Creatine Kinase Isoenzyme MM Variants in Skeletal Muscle and Plasma from Marathon Runners

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We investigated the patterns of variants of creatine kinase isoenzyme MM (CK-MM) in gastrocnemius muscle and plasma sampled from male and female long-distance runners before and after a marathon race. The proportions of CK-MM variants MM1 (PI 6.90) and MM2 (PI 6.62), identified in the skeletal muscle from both sexes, did not differ significantly from those in skeletal muscle from nonrunning controls or from heart muscle. CK-MM1 was the major (84–85%) of total CK-MM variant form. Patterns of CK-MM in plasma collected from male runners 24, 48, 72, and 96 h after the race were similar to those for female runners, but we detected two additional variants, which we designate MM1B (PI 6.76) and MM2B (PI 6.49). For both sexes the total CK-MM activities in plasma were significantly (p < 0.05) greater after the race, but the women's total CK-MM activities were significantly (p < 0.05) less than the men's. The rates of disappearance of MM1, MM2, and MM3 from plasma after the race differed significantly (p < 0.05) between men and women, MM1 clearing the fastest. Determination of the CK-MM variants in plasma after strenuous exercise may be of help in assessing CK release from injured skeletal muscle.

Additional Keyphrases: heart muscle compared • enzyme clearance • exercise, effects of • sex-related effects • isoelectric focusing • heart disease • isoforms of isoenzymes

Total creatine kinase (CK, EC 2.7.3.2) activities in plasma have been intensively investigated as an aid in evaluating damage to myocardial and skeletal muscle (1,2). Because myocardium is relatively rich in the CK-MB isoenzyme (10–25% of total CK activity), evaluation of acute injury to the myocardium has been greatly facilitated by quantification of CK-MB activity (1, 2) in plasma. Sensitive chromatographic and electrophoretic techniques reveal several variant forms (i.e., isoforms derived from the same isoenzyme but with different isoelectric points) of CK-MM (3–10), the major CK isoenzyme found in normal heart and skeletal muscle, and of CK-MB (3, 7) in plasma. Furthermore, the patterns of appearance of these variants after their release from the myocardium into the circulation have been studied in acute myocardial infarction (AMI) patients and found to be diagnostically helpful (4, 6, 10). A pure gene product from tissue, which has been designated MMt or MM1, is released into the circulation from damaged myocardium and is post-translationally converted into electrophoretically more anodally migrating CK-MM variants. A carboxypeptidase-catalyzed hydrolysis of C-terminal lysine residues in vivo has been proposed (11), involving the following scheme: MM1 (M1M1, MMt) is converted to MM2 (M1M2), which is converted to MM3 (M2M2), with the M2 subunit lacking a lysine residue. For clarity, here we have designated CK-MM1 as the pure gene product, and MM2, MM3, and MM4 as postsynthetic variants. Other investigators have designated the pure gene product as MM3 (6, 8, 9, 11), MM1 (10), or MM4 (5). Although MM4 (as we have designated it) has been identified in several studies, its origin has not yet been determined (4, 6, 10).

Previously, we reported (12) that activity profiles for CK-MM and CK-MB in plasma from male and female long-distance runners during the days after a 42.2-km marathon race were similar to those after an AMI. The proportion of CK-MB in samples of gastrocnemius muscle from long-distance runners appeared to be similar to that in myocardial muscle, and skeletal muscle was thought to be the source of increased CK-MB activities in serum from marathon runners (13, 14). Electron-microscopic evidence of exercise-induced necrosis of skeletal muscle enriched in CK-MB substantiated these observations (15). In evaluating the diagnostic significance of the leakage of CK-MM isoenzyme from diseased or damaged muscle, we considered that release of large quantities of CK-MM from exercise-damaged skeletal muscle into the circulation might overlap with the CK-MM released from the heart after an AMI.

The present study was designed to investigate the patterns of CK-MM variants in skeletal muscle and plasma obtained from men and women before and after running a marathon race. We also calculated and compared the disappearance rates of MM variants in plasma.

