Downregulation of Cytoskeletal Muscle LIM Protein by Nitric Oxide
Impact on Cardiac Myocyte Hypertrophy

Jörg Heineke, MD; Tibor Kempf, MD; Theresia Kraft, PhD; Andres Hilfiker, PhD;
Henning Morawietz, PhD; Robert J. Scheubel, MD; Pico Caroni, PhD;
Suzanne M. Lohmann, MD, PhD; Helmut Drexler, MD; Kai C. Wollert, MD

Background—In chronic heart failure, myocardial expression of the inducible isoform of nitric oxide (NO) synthase (NOS2) is enhanced, leading to a sustained production of NO. We postulated that NO modulates expression of genes in cardiac myocytes that may be functionally important in the context of cardiac hypertrophy and failure.

Methods and Results—As revealed by cDNA expression array analyses, the NO donor SNAP, which has been shown previously to inhibit agonist-induced cardiac myocyte hypertrophy, downregulates expression of the cytoskeleton-associated muscle LIM protein (MLP) in endothelin-1 (ET-1)–stimulated neonatal rat cardiac myocytes. Northern blotting and immunoblotting experiments confirmed this finding and established that SNAP negatively controls MLP mRNA (−49%, P<0.01) and protein (−52%, P<0.01) abundance in ET-1–treated cardiomyocytes via cGMP-dependent protein kinase and superoxide/peroxynitrite-dependent signaling pathways. Treatment of cardiac myocytes with IL-1β and IFN-γ downregulated MLP expression levels via induction of NOS2. Moreover, expression levels of NOS2 and MLP were inversely correlated in the failing human heart, indicating that NOS2 may regulate MLP abundance in vitro and in vivo. Antisense oligonucleotides were used to explore the functional consequences of reduced MLP expression levels in cardiac myocytes. Like SNAP, antisense downregulation of MLP protein expression (−52%, P<0.01) blunted the increases in protein synthesis, cell size, and sarcomere organization in response to ET-1 stimulation. Conversely, overexpression of MLP augmented cell size and sarcomere organization in cardiac myocytes.

Conclusions—NO negatively controls MLP expression in cardiac myocytes. Because MLP is necessary and sufficient for hypertrophy and sarcomere assembly, MLP downregulation may restrain hypertrophic growth in pathophysiological situations with increased cardiac NO production. (Circulation. 2003;107:1424-1432.)

Key Words: nitric oxide ■ cytoskeleton ■ myocytes ■ hypertrophy

In chronic heart failure and other disease states associated with inflammation and cytokine activation, myocardial expression of the inducible isoform of nitric oxide (NO) synthase (NOS2) is enhanced, leading to a sustained production of NO.1–4 NO has been shown to attenuate cardiac myocyte hypertrophy,5–8 to promote cardiac myocyte apoptosis,9,10 and to modulate cardiac myocyte contractile function.2,11 Studies in NOS2-deficient and NOS2-overexpressing mice indicate that upregulation of NOS2 is detrimental and may increase mortality in heart failure.12–14 The effects of NO in various cell types and tissues are mediated, in part, via changes in gene expression,15 raising the possibility that transcriptional effects of NO may play a role in cardiac hypertrophy and failure as well. Little is known, however, regarding the effects of NO on the gene expression profile in cardiac myocytes. In the present study, we have performed cDNA expression array analyses to identify genes that are regulated by NO in cardiac myocytes and that may be functionally important in the context of cardiac hypertrophy and failure. Using this approach, we have identified muscle LIM protein (MLP) as one gene that is downregulated by NO in cardiac myocytes. Muscle LIM protein is a member of the LIM-only class of the LIM domain protein family that is highly expressed at the Z disk in striated muscle cells.16,17 Homozygous deletion of individual LIM domain protein genes in mice results in distinct forms of cardiomyopathy and heart failure, indicating that LIM domain proteins play...
critical roles in supporting cardiac structure and function.\textsuperscript{18–20} It was recently shown that MLP expression levels are decreased in the failing human heart, suggesting that MLP may play a role in acquired forms of heart failure as well.\textsuperscript{21}

Exploiting the functional consequences of reduced MLP expression, our study reveals that MLP is necessary and sufficient for hypertrophic growth and sarcomere organization in cardiac myocytes and that downregulation of MLP contributes significantly to the antihypertrophic effects of NO.

