Prevention of Myofilament Dysfunction by Beta-blocker Therapy in Post-infarct Remodeling

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Abstract

**Background.** Myofilament contractility of individual cardiomyocytes is depressed in remote non-infarcted myocardium and contributes to global left ventricular (LV) pump dysfunction after myocardial infarction (MI). Here we investigated whether β-blocker therapy could restore myofilament contractility.

**Methods and Results.** In pigs with a MI induced by ligation of the left circumflex coronary artery, β-blocker therapy (bisoprolol, MI+β) was initiated on the first day after MI. Remote LV subendocardial biopsies were taken 3 weeks after sham or MI surgery. Isometric force was measured in single permeabilized cardiomyocytes. Maximal force ($F_{\text{max}}$) was lower, while Ca$^{2+}$-sensitivity was higher in untreated MI compared to sham (both $P<0.05$). The difference in Ca$^{2+}$-sensitivity was abolished by treatment of cells with the β-adrenergic kinase, protein kinase A (PKA). Beta-blocker therapy partially reversed basal $F_{\text{max}}$ and Ca$^{2+}$-sensitivity to sham values and significantly reduced passive force. Despite the lower myofilament Ca$^{2+}$-sensitivity in MI+β compared to untreated myocardium, the PKA-induced reduction in Ca$^{2+}$-sensitivity was largest in cardiomyocytes from myocardium treated with β-blockers. Phosphorylation of β-adrenergic target proteins (myosin binding protein C and troponin I) did not differ among groups, whereas myosin light chain 2 phosphorylation was reduced in MI, which coincided with increased expression of protein phosphatase 1. Beta-blockade fully restored the latter alterations and significantly reduced expression of protein phosphatase 2a.

**Conclusions.** Beta-blockade reversed basal myofilament dysfunction and enhanced myofilament responsiveness to PKA in remote myocardium after MI. These beneficial effects likely contribute to the beneficial effects of β-blockade on global LV function after myocardial infarction.

**Key words:** myocardial infarction, β-blockers, contractility, myofilament proteins, phosphatases
Introduction

One of the principal risk factors for the development of heart failure in humans is myocardial infarction (MI), as it results in remodeling of the myocardium, consisting of left ventricular (LV) dilation and hypertrophy, with depressed global contractility.\textsuperscript{1,2} A number of factors, including changes in cardiac structure, apoptotic cell death, abnormal energy metabolism and Ca\textsuperscript{2+}-handling disturbances have been implicated in the initiation and progression of heart failure upon MI.\textsuperscript{3,4} Recently it has been shown that cardiomyocyte contractile dysfunction in remote non-infarcted myocardium might contribute to global LV pump dysfunction following myocardial infarction.\textsuperscript{5-8}

An acute myocardial ischemic insult leads to activation of the sympathetic nervous system in order to maintain perfusion of vital organs via peripheral vasoconstriction and restoration of cardiac output. However, chronic neurohumoral activation eventually has a negative impact on cardiac function,\textsuperscript{9} as treatment of MI patients with β-blockers has been shown to improve LV dysfunction, slow the progression towards heart failure and reduce the risk of sudden death.\textsuperscript{9-11} The beneficial effects of β-blockers are attributed to their ability to reduce heart rate and myocardial contractility leading to a reduction in myocardial workload and oxygen demand.\textsuperscript{11} Moreover, β-blockers have been shown to reverse LV remodeling.\textsuperscript{9,12} However, it is unknown if and to what extent restoration of cardiomyocyte function contributes to the beneficial effects of β-blocker therapy post-MI.

In light of previous studies that demonstrated myofilament dysfunction in remodeled myocardium,\textsuperscript{5,8} we hypothesized that correction of myofilament contractile dysfunction contributes to the beneficial effects of β-blocker therapy after MI. To test this hypothesis, myofilament function and protein phosphorylation were studied in pigs with a MI induced by ligation of the left circumflex coronary artery. Beta-blocker therapy consisted of the selective β\textsubscript{1} receptor antagonist bisoprolol (0.5 mg/kg/day per os) and was initiated on the first day after MI. Remote LV subendocardial biopsies were taken 3 weeks after MI in conjunction with
echocardiographic and hemodynamic assessment of LV function at the time of biopsy procurement. To reveal beneficial effects of β-blocker therapy MI groups were compared with sham-operated pigs.

