Regulation of Expression of M, B, and Mitochondrial Creatine Kinase mRNAs in the Left Ventricle After Pressure Overload in Rats

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Pressure overload of the left ventricle induces synthesis of creatine kinase isoenzymes. To determine whether this response is associated with an altered pattern of creatine kinase gene expression, we induced arterial hypertension in rats by suprarenal aortic banding. After 4 days, left ventricular myocardium from hypertensive (n = 7) and normotensive, sham-operated (n = 5) rats was analyzed for isoenzyme activities by chromatography; M and B creatine kinase subunit protein by Western blot; and M, B, and mitochondrial creatine kinase mRNA by Northern blot. Although total creatine kinase activity increased in hypertensive (1,096 ± 214 IU/g left ventricle) compared with normotensive rats (648 ± 81 IU/g left ventricle, p < 0.01), the relative proportions of the cytoplasmic and mitochondrial isoenzymes did not change. The mass of M and B subunits increased 1.9- and 2.7-fold, respectively, in hypertensive compared with control rats. Similarly, the mRNA for M and B subunits as well as mitochondrial creatine kinase increased 2.6-, 1.6-, and 1.8-fold, respectively, in hypertensive rats compared with control rats. Thus, increased energy requirements in acute pressure overload are met by generalized induction of creatine kinase mRNA and subunit protein and not by an isoenzyme switch. (Circulation Research 1991;68:1007–1012)

Creatine kinase (EC 2.7.3.2) (CK) is an essential enzyme for maintaining the proper intracellular ratio of ATP to ADP and the size of the phosphocreatine pool and thus plays a prominent role in cardiac and skeletal muscle energy metabolism. The CK genes make up a small gene family that is differentially regulated during myogenesis and in tissues. Two genes encode highly conserved muscle (M) and brain (B) protein subunits that form three dimeric cytoplasmic isoenzymes (MM, MB, and BB). Two additional nuclear genes encode two closely related tissue-specific isoenzymes of mitochondrial CK. The MM isoenzyme predominates in skeletal muscle. The BB homodimer is the major species in brain. The MB heterodimer constitutes a variable portion of the total CK activity found in smooth and cardiac muscle. CK undergoes a developmental isoenzyme switch in heart and skeletal muscle characterized by replacement of the BB isoenzyme found in the fetal tissue with the MM isoenzyme making up the majority of CK in mature tissue. The mitochondrial isoenzyme is also developmentally regulated, appearing in heart at 6 days after birth in several species. Mitochondrial CK and the fraction of MM CK bound to the M line of the sarcomere make up a phosphocreatine shuttle to transport energy from the site of ATP generation (oxidative phosphorylation) to sites of ATP utilization, implicating mechanisms for the coordinate regulation of cytoplasmic and mitochondrial isoenzymes of CK.

Changes in the relative proportions of CK isoenzymes in myocardium have been demonstrated during pressure overload and hypertrophy of the left ventricle as well as with coronary artery disease. Myocardium from patients with left ventricular hypertrophy or coronary artery disease has been shown to accumulate MB CK. Myocardium from rats subjected to aortic banding resulting in systemic hypertension was observed to have increased total CK activity early after aortic banding, before the heart had undergone compensatory hypertrophy, with an increase in the percentage of the total CK activity comprised by the MB and BB isoenzymes and a decrease in the MM isoenzyme. In other studies, longer exposure to systemic hypertension resulting in

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compensatory hypertrophy was associated with normal total CK activity but increased proportions of MB and BB isoenzymes.13–16 The observed increases in BB and MB isoenzyme activities have been interpreted as reflecting accumulation of the B CK subunit resulting from increased expression of the fetal B CK gene.13–15 However, this has not been proven by direct analysis of gene expression. To characterize the response of the CK gene family to systemic hypertension, analyses of isoenzyme activity profiles in homogenates of myocardium have been used, the results of which are complex and potentially misleading because M and B subunits associate to form three cytoplasmic isoenzymes. Thus, the regulatory programs of the M and B CK genes can be inferred only indirectly from analyses of the activity profiles of the cytoplasmic isoenzymes.

