCR using TaqMan assays. As shown in Suppl. Table S9b, there was a good correlation of array and real-time data.

To identify pathways that might explain the different phenotypes after ISO treatment we used the advanced pathway

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Table 1. Echocardiographic assessment of cardiac function in WT and myo−/− mice with ± ISO

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>WT + ISO</th>
<th>Myo−/−</th>
<th>Myo−/− + ISO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate,</td>
<td>496 ± 66</td>
<td>661 ± 47†</td>
<td>482 ± 41</td>
<td>642 ± 59†</td>
</tr>
<tr>
<td>beats/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVID(D), cm</td>
<td>0.37 ± 0.02</td>
<td>0.39 ± 0.04†</td>
<td>0.35 ± 0.02</td>
<td>0.36 ± 0.02‡</td>
</tr>
<tr>
<td>LVID(S), cm</td>
<td>0.19 ± 0.03</td>
<td>0.21 ± 0.04*</td>
<td>0.19 ± 0.02</td>
<td>0.17 ± 0.03‡</td>
</tr>
<tr>
<td>EDV, μl</td>
<td>58 ± 9</td>
<td>69 ± 16†</td>
<td>52 ± 7</td>
<td>54 ± 9§</td>
</tr>
<tr>
<td>ESV, μl</td>
<td>11 ± 4</td>
<td>16 ± 8*</td>
<td>12 ± 4</td>
<td>9 ± 3.6†‡</td>
</tr>
<tr>
<td>SV, μl</td>
<td>46 ± 7</td>
<td>53 ± 10*</td>
<td>39 ± 4</td>
<td>46 ± 8‡</td>
</tr>
<tr>
<td>EF, %</td>
<td>77 ± 6</td>
<td>77 ± 8</td>
<td>12 ± 6</td>
<td>85 ± 6*‡</td>
</tr>
<tr>
<td>LVPW(D), cm</td>
<td>0.12 ± 0.01</td>
<td>0.15 ± 0.02*</td>
<td>0.12 ± 0.11</td>
<td>0.15 ± 0.02‡</td>
</tr>
<tr>
<td>LVPW(S), cm</td>
<td>0.17 ± 0.02</td>
<td>0.20 ± 0.02†</td>
<td>0.17 ± 0.01</td>
<td>0.22 ± 0.04‡</td>
</tr>
<tr>
<td>FS, %</td>
<td>49 ± 6</td>
<td>47 ± 8</td>
<td>45 ± 4</td>
<td>53 ± 6*</td>
</tr>
</tbody>
</table>

Data represent means ± SD of n = 9 WT and n = 12 myoglobin knockout (myo−/−) mice. ISO, isoproterenol; LVID(D), left ventricular inner diameter, diastolic; LVID(S), left ventricular inner diameter, systolic; EDV, end diastolic volume; ESV, end systolic volume; SV, stroke volume; EF, ejection fraction; LVPW(D), left ventricular posterior wall, diastolic; LVPW(S), left ventricular posterior wall, systolic; FS, fractional shortening. | P < 0.05 and † P < 0.01 vs. basal conditions. ‡ † P < 0.05 vs. wild type (WT) under the same condition.

1 The online version of this article contains supplemental material.

Fig. 2. Echocardiographic assessment of cardiac function in WT and myo−/− hearts. M-mode registrations for a WT and myo−/− heart before and after ISO treatment are shown. Note the increase in end diastolic volume (EDV) and end systolic volume (ESV) in WT but not in myo−/− hearts.
analysis tool implemented in the GeneSpring GX10 software. As shown in Fig. 6, pathway analysis revealed extensive differences depending on the genotype. Figure 6A depicts pathways that are affected by ISO in WT hearts only. The most prominent pathways and biological processes involve transforming growth factor (TGF)-β and apoptosis. These pathways were not identified in myo−/− hearts. Only a small network connected to angiogenesis was identified (Fig. 6B). Figure 6C shows that that genes affected in WT as well as myo−/− hearts could not be arranged into a major network. Only a few genes belonging to extracellular matrix proteins formed a network together with apoE and LRP and VLDLR receptors.

**DISCUSSION**

Chronic ISO treatment represents a widely used model intending to mimic the sustained adrenergic stimulation that represents an important part of the pathogenesis of maladaptive cardiac hypertrophy. The activation of β-adrenergic signaling induces in the heart many different mechanisms that contribute to the hypertrophic phenotype, including enhanced protein synthesis and proto-oncogene expression, elevated oxidative stress, and stimulation of MAP kinases and phosphatidylinositol-3-kinases (for review see Ref. 18). Other hallmarks are the development of interstitial fibrosis and cardiac necrosis.

