Chronic Treatment with Carvedilol Improves Ca$^{2+}$-Dependent ATP Consumption in Triton X-Skinned Fiber Preparations of Human Myocardium


Department of Molecular and Cellular Sport Medicine, German Sport University, Cologne, Germany (K.B., W.B.); Laboratory of Muscle Research and Molecular Cardiology, Department of Internal Medicine III, University of Cologne, Cologne, Germany (K.B., R.L., B.B., S.G., F.H., R.H.G.S.); Clinic of Cardiothoracic Surgery, University of Cologne, Cologne, Germany (U.M.); Clinic of Internal Medicine II, Hospital of Weiden, Weiden, Germany (R.H.G.S.); Department of Pathophysiology, Shanghai University of Traditional Chinese Medicine, Shanghai, China (R.L.); Clinic C–Department of Cardiology and Angiology, Hospital of the University of Münster, Münster, Germany (C.P.)

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ABSTRACT

Evidence is given that β-blocker treatment differentially influences gene expression and up-regulation of β$_1$-adrenoceptors in human myocardium. Here, we investigate whether chronic treatment with carvedilol or metoprolol may functionally alter myofibrillar function in end-stage human heart failure. Investigations were performed in Triton X (1%, 4°C, 20 h)-skinned fiber preparations of explanted hearts from patients undergoing heart transplantation due to idiopathic dilative cardiomyopathy. Five patients were not on β-adrenoceptor blocker treatment (DCM_NBB), and 5 patients received either carvedilol (DCM_CAR) or metoprolol (DCM_MET). Nonfailing (NF) donor hearts (n = 5), which could not be transplanted due to technical reasons, were investigated for comparison. Ca$^{2+}$-dependent tension (DT) development and actomyosin-ATPase activity (MYO) were measured and tension-dependent ATP consumption was calculated by the ratio of DT and MYO ("tension cost"). In addition, we measured the phosphorylation of troponin I (TNI) by back phosphorylation. Maximal DT and TNI phosphorylation were reduced, with myofibrillar Ca$^{2+}$ sensitivity of DT and MYO as well as tension cost being increased in DCM_NBB compared with NF. Metoprolol treatment restored TNI phosphorylation, decreased Ca$^{2+}$ sensitivity of tension development and of myosin-ATPase activity, but did not alter the tension-dependent ATP consumption. Carvedilol treatment improved maximal DT and significantly decreased tension-dependent ATP consumption without altering myofibrillar Ca$^{2+}$ sensitivity. TNI dephosphorylation was increased in patients treated with carvedilol. In conclusion, chronic β-adrenoceptor blockade functionally alters myofibrillar function. The more economic cross-bridge cycling in patients under carvedilol treatment may provide an explanation for the efficacy of carvedilol in the treatment of chronic heart failure patients.

The results of a recent clinical trial suggest that carvedilol may be superior to metoprolol in the treatment of heart failure patients (Poole-Wilson et al., 2003). This result may be due to the fact that myocardial contractility is decreased less when carvedilol is used instead of metoprolol as shown by some clinical trials investigating smaller patient populations (Gilbert et al., 1996; Sanderson et al., 1999; Metra et al., 2000). The reasons for the minor cardiodepressant effects of carvedilol in comparison to metoprolol are unclear. It has been shown previously that treatment of heart failure patients with β-adrenoceptor blockers results in alterations of gene expression regarding myofibrillar proteins (Lowes et al., 2002). Thus, changes in the myofibrillar response to Ca$^{2+}$ may be an explanation for the differences between the inotropic effects of β-adrenoceptor blockers. We have demonstrated previously that myofibrillar Ca$^{2+}$ sensitivity of human myocardium is not altered acutely by carvedilol or metoprolol treatment under in vitro conditions (Bundkirchen et al., 2001); however, changes in myofibrillar Ca$^{2+}$ responsiveness due to β-adrenoceptor blocker treatment may occur during chronic treatment. Therefore, we investigated Ca$^{2+}$-dependent tension and actomyosin-ATPase activity in

ABBREVIATIONS: DCM, dilative cardiomyopathy; NF, nonfailing; MET, metoprolol; CAR, carvedilol; NBB, non-β-adrenoceptor blocker treatment; pCa, −log [Ca].
chemically skinned fiber preparations of left ventricular myocardium from patients who were on treatment with carvedilol (DCM_CAR) or metoprolol (DCM_MET). Left ventricular failing myocardium from patients who did not receive β-adrenoceptor blocker treatment (DCM_NBB) as well as nonfailing (NF) left ventricular myocardium was studied in comparison.

