Mechanical Load–Dependent Regulation of Gene Expression in Monocrotaline-Induced Right Ventricular Hypertrophy in the Rat

Harald Kögler, Oliver Hartmann, Kirsten Leineweber, Phuc Nguyen van, Peter Schott, Otto-Erich Brodde, Gerd Hasenfuss

Abstract—Rats treated with monocrotaline (MCT) develop pulmonary hypertension. Their right ventricles (RVs) exhibit severe pressure overload–induced hypertrophy, whereas the left ventricles (LVs) are normally loaded. In contrast, enhanced neuroendocrine stimulation during the transition to heart failure affects both ventricles. We assessed gene expression levels of Ca^{2+}-regulating proteins in RVs and LVs of control and MCT rats in transition to heart failure to identify biomechanical load–regulated genes. In MCT RVs, both mRNA and protein levels of the Ca^{2+}-ATPase of the sarcoplasmic/endoplasmic reticulum (SERCA2a) were reduced by 36% ($P=0.001$) and 17% ($P=0.016$), respectively, compared with control RVs. Phospholamban and ryanodine receptor mRNA levels likewise were reduced (by 27% [$P=0.05$] and 21% [$P=0.011$], respectively) in MCT RVs, whereas sarcolemmal Na^{+}-Ca^{2+} exchanger expression was not altered. MCT LVs exhibited no significant expression changes compared with control LVs. Isometrically contracting MCT intact RV trabeculae showed enhanced baseline force development. Although control RV preparations exhibited a positive force-frequency relationship, MCT RVs showed a negative force-frequency relationship and blunted postrest potentiation. Contractile function of MCT LV trabeculae was normal. Maximum Ca^{2+}-activated tension was enhanced by 64% in permeabilized RV MCT preparations ($P=0.013$). β-Myosin heavy chain protein was upregulated in MCT RVs ($P<0.001$) but unaltered in MCT LVs. Degradation of troponin T was prominent in MCT RVs, a phenomenon not observed in the LV. Enhanced biomechanical load is necessary to induce the gene expression changes associated with the hypertrophic phenotype of the pressure-overloaded RV. Neuroendocrine factors, which equally affect both chambers, are not sufficient to alter the expression of Ca^{2+}-cycling proteins. (Circ Res. 2003;93:230-237.)

Key Words: hypertrophy ■ mechanical load ■ gene expression ■ calcium ■ contractility

When a heart develops hypertrophy and, subsequently, heart failure (HF), profound changes at the macroscopic and molecular level occur. This remodeling is associated with characteristic gene expression changes, such as the reactivation of a fetal gene expression program, causing the expression of atrial natriuretic factor and B-type natriuretic peptide and the switching of myosin heavy chain (MHC) isoforms from α to β and of actin isoforms from α-cardiac to α-skeletal actin. Additionally, the expression levels of Ca^{2+}-regulating proteins change in a typical way: the ATPase of the sarcoplasmic/endoplasmic reticulum (SERCA2) is downregulated in human end-stage HF and in several experimental HF models. The sarcolemmal Na^{+}-Ca^{2+} exchanger (NCX), in contrast, is upregulated. Increased biomechanical load and neuroendocrine stimulation have been implicated as potential triggers of these changes, but the relative importance of these stimuli as regulators of gene expression remains to be elucidated for each individual gene.

