Exercise Intolerance in Patients With Chronic Heart Failure and Increased Expression of Inducible Nitric Oxide Synthase in the Skeletal Muscle

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Objectives. This study was designed to analyze the effect of iNOS on mitochondrial creatine kinase (mi-CK) expression and exercise capacity in chronic heart failure (CHF).

Background. The molecular mechanisms underlying exercise intolerance in CHF are still unclear. Expression of inducible nitric oxide synthase (iNOS) and reduced phosphocreatine resynthesis have been described in skeletal muscle of patients with CHF. However, it is unknown whether these phenomena are causally related to each other and to exercise tolerance.

Methods. Thirty-eight patients with CHF and 8 healthy controls (C) underwent bicycle ergospirometry and biopsy of the vastus lateralis muscle. Expression of iNOS was quantified by immunohistochemistry and reverse-transcriptase polymerase chain reaction, mi-CK by Western-blot. Intracellular presence of NO was confirmed by immunohistochemical quantification of nitrotyrosine (NT). To corroborate clinical findings, L6 rat skeletal myoblasts were incubated with sodium nitroprusside (SNP).

Exercise intolerance is regarded as the classical hallmark of chronic heart failure (CHF). The degree to which patients are limited in their daily life by muscular fatigue and dyspnea bears great prognostic relevance and hence, has been used for classification as the New York Heart Association (NYHA) functional classes for nearly 50 years (1).

However, the molecular pathophysiology leading to exercise intolerance in CHF is still far from being understood. The traditional concept of heart failure as a “pump disorder” caused by myocardial weakness has been shaken in recent years by findings that key symptoms like exercise intolerance show only poor correlation to central hemodynamics (2,3). The focus of interest has shifted to peripheral factors, i.e. skeletal muscle metabolism and ultrastructure (4–9).

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Results. Expression of iNOS was significantly increased in CHF (4.0 ± 2.8 vs. 0.8 ± 0.7% iNOS positive tissue area, p < 0.001 vs. C) and inversely correlated to maximal oxygen uptake (r = −0.65, p < 0.001). Intracellular NO-accumulation was confirmed by increased NT levels (13.5 ± 6.5 vs. 2.0 ± 1.7% NT-positive tissue area, p < 0.001 vs. C). Mi-CK was decreased in CHF (0.84 ± 0.36 vs. 1.57 ± 0.60, p < 0.001 vs. C). The inverse correlation seen between iNOS and mi-CK expression in patients (r = −0.68, p < 0.001) was reproduced in incubation experiments with SNP.

Conclusions. Increased expression of iNOS in skeletal muscle of patients with CHF was inversely correlated with mi-CK expression and exercise capacity. Cell experiments confirmed a causal relationship via NO. These findings extend our knowledge of the pathophysiology of exercise intolerance in CHF.

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Abbreviations and Acronyms

CMP = cardiomyopathy  
CHF = chronic heart failure  
CS = citrate synthase  
DCM = dilated cardiomyopathy  
iNOS = inducible nitric oxide synthase  
LVEF = left ventricular ejection fraction  
VO_{2\text{max}} = maximal oxygen uptake  
mi-CK = mitochondrial creatine kinase  
NT = nitrotyrosine  
RT = reverse transcriptase  
SNP = sodium nitroprusside

Synthase (iNOS) is expressed in both cardiac and skeletal myocytes (16,17). We hypothesized that intracellular NO accumulation caused by enhanced iNOS expression does not only inhibit enzymes of the oxidative phosphorylation (18) but also affects mi-CK as a key enzyme for the transfer of high-energy phosphates from the mitochondrium to the cytosol.

In a previous study we demonstrated the increased expression of iNOS in skeletal muscle biopsies of patients with CHF (16). In this consecutive study, we analysed nitrotyrosine as an indirect marker of intracellular NO augmentation and mi-CK expression in CHF patients and normal inactive controls and assessed possible relations to functional work capacity.

Methods

Patient population. The study group comprised 38 male patients ≤70 years with chronic heart failure (NYHA - functional class II-IV) as a result of dilated cardiomyopathy (DCM) or ischemic heart disease and 8 age-matched subjects with atypical chest pain who were free of significant heart disease as assessed by cardiac catheterization. Their physical examination, electrocardiogram (ECG), chest x-ray, M-mode and two-dimensional echocardiography were all within normal limits. Cardiac catheterization excluded coronary artery disease and demonstrated normal left ventricular ejection fraction (LVEF). All CHF patients had clinical, radiological and echocardiographical signs of chronic heart failure and a LVEF of <40% as assessed by ventriculography.