Accordingly, the goal of this study was to determine the changes that regulatory programs of the CK genes undergo in response to acute, systemic hypertension. We measured steady-state levels of M, B, and mitochondrial CK mRNA; M and B subunit protein; and the activity of the cytoplasmic and mitochondrial isoenzymes of CK in hearts from rats subjected to suprarenal aortic banding for 4 days, an interval selected to observe changes in CK that precede left ventricular hypertrophy.

Materials and Methods

Animal Preparations

Because CK undergoes a developmental isoenzyme switch in heart, we used adult rats for our studies. Systemic hypertension was induced in male Sprague-Dawley rats (body weight, 400–500 g) anesthetized with pentobarbital (50 mg/kg i.p.) by coarctation of the upper abdominal aorta with a 4-0 silk ligature. The same operation was performed on control animals except that a ligature was not placed around the aorta. Eight aortic-banded and seven sham-operated rats were caged individually and given food and water ad libitum after surgery.

Four days after surgery, the rats were anesthetized with pentobarbital and weighed. Arterial blood pressure was measured with a saline-filled polyethylene catheter (PE50) placed in the carotid artery and connected to a pressure transducer and chart recorder. Rats with aortic constriction in which systolic blood pressure was less than 130 mm Hg or mean pressure was less than 110 mm Hg were not studied further. Control rats in which systolic blood pressure was greater than 130 mm Hg or mean pressure was greater than 110 mm Hg were also excluded from the study. After blood pressure was measured, the heart was excised, rinsed in ice-cold homogenization buffer (20 mM Tris-Cl [pH 8.0], 5 mM 2-mercaptoethanol, 10 mM EDTA), blotted dry, and weighed. The right ventricle and atria were removed. The left ventricle was sectioned transversely into three parts and frozen immediately in liquid nitrogen. The tissue was stored at −70°C.

Analysis of Enzymatic Activity of Individual Creatine Kinase Isoenzymes

One section of the left ventricle was weighed, minced with scissors, and homogenized in 2 ml/g homogenization buffer at 4°C with the use of a polytron. The homogenate was centrifuged at 2,000g for 15 minutes. The supernatant was removed and incubated with 0.1% (vol/vol) Triton X-100 for 5 minutes at 4°C to dissociate mitochondrial CK from the mitochondrial membrane. The mixture was centrifuged at 25,000g for 15 minutes, and the supernatant containing cytosolic and mitochondrial isoenzymes of CK was removed.

Isoenzymes in the supernatant fraction were separated by anion-exchange chromatography with the use of fast protein liquid chromatography (FPLC) and a high-resolution column (Mono Q HR 5/5, Pharmacia/LKB, Piscataway, N.J.) as described previously.17 The column was equilibrated with 20 mM Tris-Cl, pH 8.4. A sample of supernatant containing 1 mg protein18 was applied to the column and eluted with a linear gradient of NaCl from 0 to 400 mM (10 mmol/ml) in 20 mM Tris-Cl, pH 8.4, containing 5 mM 2-mercaptoethanol. CK activity in the column effluent was assessed on-line by incubation of the effluent at 37°C with substrates (CK S.V.R., Behring Diagnostics, San Diego) for the coupled enzyme assay of CK yielding NADPH and monitoring of absorbance at 340 nm as described previously.19 The relative proportions of activity of each individual isoenzyme were expressed as percentages of the total area under the absorbance curve.