Materials and Methods

Subjects. Eight men (ages 22 to 40 years) and six women (ages 24 to 39), long-distance marathon runners without cardiac risk factors (hypertension, smoking, hyperlipemia), and five men and four women, age-matched non-running controls, volunteered for the study after being informed of the purpose, methods, and possible complications of the procedures. Written and verbal consent were obtained for the study and were reviewed and approved by our institutional review committees. During the 10 weeks of training before the race, the male and female runners averaged 65 and 41 miles per week, respectively. None experienced clinical symptoms characteristic of AMI before, during, or after the race (16). Plasma samples were obtained 48 h before and 24, 48, 72, and 96 h after the race and stored at −40 °C until analysis. Samples were collected with EDTA as an anticoagulant, to prevent degradation of the CK-MM variants (11, 17).

Muscle preparation. Biopsies of skeletal muscle obtained by needle biopsy (18) were taken from the lateral portion of the gastrocnemius muscle 48 h before the race for the runners and at unselected times from the controls. Additionally, heart muscle was obtained at autopsy from unrelated nonrunning subjects without anatomical evidence of myocardial damage (four men) at Hennepin County Medical Center within 8 h after death. The tissues were immediately

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frozen in pentane, cooled in liquid nitrogen, and stored at −70 °C until analyzed.

*Tissue preparation.* For analysis of CK-MM variants, we homogenized, with 35 strokes at 4 °C, a precisely weighed 2- to 5-mg amount of tissue in a 15-mL Dounce glass homogenizer containing 3 mL of ice-cold pH 7.2 buffer. This buffer consisted of, per liter, 50 mmol of Tris HCl, 25 mmol of sucrose, 10 mmol of EDTA, 5 mmol of ethylene glycol bistβ-aminoethy l ether)-N,N,N',N'-tetraacetic acid, and 5 mmol of 2-mercaptoethanol. Cell fragments, including mitochondria, were removed by centrifugation (15 000 × g, 30 min). The supernates were kept on ice and analyzed the same day for total CK activity and CK-MM variant composition.

**Determination of CK activity.** Total CK activities in plasma and in muscle homogenates were measured at 30 °C with a centrifugal analyzer (Cen trifilChem 400; Baker Instruments, Pleasantville, NY) and N-acetyl-cysteine-activated reagent (Calbiochem-Behring, La Jolla, CA), by a modification of the Rosalki procedure (19). We calculated CK-MM activity by subtracting CK-MB activity (calculated as total CK activity × percent CK-MB as determined by electrophoresis) (13) from the total CK activity.

**Isoelectric focusing.** Variant patterns of CK-MM (MM1, MM1B, MM2, MM2B, MM3, MM4) were determined by flatbed isoelectric focusing as described previously (10). We used an LKB Multiphor 2 System, essentially as described by the manufacturer. Thin plates of polyacrylamide gel (Ampholine FAG plates, pH 3.5–9.5; LKB Instruments, Gaithersburg, MD) were placed on a plate cooled by a circulating water bath at 5 °C. Filter paper strips dipped in basic (1 mol/L NaOH) and acidic (1 mol/L H3PO4) solutions were placed at the cathodal and anodal ends of the gel, respectively. Muscle homogenates and plasma samples (0.5 to 2 mL), diluted to contain 500 to 800 U of activity per liter, were applied directly onto the gel, 2 cm from the cathode.

Samples were applied at the beginning of the run so that the pH gradiant formed and the CK variants were focused simultaneously. The gels were focused for 1.5 h at 30 W constant power. After the run was complete, we measured pH with a surface pH electrode at 25 °C. CK bands were made visible by placing a cellulose acetate plate saturated with CK reagent onto the gel and incubating for 15 min at 30 °C. After drying the plate, we quantified the fluorescent bands by scanning densitometry. To rule out non-CK artifacts, we also focused samples lacking the creatine phosphate substrate. In addition, we confirmed the distribution and number of CK-MM variant bands by electrophoresis on agarose gel. Samples were also focused with and without 2-mercaptoethanol (final concentration 5 mmol/L) and no differences in MM variants were detected. Nor did samples applied at either the cathodal or anodal end of the gel, or points between, show any differences in CK-MM variants.