### Methods

#### Materials

Endothelin-1 (ET-1), superoxide dismutase (SOD), the guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), and the NOS2 inhibitor 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT) were purchased from Sigma; the NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) and the SOD mimetic Mn(III)tetrakis(4-benzoic acid)porphyrin (MnTBAP) from Calbiochem; tetramethylammonium-peroxynitrite (peroxynitrite) from Alexis; the cGMP analog 8-para-chlorophenylthio-cGMP (8-pCPT-cGMP) from Biolog; and interleukin (IL)-1 and interferon (IFN)-γ from R&D Systems.

#### Cardiac Myocyte Culture

Ventricular cardiac myocytes were isolated from 1- to 3-day-old Sprague-Dawley rats (Control Animal Facility, Hannover Medical School) by Percoll gradient. Cells were plated overnight in DMEM/medium 199 (4:1) supplemented with 10% horse serum, 5% FCS, glutamine, and antibiotics. The next morning, cardiac myocytes were switched to DMEM/medium 199 supplemented with only glutamine and antibiotics (maintenance medium).\textsuperscript{7}

#### cDNA Expression Array Analysis

Total RNA was isolated by use of the Trizol reagent (Life Technologies) and treated with DNase I (1 U/L) to remove residual genomic DNA. cDNA probes were synthesized in the presence of \(\alpha^{32}\text{P}\)dATP (Amersham) by use of SuperScript II reverse transcrip-

<table>
<thead>
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<th>Name</th>
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Expression of 1176 genes was analyzed. Genes that were consistently induced or downregulated by ≥1.7-fold in 4 independent experiments are shown.

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**Figure 1.** Regulation of MLP abundance in cardiac myocytes by NO and ET-1. Cardiac myocytes were stimulated for 24 hours with SNAP (250 μmol/L) and/or ET-1 (100 nmol/L), as indicated. MLP expression levels were determined by Northern blotting (A, B) and immunoblotting (C, D). Expression levels of 18S rRNA or α-actinin were determined to confirm equal loading of RNA or protein. Typical blots are shown in A and C. Data from \(n=4\) to 5 experiments are presented in B and D.
tase (Life Technologies), purified by column chromatography, and
denatured in NaOH. Probes were hybridized for 18 hours at 68°C to
Atlas rat 1.2 nylon arrays (Clontech) containing cDNA sequences
from 1176 rat genes. The arrays were then washed under stringent
conditions, exposed to a phosphorimaging screen, and analyzed with
AtlasImage 2.0 software (Clontech).

Northern Blot and Reverse Transcription–
Polymerase Chain Reaction
MLP mRNA expression was determined by Northern blot. An MLP
cDNA probe was generated by polymerase chain reaction cloning
(forward primer nt 25 to 45, reverse primer nt 701 to 726),16
subcloned into pCR2.1 (Invitrogen) and confirmed by DNA se-
quencing. NOS2 and β-actin mRNA expression levels were deter-
mined by semiquantitative reverse transcription–polymerase chain
reaction.22

Immunoblotting
MLP protein expression was determined by immunoblotting using a
polyclonal antibody directed against the C-terminal peptide of rat
MLP (GGLTHQVEKKE).19 This antibody recognizes rat and human
MLP.16,22 A monoclonal antibody from Transduction Laboratories
was used to quantify NOS2 expression. Equal loading was confirmed
by Ponceau red staining and immunoblotting with a monoclonal
antibody directed against α-actinin (Sigma).

Human Myocardial Tissue
Left ventricular tissue samples were obtained from patients with
end-stage heart failure undergoing heart transplantation (n=31; mean
age, 54±2 years; mean left ventricular ejection fraction, 23±2%). All patients were in NYHA functional class III or IV. Tissue samples were frozen in liquid nitrogen no later than 2 hours after explantation.