Exclusion criteria were: significant valvular heart disease, uncontrolled hypertension, peripheral vascular disease, significant pulmonary disease, diabetes mellitus, chronic alcoholism, immunosuppressive therapy, renal failure, and musculoskeletal conditions limiting exercise capacity (i.e. rheumatoid arthritis). The study protocol was approved by the Ethics Committee of the University of Leipzig, and written informed consent was obtained from all subjects.

Exercise testing and respiratory variables. Exercise testing was performed on a calibrated, electronically braked bicycle in an upright position with work load increasing progressively every 3 min in steps of 25 W beginning at 25 W. Respiratory gas exchange data were determined continuously throughout the exercise test as previously described (14,19).

Skeletal muscle biopsy. Percutaneous needle biopsies were obtained at least two days before exercise testing from the middle part of the m. vastus lateralis under local anesthesia as described previously (14). The biopsies were either fixed with 4% buffered formaldehyde or snap frozen in liquid nitrogen and stored at ~80°C.

Immunohistochemical detection of iNOS. To detect specifically the inducible isoform of nitric oxide synthase in tissue sections a polyclonal anti-iNOS specific antibody (Transduction Laboratories, Lexington, Kentucky) was used and the sensitivity was enhanced by an immunohistochemical protocol using biotinylated tyramid (NEN-DuPont, Germany) as described previously (16). For consecutive duplicate biopsy samples, the variation regarding percentage iNOS-positive tissue area has been shown to be in the order of less than 10%.

Detection of iNOS by semi-quantitative RT-PCR. Expression of iNOS in skeletal muscle biopsies was assessed semiquantitatively as described by Wobus et al. (20). Total RNA was isolated from skeletal muscle biopsy samples according to the method described by Chomczynski and Sacchi (21) using a 4M Guanidinium buffer. An aliquot of the total RNA was transcribed into cDNA using random hexamer (Boehringer, Mannheim, Germany) and 200 U of reverse transcriptase (Superscript, Gibco BRL). To quantitate the expression levels an aliquot of cDNA was used for amplification in a PCR reaction containing either primers for iNOS (NOS-11: 5'-CCTTGTGTCAAGCCCTAGATACA-3' and NOS-12: 5'-TGTCAGATCTTGTCCTTGG-3') or for GAPDH (GAPDH-1: 5'-CATGGCAAATCTCCATGGCAG-3' and GAPDH-2: 5'-TGAGGACACGGGGATGCACTTCCAC-3') and GAPDH. The PCR conditions were 30 s denaturation at 95°C, 30 s annealing at 62°C, and 30 s elongation at 72°C for 40 cycles. The PCR reaction products were separated on a 1% agarose gel and stained with ethidium bromide. The band intensity was analyzed by densitometry, and the amount of iNOS was expressed as ratio of iNOS intensity/GAPDH intensity.

Immunohistochemical detection of nitrotyrosine. In addition to quantifying iNOS expression nitrotyrosine production in the skeletal muscle biopsies was assessed to confirm that nitric oxide and peroxynitrite, respectively, were present before fixation. Peroxynitrite is known to modify tyrosine residues of cellular proteins in a dose-dependent way. Therefore, the relative amount of nitrotyrosine found in biopsy samples can be used to estimate the concentration of NO/peroxynitrite even under ex vivo conditions. To detect specifically the nitration of the ortho position of tyrosine residues on paraffin sections the method recently described by Bachmaier et al. (22) was applied.

Measurement of total and isofrom specific creatine kinase. The frozen biopsy sample was homogenized with an ultra turrax in homogenization buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 100 µg/ml phenylmethylsulphonyl fluoride, 10 µg/ml trypsin inhibitor, 2 µg/ml aprotinin, 10 µg/ml leupeptin). Total creatine kinase (CK; EC
2.7.3.2) enzyme activity was measured after solubilization of the homogenate with Triton X-100 with creatinophilosphate and ADP in a coupled enzyme assay using hexokinase/glucose 6-phosphate dehydrogenase as an indicator to measure the ATP production (23). To have an internal standard for mitochondrial enzyme content citrate synthase (CS; EC 4.1.3.7) was quantified by activity measurement according to standard protocols (24).

