Vascular Reactivity in Heart Failure
Role of Myosin Light Chain Phosphatase

Syed M. Karim, Albert Y. Rhee, Allison M. Given, Michael D. Faulx, Brian D. Hoit, Frank V. Brozovich

Abstract—Congestive heart failure (CHF) is a clinical syndrome, which is the result of systolic or diastolic ventricular dysfunction. During CHF, vascular tone is regulated by the interplay of neurohormonal mechanisms and endothelial-dependent factors and is characterized by both central and peripheral vasoconstriction as well as a resistance to nitric oxide (NO)–mediated vasodilatation. At the molecular level, vascular tone depends on the level of regulatory myosin light chain phosphorylation, which is determined by the relative activities of myosin light chain kinase and myosin light chain phosphatase (MLCP). The MLCP is a trimeric enzyme with a catalytic, a 20-kDa and a myosin targeting (MYPT1) subunit. Alternative splicing of a $3^\prime$ exon results in a shift of the reading frame, which is determined by the relative activities of myosin light chain kinase and myosin light chain phosphatase (MLCP). The MLCP is a heterotrimeric enzyme (see review [7]) consisting of a central and $3^\prime$ exon results in a shift of the reading frame, whereas the activity of MLCP can be both inhibited to produce Ca$^{2+}$ desensitization (see review[15]), or stimulated to produce Ca$^{2+}$ sensitization (see review[15]).

MLCP is a heterotrimeric enzyme (see review[17]) consisting of a 37-kDa catalytic subunit, a 20-kDa subunit of unknown function, and a 130/133-kDa myosin targeting subunit (MYPT1). Alternative splicing of a central and 3′ exon generates four isoforms of MYPT1.[17,19] Specifically, exclusion of a 31-bp 3′ exon results in a shift of the reading frame of the MYPT1 transcript to encode a C-terminal LZ motif.[19] Additionally, both the activity[20] and regulation[21] of MLCP has been suggested to be isoform specific.

NO-mediated vasodilatation is one of the fundamental vascular responses.[16,22] NO is known to activate the soluble smooth muscle contraction[11] and thus vascular tone. Therefore, vascular tone is critically dependent on the activities of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP), and steady state MLC20 phosphorylation can be computed[12] as the activities of (MLCK/MLC20) and (MLCP). MLCP is a Ca$^{2+}$-calmodulin dependent enzyme, whereas the activity of MLCP can be both inhibited to produce Ca$^{2+}$ desensitization (see review[15]), or stimulated to produce Ca$^{2+}$ sensitization.[15,16]
pool of guanylate cyclase and result in an increase in cGMP, which in turn activates type I protein kinase G (PKGα). The activation of PKGα results in smooth muscle cell relaxation and a vasodilatation attributable to hyperpolarization,23 a decrease in Ca⁺⁺ flux,24,25 and an activation of MLCP.26 The importance of cGMP-mediated activation of MLCP in smooth muscle relaxation is underscored by recent results that have demonstrated that the sensitivity of smooth muscle relaxation to cGMP is regulated by the level of expression, relative to total MYPT1 expression, of the LZ⁺⁻ MYPT1 isoform19 and PKGα activation of MLCP requires a LZ⁺⁻ MYPT1.27

We have previously shown for MYPT1 that alternative mRNA splicing is both developmentally regulated and tissue specific, and thus so is MYPT1 isoform expression.18,19 This could suggest that MYPT1 splicing is modulated during disease states. In this study, we determined if the resistance to cGMP-mediated vasodilatation during CHF is attributable to a change in the relative expression of LZ⁺⁻ MYPT1 isoforms, an abnormality at the level of the smooth muscle.