Adenoviral Infection of Cardiac Myocytes
A replication-deficient adenovirus containing the cDNA of human
cGMP-dependent protein kinase type I (PKG I) was used to
overexpress PKG I in cardiac myocytes (Ad.PKG I).7,23 Control cells
were infected with a replication-deficient adenovirus encoding a
point-mutated, catalytically inactive form of human PKG I (Ad.PKG
I-K405A).7,24 Infection with Ad.PKG I augments PKG activity
12-fold compared with noninfected or Ad.PKG I-K405A–infected
cardiac myocytes.7

Antisense Oligonucleotides
Following previous published guidelines for the design of anti-
sense experiments,25 a set of 5 antisense oligonucleotides (18-mers)
directed against the ATG translation start site of rat MLP were
synthesized to selectively decrease MLP expression levels in cardio-
myocytes. Three phosphorothioate linkages were introduced at the 3’
and 5’ termini of each oligonucleotide. Cardiac myocytes were
transfected for 4 hours with antisense oligonucleotides using the

Figure 2. NO downregulates MLP
expression by cGMP-dependent and
superoxide/peroxynitrite-dependent
mechanisms. Cardiac myocytes were
incubated for 24 hours in the presence
or absence of SNAP (250 μmol/L), SOD
(1×10^3 U/mL), ET-1 (100 nmol/L), per-
oxynitrite (100 μmol/L), ODQ (100
μmol/L), and/or 8-pCPT-cGMP (500
μmol/L), as indicated. Cells in D were
infected with adenoviral vectors encod-
ing wild-type PKG I (hatched bars), cata-
lytically inactive PKG I (open bars), or no
virus (solid bars). MLP expression levels
were determined by Northern blot. Data
from n=3 to 7 experiments are
presented.
cationic reagent Lipofectamine (Invitrogen). Each oligonucleotide was initially screened for its ability to decrease MLP expression levels. Antisense oligonucleotide 4 (AS4, nt 41 to 58) downregulated MLP protein expression most potently and was used in subsequent experiments. Antisense oligonucleotides AS1, AS3, and AS5 were less effective. Antisense oligonucleotide 2 (AS2, nt 25 to 42) and a scrambled oligonucleotide corresponding to AS4 (SCR, GAC-CCTACCAGCTCTCGC) did not suppress MLP levels and served as negative controls. Control experiments using FITC-labeled AS4 and SCR oligonucleotides revealed that virtually all cardiomyocytes were successfully transfected with our protocol (not shown).

Assessment of Cardiac Myocyte Hypertrophy and Survival

Cell size was determined by planimetry. Immunostaining was performed as previously described, with a monoclonal antibody against α-actinin, from Sigma. Sarcomere organization was analyzed by a blinded observer (T. Kempf) by confocal laser microscopy in cardiac myocytes immunostained for α-actinin. A semiquantitative grading system was used: grade 1, <10% of cell area with sarcomere organization; grade 2, 10 to 50% of cell area with sarcomere organization; grade 3, 50% to 90% of cell area with sarcomere organization; and grade 4, >90% of cell area with sarcomere organization. [3H]leucine incorporation was measured as an index of total protein synthesis. Atrial natriuretic peptide (ANP) mRNA expression was assessed by Northern blot. Cell viability was determined by trypan blue exclusion, and the formation of histone-associated DNA fragments, a marker of apoptotic cell death, was quantified by cell death detection ELISA PLUS (Roche).

Plasmid Constructs and Transfection

Cardiac myocytes were Lipofectamine-transfected for 4 hours with cytomegalovirus promoter–based eukaryotic expression plasmids...
Figure A: Western blot analysis of MLP and α-actinin protein levels in control, AS2, SCR, AS4, ET-1, ET-1 + AS2, ET-1 + SCR, ET-1 + AS4, and ET-1 + AS4 samples.

Figure B: Bar graph showing MLP protein expression as a percentage of control in various conditions.

Figure C: Bar graph showing [H]leucine incorporation as a percentage of control in various conditions.

Figure D: Bar graph showing cell size in square micrometers in various conditions.

Figure E: Bar graph showing sarcomere organization grades in control, ET-1, ET-1 + SNAP, ET-1 + SCR, ET-1 + AS4 conditions.