In the present study, we used the monocrotaline (MCT) model of right ventricular (RV) hypertrophy in the rat to address this issue. A single injection of the plant alkaloid MCT causes obliterating vasculitis of lung arterioles, leading to pulmonary hypertension. The chronically elevated RV afterload causes myocardial hypertrophy, and some of the animals develop HF. Hemodynamically, this animal model is characterized by unaltered mean arterial blood pressure, indicating normal left ventricular (LV) function, in the presence of enhanced RV systolic pressure. Because the LV is normally loaded, changes in gene expression that occur in RV but not LV myocardium are assumed to be induced by biomechanical load. In contrast, both ventricles are exposed to enhanced neuroendocrine stimulation during the final transition to HF. Thus, expression changes of genes primarily regulated by neuroendocrine mechanisms are expected to occur in both ventricles to a similar degree. Using this approach, Leineweber et al demonstrated that increased...
load in the absence of neuroendocrine activation is not sufficient to induce the β₁-adrenergic receptor (β₁-AR) down-regulation typically observed in HF but that exposure to these two combined stimuli is necessary to downregulate β₁-ARs. In the present study, we report that enhanced biomechanical load is necessary for the downregulation of SERCA2, phospholamban (PLN), and the ryanodine receptor (RyR2) as well as for the upregulation of β-MHC, whereas NCX does not appear to be load-dependently expressed. The observed changes in gene expression levels are shown to be functionally relevant. A preliminary report has recently appeared.13

Materials and Methods

Animal Model

Six-week-old male Wistar rats12 received a single subcutaneous injection of MCT (MCT group, 50 mg/kg body wt) or an equal volume of solvent (control group, 1 mL/kg body wt). With an unrestricted food supply, MCT animals consume less than control animals and gain significantly less weight. To prevent MCT-induced alterations of gene expression and contractile function from being modulated by differences in the nutritional state between groups, MCT rats had an unlimited food supply, whereas the amount of food given to control animals was restricted to the quantity consumed by MCT rats on the previous day.12,14 All animal procedures were approved by the government committee for animal studies and carried out according to German laws regarding the care and use of laboratory animals.

Rat Intact Muscle Strip Preparation

Rats were euthanized on days 20 to 24 after MCT injection by halothane insufflation, and hearts were rapidly excised and retrogradely perfused with a modified Krebs-Henseleit buffer solution containing (in mmol/L) Na⁺ 140.5, K⁺ 5.1, Mg²⁺ 1.2, Ca²⁺ 0.25, Cl⁻ 124.9, SO₄²⁻ 1.2, PO₄³⁻ 2.0, HCO₃⁻ 20, glucose 10, and butanedione monoxime 20, equilibrated with carbon (95% O₂/5% CO₂), pH 7.4. Intact trabeculae or papillary muscles were isolated from the RV or LV wall and mounted isometrically in a superfusion bath between a force transducer (Scientific Instruments) and a hook connected to a micromanipulator for length adjustment. Muscle diameters were similar in all groups directly compared with each other (see online data supplement for details). Measurements were carried out at 15°C. The compositions of relaxation and activation solution are provided in an expanded Materials and Methods section in the online data supplement. Intermediate [Ca²⁺] levels were obtained by mixing appropriate amounts of relaxation and activation solutions. The free [Ca²⁺] was calculated by the computer program WinMAXC,15 and using the stability constants provided by Martell and Smith.16

Plasma NA Levels

Blood drawn from the ophthalmic venous plexus of anesthetized rats before heart excision was collected in a potassium-EDTA S-Monovette (Sarstedt), and glutathione was added at a final concentration of 1 μmol/mL plasma. Samples were centrifuged at 600g for 5 minutes at 4°C, and plasma was removed, snap-frozen in liquid nitrogen, and stored at −80°C. Plasma noradrenaline (NA) was assessed by high-pressure liquid chromatography with fluorometric detection (for details, see Schäfers et al17).

Protein Expression

RV and LV MCT and control myocardium was snap-frozen in liquid nitrogen immediately after dissection of the trabeculae for force measurements and stored at −80°C. Samples were thawed on ice in 50 μL of homogenization buffer (see online data supplement for details), homogenized, and sonicated at 4°C. Protein concentrations of the suspensions were determined according to the method of Lowry et al18, Samples of 40 μg were denatured in electrophoreses buffer (see online data supplement for details) at 95°C and subjected to SDS-PAGE. MHC isoform expression was analyzed by densitometry on a Coomassie-stained 5% SDS-acrylamide gel. Initial current was set at 15 mA and then increased to 25 mA after the bromophenol blue front line reached the separating gel. Western blotting was carried out according to standard protocols, using antibodies against calsequestrin (CS, polyclonal, Affinity Bioreagents), SERCA2a (monoclonal, Affinity Bioreagents), NCX1 (monoclonal, Santa Cruz Biotech), PLN (monoclonal, Upstate Biotechnology), and troponin T (TnT, monoclonal, Sigma). CS served as an internal standard to normalize protein levels.