Expression of mi-CK was analyzed by Western-blot using isoenzyme specific antibodies (gift from Dr. Wallimann, ETH Zürich, Switzerland) followed by densitometry. Briefly, 20 μg skeletal muscle proteins were separated on a 10% SDS-polyacrylamide gel (SDS-PAGE), followed by electrotransfer to a polyvinylidene fluoride membrane (PVDF; Serva Biochemica, Heidelberg, Germany) according to the method described by Towbin et al. (25). The transferred proteins were incubated with 1:500 dilution of a polyclonal antimitochondrial creatine kinase (mi-CK) antibody. The bound antibody was detected by a peroxidase coupled antirabbit antibody followed by a color reaction using diaminobenzidine (DAB) as substrate for peroxidase. To quantify mi-CK the stained blot was analysed by densitometry with a 1D-analysis software package (One-Dscan, Scananalytics, Billerica, Massachusetts). To compensate for blot to blot variations in staining intensity an internal standard was loaded on each blot, and the densitometry results were expressed as relation between sample intensity/internal standard intensity. The variability for duplicate measurements was less than 10%. The first ten biopsies were analysed independently by two investigators. The interobserver variability was determined as r = 0.90 for the measurement of mi-CK expression.

Cell culture experiments. The L6 rat skeletal myoblast line (gift of Dr. R. Schäfer, Department of Pathology, Charité Hospital, Humboldt University, Berlin, Germany) was maintained in Dulbecco’s modified eagle’s medium (DMEM) (Sigma Chemicals, St. Louis, Missouri) supplemented with 2 mM glutamine, 100 μU penicillin/100 μg/ml streptomycin (Biochem KG, Berlin, Germany), and 10% fetal calf serum (Biochem KG, Berlin, Germany) in T75 tissue culture flasks at 37°C with 5% carbon dioxide. L6 skeletal muscle myoblasts were grown to 80% confluence and incubated with different concentrations of sodium nitroprusside (0 mmol/L, 0.01 mmol/L, 0.1 mmol/L, 1 mmol/L). After four hours, the cells were harvested with trypsin/EDTA and the protein content was determined according to Lowry et al. (26). An aliquot of 100 μg total cell protein was separated on a 10% polyacrylamid gel, and the specific amount of mi-CK was evaluated by Western-blot analysis as described above. To control for equal protein loading the gel was stained with Commassie-blue.

Statistical analysis. Mean value ± standard deviation was calculated for all variables. Single comparisons were performed by unpaired Student’s t test. In regression analysis to determine the relation between relative amount of mitochondrial CK, iNOS expression and maximal oxygen uptake linear, exponential, logarithmic, and hyperbolic fits were tested. The method which yielded the best fit (as documented by the highest correlation coefficient) was used. A p value of less than 0.05 was considered statistically significant.

Results

Baseline characteristics (Table 1). The majority of patients was diagnosed with DCM (73.7% vs. 26.3% with ischemic heart disease. LVEF was not different between DCM or ischemic heart disease (18.1 ± 7.9% vs. 17.7 ± 8.1%, p = NS). Thirty-four CHF patients (89.5%) received angiotensin converting enzyme inhibitors, 32 (84.2%) were on digitalis, 34 (89.5%) on diuretics, and 9 (23.6%) on nitrates.

Maximal oxygen uptake (VO₂ max) as determined by bicycle ergospirometry was significantly reduced in patients with CHF (p < 0.001). A trend towards a lower exercise capacity was noted in patients with ischemic heart disease as compared to patients with DCM (14.0 ± 3.5 ml/kg/min vs. 16.4 ± 5.0 ml/kg/min, p = NS). The majority of patients was diagnosed with DCM (73.7% vs. 26.3% with ischemic heart disease. LVEF was not different between DCM or ischemic heart disease (18.1 ± 7.9% vs. 17.7 ± 8.1%, p = NS). Thirty-four CHF patients (89.5%) received angiotensin converting enzyme inhibitors, 32 (84.2%) were on digitalis, 34 (89.5%) on diuretics, and 9 (23.6%) on nitrates.