In view of the powerful adaptation of myoglobin-deficient mice we investigated to what extent myoglobin deficiency might influence the response of these mice to ISO-induced stress. We found that the degree of cardiac hypertrophy induced by ISO treatment was similar in WT and myo−/− mice. However, the increase in lung weight wet/dry ratio only in ISO-treated WT hearts revealed that the development of hypertrophy went along with lung edema, a sign of heart failure. Using two independent methods to characterize cardiac function, we showed that contractile parameters and heart rate were not different between WT and myo−/− mice. In myo−/− mice ESV and EDV were of similar size in ISO-
treated vs. untreated mice. In contrast, both parameters were elevated in ISO-treated vs. basal WT mice indicating that left ventricular dilatation occurred in WT hearts in response to ISO treatment. This went along with a reduced EF in WT hearts. Echocardiography also revealed similar levels of hypertrophy in WT and myo−/− hearts after 2 wk of ISO treatment. Moreover, EDV and ESV were shifted to the right in WT hearts, which is in accordance with the development of LV dilatation. In contrast, ESV in myo−/− was reduced under ISO, but EDV remained unaltered. Fractional shortening remained the same in WT but increased in myo−/− hearts. These results suggest that in the Echo series myo−/− hearts responded to catecholamine stimulation with elevated contractility, whereas WT hearts did not and were even dilated. Although we found differences with respect to EF in WT hearts (decrease in catheter series, unaltered in the Echo series) both experimental approaches consistently demonstrated that, surprisingly, loss of myoglobin led to a better adaptation to chronic ISO-induced cardiac stress.

What might be the basis for the beneficial effect of myoglobin deficiency in the setting of heart failure development? It appears a paradox that the loss of an oxygen transport and storage protein leads to better adaptation in the setting of heart failure. Rather, the contrary would have to be expected. To characterize ISO-induced effects on the molecular level and thereby approach to the cause for the different ISO effects, we analyzed cardiac gene expression profiles using 40K oligonucleotide arrays. Earlier studies have demonstrated that cardiac gene expression profiles may differ in a chamber-specific manner (3). Since ISO affects the whole myocardium we used total left and right ventricular RNA to avoid interference with the gene expression profile due to experimental manipulation.

The surprising finding of the these analyses was that ISO treatment led to the activation of gene expression programs that displayed substantial differences in WT and myo−/− hearts. Using stringent criteria for the definition of differentially expressed transcripts, we found that WT hearts expressed 180 genes differentially, whereas in myo−/− hearts the number of genes with altered expression amounted only to 92, i.e., 50% of the WT. When we compared these sets of genes there was an overlap of only 40 genes that were affected both in WT and in myo−/− hearts. On the other hand 140 genes were exclusively modulated in WT and 52 in myo−/− hearts, respectively.

Hierarchical cluster analysis clearly separated the four different experimental groups according to genotype and treatment conditions (WT vs. myo−/−, WT vs. WT ISO, myo−/− vs. myo−/− ISO, and WT ISO vs. myo−/− ISO). As expected on the basis of the well-described phenotypic alterations in myo−/− hearts (12, 16, 17), there exist clear differences in the cardiac gene expression profiles under basal conditions between WT and myo−/− mice.

As pointed out above ISO influenced a common set of genes in WT and myo−/− hearts. Among these genes we identified mainly well-known candidates that are usually altered in the setting of cardiac hypertrophy and remodeling, a.o., ANP, collagen (COL5A2, COL1A1, COL1A2), and other extracellular matrix proteins ([ECM1), microfibrillar-associated protein 4 (MFAP4), FN1). Further genes that have been described to be upregulated in various models of hypertrophy such as FST1 (follistatin-like protein 1) (19) or FHL1 (four and a half Lim domain protein) (10) were also found to be upregulated in WT and myo−/− hearts.

In ISO-treated WT hearts there was an additional upregulation of other genes that were not affected in myo−/− hearts, including collagen COL3A1 and several other genes involved in the formation and stabilization of extracellular matrix such as decorin (DCN), biglycan (BGN), lumican (LUM), fibrillin 1 (FBN1), elastin microfibrillar interfae 2 (EMILIN2), and microfibrillar-associated protein 5 (MFAP5). Thus, concomitant with the development of LV dilatation, genes involved in cardiac remodeling appear to be more affected in WT than in myo−/− hearts. At present, it is unclear whether the upregulation of these extracellular matrix components may cause the development of heart failure or whether they are induced to
mechanically stabilize the dilated myocardium. However, recent findings demonstrate that loss of biglycan destabilizes the myocardium and aggravates LV dilatation after myocardial infarction (2, 27), supporting the view of a stabilizing function in our model as well.