Western Blot Analysis

Western blots were performed essentially as described previously.20 An aliquot of the tissue extract prepared for analysis of isoenzymes containing 15 µg protein was fractionated by 12% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis21 and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, N.H.). The membranes were incubated with a 1:200 dilution of a goat anti-human BB CK antibody (Pel-Freez, Rogers, Ark.), which cross-reacts with rat M and B subunits with fourfold greater affinity for the B subunit (data not shown). The immune complexes were detected with rabbit anti-goat immunoglobulin G (Pel-Freez) labeled with Na125I as described.22 Because the affinity of the antibody for M and B subunits differed, relative M and B subunit mass was determined by laser densitometry (Ultrascan XL, Pharmacia/LKB) of autoradiograms with signal intensities shown to be in the linear range by comparison with a standard curve generated by analysis of known amounts of MB CK by Western blot.

RNA isolation. Total cellular RNA was extracted from another section of the left ventricle with proteinase K23 and quantified by absorbance at 260 nm. The integrity of each RNA preparation was evaluated by electrophoresis through 1.2% agarose gels after denaturation in methyl mercuric hydroxide.
**Northern blot hybridization analysis.** Ten micrograms of total cellular RNA was fractionated on 1.5% agarose gels containing 6% formaldehyde and transferred to GeneScreen membranes (Du Pont/New England Nuclear, Boston). The membranes were prehybridized in a solution consisting of 50% deionized formamide (Bethesda Research Laboratories, Gaithersburg, Md.), 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 0.2% Ficoll, 0.05 M Tris-HCl (pH 7.5), 1 M NaCl, 0.1% NaPP, 1.0% SDS, 10% dextran sulfate (Pharmacia/LKB), and 100 μg/ml denatured sonicated salmon sperm DNA for at least 6 hours at 42°C and hybridized in the same solution with 3–6×10⁶ dpm/ml ³²P-labeled probe for 16–24 hours at 42°C.

The membranes were washed twice with a solution of 2× SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) with 1% SDS at room temperature for 5 minutes and once with a solution of 2× SSC and 1.0% SDS for 30 minutes at 65°C. Autoradiograms were prepared with Kodak XAR-5 film and Cronex Lightning-Plus intensifying screens (Du Pont/New England Nuclear) at −80°C. The relative abundance of M, B, and mitochondrial CK mRNAs was determined by laser densitometry of autoradiograms or by radioisotopic scanning of nitrocellulose membranes with an AMBIS radioanalytic imaging system (AMBIS Systems, San Diego). To remove the hybridized probe, membranes were boiled in a solution of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, with 1% SDS for 20 minutes.

**cDNA and oligonucleotide probes.** Subunit-specific M, B, and mitochondrial CK probes were prepared from the untranslated regions of the cDNAs that show no significant nucleotide identity. A cDNA clone encoding rat M CK was isolated from a library prepared from rat atrial mRNA in the vector λgt10 (obtained from R. Weigand, Monsanto Chemical Co., St. Louis). The identity of the rat M CK cDNA was confirmed by comparison with the published sequence. An M-specific cDNA probe was generated after an Apa I/HindIII digest of the rat M cDNA. The 185-base-pair fragment comprised 115 base pairs of 3’-untranslated region and 70 base pairs of coding region. Although the probe contained coding sequence, it did not hybridize to RNA prepared from rat brain, in which B subunit mRNA is expressed at high levels under the conditions of these experiments and is thus M specific (data not shown). A synthetic oligonucleotide specific for B subunit mRNA, and comprising the complement of the first 50 bases of the 3'-untranslated region of the published rat B CK cDNA sequence, was synthesized on an Applied Biosystems 380B automated DNA synthesizer in the Protein Chemistry Facility at Washington University, St. Louis. A cDNA clone encoding the sarcamere-specific isoform of mitochondrial CK was isolated from a rat heart-derived cDNA library and identified by comparison with the published sequence of human heart mitochondrial CK mRNA. A 150-base-pair fragment derived from the 5'-untranslated region of the rat mitochondrial cDNA, which shows no significant homology with the M or B CK cDNAs, was used as a probe for Northern blot analysis.