Materials and Methods

Animal Model of Congestive Heart Failure

We used a well-accepted and studied model of CHF, the rat infarct model, which many others have demonstrated has signs of CHF including a decrease in left ventricular function (LVF), dilation of the LV, hypervolemia, pleural effusions, and ascites.28–31 Briefly, using a surgical protocol approved by the IACUC of Case Western Reserve University, Sprague Dawley rats were placed under general anesthesia using a combination of IP ketamine, xylazine, and acepromazine, and then intubated and placed on a mechanical ventilator (Harvard Apparatus). A left thoracotomy was performed, and the heart was exposed. The anterior coronary arteries were identified at the base of the ventricle and ligated using 6–0 suture. The chest was then closed and the animals were allowed to recover in a temperature-regulated environment under close observation before being returned to the Animal Research Center. Additionally, a group of animals underwent sham surgery, in which the procedure was identical except that only the heart was exposed before the chest was closed.

In control and postmyocardial infarction rats, left ventricular function was assessed by echocardiography at 2-week intervals. Images were collected using a cardiac ultrasound system (Acuson Sequoia) equipped with a 13-MHz linear array transducer. Two-dimensional digital loop images were obtained in the parasternal long (ESD) and parasternal short axis, and subcostal views, and measurements of diastolic wall thickness, chamber dimensions in both systole (EDD), and diastole (EDD) were made with digital calipers, as previously described.29 Fractional shortening was calculated as [(EDD – ESD)/EDD]×100, and left ventricular ejection fraction (LVEF) was estimated from wall motion and thickening.32 All other studies were performed at 8 weeks after the infarction.

Mechanical Studies

Small smooth muscle strips isolated from the aorta, iliac artery, and portal vein were used from control and CHF rats to determine the dose-response relationship to 8-BrcGMP. The methods for tissue preparation and composition of all solutions have been described in detail.33,34 Briefly, the smooth muscle was placed in Ca⁺⁺ free physiological saline solution (NaCl 140 mmol/L, KCl 4.7 mmol/L, Na₂HPO₄ 2.25 mmol/L, KCl 1.2 mmol/L, glucose 5.6 mmol/L, MOPS 2 mmol/L, and EDTA 0.5 mmol/L at a pH of 7.4), and then cleaned of connective tissue. Smooth muscle strips of approximately 600 to 800 µm in length, 200 to 600 µm in width, and ∼50 µm thick (muscle cross-sectional area was calculated as width×thickness) were isolated and clamped at both ends in aluminum foil T-clips. The tissue was then skinned at room temperature for 10 minutes at pCa 9 (−log([Ca⁺⁺])) with 1% triton-X100 (MgATP 5 mmol/L, K₄EGTA 5 mmol/L, KMS 25 mmol/L, MgCl₂ 6.9 mmol/L, creatine phosphate 25 mmol/L, BES 5 mmol/L, and glutathione 2 mmol/L). After skinning, the smooth muscle strips were transferred to a mechanics workstation and mounted between a piezoresistive force transducer (Sensor One, San Francisco, Calif) and a length driver (Polytec PI, Auburn, Mass). The strips were stretched to L₀, the length for optimum force production, in pCa 9 relaxing solution (MgATP 5.47 mmol/L, K₄EGTA 10 mmol/L, KMS 56.5 mmol/L, MgCl₂ 7.22 mmol/L, creatine phosphate 25 mmol/L, BES 25 mmol/L, and CaCl₂ 0.02 mmol/L). The smooth muscle strips were then maximally Ca⁺⁺ (pCa 4) activated (MgATP 5.64 mmol/L, K₄EGTA 10 mmol/L, KMS 35.8 mmol/L, MgCl₂ 6.97 mmol/L, CP 25 mmol/L, BES 25 mmol/L, and CaCl₂ 10.3 mmol/L). After force reached a steady state, the dose-response relationship to 8-Br-cGMP (Calbiochem) added to the pCa 4 activating solution was determined.