Quantification of mRNA Expression

DNA-free total RNA was extracted using a Qiagen RNeasy kit and an RNase-Free DNase Set. First-strand cDNA synthesis was performed with the reverse transcriptase SuperScript II and random primers according to the supplier’s instructions (Invitrogen). Polymerase chain reaction (PCR) was performed with a real-time PCR LightCycler (Roche) in a final volume of 20 μL in glass capillaries (see online data supplement for details). After initial denaturation at 95°C for 30 seconds, the samples underwent 45 cycles of 94°C for 0 seconds (recycling immediately after peak), 60°C for 5 seconds, and 72°C for 10 seconds. Emission at 520 nm was measured every cycle at 87°C for SERCA2a, at 83°C for CS and PLN, at 82°C for NCX, and at 85°C for RyR. For details on the primer pairs used, see the online data supplement.

Data Analysis and Statistics

Force was converted to tension by normalizing to the cross-sectional area of each preparation. To characterize the tension-[Ca²⁺] relationship in skinned fiber preparations, tension data and the respective free [Ca²⁺] were fitted to a Hill equation as follows: Fₜ = Fₜₘₐₓ × (Fₜₘₜ₋₉ₖ)/(Ca⁹⁺)² + [Ca⁹⁺], where Fₜ is actual tension, Fₜₘₜ is resting tension, Fₜₘₜ is maximal Ca²⁺-activated tension, [Ca⁹⁺] is the actual [Ca²⁺], Ca₉⁺ is the [Ca²⁺] required for development of half-maximal tension, and n is the Hill coefficient. Data are expressed as mean ± SEM. Statistical analysis used a

Myofilament Ca²⁺ Responsiveness

Thin RV trabeculae were permeabilized overnight (minimum 10 hours) at 4°C in relaxation solution containing 1% (vol/vol) Triton X-100. Smaller bundles were dissected from these trabeculae, mounted isometrically using T-clips, and stretched to the length at which passive tension just began to increase. This corresponded to a sarcomere length of 1.9 μm (laser light diffraction), without a difference between groups. Measurements were carried out at 15°C. The composition of relaxation and activation solution are provided in an expanded Materials and Methods section in the online data supplement. Intermediate [Ca²⁺] levels were obtained by mixing appropriate amounts of relaxation and activation solutions. The free [Ca²⁺] was calculated by the computer program WinMAXC15 and using the stability constants provided by Martell and Smith.16

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repeated-measures ANOVA, followed by the Bonferroni correction for multiple comparisons, or (for gene-expression analyses) an unpaired Student t test. Two-sided values of \( P < 0.05 \) were considered statistically significant.

**Results**

**Animal Model**

MCT rats were euthanized on days 20 to 24 after injection of MCT, when they showed lethargy, accelerated breathing, and ruffled fur. At that time, neither ascites nor pleural effusions were observed in any of the MCT animals. Control rats injected with saline solution on the same day were euthanized accordingly. Although we adjusted the intake of food by the control group to the spontaneously reduced intake by MCT according to the weight/body weight in the MCT group (231.5 g [control], 225.6 g [MCT], \( P < 0.001 \); n = 10 each). These organ weight data are fully compliant with earlier reports.