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mal oxygen uptake during ergospirometry ($r = -0.65$, $p < 0.001$) (Fig. 2A), indicating that aerobic exercise tolerance decreases with increasing iNOS expression in the skeletal muscle of patients with CHF. There was no difference in iNOS expression with respect to the etiology of CHF (DCM vs. ischemic heart disease).

Expression of mi-CK in the skeletal muscle of patients with CHF. A significant reduction of mi-CK expression was observed in the skeletal muscle of patients with CHF as compared with controls ($p < 0.001$, Table 2) and there was no difference in mi-CK with respect to the etiology of cardiomyopathy (DCM: 0.88 ± 0.38 vs. ischemic heart disease: 0.73 ± 0.32, $p = NS$). Total cellular specific CK activity as quantified by enzymatic activity assays remained unchanged in CHF ($p = NS$). Changes in mi-CK could reflect either a specific reduction in the relative amount of mi-CK in otherwise normal mitochondria or an overall decrease of mitochondrial-based enzymes. To distinguish between the two possibilities, citrate synthase activity was measured. No differences in citrate synthase activity levels were observed between patients and controls (0.11 ± 0.09 vs. 0.11 ± 0.05 mU/mg) indicative of no change in overall mitochondrial enzyme contents (Table 2).

### Table 2. Alterations in Skeletal Muscle Biopsies From CHF Patients

<table>
<thead>
<tr>
<th></th>
<th>CHF</th>
<th>Healthy Controls</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td>iNOS (% tissue area)</td>
<td>4.0 ± 2.8</td>
<td>0.8 ± 0.7</td>
<td>0.002</td>
</tr>
<tr>
<td>NT (% tissue area)</td>
<td>13.5 ± 8.5</td>
<td>2.0 ± 1.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>total CK (IU/mg)</td>
<td>5.1 ± 3.6</td>
<td>6.9 ± 3.0</td>
<td>NS</td>
</tr>
<tr>
<td>mi-CK (relative amount)</td>
<td>0.84 ± 0.36</td>
<td>1.57 ± 0.60</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CS (IU/mg)</td>
<td>0.11 ± 0.09</td>
<td>0.11 ± 0.05</td>
<td>NS</td>
</tr>
</tbody>
</table>

CHF = chronic heart failure; total CK = total creatine kinase activity; mi-CK = relative amount of mitochondrial creatine kinase; iNOS = inducible NO-synthase; NT = nitrotyrosine; CS = citrate synthase activity.

Correlation between iNOS and mi-CK. Expression of iNOS was inversely related with the relative amount of mi-CK ($r = -0.68$, $p < 0.001$) (Fig. 2B). To analyse whether VO$_2$max is correlated with mi-CK, data from all patients and controls were entered into a model of linear, potential, exponential, logarithmic and hyperbolic regression analysis. Linear correlation proved to give the best fit with a correlation coefficient of $r = -0.60$ ($y = 0.11 + 0.05x$). Higher levels of mi-CK are associated with enhanced aerobic exercise tolerance.

Cell culture experiment: reduction of mi-CK expression by sodium nitroprusside incubation of L6 skeletal myoblasts (Fig. 3). After incubation with SNP for 4 h we found no significant difference in cell viability in association with increasing SNP concentration. In three consecutive sets of cell culture experiments mi-CK expression without SNP was arbitrarily set as 100%. The relative mi-CK expression with increasing con-
centrations of SNP decreases from 100% to 64.2 ± 12.8% with 0.01 mmol/L SNP, to 34.2 ± 4.7% with 0.1 mmol/L SNP, and to 11.6 ± 4.4% with 1 mmol/L SNP (Fig. 3). This dose-response curve demonstrates the same hyperbolic correlation seen between SNP and mi-CK expression in iNOS transfected L6 skeletal myoblasts. Mean of three experiments, mean ± SD.