Western Blots

Western blots were performed as previously described.21,22 Briefly, total protein was extracted from the smooth muscle tissue samples in SDS sample buffer and resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with an acrylamide/bisacrylamide ratio of 29:1. MYPT1 was resolved using 6% gels, whereas PKG1 was resolved using 10% gels, and protein loading, unless otherwise noted, was normalized for the actin band. After SDS-PAGE separation, protein bands were electrophoretically transferred to a nitrocellulose membrane (Schleicher and Schuell) in buffer containing 25 mmol/L Tris-HCl, 192 mmol/L glycine, and 10% methanol (v/v). Antibodies used were a polyclonal anti-MYPT1 (Convance), a monoclonal anti-MYPT1 (Convance), a monoclonal anti-MYPT1 (Convance), a monoclonal anti-MYPT1 (Convance), a monoclonal anti-MYPT1 (Convance), a monoclonal anti-MYPT1 (Convance), a monoclonal anti-MYPT1 (Convance), and anti-PKG1β (Stressgen Biotechnologies Corp), and anti-PKG1α (Stressgen Biotechnologies Corp). Secondary antibodies specific to primary antibody were alkaline-phosphatase-conjugated. Densitometry was used to determine the intensities of the bands using an automated unit consisting of a flat bed scanner and analysis software (Amersham).

Statistics

All values reported in the text are mean±SEM and a total of n=6 to 10 animals are included in each experimental group. Differences between means were determined using an ANOVA and P<0.05 was taken as significant. There was no difference between the data for control and sham surgical animals, and the data are labeled as control.

Results

Left Ventricular Function

Two-dimensional echocardiography was performed on both control and CHF rats. The control animals had LVEF of 65±5%. After ligation of the LAD, the anterior wall and septum became hypokinetic to akinetic, the left ventricular end diastolic dimension increased, and the LVEF fell to 30±5% (n=20, Figure 2, Table 1). Further, the CHF animals would stand in their cage with their noses elevated, which is...
were all significantly ($P<0.01$) different in the CHF rats compared to controls.

Fractional shortening 36.0
End systolic dimension 0.59

TABLE 1. Left Ventricular Function

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<th>Controls</th>
<th>CHF</th>
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<tr>
<td>End diastolic dimension</td>
<td>0.87±0.04</td>
<td>1.04±0.10</td>
</tr>
<tr>
<td>End systolic dimension</td>
<td>0.59±0.04</td>
<td>0.77±0.04</td>
</tr>
<tr>
<td>Fractional shortening</td>
<td>36.0±2.9</td>
<td>25.8±2.7</td>
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End diastolic dimension, end systolic dimension, and fractional shortening were all significantly ($P<0.01$) different in the CHF rats compared to controls.

Mechanical Studies

After Ca$^{2+}$ activation of permeabilized smooth muscle strips, force rose to a steady state in both control and CHF animals. There was no significant difference in the maximum force per cross-section between the control and the CHF rats (Table 2), suggesting that tissues were permeabilized to similar extents. In both controls and CHF animals, the aorta generated the highest force, whereas the iliac artery developed the lowest force.

Overall, the smooth muscles from the control animals, compared with CHF animals, were significantly more sensitive to 8-Br-cGMP (Figure 3). In skinned control aortic strips, 1 nmol/L 8-Br-cGMP produced a 58±14% ($n=6$) relaxation compared with only 8±3% ($n=5$, $P<0.05$) in the CHF animals; in control aorta complete relaxation was produced by 10 μmol/L 8-Br-cGMP, whereas in CHF aortic strips 100 μmol/L 8-Br-cGMP only produced a 47±19% relaxation. Similarly for the iliac artery, 10 nmol/L 8-Br-cGMP completely relaxed control strips ($n=6$), compared with a 30±6% ($n=5$) relaxation in the CHF rats. Further, 10 nmol/L 8-Br-cGMP only produced a 66±12% relaxation iliac artery strips from CHF rats (Figure 3). The portal vein was relatively insensitive to 8-Br-cGMP in both control and CHF rats; 1 nmol/L 8-Br-cGMP produced a 3±1% relaxation for both the control ($n=5$) and CHF ($n=5$) animals, whereas 100 μmol/L 8-Br-cGMP produced a 52±9% relaxation in the portal vein from control rats compared with a 37±12% relaxation in CHF rats (Figure 3). Although the relaxation response of the portal vein to higher concentrations of 8-Br-cGMP was decreased in the CHF rats, these differences were not statistically significant.