We assessed plasma NA levels in a separate set of reports. We examined isometrically contracting intact muscle strips isolated from the RVs and LVs of both control and MCT rats. Figure 2 summarizes FFR and postrest behavior data. Between 2 and 7 Hz, there was a steady increase in developed tension in control RV preparations (n = 8), indicating a positive FFR in this group (Figure 2A). The same test carried out in MCT RV preparations (n = 7) revealed two striking differences (Figure 2A). First, developed tension at the baseline stimulation rate of 2 Hz was significantly enhanced in the MCT group, from 3.9 ± 0.9 to 10.6 ± 2.0 mN/mm² (\( P = 0.017 \)). Second, developed tension monotonously decreased with an increasing stimulation rate, indicating a negative FFR. The relationships exhibited a highly significant difference between groups (\( P < 0.001 \)). FFRs were superimposable in control LV preparations (n = 6) and control RV preparations (Figures 2A and 2B). In contrast, MCT LV preparations (n = 7) were largely different from their RV counterparts: Baseline developed tension was similar to that of control LVs; also, the overall FFR was positive in LV preparations from MCT rats (Figure 2B). Multiple linear regression analysis revealed no significant difference between MCT and control LVs.

During the examination of postrest behavior, control RV preparations (n = 8) showed strong rest potentiation, with a maximum postrest twitch amplitude (3.7 ± 0.5-fold potentiation) found after an interval of 30 seconds (Figure 2C). The postrest behavior of control LV preparations (n = 5) was...
MCT RVs, RT50 was significantly higher than in control preparations over the entire range of stimulation rates (Figure 3C, P<0.001), indicating diastolic dysfunction. In contrast, MCT LV preparations exhibited RT50 values similar to those of control LV preparations. Thus, the impairment of relaxation in MCT rats was restricted to the RV.

**Myofilament Ca**\textsuperscript{2+} Responsiveness

The results obtained on intact multicellular muscle strips indicate that Ca\textsuperscript{2+} handling is dysfunctional in MCT RV myocardium. Nevertheless, baseline tension development was enhanced in MCT RV preparations. A potential cause for this apparent discrepancy would be an increase in myofilament Ca\textsuperscript{2+} responsiveness, thereby enabling the myocardium to produce adequate force despite reduced activating [Ca\textsuperscript{2+}].

Therefore, we compared the tension-[Ca\textsuperscript{2+}] relationships of MCT and control RV myocardium in detergent-skinned trabeculae (Figures 4A and 4B). Maximum Ca\textsuperscript{2+}-activated tension was significantly enhanced by 64% in MCT RVs (n=8) compared with control RVs (n=13, P=0.013). Figure 4B presents the same data normalized for resting and maximum Ca\textsuperscript{2+}-activated tension. It becomes obvious that there is a slight, but significant, leftward shift of the tension-[Ca\textsuperscript{2+}] curve of MCT RV fibers (ΔpCa 0.08, P=0.014), indicating Ca\textsuperscript{2+} sensitization, compared with control RV fibers.

**Expression of Ca**\textsuperscript{2+}-Regulating Proteins

We quantitatively analyzed mRNA and protein expression levels of several target genes involved in the maintenance of Ca\textsuperscript{2+} homeostasis in RV and LV myocardium from control and MCT rats. In MCT RVs compared with control RVs, the SERCA2a/CS mRNA ratio was reduced by 36% (n=8 [MCT] versus n=7 [control], P=0.001), whereas in MCT LVs (n=8), the SERCA2a/CS mRNA ratio was similar to that of the respective control LVs (n=7, Figure 5A). Also, at the protein level, the SERCA2a/CS ratio in MCT RVs (n=6) compared with control RVs (n=6) was significantly reduced (~17%, P=0.016), whereas the SERCA2a/CS ratio was unchanged in the LVs (Figure 5B). A representative immunoblot from RV myocardium is shown in Figure 5C. We next examined RyR mRNA expression and likewise found that the RyR/CS mRNA
ratio was reduced in MCT RVs (n=8) compared with control RVs (28%, n=7; P=0.01), whereas in the LV, the RyR/CS mRNA ratio was not different between groups (n=8 and n=7, respectively; Figure 6A). Also, the PLN/CS mRNA ratio had decreased in MCT RV myocardium (27%; n=8 and n=7, respectively; P=0.049), whereas in the LV, no significant change was observed (n=8 and n=7, respectively; Figure 6B).