### Discussion

Four key messages emerge from this study: 1) The expression of iNOS is increased in the skeletal muscle of patients with CHF. Expression of iNOS augments intracellular generation of NO. This can be confirmed in biopsy samples by quantification of nitrotyrosine, which serves as an indicator of NO/peroxynitrite associated protein modifications; 2) The relative amount of mitochondrial creatine kinase, a key enzyme for rapid energy transfer from mitochondria to cytosol, was significantly reduced in patients with CHF as compared with age-matched controls; 3) Functional capacity of patients with chronic heart failure as determined by maximal oxygen uptake during ergospirometry was inversely correlated to the extent of iNOS expression and positively correlated to the relative amount of mi-CK present in skeletal muscle; 4) Mitochondrial CK was inversely correlated to iNOS expression suggesting that NO produced by iNOS attenuates mitochondrial energy transfer. This finding was corroborated by incubation of myoblasts with ascending concentrations of sodium nitroprusside (SNP).

Taken together, these findings extend our knowledge of the pathophysiology of exercise intolerance in patients with CHF.

### Increase of iNOS expression in the skeletal muscle.

In the present study, the expression of iNOS in skeletal myocytes was assessed with two independent methods: Immunohistochemistry and RT-PCR. In addition, nitrotyrosine was quantified immunohistochemically to demonstrate the biochemical relevance of enhanced iNOS expression. All methods employed yielded similar results: iNOS is expressed in the skeletal muscle of patients with CHF in quantities large enough to produce NO concentrations which modify proteins and may interfere with normal aerobic metabolism.

### Reduction of mi-CK expression in the skeletal muscle of CHF patients.

An increased rate of phosphocreatine depletion associated with early acidosis during physical exercise was found by 31P magnetic resonance spectroscopy (MRS) in patients with CHF (8–10,27). This observation suggests a delayed resynthesis of high energy compounds (8). In the present study, the relative amount of mitochondrial creatine kinase was reduced by 46% in CHF patients as compared with controls. The relative amount of mi-CK was correlated to maximal oxygen uptake irrespective of age and etiology of disease. The reduction of mi-CK expression might explain the delayed resynthesis of phosphocreatine during exercise in skeletal muscle observed in patients with long-term heart failure (8). The reduced expression of mi-CK could also reflect an overall decrease in mitochondrial-based enzymes. However, we found no reduction in citrate synthase activity, a mitochondrial-based enzyme involved in the citric acid cycle.

### Effects of iNOS on mi-CK and exercise tolerance.

A major finding of the present study is the inverse relation between exercise capacity as determined by ergospirometry and iNOS, demonstrating that an increased expression of iNOS is associated with a lower maximal oxygen uptake and earlier fatigue. The inverse correlation observed between iNOS and exercise capacity was mirrored by the correlation between iNOS and mi-CK expression in skeletal muscle (Fig. 2b)—a finding that is consistent with previous observations concerning the negative effect of NO on key enzymes of energy metabolism, i.e. NADH ubiquinone oxidoreductase or cytochrome c-oxidase (28,29).

### Cell culture experiments and biochemical mechanisms.

The inverse relation between iNOS and mi-CK expression in skeletal muscle of patients with CHF was corroborated by in vitro experiments. After incubation of L6 skeletal myoblasts with ascending concentrations of SNP, mi-CK content was significantly reduced. The hyperbolic dose-response relation between SNP and mi-CK is paralleled by the clinical hyperbolic correlation between iNOS positive tissue area and mi-CK expression.

Three possible biochemical interactions between NO and cellular enzymes may lead to the observed decrease in cellular mi-CK content: 1) Nitrosylation of thiole residues could lead to conformational changes thereby reducing CK activity in vitro (30,31). However, this mechanism would not explain the change in mi-CK content. 2) Active mi-CK has an octamer which is no longer membrane-
bound (11). It is a well known phenomenon that unbound proteins are more prone to be attacked by proteases as compared with their bound form (32). 3) NO might directly influence the transcription and/or translation of mi-CK thus altering cellular mi-CK content. However, this change of enzyme expression has to be subtype selective as the total CK activity remained unchanged in the presence of elevated NO levels.

Clinical implications. This study provides new insight into the pathophysiology of intrinsic skeletal muscle alterations in patients with chronic heart failure. There is new evidence that nitric oxide produced by iNOS contributes to the metabolic derangements in skeletal muscle cells. In addition to the inhibitory effects of NO on key enzymes of the respiratory chain, we demonstrate that mi-CK expression is reduced in the presence of high NO concentrations. This finding implies a reduction of energy transfer from the mitochondrion to the cytosol.

Both mechanisms—reduced aerobic energy production and transfer—may be responsible for the early muscular fatigue and exercise intolerance in CHF.

References
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