**Materials and Methods**

**Cardiac Tissue.** Failing left ventricular tissue was obtained during cardiac transplantation. Patients suffered from heart failure clinically classified as New York Heart Association class IV on the basis of clinical symptoms and signs as judged by the attending cardiologist shortly before operation. All patients gave written informed consent before surgery. Only male patients aged between 40 and 60 years were included for the present study. The treatment with carvedilol and metoprolol, respectively, had been administered for approximately 2 to 12 months. The patients' characteristics are given in Table 1. Drugs used for general anesthesia were flunitrazepam and pancuronium bromide with isoflurane. Cardiac surgery was performed on cardiopulmonary bypass patients with cardioplegic arrest during hypothermia. The cardioplegic solution (a modified Bretschneider solution) contained 15 mM NaCl, 9 mM KCl, 4 mM MgCl₂, 180 mM histidine, 2 mM tryptophan, 30 mM mannitol, and 1 mM potassium dihydrogen oxoglutarate.

Nonfailing human myocardium was obtained from donor hearts, which were rejected for further transplantation due to technical reasons. The mean age of the donor group was 50.6 ± 2.8 years. No cardiac catheterization had been performed in the organ donor group, but none of the donors had a history of heart disease and all had normal left ventricular function as measured by echocardiography. The study was approved by the local ethics committee.

**Chemically Skinned Left Ventricular Fibers.** Left ventricular muscle fibers were prepared with minor modifications as described previously (Brixius and Schwinger, 2000). In brief, frozen cardiac tissue was slowly and carefully thawed and small fiber bundles (diameter approximately 5–8 mg) were homogenized at 4°C with an Ultra-Turrax T8 glass-Teflon-Potter homogenizer (B. Braun AG, Melsungen, Germany). The ratio of suprabasal ATPase activity and force in the steep part of the respective Ca²⁺-dependence curve was assumed as a measure for the "tension cost," because these parts reflect the Ca²⁺-dependent linear changes.

**Immunocytochemistry and Measurement of Sarcomere Length.** Skinned fibers of human hearts, prepared as described above, were used for immunocytochemical labeling of the Z-lines by α-actinin staining. After three washes in 0.1 mM phosphate-buffered saline buffer, the skinned fiber preparations were incubated in a 1:800 dilution of mouse anti-rat α-actinin antibody for 1 h at room temperature, followed by treatment with a secondary biotinylated goat anti-mouse antibody (1:400) for 1 h and Cy3-labeled extravidin (1:600) for 1 h (Ji et al., 1999). Afterward, the skinned fibers were washed with 0.1 M Tris-buffered saline and stored at −20°C until the sarcomere length measurements.

The measurements of sarcomeric length were performed using a Zeiss Axiovert 135 fluorescence microscope (Zeiss, Oberkochen, Germany), a Sony three chip camera, and computer-assisted imaging software (Optimas 6.01). For investigation of the sarcomere length, the skinned fibers were fixed at slack position in relaxation solution. The distance of 10 to 15 actinin/Cy3-labeled Z-lines was measured at 10 different areas of each skinned fiber using a 40× Neofluar objective (Zeiss). The sarcomeric length was calculated by dividing the measured distance by the number of spaces between labeled Z-lines. The mean of sarcomeric length for each skinned fiber was calculated from all investigated areas. The experiments were performed as described previously (Brixius and Schwinger, 2000). Average sarcomeric length was 1.95 ± 0.04 μm.