**Kinetics of formation of CK variants.** We calculated the disappearance rates (Kd) and half-lives of the variants from data on the slope of the plot of the log of enzyme activity vs time, assuming first-order elimination kinetics (20). The Kd for MM1, MM2, MM3, and total MM activities was calculated from values beginning 24 h after the race until 96 h after the race. The activities for MM1, MM2, and MM3 used in this calculation were determined from the product of total CK activity multiplied by the percentage of MM variant present.

**Statistics.** To determine the significance of changes in MM variant activities, we used a one-way analysis of variance (ANOVA) with repeated measures. Tukey's Ω procedure was used to locate the source of the difference. The level of significance was set at 0.05. Descriptive statistics (mean ± SD) were calculated for all dependent variables.

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**Results**

We identified two CK-MM variants, MM1 and MM2, with respective isoelectric points of 6.90 and 6.62, in skeletal muscle of both men and women runners. There were no significant differences in the mean (±SD) percentages of CK-MM variants between the men's (MM1, 84 ± 11%; MM2, 16 ± 11%) and women's (MM1, 85 ± 5%; MM2, 15 ± 5%) skeletal-muscle samples. Also, the mean percentages of CK-MM variants in control muscles from nonrunners were similar (MM1, 89 ± 8%; MM2, 11 ± 8%). In the men's skeletal muscle the mean total CK-MM activity was 2552 ± 285 U/g wet weight of tissue but was substantially less in skeletal muscle obtained from women runners 1598 ± 186 U/g. In comparison, the mean total CK-MM activity in fresh autopsy-derived heart muscle was 357 ± 48 U/g. Further, the percentages of myocardial CK-MM variants (MM1, 88 ± 7%; MM2, 12 ± 7%) did not differ significantly from the CK-MM variants observed in the skeletal muscle from the runners.

Table 1 shows the plasma activities and distributions of CK-MM variants 48 h before and 24, 48, 72, and 96 h after the marathon for the male runners. CK-MM was significantly (p < 0.05) greater 24 h (25-fold) and 48 h (13-fold) after the race than before the race. Figure 1 shows a representative isoelectric focusing pattern for CK-MM in plasma from a male runner. Three major CK-MM variants MM1, MM2, and MM3 (pl 6.36) were detected, both before and after the race. After the race, the men's plasma contained three additional variants: MM4 (pl 6.20) and two variants not previously described, MM1B (pl 6.76) and MM2B (pl 6.49). Isoelectric focusing of fivefold-concentrated specimens of plasma taken before the race confirmed that neither CK-MM1B nor MM2B was detectable before the race. MM1 peaked at 24 h, while MM3 progressively in-
creased at 72 and 96 h after the race. The MM1:MM3 ratio (pure gene product:postsynthetic product) never exceeded 1.0 after the race.

Table 1 also shows the activities and distributions of CK-MM variants in plasma from the women, before and after the marathon. Plasma CK-MM was significantly (p < 0.05) greater 24 h (7.3-fold), 48 h (3.2-fold), and 72 h (2.2-fold) after the race than before. Further, CK-MM activities in plasma collected from female runners 24 h and 48 h after the race were significantly lower (p < 0.05) than the men's.

Figure 2 shows a typical scanning densitometric tracing of an isoelectric focusing pattern for CK-MM in plasma sampled from a female runner before and after the race. Three major CK-MM variants were present before the race: MM1, MM2, and MM3. The women's CK-MM variant pattern in plasma after the marathon race was similar to that for men except that the CK-MM1B and CK-MM2B variants were not detected in any of the plasma samples from the women. The pattern exhibited a cathodal to anodal shift as follows: MM1 → MM2 → MM3. MM1 peaked at 24 h, MM3 at 96 h. The MM1:MM3 ratios at 24 h and 48 h were 1.87 and 1.90, respectively. The plasma CK-MM variant patterns for both women and men runners had become nearly normal eight days after the race. Also, the increases in plasma CK-MM1 in both men and women 24 h after the race imply a release of CK-MM1 from skeletal muscle into the circulation.