Figure F: Fluorescence images of control, ET-1, ET-1 + SNAP, ET-1 + SCR, ET-1 + AS4 samples.
encoding β-galactosidase or MLP. Transfected cells were identified by histochemical staining for β-galactosidase or by immunostaining for β-galactosidase (polyclonal antibody from Invitrogen). ANP promoter activity was determined in cells transfected with a luciferase reporter plasmid driven by 3003 bp of the rat ANP gene 5′ flanking region.

**Statistical Analysis**

Data are presented as mean±SEM. Differences between groups were analyzed by 1-way ANOVA and Student-Newman-Keuls post hoc test. Linear regression analysis was performed to test for a correlation between 2 variables. A 2-tailed probability value of P<0.05 was considered to indicate statistical significance.

**Results**

**cDNA Expression Array Analyses Reveal Downregulation of MLP by NO in Cardiac Myocytes**

Radiolabeled cDNA probes were generated from cardiac myocytes stimulated for 24 hours with the prohypertrophic polypeptide ET-1 (100 nmol/L)27 or ET-1 and the NO donor SNAP (250 μmol/L). At this concentration, SNAP promotes antihypertrophic but not proapoptotic effects in our experimental system.28 The cDNA probes were hybridized to nylon arrays containing cDNA fragments from 1176 rat genes. Altogether, 10 genes were identified that were induced or downregulated (Table). Expression of MLP was downregulated by SNAP in ET-1–treated cardiac myocytes. Subsequent experiments were performed to explore the molecular mechanisms and functional consequences of this observation.

**MLP Is Downregulated by NO and Induced by ET-1 in Cardiac Myocytes**

Northern blotting and immunoblots confirmed the array data and established that MLP mRNA and protein abundance is downregulated by SNAP in ET-1–treated cardiomyocytes (Figure 1, A through D). A similar trend was observed in the absence of ET-1. Dose-response experiments revealed that stimulation with 50 μmol/L SNAP is required for MLP downregulation (not shown). Notably, ET-1 stimulation was associated with a significant increase in MLP abundance (Figure 1, A through D). Taken together, induction (by ET-1) and suppression (by NO) of cardiac myocyte hypertrophy are associated with augmented and reduced MLP levels, respectively.

**NO Downregulates MLP by cGMP-Dependent and Superoxide/Peroxynitrite-Dependent Mechanisms**

In general, NO can alter gene expression via cGMP-dependent and cGMP-independent signaling pathways. PKG I has been identified as a critical downstream target of cGMP in this regard.28 One important cGMP-independent pathway involves the reaction of NO with superoxide to form peroxynitrite.29 The suppressive effects of SNAP on MLP abundance in ET-1–treated cardiomyocytes were partially reduced by the superoxide scavenger SOD (Figure 2A) and by the SOD mimic MnTBAP (not shown), indicating that superoxide is involved in the inhibitory effects of NO. Treatment of cardiac myocytes with SOD alone (without SNAP) had no significant effect on MLP abundance, indicating that superoxide itself does not control MLP expression (Figure 2A). Peroxynitrite, when added directly to the cells, significantly suppressed MLP expression levels in ET-1–stimulated cardiomyocytes (Figure 2B). The data presented in Figure 2, A and B, support the concept that NO suppresses MLP expression, in part, by reacting with superoxide and promoting peroxynitrite formation. As shown in Figure 2C, NO–mediated downregulation of MLP in ET-1–treated cells was also partially blunted by the guanylyl cyclase inhibitor ODQ, indicating that cGMP-dependent mechanisms also contribute to the inhibitory effects of NO. The suppressive effects of NO were completely reversed by combined treatment with ODQ and SOD (Figure 2C). Taken together, suppression of MLP abundance by NO in ET-1–treated cardiac myocytes is mediated via cGMP-dependent (Figure 2C) and superoxide/peroxynitrite-dependent (Figure 2, A and B) pathways.