**Measurement of Force and Actomyosin-ATPase Activity.** Force and actomyosin-ATPase activity were simultaneously measured as described before (Guth and Wojciechowski, 1986; Brixius and Schwinger, 2000; experimental setup, Scientific Instruments, Heidelberg, Germany). The actomyosin-ATPase activity was measured using a linked NADH-fluorescence assay. The relaxation solution contained 20 mM imidazole, 10 mM Na₂ATP, 5 mM NaN₃, 5 mM EGTA, 12.5 mM MgCl₂, 5 mM phospho(enol)-pyruvate, 0.6 mM NADH, 0.2 mM P₅-P₅-di(adenosine 5’)-pentaphosphate (myokinase inhibitor), and 25 mM cyclopiazonic acid, together with 100 U/ml pyruvate kinase and 125 U/ml lactate dehydrogenase. The contraction solution contained calcium EGTA (5 mM) instead of EGTA. Both solutions were mixed by a gradient mixer so that Ca²⁺ was successively increased every 30 s. Free Ca²⁺ concentration was determined by calculator programs designed for experiments in skinned muscle cells (Fabiani and Fabiani, 1979). Measurement of developed tension and myosin-ATPase activity started 3 s after the solution was exchanged. Developed tension and myosin-ATPase activity had reached a stable plateau at that time. By subtracting the basal ATPase activity obtained in the relaxation solution from the measured ATPase activity, the suprabasal ATP-splitting rate was obtained. The ratio of suprabasal ATPase activity and force in the steep part of the respective Ca²⁺-relaxation was assumed as a measure for the “tension cost,” because these parts reflect the Ca²⁺-dependent linear changes.

**Back Phosphorylation.** Freeze-clamped skinned fibers (approximately 5–8 mg) were homogenized at 4°C with an Ultra-Turrax T8 (Janke and Kunkel KG, IKA-Werke, Staufen i. Breisgau, Germany) three times for 20 s, followed by three strokes for 30 s with a glass-Teflon-Potter homogenizer (B. Braun AG, Melsungen, Germany) in 3 times the volume of chilled preparation buffer containing

### Table 1

<table>
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<th>No.</th>
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<th>Age</th>
<th>LVDEP</th>
<th>EF</th>
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<td>×</td>
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<td>×</td>
</tr>
<tr>
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<td>18</td>
<td>2.6</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>×</td>
</tr>
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</table>

EF, ejection fraction (%); LVDEP, left ventricular end-diastolic pressure (mm Hg); CI, cardiac index (l·min⁻¹·m⁻²); NI, nitrates; Diu, diuretics; Gly, glycosides; ACE, angiotensin-converting enzyme inhibitors; AT1, AT₂, antagonists.
5 mM histidine-HCl, 0.2 mM dithiothreitol, 25 mM NaF, 10 mM EDTA, 50 mM NaH2PO4, and 0.1 mM phenylmethylsulfonyl fluoride (pH 7.4). The homogenates were stored at -80°C. For back phosphorylation, 40 μg of protein (final verification by Bradford’s assay (Bradford, 1976)) were phosphorylated in a medium containing 40 mM histidine-HCl, 100 mM NaCl, 10 mM MgCl2, 15 mM NaF, 1 mM EDTA, 1% Triton X, 100 μg of BSA, and 0.5 U/mL catalytic subunit of protein kinase A in the presence of 50 μM [γ-32P]ATP (pH 6.8). The reaction was stopped after 10 min with ice-cold stop solution containing 50 mM H3PO4, 0.5 mM ATP, and 15% trichloroacetic acid. After centrifugation (2000g, 20 min), the precipitate was directly processed for electrophoresis in sample buffer (50 mM H3PO4, 5 M EDTA, 1% mercaptoethanol, 2% SDS, 10% glycerol, and a trace of bromphenol blue as tracking dye, pH 6.8 adjusted with Tris). As molecular mass marker, gel electrophoresis as described previously (Swank and Munkres, 1971). Gels were stained with Coomassie blue and destained with methanol/acetic acid/water (3:1:6 v/v). As molecular mass marker, the gel electrophoresis was described as described previously (Swank and Munkres, 1971). Gel bands were excised from X-ray films. Autoradiography using X-OMAT film (Eastman Kodak, Rochester, NY) and intensifying screens permitted the detection of 32P-labeled proteins on the gels. Densitometric units of the signals were measured using computerized imaging system. Previous measurements reveal evidence that the troponin I content is similar between nonfailing and DCM (nonischemic) failing hearts. Therefore, we assume that the protein content of our preparations reflects that of the myofibrillar proteins.

Materials. All chemicals were of analytical grade or the best grade commercially available. The 30% acrylamide/bisacrylamide and kaledioscope stained marker were from Bio-Rad (Hercules, CA) was used. The gels were exposed to X-ray films. Autoradiography using X-OMAT film (Eastman Kodak, Rochester, NY) and intensifying screens permitted the detection of 32P-labeled proteins on the gels. Densitometric units of the signals were investigated by scanning the respective bands for troponin I of the whole autoradiogram. The band intensities were evaluated by densitometric scanning using a computerized imaging system. Previous measurements reveal evidence that the troponin I content is similar between nonfailing and DCM (nonischemic) failing hearts. Therefore, we assume that the protein content of our preparations reflects that of the myofibrillar proteins.

