Downregulation of the Na^+-Creatine Cotransporter in Failing Human Myocardium and in Experimental Heart Failure

Stefan Neubauer, MD; Helga Remkes; Matthias Spindler, MD; Michael Horn, PhD; Frank Wiesmann, MD; Jürgen Prestle, PhD; Bernd Walzel; Georg Ertl, MD; Gerd Hasenfuss, MD; Theo Wallimann, PhD

Background—The failing myocardium is characterized by depletion of phosphocreatine and of total creatine content. We hypothesized that this is due to loss of creatine transporter protein.

Methods and Results—Creatine transporter protein was quantified in nonfailing and failing human myocardium (explanted hearts with dilated cardiomyopathy [DCM; n=8] and healthy donor hearts [n=8]) as well as in experimental heart failure (residual intact left ventricular tissue, rats 2 months after left anterior descending coronary artery ligation [MI; n=8] or sham operation [sham; n=6]) by Western blotting. Total creatine content was determined by high-performance liquid chromatography. Donor and DCM hearts had total creatine contents of 136.4±6.1 and 68.7±4.6 nmol/mg protein, respectively (*$P<0.05$); creatine transporter protein was 25.4±2.2 optical density units in donor and 17.7±2.5 in DCM (*$P<0.05$). Total creatine was 87.5±4.2 nmol/mg protein in sham and 65.7±4.2 in MI rats (*$P<0.05$); creatine transporter protein was 139.0±8.7 optical density units in sham and 82.1±4.0 in MI (*$P<0.05$).

Conclusions—Both in human and in experimental heart failure, creatine transporter protein content is reduced. This mechanism may contribute to the depletion of creatine compounds and thus to the reduced energy reserve in failing myocardium. This finding may have therapeutic implications, suggesting a search for treatment strategies targeted toward creatine transport. **(*Circulation*. 1999;100:1847-1850.)**

Key Words: sodium ■ creatine ■ myocardium ■ heart failure

The failing myocardium shows depletion of phosphocreatine and total creatine content.[1–3] This may contribute to the development of cardiac failure by limiting myocardial energy reserve.[4] Although this has been known for decades, the mechanism of creatine/phosphocreatine depletion in heart failure is still largely unknown. Creatine is not synthesized by cardiomyocytes[5] but rather is taken up from the serum via the action of a specific Cl^-- and Na^+-dependent creatine cotransporter (the creatine transporter), a recently cloned protein expressed at high levels in skeletal muscle, heart, and nervous tissue.[6,7] In the cardiomyocyte, creatine is in part phosphorylated to phosphocreatine via creatine kinase, the latter being important for temporal and spatial energy buffering.[8] At concentrations of creatine in the serum of 30 to 100 μmol/L, rising sharply after ingestion of creatine with food,[5] the creatine transporter, with a K_m for creatine of 25 to 80 μmol/L, normally operates near or at saturation. In addition, creatine degradation to creatinine is nonenzymatic and is most likely not regulated.[9] Thus, total heart creatine content should be determined mainly by the amount and the activity of creatine transporter. Our hypothesis, therefore, was that in heart failure, depletion of creatine and phosphocreatine may be due to reduced expression and/or accumulation of creatine transporter. If true, this may identify one pathomechanism that contributes to contractile dysfunction in heart failure.

Methods

Human Heart Samples

Nonfailing donor hearts (age 52±4 years; n=8) and hearts from patients with end-stage heart failure undergoing cardiac transplantation due to dilated cardiomyopathy (DCM, age 56±3 years; left ventricular ejection fraction 25±3%; n=8) were investigated. Excised hearts were rinsed immediately in cardioplegic solution containing 30 mmol/L 2,3-butanedione monoxime. Transmural tissue samples from the left ventricular free wall were rapidly frozen and stored at −80°C. Aliquots of ≈50 mg were homogenized as described below.

Rat Model of Chronic Myocardial Infarction

Infarcts (left anterior descending coronary artery ligation: n=8 surviving animals) or sham operations (n=6) were carried out in

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From the Medizinische Universitätsklinik Würzburg (S.N., H.R., M.S., M.H., F.W., G.E.) and the Abteilung für Molekulare Kardiologie, Göttingen University (J.P., G.H.), Germany, and the Institute for Cell Biology, ETH-Hönggerberg, Zürich, Switzerland (B.W., T.W.).