The results of the present study show that chronic β-blocker therapy after MI largely prevents myofilament dysfunction and cellular hypertrophy, which explains at least part of its clinical benefit in patients with a recent MI.
Methods

Myocardial Infarction and β-blocker Therapy

Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication 86-23, revised 1996), and with approval of the Animal Care Committee of the Erasmus Medical Center. Twenty-five 2-3 month-old Yorkshire-Landrace pigs of either sex entered the study. Pigs were sedated (ketamine, 20 mg/kg IM, and midazolam, 0.5 mg/kg i.m.), anesthetized (thiopental, 10 mg/kg i.v.), intubated and ventilated with O₂ and N₂O to which 0.1%-1% (vol/vol) isoflurane was added. Anesthesia was maintained with midazolam (2 mg/kg followed by 1 mg/kg per hour i.v.) and fentanyl (10 μg/kg per hour IV). Under sterile conditions, the chest was opened via the fourth left intercostal space and a 4 cm-incision was made in the pericardium at the site of the origin of the left circumflex coronary artery (LCx). The LCx was dissected out and a suture was placed around it. The LCx was permanently ligated to produce a myocardial infarction (MI, n=17), whereas in the animals from the sham group (n=8) the suture was removed. The chest was closed and the animals were allowed to recover, receiving analgesia (0.3 mg buprenorphine IM) for 2 days and antibiotic prophylaxis (25 mg/kg amoxicillin and 5 mg/kg gentamycin IV) for 5 days.

Prior to induction of infarction, MI pigs were randomly assigned to receive no treatment (MI, n=9) or beta-blocker treatment with bisoprolol (MI+β, n=8), excluding any bias towards differences in acute infarct size between the two MI groups. Bisoprolol was administered orally by mixing bisoprolol powder with regular pig chow. The drug was started at a dosage of 0.125 mg/kg p.o. and increased (by 0.125 mg/kg per day) in three days to a dosage of 0.5 mg/kg p.o. per day. This dosage was maintained until the day before sacrifice. Since pigs lack a native coronary collateral circulation, myocardial infarct size reaches its plateau at ~90% of the risk area within 2h after onset of coronary ligation. To prevent a potential effect of β-blockade on
acute infarct size, bisoprolol treatment was initiated at ~24 hrs after the onset of permanent LCx ligation.

Six animals died during the period before sacrifice. One sham and two MI pigs (one MI and MI+β) died during the surgical procedures or overnight after surgery, i.e. prior to treatment initiation. One animal (MI group) died at one week after surgery, and two animals died on the day of sacrifice due to acute pump failure, one after sedation (MI group) and one after induction of anesthesia (MI+β group). Thus the number of animals included in this study amounted to 7 in the sham group, 6 in the MI group and 6 in the MI+β group.

In Vivo Measurements and Storage of Cardiac Tissue Samples

Three weeks after surgery, pigs were sedated (ketamine, 20 mg/kg intramuscularly (IM), and midazolam, 0.5 mg/kg IM). 2D echocardiographic recordings of the LV short axis at midpapillary level were obtained (ALOKA ProSound SSD-4000; Japan) and stored for off-line analysis. LV end-diastolic cross-sectional area (EDA) and end-systolic cross-sectional area (ESA) were determined, and ejection fraction (EF) was calculated as (EDA-ESA)/EDAx100%.

Subsequently, pigs were anesthetized (pentobarbital, 20 mg/kg IV), intubated and ventilated with O₂ and N₂. Anesthesia was maintained with pentobarbital (10-15 mg/kg/h IV). Animals were instrumented to allow closed-chest monitoring of heart rate, cardiac output, mean aortic and pulmonary artery blood pressures, and LV pressure, its first derivative dP/dt at LV pressure of 40 mmHg (LVdP/dtP40) and the time constant of exponential LV pressure decay (tau). Then, a midline sternotomy was performed and the heart was suspended in a pericardial cradle. To determine myofilament function and protein phosphorylation, 2-3 thru-cut (transmural, 0.5 mg wet weight) needle biopsies were taken from the LV anterior free wall myocardium (in MI pigs: remodeled non-infarcted tissue) and immediately frozen and stored in liquid nitrogen. Subsequently, hearts were arrested, quickly excised and subendocardial samples were frozen in liquid nitrogen for histological analysis of cross-sectional cardiomyocyte area.
Cardiomyocyte Measurements

Single cardiomyocytes were obtained via mechanical isolation\textsuperscript{16} from the subendocardial half of the biopsies, and incubated for 5 min with Triton X-100 (0.5\%) to remove all membranes. Isometric force was measured at various calcium concentrations at 15°C and sarcomere length of 2.2 μm. Passive force development was measured at sarcomere lengths of 1.8, 2.0 and 2.2 μm. Composition of solutions used for cell isolation and force measurements have been described in detail previously.\textsuperscript{16} In a number of cells force measurements were repeated after incubation with the catalytic subunit of protein kinase A (PKA, 40 min; 20°C; 100 U/mL; Sigma). In addition, force measurements were repeated upon incubation with protein phosphatase 1 (PP1, 60 min; 20°C; 0.5 U/mL; 14-110; Upstate) is a number of sham cardiomyocytes.