Statistics. All values are means ± S.E.M. unless otherwise noted. One-way ANOVA was used to test significance. p values of <0.05 were accepted as significant. pCa force as well as pCa actomyosin-ATPase activity (EC50 for Ca2+ concentration giving half-maximal activation (inhibition), and nH is the fractional force, or actomyosin-ATPase activity, pCa50 is the Ca2+ concentration giving half-maximal activation (inhibition), and H is the Hill’s coefficient of the Hill equation (Hill, 1910) as follows: Y = Ca2+/[pCa50]0.5 + Ca2+). The concentration needed for maximal Ca2+ activation of tension development or myosin-ATPase activity (EC50 for Ca2+), all Hill coefficients, and the tension cost (ratio of ATPase activity and tension development) were analyzed by GraphPad Prism (GraphPad Software Inc., San Diego, CA).

Results

Nonfailing Versus Failing Myocardium in the Absence of β-Adrenoceptor Blocker Treatment. The present study investigated the influence of chronic β-adrenoceptor blocker treatment on myofibrillar function in human myocardium. Table 2 summarizes the results obtained in Triton X-skinned fiber preparations by simultaneous measurements of Ca2+-dependent force and actomyosin-ATPase activity in DCM_NBB in comparison to NF myocardium.

In DCM_NBB, maximal Ca2+-dependent tension was significantly decreased compared with NF. This was accompanied by a significant increase in Ca2+ sensitivity of tension and actomyosin-ATPase activity in DCM_NBB compared with NF. There was no difference in Ca2+-activated actomyosin-ATPase activity between the two groups.

Tension Development after Chronic Treatment with Carvedilol or Metoprolol. Figure 1 summarizes the results obtained for Ca2+-dependent tension development in human nonfailing and failing myocardium with and without β-blocker treatment. The depression of maximal Ca2+-dependent tension in DCM_NBB was not restored in patients chronically treated with metoprolol. However, metoprolol significantly shifted the Ca2+-concentration/tension relationship to the right (EC50 of Ca2+ tension ECM_DCM, 0.60 ± 0.03); i.e., metoprolol decreased the Ca2+ sensitivity of human failing myocardium to values similar to those obtained in human nonfailing hearts (Table 2). In contrast to metoprolol, carvedilol significantly increased maximal Ca2+-dependent tension (although the tension development was not fully restored compared with human nonfailing myocardium), without altering the Ca2+ sensitivity of myofibrillar tension (EC50 of Ca2+ tension ECM_CAR, 0.52 ± 0.05). There were no significant alterations between the Hill coefficients of the four groups (nH of tension of NF, 2.24 ± 0.06; DCM_NBB, 1.94 ± 0.04; DCM_MET, 1.90 ± 0.04; DCM_CAR, 2.06 ± 0.04). This means that after chronic treatment with carvedilol, the Ca2+ sensitivity of human failing myocardium is still significantly increased in failing compared with nonfailing hearts.

Actomyosin-ATPase Activity. In human heart failure, the ATP turnover is a critical point for the pathophysiology of the disease, because the ATP supply is hampered in the failing human myocardium. Both carvedilol and metoprolol decreased the maximal Ca2+-dependent actomyosin-ATPase activity (Fig. 2, left). In addition, metoprolol (EC50 of Ca2+ ATPase ECM_MET, 0.54 ± 0.03), but not carvedilol (EC50 of Ca2+ tension ECM_CAR, 0.44 ± 0.03), induced a rightward shift of the Ca2+ sensitivity of the actomyosin-ATPase activity (Fig. 2, right; Table 2). Hill coefficients of the Ca2+/actomyosin-ATPase activity were similar for all four groups (nH of ATPase NF, 1.33 ± 0.06; DCM_NBB, 1.37 ± 0.04; DCM_MET, 1.34 ± 0.06; DCM_CAR: nH, 1.40 ± 0.04).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NF (n = 5 Men)</th>
<th>DCM_NBB (n = 5 Men)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tension</td>
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<td></td>
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<tr>
<td>Maximum (mN/mm²)</td>
<td>21.4 ± 1.9</td>
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<td>EC50 Ca²⁺ (μM)</td>
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<td>Actomyosin-ATPase activity</td>
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<td>Maximum (μM ADP/μs)</td>
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<td>44.5 ± 1.6 *</td>
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<tr>
<td>EC50 Ca²⁺ (μM)</td>
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<tr>
<td>Tension-dependent ATP consumption</td>
<td>α ([μM ADP × s⁻¹ × (mm²/mM)⁻¹]</td>
<td>1.79 ± 0.03</td>
</tr>
</tbody>
</table>

α, slope of the myosin-ATPase/tension relationship; EC50 Ca²⁺, Ca²⁺ concentration at which a 50% increase of tension or myosin-ATPase activity was achieved.