Correspondence to Stefan Neubauer, MD, Medizinische Universitätsklinik, Josef-Schneider-Straße 2, 97080 Würzburg, FRG. E-mail s.neubauer@mail.uni-wuerzburg.de

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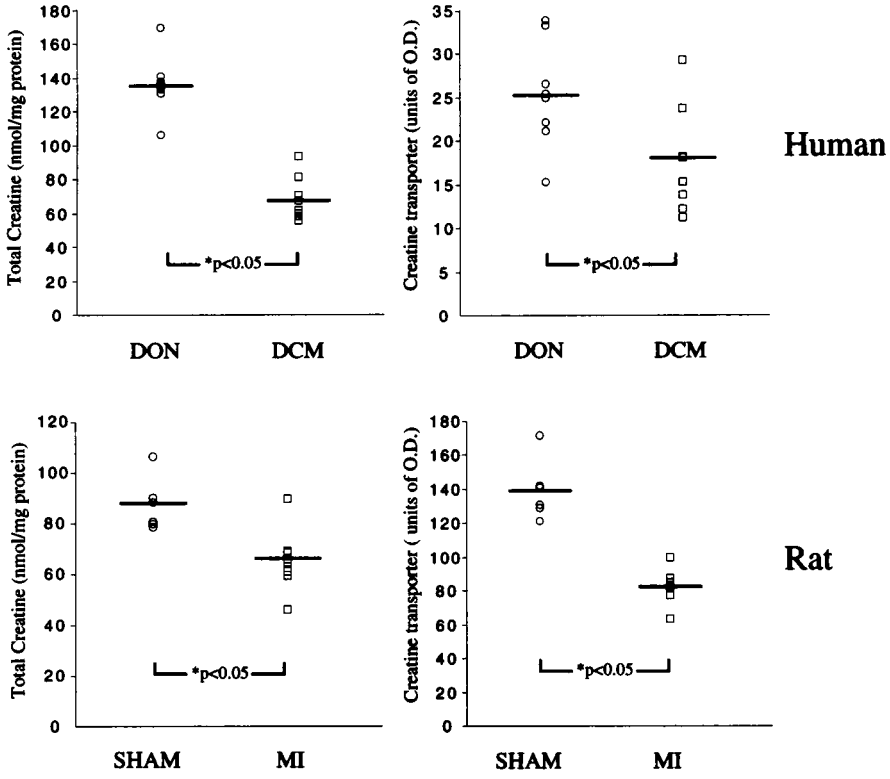

Figure 1. Creatine transporter protein and total creatine measurements. In human and in experimental heart failure, reductions of both creatine transporter and creatine were found. *$P<0.05$ failing vs nonfailing. DON indicates donor; and O.D., optical density.

12-week-old Wistar rats as described.[3] Two months later, rats were reanesthetized, and pressure was measured in the right carotid artery and in the left ventricle under spontaneous respiration as described.[10] Thereafter, hearts were isolated and buffer-perfused for 2 minutes to rinse off blood. Finally, an ≈100-mg biopsy of noninfarcted left ventricular tissue was rapidly frozen. Investigations were approved by local authorities.

Western Blotting

A Western blot for quantification of creatine transporter was established by our group using antibodies raised against a COOH terminal 15-mer synthetic peptide that specifically recognizes two 55-kDa and 70-kDa polypeptides.[5] These 2 protein bands were downregulated and upregulated in skeletal muscles after long-term feeding with creatine and guanidinopropionate (a competitive blocker of creatine entry into muscle cells), respectively, demonstrating that both polypeptides are indeed related to creatine transport.[5]

Preparation of Rat Tissue Extracts

Heart tissue from sham-operated and infarcted rats was removed and placed in cold (4°C) MSH buffer (220 mmol/L D-mannitol, 70 mmol/L sucrose, 10 mmol/L HEPES, pH 7.4). The tissue was homogenized in 2 to 3 vol MSH at 4°C. The pellet of a first centrifugation at 20 000*g* for 20 minutes was resuspended in MSH buffer. The creatine transporter protein from this pellet was extracted for 30 minutes on ice in the presence of 1% Triton X-100. Extracted proteins were then centrifuged at 10 000*g* for 10 minutes. Supernatants served as tissue extracts and were kept at −80°C. Noncollagen protein concentration was determined as described.[3]

Electrophoretic Techniques and Immunoblotting

SDS-PAGE was performed according to Laemmli[11] on a 12% polyacrylamide gel, with 10 μg of tissue extract protein loaded per lane. After electrophoresis, separated proteins were blotted semidry onto nitrocellulose paper. Unspecific sites were blocked by a solution containing 3% fat-free milk powder in PBS. Papers were labeled with polyclonal anti–COOH-terminal anti–creatine transporter protein antibodies characterized previously[5] (at 1:2500 dilution in blocking solution) for 2 to 3 hours at 22°C. After 3 washes with blocking buffer, membranes were incubated with the secondary antibody (goat anti-rabbit IgG conjugated with horseradish peroxidase and diluted 1:5000 in blocking buffer). For detection, peroxidase reaction was carried out by enzyme-linked chemiluminescence (Amersham RPN 2106) and exposure to x-ray film for 5 to 20 seconds. The sum of both 55-kDa and 70-kDa polypeptide bands was quantified by computerized blot scanning and measurement of optical density. To improve accuracy, each sample was analyzed 4 times, and average values were calculated.