Myofilament Protein Composition

ProQ Phosphostaining

Subendocardial tissue (~0.5-1.0 mg dry weight) from needle biopsies was TCA (tri-chloro acetic acid)-treated as described previously.\textsuperscript{17} Phosphorylation status of myofilament proteins was determined using Pro-Q Diamond phosphostaining (Molecular Probes). Samples were separated on a gradient gel (Criterion Tris-HCl 4-15\% gel, BioRad) and proteins were stained for one hour with Pro-Q Diamond Phosphoprotein Stain. Fixation, washing and de-staining were performed according to the manufacturer’s guidelines. Staining was visualized using the LAS-3000 Image Reader (FUJI; 460 nm/605 nm Ex/Em; 2 min illumination) and signals were analyzed with AIDA. All protein signals were within the linear range. Subsequently gels were stained overnight with SYPRO Ruby stain (Molecular Probes) and visualized with the LAS-3000 (460 nm/605 nm Ex/Em; 2 s illumination). Since illumination of Pro-Q Diamond-stained gels for 2 seconds did not reveal any signal, the signals obtained upon SYPRO Ruby staining are not tainted by Pro-Q signals.
**Effect of Protein Phosphatase 1 on Myofilament Protein Phosphorylation**

Triton-treated (0.5%) sham samples (1 mg dry weight) were incubated with PP1 and subsequently treated with TCA and analyzed using Pro-Q Diamond stained gradient gels as described above.

**Differential Myosin Binding Protein C Phosphorylation**

Cardiac myosin binding protein C (cMyBP-C) composition was analyzed by 2D-gel electrophoresis as described recently by Yuan et al.\(^\text{18}\) Approximately 75 μg of biopsy tissue was loaded and proteins were stained with SYPRO Ruby.

**Western Immunoblotting**

Gel electrophoresis and Western immunoblotting was performed to analyze phosphorylation (at Ser-23/24, i.e. PKA sites, rabbit polyclonal Ab, dilution 1:500, Cell signaling) and degradation (clone 8I-7, mouse monoclonal Ab, dilution 1:1000, Spectral Diagnostics)\(^\text{8}\) of cardiac troponin I (cTnI). The following antibodies were used to detect degradation/expression of cMyBP-C (C0C2, rabbit polyclonal Ab, diluted 1:1000; kindly provided by Dr. S. Winegrad, University of Pennsylvania, Philadelphia, USA), cardiac troponin T (cTnT, JLT12, mouse monoclonal Ab, diluted 1:250, Sigma) and myosin light chain 2 (MLC-2, F109.3E1, mouse monoclonal Ab, diluted 1:200, Alexis biochemicals), phosphorylation of cMyBP-C (P-cMyBP-C, Ser-282, rabbit polyclonal Ab, dilution 1:1000), protein kinase C α expression (PKCa, 664-672, rabbit polyclonal Ab, dilution 1:500, Research & Diagnostic Antibodies) and activity (p-PKCa, Ser-657, sc-12356, rabbit polyclonal Ab, dilution 1:200, Santa Cruz Biotechnology), expression level of protein phosphatase 1 (PP1, sc-7482, mouse monoclonal Ab, dilution 1:200, Santa Cruz Biotechnology) and protein phosphatase 2a (PP2a, rabbit polyclonal, dilution 1:500, Upstate). Signals were normalized to actin to correct for differences in protein loading. For normalization of PP2a calsequestrin was used (CSQ, rabbit polyclonal, dilution 1:2000; Santa Cruz Biotechnology).
**Titin Isoform Composition**

Heart tissue was homogenized in modified Laemmli buffer, proteins were separated on Agarose-strengthened 2% SDS-PAGE as described previously. Protein bands were visualized by Imperial protein stain, scanned, and analyzed densitometrically.

**Statistical Analysis**

Data are given as means±SEM. Data of the three experimental groups (sham, MI and MI+β) were compared using 1-way ANOVA and post-hoc Bonferroni analysis. Effects of PKA and PP1 were tested by paired student t-tests. Significance was accepted when P<0.05.
Results

Improvement of Global LV Function by Early Beta-blocker Therapy

Three weeks after infarction, significant LV remodeling had occurred, reflected by LV dilation and hypertrophy of the surviving myocardium (Table 1). Thus, despite loss of ~25% of viable myocardium, LV weight was maintained while a 20% increase in cardiomyocyte cross-sectional area was observed in MI compared to sham. LV systolic dysfunction was reflected in lower levels of ejection fraction and stroke volume (Table 1). The consequent decrease in cardiac output resulted in elevated LV end-diastolic pressure and elevated pulmonary artery pressure that was associated with a trend towards an increase in RV weight.

Treatment of MI pigs with bisoprolol had no significant effects on LV mass or lumen area. However, bisoprolol lowered heart rate and fully restored stroke volume, so that the MI-induced decrease in cardiac output was prevented (Table 1).