* p < 0.05 vs. NF.
Tension-Dependent Myofibrillar ATP Consumption and β-Adrenoceptor Blocker Treatment. The tension-dependent ATP consumption was evaluated by the ratio of Ca\textsuperscript{2+}-dependent tension and suprabasal actomyosin-ATPase activity for the very steep part of the Ca\textsuperscript{2+}/tension, namely, actomyosin-ATPase relationship. A linear line fit was done for all data points obtained in the different groups. Figure 3 shows the results. Metoprolol treatment did not significantly alter the tension-dependent ATP consumption in human failing myocardium. In contrast, carvedilol completely restored the increased tension-dependent ATP consumption. The tension-dependent ATP consumption of Triton X-skinned fiber preparations obtained from human failing myocardium of patients who had undergone chronic treatment with carvedilol was similar to that of human nonfailing myocardium.

Troponin I Phosphorylation. It has been shown that the increased myofibrillar Ca\textsuperscript{2+} sensitivity of human failing myocardium may be due to alterations in the phosphorylation of contractile proteins, e.g., troponin I (van der Velden et al., 2003a,b). Therefore, we investigated the phosphorylation status of troponin I using the back phosphorylation technique. Figure 4 presents the original blots as well as the summarized data. Although the phosphorylation varied within the different samples of one group, back phosphorylation of troponin I was significantly increased in myocardium of heart failure patients who had not been treated with β-adrenoceptor blockers compared with nonfailing myocardium, which indicates a higher phosphorylation status in nonfailing myocardium. Although chronic treatment with metoprolol reversed this situation in DCM, the dephosphorylation of troponin I was further decreased in human failing myocardium in hearts of patients chronically treated with carvedilol.

Discussion

The present study investigated Ca\textsuperscript{2+}-dependent tension and myosin-ATPase activity in patients suffering from di-
In the present study it was shown that carvedilol did not alter the increased myofibrillar Ca\(^{2+}\) sensitivity in heart failure patients without β-adrenoceptor blocker treatment. These findings are in agreement with previous results from our group (Brixius et al., 2002) and have also been confirmed in single-cell preparations by different groups (Wolff et al., 1996; van der Velden et al., 2002). As shown previously, chronic treatment with metoprolol reversed the hyperphosphorylation of the ryanodine receptor and restored the stoichiometry of the ryanodine receptor macromolecular complex (Reiken et al., 2003). The present study shows that metoprolol treatment also restores the functional integrity of the myofibrillar system, because metoprolol treatment improved the phosphorylation of tropinin I, and this improvement was paralleled by a decrease in myofibrillar Ca\(^{2+}\) sensitivity. Although these alterations may be advantages for the diastolic cardiac function and may prevent cardiac Ca\(^{2+}\) overload, metoprolol did not alter the myofibrillar ATP consumption and thus did not improve myofibrillar economy, at least under the in vitro system of skinned fiber preparations.

In contrast, carvedilol significantly reduced tension-dependent ATP consumption. This may be of especial advantage in a situation of ATP deprivation, as has been described in failing myocardium (Hearse, 1979). In addition, carvedilol treatment increased maximal Ca\(^{2+}\)-dependent tension development. These effects of carvedilol may be the result of its antioxidant effects (Flesch et al., 1999; Arumanayagam et al., 2001; Nakamura et al., 2002). As shown previously, chronic treatment with metoprolol but not carvedilol is a scavenger of radicals and thus unfolds antioxidant properties that may significantly contribute to its beneficial effects in heart failure (Flesch et al., 1999; Arumanayagam et al., 2001; Nakamura et al., 2002). An alternative explanation may be an altered expression of myofibrillar proteins under chronic treatment with carvedilol. Thus, it has been shown in previous studies that carvedilol treatment results in an up-regulation of the α-myosin heavy chain mRNA and a down-regulation of the β-myosin heavy chain mRNA (Lowes et al., 2002). However, whether these alterations also occur on the protein level has to be investigated in further studies. As shown in this study, carvedilol did not alter the increased myofibrillar Ca\(^{2+}\) sensitivity and even increased the dephosphorylation of the troponin I protein. A very effective suppression of the β-adrenergic system by carvedilol has been described previously, and this β-adrenergic suppression may also have been present after the carvedilol treatment was stopped (Maack et al., 2000).