**Cytokine-Induced NOS2 Downregulates MLP Expression Levels in Cardiac Myocytes**

Consistent with a previous report,40 stimulation with IL-1β and IFN-γ induced NOS2 expression in cardiac myocytes under basal conditions and during ET-1 stimulation (Figure 3A). At the same time, IL-1β/IFN-γ significantly suppressed MLP expression levels in ET-1–treated cells (Figure 3B). A
similar trend was observed in the absence of ET-1. Coinculation of cytokine-stimulated cardiac myocytes with the NOS2 inhibitor AMT reversed the inhibitory effects on MLP abundance in ET-1–treated cells, indicating that IL-1β/IFN-γ suppressed MLP levels via NOS2 (Figure 3B).

NOS2 and MLP Expression Levels Are Inversely Correlated in the Failing Human Heart

To start exploring whether NOS2 regulates MLP abundance in the failing human heart, NOS2 and MLP protein expression levels were determined in left ventricular myocardium from patients with end-stage heart failure caused by ischemic or dilated cardiomyopathy. As shown in Figure 4, NOS2 and MLP protein levels were inversely correlated in the failing human heart (n=31, r=−0.43, P<0.01). An inverse correlation was also seen in a separate analysis of patients with ischemic cardiomyopathy (n=14, r=−0.55, P<0.05). A similar trend was observed in patients with dilated cardiomyopathy (n=17, r=−0.41, P=0.1).

Antisense Downregulation of MLP Suppresses Cardiac Myocyte Hypertrophy

MLP antisense oligonucleotides were used to investigate the functional consequences of MLP downregulation in cardiac myocytes. As shown in Figure 5, A and B, transfection of cardiac myocytes with AS4 but not AS2 or a scrambled oligonucleotide suppressed MLP protein abundance in ET-1–treated cardiomyocytes by 52%, which was comparable to the degree of downregulation induced by SNAP (Figure 1). Antisense downregulation of MLP significantly suppressed ET-1–induced cardiac myocyte hypertrophy, as shown by a reduction of [3H]leucine incorporation (Figure 5C), cell size (Figure 5D), and sarcomere organization (Figure 5, E and F). ET-1 induction of ANP mRNA expression was not suppressed by antisense downregulation of MLP (not shown). Antisense downregulation of MLP in cardiomyocytes did not reduce cell viability (trypan blue exclusion) and did not promote formation of histone-associated DNA fragments, a marker of apoptotic cell death (not shown).

MLP Promotes Increases in Cell Size and Sarcomere Organization

Overexpression of MLP from a cytomegalovirus promoter–based expression plasmid promoted significant increases in cell size (Figure 6A) and sarcomere organization (Figure 6B), consistent with a direct hypertrophic effect of MLP. The growth-promoting effects of MLP were not inhibited by SNAP (Figure 6, A and B). However, overexpression of MLP reversed the inhibitory effects of SNAP on cell size in ET-1–treated cardiac myocytes (Figure 6A). Overexpression of MLP did not activate ANP transcription, as shown by cotransfection of the MLP expression plasmid with an ANP promoter–luciferase reporter plasmid (not shown).

Discussion

Biological functions of NO are mediated in part via transcriptional effects. To identify NO-regulated genes that may be functionally important in the context of cardiac hypertrophy and failure, we analyzed the expression profile of 1176 genes in cardiac myocytes stimulated with the prohypertrophic polypeptide ET-1 in the presence or absence of the NO donor SNAP. Several genes were identified that were differentially regulated by NO (Table). One gene that was consistently downregulated by NO in ET-1–treated cardiac myocytes was MLP. The present study provides an in-depth analysis of the functional consequences of this observation.

Northern blotting and immunoblotting experiments confirmed and extended the cDNA expression array data by showing that NO downregulates MLP expression in cardiac myocytes at both the mRNA and protein levels. NO has been shown previously to inhibit cardiac myocyte hypertrophy, at least in part, via cGMP and its activation of PKG I. Similarly, our data imply a cGMP/PKG I-dependent pathway in the negative regulation of MLP abundance in cardiac myocytes. In addition, reaction of NO with superoxide leading to peroxynitrite formation appears to contribute to the inhibitory effects of NO on MLP expression levels. Combined blockade of both pathways with a guanylyl cyclase inhibitor and SOD completely prevented the inhibitory ef-
fects of NO on MLP expression, indicating that both pathways are operating in concert.