Improvement of Myofilament Function by Early Beta-blocker Therapy

Similar to our previous observations, maximal force development (F\(_{\text{max}}\); Figure 1A) of cardiomyocytes isolated from post-MI remodeled hearts was significantly decreased by 47% compared to sham, while myofilament responsiveness to Ca\(^{2+}\) was significantly increased as illustrated by the leftward shift of the force-calcium relation and the lower EC\(_{50}\) and in MI (Figures 1C,D). Beta-blocker therapy partially reversed the reductions in F\(_{\text{max}}\) and EC\(_{50}\) in MI, while passive force (F\(_{\text{pas}}\)) was significantly reduced at a sarcomere length of 2.2 μm compared to both sham and untreated MI (Figure 1B). The steepness of the force-calcium relation (nHill) did not significantly differ among the three groups (nHill: 3.49±0.11, 3.16±0.18 and 3.38±0.20 in sham, MI and MI+β, respectively).

To assess whether the effects on cardiomyocyte force characteristics are related to differences in β-adrenergic signaling among the three groups, force measurements were repeated after incubation with exogenous PKA. F\(_{\text{max}}\) slightly (~10%) but significantly increased...
in sham and MI+β upon PKA, while it did not change in untreated MI. Hence, the difference in $F_{\text{max}}$ between groups remained (Figure 2A). $F_{\text{pas}}$ was significantly reduced after PKA in MI+β cells and remained lower compared to MI and sham cells (Figure 2B). In all groups, PKA significantly reduced Ca$^{2+}$-sensitivity of the myofilaments, but analogous to the effect on $F_{\text{max}}$ the shift in $EC_{50}$ varied among the groups, being smallest in sham cells and largest in MI+β cells (Figure 2C). Post-hoc Bonferroni analysis showed that PKA abolished the difference in Ca$^{2+}$-sensitivity between sham and MI cells, while $EC_{50}$ was significantly higher in MI+β compared to MI cells (Figure 2C,D). Steepness of the force-Ca$^{2+}$ relation was not significantly altered by PKA ($n_{\text{Hill}}$ after PKA: 3.55±0.28, 3.10±0.12 and 3.90±0.20 in sham, MI and MI+β, respectively).

Unaltered Phosphorylation of Target Proteins of the Beta-adrenergic Pathway

To investigate whether changes in myofilament protein phosphorylation may explain the differences in myofilament force characteristics, proteins were separated on 1D gradient gels and stained with Pro-Q Diamond (Figure 3A) and SYPRO Ruby (Figure 3B). Phosphorylation status of the major target proteins of PKA, cMyBP-C and cTnI, did not differ among the three groups. In addition, no difference was seen in desmin and cardiac troponin T (cTnT) phosphorylation (Figure 3C). Western immunoblot analysis with a specific antibody directed against phosphorylated PKA-sites in cTnI did not reveal a significant difference among the groups. Similarly, analysis of cMyBP-C phosphorylation at Ser-282 did not differ among the three groups (data not shown). In contrast, myosin light chain 2 (MLC-2) phosphorylation was lower in MI than in sham and MI treated with β-blockers (Figure 3C).

Differences in myofilament $F_{\text{max}}$ have been associated with changes in cMyBP-C phosphorylation, involving Ca$^{2+}$-regulated and PKA-mediated phosphorylation. Thus, although no significant differences were observed in total cMyBP-C phosphorylation, cMyBP-C could be differentially phosphorylated by the combined actions of kinases and phosphatases. To resolve this issue, cMyBP-C was separated by 2D-gel electrophoresis (Figure 4A). In agreement with
the observation by Yuan et al.\textsuperscript{18} in dog myocardium, multiple spots were found at the level of cMyBP-C (Figure 4A,B), which represent differentially phosphorylated forms. No significant difference was found in the pattern of cMyBP-C spots among the 3 groups as shown in Figure 4C.

Absence of Myofilament Protein Degradation

Previously, minute amounts of a cTnI degradation product were found in remodeled post-infarct myocardium.\textsuperscript{5} Western immunoblot analysis using clone 8I-7, which is directed against the central region of cTnI, did not reveal degradation of cTnI in any of the subendocardial needle biopsies (Figure 5A). Moreover, no degradation products were observed for cMyBP-C, cTnT and MLC-2 (Figure 5B). As the reduced phosphorylation of MLC-2 in MI (Figure 3C) may be the resultant of reduced MLC-2 expression, expression of MLC-2 protein was determined relative to Ponceau-stained actin. MLC-2 expression was similar in all groups (Figure 5C), and therefore does not underlie alterations in MLC-2 phosphorylation. Similarly, expression of cTnI, cMyBP-C and cTnT (relative to Ponceau-stained actin) did not differ among groups (not shown).

Unaltered Titin Isoform Composition

To investigate whether a shift in titin isoform composition underlies the reduction in $F_{\text{pas}}$ observed in MI+$\beta$, the stiff N2B and compliant N2BA titin isoforms were analyzed using SDS-PAGE. The percentage of the more compliant N2BA titin isoform did not significantly differ among groups (Figure 5D).