Limitations of the Present Study. In contrast to our previous studies, we observed a significant decrease in maximal Ca\(^{2+}\)-activated force in human failing myocardium. One difference between our present study and the previous studies is that for the present study, the myocardial samples had been frozen and were thawed for the preparation of skinned fibers. The freezing and thawing treatment of the fibers may have altered myofibrillar function. Nevertheless, functional data obtained in the present samples are in the range of studies obtained in rat myocardium, in which a rightward shift of the Ca\(^{2+}\)-tension relation has been shown (Konhilas et al., 2002). However, the isoform composition of myosin differs between human and rat, especially under pathophysiological conditions. In rat myocardium, a reexpression of the α-myosin heavy chain may be the underlying reason for the rightward shift of the Ca\(^{2+}\)-tension relation (De Sousa et al., 1999).

Although the β-blockers metoprolol and carvedilol have been shown to be beneficial for heart failure patients and to significantly prolong the survival of these patients to a similar extent (for review, see Domanski et al., 2003), the pharmacodynamic profile of the two drugs is different. Thus, carvedilol but not metoprolol is a scavenger of radicals and thus unfolds antioxidant properties that may significantly contribute to its beneficial effects in heart failure (Flesch et al., 1999; Arumanayagam et al., 2001; Nakamura et al., 2002). As shown previously, chronic treatment with metoprolol reversed the hyperphosphorylation of the ryanodine receptor and restored the stoichiometry of the ryanodine receptor macromolecular complex (Reiken et al., 2003). The present study shows that metoprolol treatment also restores the functional integrity of the myofibrillar system, because metoprolol treatment improved the phosphorylation of troponin I, and this improvement was paralleled by a decrease in myofibrillar Ca\(^{2+}\) sensitivity. Although these alterations may be advantages for the diastolic cardiac function and may prevent cardiac Ca\(^{2+}\) overload, metoprolol did not alter the myofibrillar ATP consumption and thus did not improve myofibrillar economy, at least under the in vitro system of skinned fiber preparations.
those obtained in fresh preparations (Schwinger et al., 1994). In addition, by using this method, we were able to perform investigations in a very clearly defined patient collective (all male, aged between 40 and 60 years). The reason for the alterations in the maximal tension development by different treatment of the fibers has to be investigated in further studies.

A further limitation of the study is the high variability inherent to studies in humans. However, to avoid this issue, we have selected patients from a group of more than 200 heart transplantations with definite criteria. The groups we investigated consisted only of male patients between the ages of 40 and 60 years, who were on a definite pharmacological treatment (i.e., either no β-blocker, carvedilol, or metoprolol).

Thus, although the groups are very small, they are very homogenous regarding the patients included.

In conclusion, a more detailed understanding of the molecular consequences of β-blockers may be of importance for a differential therapy of heart failure patients. Thus, in a situation of increased β-adrenergic suppression induced by carvedilol, it may be contraindicated to treat the patients with Ca²⁺-dependent positive inotropes, e.g., digitalis, because the risk for Ca²⁺ overload of the cardiomyocytes may be increased. Furthermore, additional treatment with a Ca²⁺-sensitizer like levsimendan may be unfavorable in addition to carvedilol, because the myofibrillar Ca²⁺ sensitivity may be increased to such an extent that diastolic dysfunction may result. A recent clinical trial has demonstrated that levisimendan as well as carvedilol improves cardiac function. Thus, further studies are needed to more clearly define the pharmaco-molecular mechanisms underlying β-blocker treatment.

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Address correspondence to: Prof. Dr. med. Robert H.G. Schwinger, Laboratory of Molecular Research and Molecular Cardiology, Clinic III for Internal Medicine, University of Cologne, Joseph-Stelzmann-Str. 9, D-50924 Cologne, Germany. E-mail: robert.schwinger@medizin.uni-koeln.de.