Measurement of Total Creatine Content

Human and rat left ventricular tissue was analyzed for total creatine content by high-performance liquid chromatography and for noncollagen protein as previously described.[12]

Statistical Analysis

Failing and nonfailing groups were compared by an unpaired *t* test (significance level, $P<0.05$). Correlations were tested with linear regression.

Results

Human Myocardium

In human donor hearts, total creatine content was 136.4±6.1 nmol/mg protein. In DCM, creatine was significantly decreased, by 50% (Figure 1). Figure 2 shows typical Western blots of donor and failing hearts, showing substantial reduction of creatine transporter protein content in failing human myocardium, involving both the 55- and 70-kDa polypeptides. On average, creatine transporter was significantly decreased, by 30% (Figure 1). Linear regression for creatine transporter and creatine content revealed a correlation coefficient of $r=0.51$.

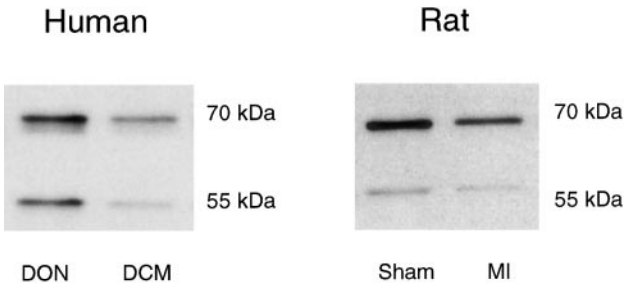

Figure 2. Typical examples of Western blots for creatine transporter, demonstrating reduced expression in heart failure.

Rat Myocardium

Left ventricular end-diastolic pressure was 5±1 mm Hg in sham and 23±1 mm Hg (range 21 to 29 mm Hg) in failing rats (*$P<0.05$). Left ventricular systolic pressures were 118±3 and 123±2 mm Hg, respectively (P=NS).

Left ventricular total creatine content was significantly decreased, by 25%, in failing rats (Figure 1). Figure 2 also demonstrates typical Western blots, indicating a substantial reduction of creatine transporter protein content in failing rat myocardium. On average, creatine transporter was decreased by 41%. Linear correlations were found both between total creatine content and creatine transporter (r=0.62) and between left ventricular end-diastolic pressure and creatine transporter (r=0.86).

Discussion

In this study, we show that both total creatine and creatine transporter protein are significantly reduced in human and experimental heart failure. Furthermore, creatine content and creatine transporter were linearly correlated in both humans and rats. In rats, there also was close correlation between the extent of heart failure (left ventricular end-diastolic pressure) and creatine transporter reduction. Because left ventricular ejection fraction was not measured in donors and because ejection fractions in DCM patients covered a narrow range (25±3%), correlation between the extent of mechanical dysfunction and creatine transporter could not be examined for the human samples. The creatine transporter was reported to have a K_m value for creatine of ≈50 μmol/L,[5] whereas fasting and postprandial creatine serum levels are 0.2 to 0.5 and up to 3 mmol/L,[13] respectively. Thus, the creatine transporter normally operates at or close to saturating levels of extracellular creatine. Because, in addition, degradation of creatine is nonenzymatic and most likely not regulated, these findings strongly suggest that one cause of creatine depletion in failing myocardium is a reduction of the expression and accumulation of creatine transporter. The fact that correlation coefficients for creatine and creatine transporter were 0.51 and 0.62, however, only suggests that other additional mechanisms regulating creatine content exist.

Although creatine transporter downregulation most likely contributes to creatine depletion in heart failure, the reasons for the loss of creatine transporter are currently unknown. At present, few data exist on the mechanisms regulating this transporter. Because the creatine transporter is a Na^+-cotransporter,[7] in cell culture, creatine transporter kinetics were acutely dependent on Na^+/K^+-ATPase activity. Also, β_2-receptor stimulation and thyroid hormone[14] stimulate creatine uptake. Both in human muscle cells and in G8 myoblasts, creatine transporter activity was downregulated by high and upregulated by low extracellular creatine concentrations.[15] However, in heart failure, serum creatine levels remain unchanged,[16] and none of these findings can explain the decrease of creatine transporter observed in failing myocardium. Thus, the mechanisms responsible for creatine transporter downregulation in heart failure remain to be determined.