Probes derived from cDNAs were labeled with [α-³²P]dCTP to a specific activity of 1–2×10⁶ dpm/μg by the random primer method. Synthetic oligonucleotides were 5’-end labeled to a specific activity of 1–5×10⁶ dpm/pmol with [γ-³²P]ATP and T4 polynucleotide kinase (Bethesda Research Laboratories).

### Statistical Analysis

Results are expressed as mean±SD. Experimental and control group data were compared using a two-sided, unpaired t test.

## Results

### Blood Pressure, Heart Weight, and Body Weight

One normotensive aortic-banded rat and two hypertensive control rats were excluded from further analysis. Among the remaining rats, systolic, diastolic, and mean arterial blood pressures were elevated significantly in those with aortic banding compared with control animals (Table 1). Heart weight, left ventricular weight, and the ratio of left ventricular weight to body weight did not differ between groups 4 days after surgery (Table 1).

### Creatine Kinase Activity and Isoenzyme Distribution

Total CK activity increased significantly in the left ventricle of rats with hypertension induced by aortic banding (Table 1). When normalized to left ventricular weight, CK activity was increased nearly twofold in hypertensive rats (Table 1). Increased CK activity occurred with no change in left ventricular mass or protein content per gram of left ventricular tissue.

CK isozymes were well resolved by anion-exchange chromatography, facilitating their quantification (Figure 1). The relative percentage of total CK activity comprised by the three cytoplasmic isozymes and mitochondrial CK is shown in Figure 2. Although total CK activity in left ventricular homogenates increased in hypertensive rats compared with controls, the relative proportions of cytoplasmic and

### Table 1. Blood Pressures, Left Ventricular Weights, and Creatine Kinase Protein

<table>
<thead>
<tr>
<th></th>
<th>Control (n=5)</th>
<th>Aortic-banded (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood pressure (mm Hg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>120.0±5.8</td>
<td>154.0±15.2*</td>
</tr>
<tr>
<td>Diastolic</td>
<td>86.3±11.1</td>
<td>119.0±13.9*</td>
</tr>
<tr>
<td>Mean</td>
<td>100.0±14.7</td>
<td>132.4±12.3*</td>
</tr>
<tr>
<td>LV wt (mg)</td>
<td>1,034.0±88.5</td>
<td>939.0±74.1</td>
</tr>
<tr>
<td>LV wt/body wt (mg/g)</td>
<td>2.33±0.14</td>
<td>2.31±0.26</td>
</tr>
<tr>
<td>Total creatine kinase (IU/g LV)</td>
<td>647.6±81.1</td>
<td>1,095.6±214*</td>
</tr>
<tr>
<td>Total protein (mg/g LV)</td>
<td>19.4±5.5</td>
<td>18.8±5.10</td>
</tr>
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Values are mean±SD. LV, left ventricle. *p<0.01 compared with control.
mitochondrial isoenzymes of CK did not differ between groups.

Western Blot Analysis

The M and B subunits of CK were well separated after electrophoresis in SDS-polyacrylamide gels (Figure 3A). Laser densitometry of autoradiograms showed that M subunit protein increased 1.9-fold and B subunit protein increased 2.7-fold in hypertensive compared with normotensive rats (Figure 3B). Because antibodies specific for the mitochondrial subunit were not available, mitochondrial CK was not analyzed by Western blot.

Northern Blot Analysis

Northern blots of RNA extracted from normal and hypertensive rat hearts were probed, stripped, and reprobed consecutively for M, B, and mitochondrial CK mRNA (Figure 4A). Because of the high degree of sequence identity in the coding regions of the cytoplasmic and mitochondrial CK mRNAs, we prepared probes from the untranslated regions that show no significant DNA sequence identity. Analysis of autoradiograms by laser densitometry showed that steady-state levels of all three subunit mRNAs increased (M CK mRNA, 2.6-fold; B CK mRNA, 1.6-fold; mitochondrial CK mRNA, 1.8-fold) in hypertensive compared with control groups (Figure 4B). Because hybridization with the M CK–specific cDNA probe resulted in an intense autoradiographic signal, radioisotopic scanning of the membrane was used as an additional method to quantify M CK mRNA levels. The results of analysis by radioisotopic scanning and laser densitometry agreed closely.