Increased PP1 in MI and Reduced PP2a upon $\beta$-blockade

Recently, Belin et al.\textsuperscript{21} showed that augmented PKC\textsubscript{\(\alpha\)}-induced protein phosphorylation might contribute to myofilament dysfunction in congestive heart failure. Western immunoblot analysis did not reveal differences in PKC\textsubscript{\(\alpha\)} expression (relative to ponceau-stained actin: 0.36±0.10,
0.29±0.02, 0.31±0.03) and activity (i.e. p-PKCa: 1.11±0.13, 1.15±0.24, 1.18±0.28) between sham, MI and MI+β, respectively (n=5 in all groups). In contrast analysis of protein phosphates revealed a significantly increased PP1 expression in MI by 80% compared to sham, which was prevented by β-blockade (Figure 6A) and a significant reduction in PP2a in MI treated with β-blockers (Figure 6B).

**PP1 Increases Myofilament Ca²⁺-sensitivity**

To investigate if altered baseline force characteristics in MI (i.e. reduced F\text{max} and reduced EC\text{50}; Figure 1A,D) may be the resultant of increased PP1 force was measured in sham cardiomyocytes before and after PP1 treatment. PP1 slightly increased F\text{max} (P=0.07; Figure 7A), but did not alter F\text{pas} (before vs. after: 4.9±0.7 and 4.5±0.7 kN/m²). Ca²⁺-sensitivity significantly increased upon PP1 treatment illustrated by the reduction in EC\text{50} (Figure 7A), similar to the value observed in MI (Figure 1D). In addition, a significant decrease was observed in nHill (before vs. after: 3.64±0.17 and 3.09±0.13).

Pro-Q Diamond staining (Figure 7B) of sham myocardium incubated with and without PP1 revealed a reduction in MLC-2 phosphorylation by 71% (Figure 7C). PP1 also decreased cTnI phosphorylation by 45%, while the effects on phosphorylation of the other proteins (cMyBP-C, desmin and cTnT) was only minor.
Discussion

The present study was designed to determine the effects of β-blocker therapy on cardiomyocyte function after myocardial infarction in a large animal model. Early β-blocker treatment attenuated global LV dysfunction after MI. At the cardiomyocyte level, β-blockade blunted not only the MI-induced cardiomyocyte hypertrophy but also prevented the MI-induced decrease in maximum force development and increase in myofilament Ca\(^{2+}\)-sensitivity. The implications of these findings and their molecular underpinnings are discussed in detail.

Beta-blocker Therapy Improved Cardiomyocyte Function after MI

Maximal Force Development In accordance with previous studies\(^5\)\(^-\)\(^8\) our data indicate that a reduction in maximal force generating capacity (F\(_{\text{max}}\)) of single permeabilized non-infarcted cardiomyocytes may contribute to systolic dysfunction of remodeled myocardium. Different mechanisms have been implicated in the reduction of F\(_{\text{max}}\), including cTnI degradation.\(^5\) Degradation of cTnI could result from intermittent subendocardial ischemia, due to recurrent subendocardial hypoperfusion during increased physical activity,\(^1\) or could be stretch-mediated secondary to the increased LV end-diastolic pressures.\(^22\) However, using selective exchange of endogenous full length cTnI by truncated cTnI, Narolska et al.\(^23\) did not observe an effect on maximal force. Moreover, in the present study (using an in vivo needle biopsy approach that allows faster procurement of subendocardial tissue compared to the ex vivo approach in our previous study\(^5\)) we did not observe any cTnI degradation (Figure 5A) in remote myocardium of MI pigs, or degradation products for cMyBP-C, cTnT or MLC-2 (Figure 5B). On the basis of these data we conclude that reduced F\(_{\text{max}}\) in remodeled myocardium is not caused by protein proteolysis.

Alternatively, the decrease in F\(_{\text{max}}\) could be the result of neurohumoral activation.\(^5\),\(^21\) We have previously shown\(^6\) that in vivo cardiac responsiveness to the aggravated exercise-induced increases in noradrenaline was blunted in pigs with a myocardial infarction, consistent with
down-regulation, uncoupling and/or downstream signaling defects of the β-adrenergic receptors. Stimulation of the β-adrenergic pathway activates PKA-mediated phosphorylation of the sarcomeric proteins cMyBP-C, cTnI and titin, of which cMyBP-C is believed to exert an effect on the maximal force generating capacity of myofilaments. Recently, reduced cMyBP-C phosphorylation was observed in human heart failure and altered cMyBP-C phosphorylation has been implicated in contractile dysfunction. However, in the present study phosphorylation level of cMyBP-C did not differ among groups and exogenous PKA did not enhance basal $F_{\text{max}}$ in MI. In a previous study McClellan et al. observed that phosphorylation of cMyBP-C by Ca$^{2+}$-calmodulin-dependent kinase was prerequisite for a PKA-mediated increase in $F_{\text{max}}$. Moreover, phosphorylation at Ser-282 enables cMyBP-C phosphorylation at other sites. As exogenous PKA significantly increased $F_{\text{max}}$ only in sham and β-blocker treated MI pigs, differential cMyBP-C phosphorylation might explain the differences in $F_{\text{max}}$. Our 2D analysis of MyBP-C did however not reveal diverse phosphorylation patterns among the three groups. Moreover, phosphorylation at Ser-282 did not differ among the groups. Taken together, these findings indicate that alterations in cMyBP-C phosphorylation do not underlie the reduction in $F_{\text{max}}$ in remote myocardium, and cannot explain the positive effect of β-blockade thereon.