We investigated whether NCX mRNA expression is altered in the hearts of MCT rats. Neither in the RV nor in the LV was there a significant change in NCX/CS mRNA expression (n=8 and n=7, respectively; Figure 6C); also, at the protein level, no significant difference was observed between groups (n=6 each, Figure 6D).

Discussion
In HF, the myocardium is subjected to a combination of increased biomechanical load and enhanced neuroendocrine...
LVs.
sively in MCT RV and is absent in con RVs and MCT or con
TnT. A prominent lower molecular mass band appears exclu-
Figure 7. Myofilament proteins. A, Coomassie-stained SDS-
PAGE gel showing MHC isoform composition of MCT and con-
trol RVs. B, Fraction of total MHC present as β-isoform in con-
trol (con) and MCT RV myocardium (n=7/7, *P<0.01). C, 
Representative Western blot with antibodies against cardiac
MHC/α-MHC, β-MHC, and stimulated adenylyl cyclase activity 
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phosphorylation. Consistent with these assumptions, we dem-
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from MCT RV (1) increased relaxation time, (2) negative 
FFR and blunted postrest potentiation, and (3) decreased 
contractile reserve after treatment with isoproterenol, whereas 
none of these functional changes were observed in MCT LV 
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rate or β-AR stimulation. Therefore, the observed alterations 
in contractility reflect intrinsic myocardial properties, and we 
feel confident to conclude that the biomechanical load– 
duced downregulation of SERCA2a expression in this 
model is functionally relevant. This is also supported by a 
finding reported previously and indicating that in myocardium 
during reperfusion, the diastolic decline in [Ca2+]i, is 
prolonged. Potential consequences of RyR downregulation 
are more difficult to predict, especially because it has recently 
become obvious that in HF, RyR function is modulated by 
force-mediated load in this animal model remains the primary factor expected to alter gene expression.

What are the functional consequences of these expression changes? We did not directly assess the Ca2+ uptake function of the SR. However, it can be assumed that decreased abundance of SERCA2a, which was confirmed at the protein level, will (1) slow the decline in [Ca2+]i (and thus, in force) during relaxation, (2) cause a net loss of Ca2+ from the cell due to alternative transsarcolemmal elimination via NCX, and (3) limit the maximum inotropic response to β-AR stimulation, which is mainly mediated by enhanced Ca2+ uptake into the SR after disinhibition of SERCA2a activity after PLN phosphorylation. Consistent with these assumptions, we dem-
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cannot be completely excluded, the difference in biomechanical 
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expected to alter gene expression.

We12 have recently shown that both the activity and density of the NA transporter are reduced in hypertrophied MCT RV myocardium. It might be argued that the activity and density of the NA transporter are reduced in hypertrophied MCT RV myocardium. It might be argued that this, in principle, could cause local RV synaptic cleft NA levels to increase to levels higher than in the LV despite identical plasma NA levels. This, in turn, would imply that it may not be correct to assume an increased biomechanical load to be the sole factor altered 
within the RVs of MCT rats. However, the transition to HF in these animals is characterized by a reduction in β-AR expression,12 such that local downregulation of the NA transporter will to some extent be offset by a reduced sensitivity of the β-AR cascade in RV myocardium of MCT rats. Consistent with this notion, hypertrophied hearts of MCT rats exhibit a reduction in basal 
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stimulation. The relative contributions of load and humoral 
factors in mediating the gene expression changes typically 
associated with this condition are unclear. Therefore, we 
sought to resolve whether or not enhanced biomechanical 
load is a necessary prerequisite for this molecular remodeling. 
In the present study, we report that in rats with MCT-induced 
RV hypertrophy, RV myocardium exhibits a gene expression 
pattern distinct from that of LV myocardium despite plasma 
NA levels that were elevated to about the same extent as 
in MCT LV myocardium compared with myocardium of 
saline-treated control rats. This suggests that the enhanced 
mechanical load on the RV due to pulmonary hypertension is 
necessary to induce these changes. NCX, on the other hand, 
was not differentially expressed, indicating that the regulation 
of NCX expression in this experimental model is not sensitive 
to mechanical load.