Protein Expression and Message

To determine whether MYPT1 was associated in a change in protein expression of MYPT1 isoforms, we performed Western blots using both a polyclonal MYPT1 antibody and a monoclonal antibody selective for only LZ$^+$ MYPT1 isoforms. As can be seen (Figure 4A), in smooth muscle from

<table>
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<th>Controls</th>
<th>CHF</th>
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<tbody>
<tr>
<td>Aorta</td>
<td>16.0±2.2</td>
<td>15.1±0.7</td>
</tr>
<tr>
<td>Portal vein</td>
<td>10.9±0.8</td>
<td>12.4±2.6</td>
</tr>
<tr>
<td>Iliac artery</td>
<td>7.2±0.9</td>
<td>9.8±3.4</td>
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There was no significant difference between the maximal Ca$^{2+}$ activated force (mN/mm$^2$) in smooth muscle from control or CHF animals ($n=5–6$ in each group).
the CHF rats compared with control tissues for the same level of total MYPT1, the expression of the LZ/MYPT1 isoform decreased markedly in the iliac artery and modestly in the aorta. The ratio of the density of LZ/MYPT1 band/total MYPT1 band was calculated to quantify the relative expression of the LZ/MYPT1 isoform. However, this quantification uses two antibodies with different affinities, and thus this ratio is only a relative measure of protein expression. In control tissues, the relative expression of the LZ/MYPT1 isoform was highest in the iliac artery (Figure 4B). Compared with the control tissues, in the CHF animals, the expression of the LZ/MYPT1 isoform significantly decreased (P<0.05) in both the aorta and iliac artery, but the decrease was more pronounced (P<0.05) in the iliac artery compared with the aorta (30% versus 20%, respectively). The expression of the LZ/MYPT1 isoform in the portal vein was low, and did not change after the development of CHF.

To confirm that this decrease in protein expression in the aorta and iliac artery was accompanied by a change in the relative mRNA levels, we used RT-PCR to determine the relative mRNA levels for LZ+ and LZ− MYPT1 transcripts (Figure 5). In control animals, the expression the LZ+ MYPT1 transcript predominated in aorta (94±6%) and iliac artery (67±4%). Eight weeks after ligation of the anterior coronary artery in the CHF animals there was a decrease (P<0.05) in the expression of the LZ+ MYPT1 transcript to 79±6% in aorta and 37±5% in the iliac artery (Figure 5). Thus for both the aorta and iliac artery, CHF was associated with a significant decrease in the expression of both MYPT1 LZ+ transcript and protein.

We also used Western blots to determine whether a change in the expression of PKGI or a switch in PKGI isoform expression, from PKGIα to PKGIβ, accompanied CHF. Western blots using a nonspecific PKGIα/β antibody showed the presence of only a single protein band at 78 kDa in smooth muscle from both control and CHF rats (Figure 6). The intensity of this band was similar (P>0.05) in control and CHF tissues, suggesting that CHF is not accompanied by a change in total PKGI expression. In addition in both control and CHF smooth muscle, a PKGIβ-specific antibody did not detect protein, which suggests that PKGI expression is exclusively PKGIα.