Impaired cardiac function has been ascribed to increased expression and activity of protein kinase C (PKC) isoforms in myocardial disease. In particular PKC-mediated phosphorylation of troponin subunits has been implicated in reduced maximal force production of myofilaments. Recently, Belin et al. observed up-regulation of PKCα, increased cTnI phosphorylation, preserved PP1 expression, and a reduction in $F_{\text{max}}$ and in myofilament Ca$^{2+}$-sensitivity in a rat model of end-stage congestive heart failure ~34 weeks after MI. Myofilament dysfunction was largely reversed by exogenous PP1 treatment, indicative for hyperphosphorylation of myofilament proteins. However, myofilament function was unaltered early after MI (~12 weeks), which contrasts with our data. In our pig model, a reduction in $F_{\text{max}}$ in remodeled myocardium 3 weeks after MI coincided with reduced phosphorylation of MLC-2
and increased PP1 expression, while no differences were found in basal phosphorylation of cTnT and cTnI. Moreover, no difference was found in PKCα expression and activity, and previous analysis of differential cTnT phosphorylation with 2D-gel electrophoresis did not reveal differences between MI and sham. The increased expression of PP1 implies that hypo- rather than hyperphosphorylation could be involved in altered myofilament contractility. The increase in PP1 is consistent with previous observations in failing human myocardium, in which increased PP1 activity has been observed. Beta-blockers lowered expression levels of both PP1 and PP2a and reversed the reduction in MLC-2 phosphorylation. Hence, restoration of $F_{\text{max}}$ by $\beta$-blockade may be explained by alterations in phosphatase rather than in kinase activities.

**Ca$^{2+}$-sensitivity.** In healthy myocardium, myofilament protein phosphorylation by PKA reduces myofilament Ca$^{2+}$-sensitivity upon adrenergic stimulation and thereby exerts a positive lusitropic effect. The increased myofilament Ca$^{2+}$-sensitivity in post-MI remodeled myocardium is thought to originate from reduced $\beta$-adrenergic mediated cTnI and cMyBP-C phosphorylation. Accordingly, treatment of cardiomyocytes with exogenous PKA induced a larger shift in EC$_{50}$ in MI compared to sham and abolished the initial difference in Ca$^{2+}$-sensitivity. Beta-blockade largely prevented the enhanced myofilament Ca$^{2+}$-sensitivity, suggesting that defective PKA signaling was prevented. Surprisingly, however, PKA responsiveness was further enhanced in MI pigs receiving $\beta$-blockade. Moreover, the increase in PKA responsiveness in MI pigs either without or with $\beta$-blockade could not be explained by alterations in cMyBP-C phosphorylation or in cTnI phosphorylation. Taken together these findings suggest that the beneficial effects of $\beta$-blockade on Ca$^{2+}$-sensitivity occurred independently of alterations in PKA signaling and phosphorylation of its myofilament target proteins cMyBP-C and cTnI.

**Passive Force.** The decrease in LV end diastolic pressure in the $\beta$-blocker treated pigs may, at least in part, be the result of reduced cardiomyocyte stiffness. The latter could not be explained by a shift in titin isoform composition. PKA-mediated phosphorylation of titin has been shown to reduce passive force development. Moreover, the PKA-mediated $F_{\text{pas}}$ reduction...
depends on isoform composition, as it is larger in tissue expressing predominantly stiff N2B titin isoform. The β-blocker induced decrease in \( F_{\text{pas}} \) may involve augmented phosphorylation status of titin isoforms resulting from reductions in both PP1 and PP2a and warrants further research. 

**Importance of Phosphatases in Altered Myofilament Function**

A recent study in rats with ischaemic heart failure revealed impaired contractile function and reduced MLC-2 phosphorylation, which were both restored by exercise training. Similarly, in MI mice, exercise prevented the reduction in \( F_{\text{max}} \), which was associated with an increase in MLC-2 phosphorylation. Thus, the reduction in maximal force generating capacity of myofilaments may be due to reduced MLC-2 phosphorylation post-MI. To reveal if altered myofilament function and reduced MLC-2 phosphorylation in post-MI remodeled myocardium can be explained by the increased PP1 expression, sham myocardium was treated with exogenous PP1. PP1 decreased MLC-2 phosphorylation and lowered \( E_{50} \) in sham cells (Figure 7A) to the value observed in MI cardiomyocytes (Figure 1D). Thus, the increase in PP1 in MI myocardium may underlie enhanced Ca\(^{2+}\)-sensitivity and reduced MLC-2 phosphorylation. However, \( F_{\text{max}} \) remained unaltered. The absence of an affect on \( F_{\text{max}} \) may be explained by the fact that exogenous PP1 treatment did not solely dephosphorylate MLC-2, but also lowered cTnI phosphorylation. Similarly, PP1 treatment of human cardiac tissue reduced phosphorylation of MLC-2 and cTnI, while PP2a only reduced cTnI. To fully understand the consequences of altered protein phosphatases for myofilament function, it remains to be investigated which myofilament proteins are targets for protein phosphatases in vivo.