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crease in RyR density in the junctional SR could reduce the gain of Ca\(^{2+}\)-induced Ca\(^{2+}\) release, but this was not tested in the present study. A downregulation of PLN may be considered as a compensatory response that in the presence of reduced SERCA2a levels will serve to maintain the SERCA2a/PLN stoichiometry.

An interesting feature of the contractile phenotype of intact MCT RV trabeculae was the considerably enhanced baseline force development (1.25 mmol/L Ca\(^{2+}\), 2-Hz stimulation, and absence of inotropic intervention), which seemingly contradicts the defective Ca\(^{2+}\) cycling. Our observation of a substantially increased maximum Ca\(^{2+}\)-activated tension and slight, but significant, Ca\(^{2+}\) sensitization in permeabilized preparations provides a reasonable explanation for this phenomenon, inasmuch as enhanced Ca\(^{2+}\) responsiveness allows for the maintenance of adequate force development despite reduced levels of [Ca\(^{2+}\)]. Interestingly, other studies investigating the effect of RV pressure overload on myofilament function did not observe enhanced Ca\(^{2+}\) responsiveness, in disagreement with our findings. However, in one of the studies using pulmonary artery banding in ferrets, the degree of hypertrophy was rather mild, raising the possibility that the biomechanical stimulus was not sufficient to trigger myofilament alterations. On the other hand, the other study, which used pulmonary artery banding in rats, examined myofilament phenotype after long-term pressure overload with overt clinical signs of HF. This makes direct comparison with the present study difficult. We cannot exclude the possibility that in our model of MCT-induced RV hypertrophy, if animals survive for several months to reach a state of end-stage HF, a different myofilament phenotype might also arise.

The MHC isoform composition has been shown to affect the Ca\(^{2+}\) responsiveness of the myofilaments. Therefore, we probed MCT myocardium for changes in MHC isoform composition and, consistent with findings in other myocardial hypertrophy models in the rat, observed significant up-regulation of β-MHC at the protein level. It has been proposed that expression of the slowly cycling β-MHC by increasing the duty cycle would increase the force-time integral for a given Ca\(^{2+}\) saturation of troponin C, thereby causing Ca\(^{2+}\) sensitization of the myofilaments, and a leftward shift of the pCa-tension relationship of the same order of magnitude observed in the present study has been reported in hypothyroid rats expressing high levels of β-MHC. However, this concept has recently been challenged, and an increase in maximum Ca\(^{2+}\)-activated tension, as reported in the present study, has not been found to be associated with β-MHC expression. Thus, additional alterations of myofilament composition are likely to exist in MCT RV myocardium. Because an isoform shift of TnT has been reported to occur in failing human hearts, we examined whether similar changes can be found in MCT-induced RV hypertrophy. Only 1 TnT band could be detected in control RV and control and MCT LV myocardial homogenates, whereas an additional prominent band with a molecular mass of ~27 kDa was consistently observed in MCT RV samples. We have recently reported that treatment with reactive oxygen species induces specific proteolysis of TnT in rabbit myocardium. Whether this band represents a different isoform or a proteolytic fragment is currently under investigation. The potential functional significance of this change, especially with respect to the observed changes in myofilament Ca\(^{2+}\) responsiveness, remains to be elucidated.

In summary, we find downregulated SERCA2a, RyR, and PLN expression as well as upregulation of β-MHC in pressure overload-induced RV hypertrophy in the rat. Neuroneuroendocrine activation alone is not sufficient; enhanced biomechanical load is necessary to induce these changes. MCT-induced pulmonary hypertension along with consecutive RV hypertrophy and failure is an experimental tool ideally suited to investigate the relative roles that mechanical and humoral stimuli play in the regulation of gene expression in the myocardium.

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