Does the reduction of the creatine transporter constitute a pathophysiological or an adaptive mechanism in heart failure? The former would have to be mediated by the reduction of total creatine content. Although parallel reductions of both phosphorylated and nonphosphorylated creatine would not affect free ADP or the free energy change of ATP hydrolysis, they substantially reduce the rate and extent of intracellular ATP transfer via the creatine kinase reaction.[3] Furthermore, a reduction of cellular creatine levels would reduce creatine-stimulated respiration[17] and thus compromise the rate of mitochondrial energy production. In line with this observation, contractile reserve is limited when creatine levels are substantially depleted.[18] Whether the depletion of total and phosphorylated creatine is a mechanism that directly contributes to heart failure has been a matter of intense debate (see Reference [4] for review), but this question is still not answered unequivocally. At the same time, it is also conceivable that reduction of total creatine in heart failure is an adaptive response slowing ATP delivery for contraction, thereby preserving energy to maintain cell viability.

If reductions of creatine content do in fact play a causal role in heart failure, then strategies to maintain the creatine transporter at normal or even supernormal levels might be a promising new treatment strategy for heart failure. At present, this remains speculative, but it may be achievable either via gene transfer or transgenic overexpression. Such studies will reveal whether providing additional creatine transporter molecules to the failing cardiomyocyte has protective effects.

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References

1. Ingwall JS, Kramer MF, Fifer MA, Lorell BH, Shemin R, Grossman W, Allen PD. The creatine kinase system in normal and diseased human myocardium. *N Engl J Med.* 1985;313:1050–1054.
2. Nascimben L, Ingwall JS, Pauletto P, Friedrich J, Gwathmey JK, Saks V, Pessina AC, Allen PD. The creatine kinase system in failing and non-failing human myocardium. *Circulation.* 1996;94:1894–1901.
3. Neubauer S, Horn M, Naumann A, Tian R, Hu K, Laser M, Friedrich J, Gaudron P, Schnackerz K, Ingwall JS, Ertl G. Impairment of energy metabolism in intact residual myocardium of rat hearts with chronic myocardial infarction. *J Clin Invest.* 1995;95:1092–1100.
4. Ingwall JS. Is cardiac failure a consequence of decreased energy reserve? *Circulation.* 1993;87(suppl VII):VII-58–VII-62.
5. Guerrero-Ontiveros M-L, Wallimann T. Creatine supplementation in health and disease: effects of chronic creatine ingestion in vivo: down-regulation of the expression of creatine transporter isoforms in skeletal muscle. *Mol Cell Biochem.* 1998;184:427–437.

6. Guimbal C, Kilimann MW. A Na^+-dependent creatine transporter in rabbit brain, muscle, heart and kidney: cDNA cloning and functional expression. *J Biol Chem*. 1993;268:8418–8421.
7. Schloss P, Mayser W, Betz H. The putative rat choline transporter CHOT1 transports creatine and is highly expressed in neural and muscle-rich tissues. *Biochem Biophys Res Commun*. 1994;198: 637–645.
8. Wallimann T, Wyss M, Brdiczka D, Nicolay K, Eppenberger HM. Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the "phosphocreatine circuit" for cellular energy homeostasis. *Biophys J*. 1992;281:21–40.
9. Wyss M, Wallimann T. Creatine metabolism and the consequences of creatine depletion in muscle. *Mol Cell Biochem*. 1994;133/134:51–66.
10. Gaudron P, Hu K, Schamberger R, Budin M, Walter B, Ertl G. Effect of endurance training early or late after coronary occlusion on left ventricular remodeling, hemodynamics, and survival in rats with chronic transmural myocardial infarction. *Circulation*. 1994;89:402–412.
11. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970;227:680–685.
12. Horn M, Frantz S, Remkes H, Laser A, Urban B, Mettenleiter A, Schnackerz K, Neubauer S. Effects of chronic dietary creatine feeding on cardiac energy metabolism and on creatine content in heart, skeletal muscle, brain, liver and kidney. *J Mol Cell Cardiol*. 1998;30:277–284.
13. Greenhaff PL. The nutritional biochemistry of creatine. *J Nutr Biochem*. 1997;8:610–618.
14. Odoom JE, Kemp GJ, Radda GK. The regulation of total creatine content in a myoblast cell line. *Mol Cell Biochem*. 1996;158:179–188.
15. Loike JD, Zalutsky DL, Kaback E, Miranda AF, Silverstein SC. Extracellular creatine regulates creatine transport in rat and human muscle cells. *Proc Natl Acad Sci U S A*. 1988;85:807–811.
16. Sanbe A, Tanonaka K, Hanaoka Y, Katoh T, Takeo S. Regional energy metabolism of failing hearts following myocardial infarction. *J Mol Cell Cardiol*. 1993;25:995–1013.
17. O'Gorman E, Beutner G, Wallimann T, Brdiczka D. Differential effects of creatine depletion on the regulation of enzyme activities and on creatine-stimulated respiration in skeletal muscle, heart, and brain. *Biochim Biophys Acta*. 1996;1276:161–170.
18. Tian R, Ingwall JS. Energetic basis for reduced contractile reserve in isolated rat hearts. *Am J Physiol*. 1996;270:H1207–H1216.

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