Rather than a direct effect of phosphatase on cellular target proteins, PP2a has been shown to alter phosphorylation of the calcium handling protein phospholamban via regulation of inhibitor-1 (I-1), the protein inhibitor of PP1. Inhibitor-1, which phosphorylation status is regulated by kinases and phosphatases, plays a pivotal role in the
phosphorylation/dephosphorylation balance. PKA-mediated phosphorylation of I-1 upon β-AR stimulation augments blockade of PP1, shifting the balance to phosphorylation. Alternatively, PP2a dephosphorylates and inactivates I-1 and thereby releases blockade of PP1, shifting the balance to dephosphorylation. In line with these observations, increased PP1 expression as observed in MI would counteract β-AR signaling, while the β-blockade induced decrease in PP2a would enhance β-AR responsiveness via augmented phosphorylation of I-1. The latter corresponds with the enhanced myofilament responsiveness to exogenous PKA in MI treated with β-blockers. Future investigations are warranted to explore the effects of chronic β-blockade on β-AR responsiveness of intact cardiomyocytes and to determine the role of I-1 and its phosphorylation status in the effects of chronic β-blockade.

**Methodological Considerations**

Since the acute effect of beta-blockade in post-MI remodeled pig hearts is a reduction in global LV function, it follows that an improvement in LV pump function produced by chronic beta-blockade must stem from molecular alterations at the cardiomyocyte level. Interestingly, we did not observe a significant effect of beta-blockade on global LV remodeling or cardiomyocyte hypertrophy, consistent with the concept that chronic beta-blockade acted through a primary effect on cardiomyocyte (i.e. myofilament) function that resulted in an improvement of global LV function. Nevertheless, it must be acknowledged that in addition to the correction of myofilament dysfunction, several other mechanisms may also have contributed to the beneficial effect of beta-blocker therapy on cardiac performance after MI. For example, we have previously shown that apoptosis and interstitial collagen deposition are increased in post-MI remodeled myocardium, blunting of which could have contributed to the favorable effects of β-adrenergic receptor blockade. Similarly, it could be speculated that beta-blocker therapy may have acted in part via restoring myocardial oxygen balance within the remodeled myocardium. Finally, correction of impaired calcium handling may improve cardiomyocyte contractility. We did not
observe changes in expression of SERCA2a and phospholamban among groups (data not shown), but cannot exclude that alterations in phosphorylation status of calcium handling proteins contributed to an improvement in calcium handling. These issues should be subject of further studies.

Conclusions and Clinical Implications

The present study demonstrates that β-blocker therapy prevents myofilament dysfunction of surviving cardiomyocytes in a large animal model of post-MI remodeling. Furthermore, β-blockade reduced passive cardiomyocyte stiffness and enhanced the myofilament Ca\(^{2+}\)-responsiveness to exogenous PKA stimulation, which may benefit in vivo myocardial relaxation during increased cardiac stress. Our data indicate that enhanced protein phosphorylation by altered expression levels of protein phosphatases underlie restoration of myofilament dysfunction and reduced passive stiffness at the cellular level. These beneficial effects likely contribute to the observed improvements in global LV function in clinical investigations.\(^{2,49,50}\)
**Funding sources**

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**Disclosures**

None.
References


Legends

Figure 1. **Myofilament function.** Force characteristics in cells from sham (n=13), MI (n=9) and MI+β (n=10) endocardial tissue. Maximal force (Fmax) was significantly lower in MI cells (white bar) compared to sham (black bar) and MI+β (grey bar) cells (A). Passive force (Fpas) was significantly reduced in MI+β compared to sham and MI (C). The reduction in EC$_{50}$ in MI was reversed by β-blocker therapy (C,D). *P<0.05, MI vs. sham; †P<0.05, MI+β vs. MI in post-test Bonferroni analysis. n, number of cardiomyocytes.