Discussion

Our results show that systemic hypertension results in an increase in steady-state levels of M, B, and mitochondrial CK mRNAs with a concomitant increase in M and B subunit protein, total CK activity, and the activity of the cytoplasmic and mitochondrial isoenzymes of CK. These changes occurred early after aortic banding, before left ventricular hypertrophy ensued, as evidenced by the lack of change in the ratio of left ventricular weight to body weight. The absence of an increase in the total protein content of the left ventricle after aortic banding indicates that the increase in CK activity does not reflect a generalized increase in all myocardial proteins. Nevertheless, results of Western blot analysis indicate that M and B subunit mass were increased in response to hypertension. The results of Northern blot analysis with M, B, and mitochondrial CK–specific probes indicate that the increase in isoenzyme protein is mediated at the level of mRNA abundance. Although posttranscriptional effects on mRNA stability cannot be ruled out, both the M and B CK genes are regulated at the level of transcription in myogenic cell lines, and it is likely that the stimulus of hypertension results in an increase in transcription of the CK genes. Thus, the response of the myocardium to acute hypertension is characterized by an increase in expression of the M, B, and mitochondrial CK genes and not by a selective increase in expression of the B gene. Steady-state levels of M and B CK mRNA have not been analyzed in hearts that have undergone compensatory hypertrophy in response to chronic systemic hypertension. Hence, development of left ventricular hypertrophy in response to chronic systemic hypertension with increased proportions of MB and BB isoenzymes in myocardium may be
associated with a selective increase in expression of the B CK gene.

In the only other reported study in which CK isoenzymes were measured in hearts subjected to systemic hypertension for intervals too brief to induce left ventricular hypertrophy, Meerson and Javich also found that total CK activity in myocardium was increased. However, they reported a decrease in the percentage of total CK activity comprised by MM and an increase in the activity of MB and BB isoenzymes when whole heart homogenates were analyzed by electrophoresis in agarose gels. Although the reasons for this difference in results are not obvious, the CK isoenzyme distribution reported by Meerson and Javich for hearts from age-matched control rats showed a much higher percentage of total CK activity comprised by MB than what we observed or others have reported, suggesting difficulty in quantification of MB CK by their method. Because M and B subunit mass and mRNA were not measured, additional comparisons with our results cannot be made.

The last trimester of development is characterized by a marked increase in total CK activity in heart as both the MB and MM isoenzymes accumulate and replace the BB isoenzyme. The increase in MB CK reflects an increase in synthesis of both M and B subunits mediated by coordinate induction of M and B CK mRNA. Interestingly, differentiation of myogenic cell lines in culture is also characterized by an increase in all three cytoplasmic isoenzymes of CK with induction of M and B mRNA as myoblasts fuse to form multinucleated myotubes (J. Billadello, unpublished data, 1990). Thus, increased expression of mRNAs encoding the M and B subunits of CK in response to acute pressure overload is consistent with recurrence of the fetal pattern of CK gene expression, which is characterized by coordinate regulation of the M and B CK genes and not by selective upregulation of the fetal B CK gene. In contrast, the fetal-type isogenes encoding skeletal α-actin, β-tropomyosin, β-myosin heavy chain, and...
atrial natriuretic factor are selectively induced in heart during the early period after acute pressure overload (2 days to 1 week), coinciding with the interval of increased wall stress. Expression of these genes decreases toward control levels with normalization of systolic wall stress as hypertrophy ensues by the second week after surgery. Accordingly, re-expression of fetal-type contractile protein genes as well as the fetal pattern of CK genes in response to pressure overload may represent a generalized adaptive response to hemodynamic stress. The functional significance of the appearance of fetal isoforms of myocardial proteins in the heart with acute pressure overload requires further investigation.

Acknowledgments

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