MLP is a member of the LIM-only class of the LIM domain protein family, which is highly expressed in striated muscle cells, in which it induces myogenic differentiation. In cardiac myocytes, MLP is localized at the Z disk, where it anchors the sarcomere to the sarcolemma by interacting with α-actinin and β-spectrin. As shown in our study, MLP expression levels are dynamically regulated in cardiac myocytes and play a critical role in the modulation of hypertrophic growth responses. Stimulation of cardiac myocyte hypertrophy by ET-1 was associated with an increase in MLP expression levels. Importantly, this increase in MLP abundance was required for some aspects of the hypertrophic response to ET-1, because a specific antisense oligonucleotide that blunted the upregulation of MLP significantly suppressed ET-1-induced increases in protein synthesis, cell size, and sarcomere organization. By contrast, upregulation by ET-1 of the embryonic marker gene ANP appeared to be mediated via MLP-independent mechanisms. Remarkably, the effects of antisense downregulation of MLP on these distinct hypertrophy markers are virtually identical to the antihypertrophic effects mediated by SNAP. Therefore, MLP downregulation may be one important mechanism contributing to the antihypertrophic effects of NO in cardiac myocytes. In line with this conclusion, overexpression of MLP reversed the inhibitory effects of SNAP on cell size in ET-1-treated cardiac myocytes. Overexpression of MLP by itself promoted increases in cardiac myocyte size and sarcomere organization, indicating that enhanced expression of MLP is not only necessary (as shown in our antisense experiments) but also sufficient to induce morphological markers of cardiac myocyte hypertrophy. Interestingly, MLP overexpression did not augment ANP promoter activity, confirming that ANP is regulated by MLP-independent pathways. Like MLP, overexpression of α-actinin-associated LIM protein has been shown to promote sarcomere organization in cardiac myocytes. The precise molecular mechanisms that couple MLP (and other LIM-domain proteins) to hypertrophy and sarcomere assembly remain to be elucidated. Conceptually, LIM domain proteins may act as scaffold proteins and may thereby facilitate sarcomere assembly and cell enlargement. Furthermore, it has been proposed that LIM domain proteins may trigger as yet unknown downstream signaling events. Activation of such signaling pathways may provide additional means of promoting hypertrophy.

A growing body of evidence shows that the cytoskeleton plays an important role in transmitting growth signals from the cell membrane to the nucleus. Mutations in cytoskeletal protein genes are the molecular basis for many inherited forms of dilated cardiomyopathy. How well these genetically based forms of chamber dilation reflect events in the more prevalent forms of heart failure remains an unresolved question. However, quantitative (expression levels) and qualitative (disorganization) changes within the cytoskeleton have been described in acquired forms of heart failure. In this context, it has been reported that MLP levels are decreased by ~50% in patients with end-stage heart failure caused by ischemic or dilated cardiomyopathy. In our study, protein expression levels of NOS2 and MLP were inversely correlated in the failing human heart. Although a causal relationship cannot be inferred with certainty from these data, it is conceivable that NOS2-derived NO suppresses MLP abundance in the failing human heart. In support of this concept, upregulation of NOS2 by IL-1β and IFN-γ suppressed MLP expression levels in cultured cardiac myocytes. It is not clear whether low MLP levels contribute to the process of heart failure progression, are an epiphenomenon, or are part of a compensatory response. Considering that increased MLP levels are necessary for cardiac myocyte hypertrophy, downregulation of MLP may restrain hypertrophic growth in the failing heart. Intriguingly, mice with a homozygous deletion of MLP, ie, a complete loss of MLP expression, develop ventricular dilation and failure, although the molecular mechanisms involved remain largely unknown. To further define the consequences of a partial MLP deficiency that is observed in response to NO stimulation and in the failing human heart, future studies should determine the impact of a heterozygous deletion of MLP on cardiac remodeling and hypertrophy.

In conclusion, our study reveals a link between NO and cytoskeletal alterations in cardiac myocytes that may be functionally important in the setting of cardiac hypertrophy and failure.

Acknowledgments

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References


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