Among the genes specifically altered by ISO in myo−/− hearts one set linked to substrate metabolism was identified. The up-regulation of PHKA1 encoding a subunit of phosphorylase kinase suggests a higher capacity of the heart to perform glycogenolysis. In addition, a marked downregulation of PDK4, the regulated pyruvate dehydrogenase kinase, suggests a lower inhibition of pyruvate dehydrogenase and therefore an elevated carbohydrate utilization by ISO-treated myo−/− hearts (25). Moreover, genes encoding enzymes involved in fatty acid metabolism (enoyl CoA hydratase, isovaleryl CoA hydratase, and dienoyl CoA reductase) were found to be downregulated. In an earlier study we could show by 13C-NMR spectroscopy and positron emission tomography that loss of myoglobin induced a change in cardiac substrate selection. Whereas WT hearts predominantly used fatty acids as an energy source, myo−/− hearts switched to enhanced glucose utilization and reduced fatty acid consumption (8). The preferred use of oxygen-rich glucose may represent an adaptive mechanism to the loss of the myoglobin-associated oxygen storage and transport capacity. Thus, it appears that ISO stimulation might further augment the metabolic remodeling observed in myo−/− hearts already under basal conditions.

An intriguing question is whether gene expression analysis might reveal some plausible candidates that could act as master regulators responsible for the adaptive phenotype of myo−/− hearts. At present, we can only speculate, but some of the differentially expressed genes found in myo−/− hearts after ISO treatment are of special interest. For example, the upregulation of protein kinases PIM3 and SNF1LK is an interesting observation. Pim3 mediates antiapoptotic effects by phosphorylation of the proapoptotic protein Bad (21). SNF1-like kinase has recently been shown to be upregulated in skeletal muscle of mice in response to ISO administration. It inactivates the class II histone deacetylases HDAC4 and HDAC5 and thereby contributes to muscular repair (1). Thus, it is conceivable that a similar function may be executed in cardiac muscle. Since modulation of HDAC activity is associated with more general changes of gene expression SNF1LK represents an interesting candidate that might be causally involved in the adaptation of myo−/− hearts to ISO-induced cardiac stress.

Cluster analysis revealed another interesting feature of the gene expression profiles. Approximately half of the genes that were differentially expressed in WT hearts in response to ISO were found in the same subcluster with genes that are differentially expressed in myo−/− hearts already under basal conditions. Thus, it appears that part of the genetic program that was activated in WT mice by ISO was already executed in
myo−/− mice most likely as part of the well-compensated phenotype. Thus, WT and myo−/− mice had intrinsically different starting conditions when we induced ISO stress. The well-adapted phenotype of myo−/− hearts may therefore result at least in part from the already activated adaptive gene expression program. The genes in these subclusters may be linked to miscellaneous biological functions. The downregulation of set and mynd domain protein 1 (SMYD1) that represents a histone methyltransferase involved in cardiogenesis (15, 20) is of special interest, as is the idea for the SNF1LK-dependent inactivation of HDACs; downregulation of SMYD1 may pose major alterations on cardiac chromatin structure and gene expression that might contribute to the activation/inactivation of specific sets of genes that mediate efficient adaptation of myo−/− hearts to ISO-induced cardiac stress.

To gain further insight into mechanisms that might explain the different phenotypes we performed an exploratory pathway analysis. By this approach we identified substantial differences between WT and myo−/− hearts. Genes specifically affected in WT hearts clustered around two nodes with TGF-

Fig. 6. Result of the pathway analysis: A: networks specifically regulated in WT mice after ISO treatment. B: networks specifically regulated in myo−/− mice after ISO treatment. C: networks regulated in WT and myo−/− mice after ISO treatment. All direct interactions are presented: purple square, regulation; rose square, protein modification; red square, expression; blue square, binding; turquoise rhombus, metabolism.
apoptosis in their centers. TGF-β-signaling as well as apoptosis are involved in cardiac remodeling. Therefore, the identification of both processes is consistent with the phenotype of cardiac dysfunction in WT hearts. In myo—/— hearts, however, these large networks were not identified. In contrast, only a small specific network related to angiogenesis was identified. Thus, our pathway analysis confirms that myo—/— hearts activate a distinct gene expression profile. The lack of activation of the profibrotic and apoptotic pathways may explain the conserved cardiac function in myo—/— mice.

In summary, our data show that the genetic background (WT, myo—/—) has a major impact on the capacity of the heart to adapt to cardiac stress. The adaptation on the functional level is associated with alterations of the cardiac gene expression profiles that appear to be quite specific for WT and myo—/— hearts, and 2) another subset of genes that is differentially expressed in WT hearts in response to ISO was already altered in myo—/— hearts under basal conditions. It is tempting to speculate that (parts of) these sets of genes specifically altered in myo—/— hearts may have provided them with protection from ISO-induced stress. At present we cannot answer the question which genes are responsible for the adaptive phenotype of myo—/— hearts, but with SNF1LK and MYD1, interesting candidates have been identified. Thus, functional analysis of single candidates defined here may help to uncover novel mechanisms of cardiac protection.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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