Figure 2. **Effect exogenous PKA.** PKA treatment of cells significantly increased F$_{max}$ in sham (n=5) and MI+β (n=9) cells, while it had no effect in MI (n=5) (A). PKA significantly reduced F$_{pas}$ in MI+β (B). PKA increased EC$_{50}$ in all groups (C). The PKA-induced shift in EC$_{50}$ was significantly different among groups and abolished the difference in EC$_{50}$ between sham and MI cells, while EC$_{50}$ was significantly higher in MI+β than in MI cardiomyocytes after PKA (C,D). *P<0.05, MI vs. sham; †P<0.05, MI+β vs. MI in post-test Bonferroni analysis. ‡P<0.05, before vs. after PKA in paired t-test. n, number of cardiomyocytes.

Figure 3. **Myofilament protein phosphorylation.** Biopsy samples (20 μg/lane) were separated on a 4-15% gradient gel stained with Pro-Q Diamond (A). The gels were subsequently stained with SYPRO Ruby (B). ProQ diamond stained signals (cardiac myosin binding protein C, cMyBP-C; desmin; cardiac troponin T, cTnT; cardiac troponin I, cTnI and myosin light chain 2, MLC-2) were divided by the SYPRO-stained cMyBP-C protein band to correct for differences in protein loading. Abbreviations: M, molecular weight marker (PeppermintStick Phosphoprotein marker in which ovalbumin and β-casein are phosphorylated); MHC, myosin heavy chain. One-way ANOVA did not reveal significant differences among the three groups in phosphorylation
status of cMyBP-C, desmin, cTnT and cTn, while MLC-2 phosphorylation was significantly lower in MI compared to sham (C). *P<0.05, MI vs. sham in post-test Bonferroni analysis.

**Figure 4. Differential phosphorylation of cMyBP-C.** Analysis of cMyBP-C phosphorylation by 2D gel electrophoresis (A) revealed multiple spots (B) at the level of cMyBP-C, which intensities did not differ among the three groups (C; n=5 in all groups).

**Figure 5.** Protein analysis did not reveal degradation of cTnI (A). In addition, no degradation products were found for cMyBP-C, cTnT and MLC-2 (B). Protein expression of MLC-2 relative to Ponceau-stained actin did not differ among groups (C). No differences were found in titin isoform composition (D).

**Figure 6.** A. Western immunoblot analysis of protein phosphatase 1 (PP1) normalized to ponceau-stained actin revealed increased PP1 expression in MI, while protein phosphatase 2a (PP2a) normalized to calsequestrin (CSQ) was significantly reduced in β-blocker treated animals (B). *P<0.05, MI vs. sham; †P<0.05, MI+β vs. sham in post-test Bonferroni analysis.

**Figure 7.** Functional effects of PP1 were studied in sham cardiomyocytes (n=8). A. PP1 did not affect F_{max}, but significantly reduced EC_{50}. B. 1D gradient gel of sham myocardium without (control, C) and with PP1, stained with Pro-Q Diamond and SYPRO. C. PP1 treatment reduced phosphorylation of cTnI and MLC-2 (data normalized to control). Abbreviation: MHC, myosin heavy chain. ‡P<0.05, before vs. after PP1 in paired t-test.
Table 1. Anatomical and hemodynamical data

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| **Hemodynamic data** |            |          |            |             |
| Cardiac output (CO, L/min) | 4.5±0.3 | 3.1±0.1* | 4.1±0.3† | # |
| Heart rate (HR, bpm)     | 122±6     | 141±19   | 105±6      |             |
| Stroke volume (SV, mL)   | 38±3      | 23±3*    | 40±4†      | # |
| Mean aortic pressure (mmHg) | 91±10    | 100±5    | 100±5      |             |
| Mean pulmonary artery pressure (mmHg) | 18±2 | 28±3* | 25±2 | # |
| Systolic pulmonary artery pressure (mmHg) | 24±1 | 35±4* | 34±1* | # |
| LV end-diastolic pressure (mmHg) | 5±3 | 16±3* | 12±2 | # |
| LVdP/dtP40 (mmHg/s)      | 1760±190  | 1350±110 | 1600±150   |             |
| Tau (ms)                | 34±1      | 46±6     | 47±4       | P=0.09      |

#P<0.05, significantly different among the three groups (sham, MI, MI+β) by 1-way ANOVA.

*P<0.05, MI and MI+β vs. sham; †MI+β vs. MI in post-test Bonferroni analysis. n, number of animals.
Figure 1
Figure 2
Figure 3

C.  

- cMyBP-C
- Desmin
- cTnT
- cTnI
- MLC-2

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</table>
Figure 4
Figure 5

A. 

B. 

C. 

D. 

Figure 5
Figure 6
Figure 7

A. 

- Before PP1
- After PP1

F_max (kN/m²)

Before PP1: 50
After PP1: 40

EC50 (μM)

Before PP1: 3.0
After PP1: 2.0

‡

B. 

C MyBP-C - Desmin - cTnT - cTnI - MLC-2

Pro-Q

SYPRO

Phosphorylation relative to control

cMyBP-C - Desmin - cTnT - cTnI - MLC-2

PP1 treatment

‡
Prevention of Myofilament Dysfunction by Beta-blocker Therapy in Post-infarct Remodeling

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