Discussion

NO-mediated vasodilatation is a fundamental response of the vasculature. The basic mechanism governing NO-
mediated smooth muscle relaxation has been delineated. NO is known to stimulate guanylate cyclase to increase cGMP which in turn activates PKGI. PKGI has multiple targets in smooth muscle, which all result in smooth muscle relaxation by different mechanisms including phosphorylation of the maxi K+ channel to produce hyperpolarization, both Ca2+ channels and the SERCA to decrease intracellular Ca2+, and an activation of MLCP. However, there is diversity in the response of smooth muscle to NO in normal tissues, as well as a blunted response in CHF. The mechanism responsible for differential sensitivity to NO has been suggested to be attributable to changes in the relative expression of LZ+/LZ− MYPT1 isoforms. Surks et al have demonstrated that PKGIα activates MLCP phosphatase activity, and postulated that this was attributable to a LZ-LZ interaction of the NH2-terminal LZ of PKGIα with the COOH-terminal LZ of MYPT1. Moreover, we have previously demonstrated that the sensitivity of skinned smooth muscle to cGMP-mediated relaxation correlates with the expression of the LZ+ MYPT1 isoform. Thus, alternative MYPT1 splicing to produce changes in the relative expression of LZ+/LZ− MYPT1 isoforms could explain a decrease in sensitivity to NO-mediated vasodilatation in CHF.

In our animals 8 weeks after anterior coronary artery ligation, echocardiography (Figure 2) demonstrated a significant impairment of LV systolic function and dilatation of the left ventricle (Table 1). Ligation of the LAD to produce an anterior myocardial infarction is well accepted to produce a model of CHF and LV remodeling in rats. The alterations in LVF observed in our CHF animals is similar to that demonstrated by others.

In the rats used in this study, CHF was associated with a change in the relative expression of LZ+/LZ− MYPT1 isoforms with a significant decrease in the expression at the protein level of the LZ+ MYPT1 isoform in both the aorta and the iliac artery (Figure 4). The decrease in LZ+ MYPT1 isoform expression was more prominent in the more distal vessel (iliac artery), compared with a conduit vessel (aorta), and could suggest that the modulation of LZ+ MYPT1 isoform expression in resistance vessels could be even more dramatic. Furthermore, the sensitivity to cGMP-mediated smooth muscle relaxation (Figure 3) scaled with the relative expression of the LZ+ MYPT1 isoform (Figure 4). The expression of the LZ+ MYPT1 isoform and sensitivity to cGMP-mediated relaxation listed from highest to lowest is control iliac artery, control aorta, CHF iliac artery, CHF aorta, and portal vein. Further the decrease in LZ+ MYPT1 isoform expression in both the aorta and iliac artery correlates with the decrease in the sensitivity to cGMP mediated smooth muscle relaxation. Thus, similar to normal tissues, the sensitivity to cGMP-mediated relaxation is determined, at least in part, by the expression of the LZ+ MYPT1 isoform. The sensitivity to cGMP-mediated smooth muscle relaxation was low in the portal vein consistent with the expression of the LZ− MYPT1 isoform, but similar to that observed in the CHF aorta. These data could suggest that there could be a threshold level of LZ+ expression before a tissue is sensitive to cGMP.

PKGI is expressed in three different isoforms (PKGIα, PKGIβ, and PKGIII) in mammalian smooth muscles, and this group has suggested that only PKGIα, but not PKGIβ, dimerizes with the MYPT1 to activate MLCP activity. We demonstrated that compared with control tissue, CHF was neither associated with a change in the total PKGI expression nor a change in PKGI isoform expression, which is exclusively PKGIα in both control and CHF smooth muscle (Figure 6). These data rule out the possibility that the

**Figure 5.** Relative mRNA Levels. Bar graph of the RT-PCR results showing relative mRNA level of the LZ+ MYPT1 transcript (n=5 to 6) in aortic and iliac artery smooth muscle of control and CHF rats. Inset, Representative agarose gel showing transcript pattern of MYPT1 in aortic (A) and iliac artery (IA) smooth muscle in control and CHF rats. Lower band is 3′ exon out, or the LZ− MYPT1 transcript.

