Brief Rapid Communication

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

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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\(^6\) 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.\(^6\) 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 \(\mu\)mol/L, rising sharply after ingestion of creatine with food,\(^3\) the creatine transporter, with a \(K_a\) for creatine of 25 to 80 \(\mu\)mol/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 \(-80^\circ\)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
12-week-old Wistar rats as described. 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. 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. 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.

**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 20,000g 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 10,000g for 10 minutes. Supernatants served as tissue extracts and were kept at −80°C. Noncollagen protein concentration was determined as described.

**Electrophoretic Techniques and Immunoblotting**

SDS-PAGE was performed according to Laemmli 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 (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.

**Statistical Analysis**

Failing and nonfailing groups were compared by an unpaired t test (significance level, \( P \). 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 \( r = 0.51 \).
transporter. Because the creatine transporter is a Na\(^+\)K\(^-\)ATPase activity. Also, \(\beta_2\)-receptor stimulation and thyroid hormone 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. However, in heart failure, serum creatine levels remain unchanged, 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. Furthermore, a reduction of cellular creatine levels would reduce creatine-stimulated respiration and thus compromise the rate of mitochondrial energy production. In line with this observation, contractile reserve is limited when creatine levels are substantially depleted. 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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