**Figure 6.** PKGI expression. Western blots of the aorta, portal vein, and iliac artery smooth muscle from control (right three lanes) and CHF (left three lanes) rats using a nonspecific PKGIα/β antibody (top) and PKGIβ specific antibody (bottom). A positive control for PKGIβ is in the lane labeled NM cells. Arrows indicate the protein recognized by its specific antibody. Nonspecific PKGI antibody detected a single protein band and the intensity of this band is similar (P>0.05) in smooth muscle from control and CHF rats (n=5 to 6). There was no protein detected with the specific antibody to PKGIβ, indicating that expression is exclusively PKGIα in smooth muscle isolated from both control and CHF rats.
observed decrease in sensitivity to cGMP-mediated smooth muscle relaxation in the present study was attributable to a change in PKGI expression. In addition, our experiments were performed in skinned smooth muscle, and thus we can also rule out neurohormonal activation, differences in resting membrane potential or differences in Ca2+ flux as contributors to the decrease in sensitivity to cGMP.

Using short GST fusion proteins, Surks et al have shown that MYPT1 is able to dimerize with PKGIα, only if both MYPT1 and PKGI express a LZ. However, we have recently shown that both full-length avian LZ- and LZ+ MYPT1 isoforms will bind PKGIα, suggesting that the PKGI-MYPT1 interaction is not mediated by the C-terminal LZ domain of MYPT1, but rather, possibly mediated by a MYPT1 coiled-coil domain present at aa 888–928. Although the LZ is not required for the interaction of MYPT1 with PKGIα, we did demonstrate that a LZ+ MYPT1 isoform is required for PKGIα to activate MLCP activity. Although the exact mechanism by which PKGIα activates MLCP activity has yet to be elucidated, it could be attributable to coiled-coil interaction of PKGIα with MYPT1 and subsequent MYPT1 phosphorylation. Nonetheless, the decrease in LZ+ MYPT1 expression observed during CHF in the present study would suggest that the decrease in sensitivity to NO-mediated vasodilatation observed in this clinical syndrome lies at least partially at the level of the smooth muscle and is a consequence of the decrease in LZ+ MYPT1 expression.

Most effective therapies that result in a decrease in mortality in patients with CHF are aimed at the vasculature, which suggests that there is an underlying abnormality of vascular function associated with this clinical syndrome. However until the present study, no data existed to suggest a possible molecular mechanism for the abnormality of vascular function. Others have demonstrated a decrease in the sensitivity of smooth muscle relaxation to nitroprusside in the rat infarct model of CHF, suggesting that in this model there is an abnormality in NO signaling at the level of the smooth muscle, independent of endothelial function. Additionally, the skinned smooth muscle strips used in the present study lack an endothelium and thus the decrease in sensitivity to cGMP mediated relaxation in the present study, reflects the abnormality in NO signaling in this animal model of CHF. Further, we have previously shown that cGMP/PKG-mediated activation of MLCP activity requires the expression of a LZ+ MYPT1 subunit. Thus during the clinical syndrome of CHF, the data in the present article suggest that the resistance to NO-mediated vasodilatation lie at least in part at the level of the smooth muscle and is a consequence of a modulation of MYPT1 LZ+ isoform expression, and specifically attributable to a decrease in the expression of LZ+ MYPT1 isoform.

An increase in blood flow is known to increase the shear stress on the endothelial cells and stimulate the production of NO, which stimulates PKGI and a resulting increase in cGMP, which activates PKGI and results in vasodilatation. Our results demonstrate that the vessel’s sensitivity to NO-mediated vasodilatation is determined, in part, by the relative expression of the LZ+ MYPT1 isoform. Thus, the decrease in LZ+ MYPT1 expression observed in our animal model of CHF would thus result in a decrease in the sensitivity to NO, and would be expected to blunt flow-mediated vasodilatation. This would increase vascular tone and thus could produce a resting vasoconstriction, another vascular abnormality associated with the clinical syndrome of CHF.

The signaling pathway to explain a change in LZ+/− MYPT1 isoform expression both during development and CHF (present study) is unknown. However, pharmacologically targeting this pathway with small molecule inhibitors or other therapeutic agents aimed at blocking MYPT1 isoform switching or directly activating the MLCP, could potentially open a new, and possibly more effective avenue for treating patients with CHF.

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