Table 2 shows the mean disappearance rates from plasma and the half-lives for the MM variants after the race. Clearances for total CK-MM, MM1, or MM2 did not differ significantly between men and women. However, for the men, clearance rates were significantly (p < 0.05) different between MM1, MM2, and MM3, as they also were for MM1 and MM2 in the women (p < 0.05). In both the men and women, CK-MM1 was cleared fastest from the circulation, followed by MM2 and then MM3.

Discussion

Creatine kinase MM variants have been utilized for appraising release of new enzyme from the heart after AMI.

![Diagram of CK-MM variants](Image)

Table 2. Mean (and SD) Rates of Disappearance of CK-MM Variants from Plasma

<table>
<thead>
<tr>
<th>Values</th>
<th>Men</th>
<th>Women</th>
</tr>
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<tbody>
<tr>
<td>CK-MM1</td>
<td>0.977</td>
<td>0.991</td>
</tr>
<tr>
<td>CK-MM2</td>
<td>24.3</td>
<td>19.1</td>
</tr>
<tr>
<td>CK-MM3</td>
<td>31.9</td>
<td>24.5</td>
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</table>

Values of Kd (disappearance rate) and r (correlation coefficient of the monoeponential regression) are for men and six women. *Significant (p < 0.05) difference between men's MM1, MM2, and MM3 clearances and half-lives. **Significant (p < 0.05) difference between women's MM1 and MM2 clearances and half-lives. NA: not enough data points.

(d, 6, 10). After strenuous exercise such as a marathon race, CK-MM variant analysis might be useful for determining the extent and timing of damage to skeletal muscle. This report describes for the first time the measurement of skeletal muscle and plasma CK-MM variants obtained from long-distance runners before and after the stress of marathon racing. The similarities between the CK-MM variant composition in skeletal muscle and heart muscle as well as variant patterns in plasma and clearance rates from plasma after marathon racing and AMI must be recognized.

In the pattern of CK-MM isoenzyme variants in human heart muscle, CK-MM1 predominates. It is designated by investigators as a pure gene product (3, 4, 6, 10, 11). Our findings of 88% MM1 and 12% MM2 in fresh autopsy-derived heart muscle agree with these observations. Additionally, we show that the gastrocnemius muscle in long-distance runners resembles heart muscle with respect to cytoplasmic CK-MM variant. The mean percentages of CK-MM1 (84%, 85%) and MM2 (16%, 15%) in skeletal muscle from male and female runners, respectively, did not significantly differ from that in human heart muscle. These results and parallel findings of similar CK-MB compositions in exercised, trained skeletal muscle and heart muscle (12). The composition differences between skeletal and heart muscle with respect to CK-MM variant was reflected in the absolute values for CK-MM, i.e., the activities per gram of tissue.

Heart muscle contained 357 U of CK-MM activity per gram; skeletal muscle obtained from male runners contained 2552 U/g, skeletal muscle from the women 1596 U/g. Although greater CK-MM activity in skeletal muscle vs heart muscle has been previously documented (1, 2), this report describes for the first time the significant sex-related difference in CK-MM activity in skeletal muscle. This may be one of the major reasons why total CK activities in plasma of women runners after a marathon race were significantly less than the case for men runners (Table 1) (21).

The resolution of CK-MM variants from runners' skeletal muscle into two variants, MM1 and MM2, contrasts with the findings of Gulsli and Jacobs (22). They described the separation, by isoelectric focusing, of 21 variants of CK-MM from human psoas muscle, compatible with the presence of six different M subunits. Our results agree with reports (8, 11) of only two subunits, M1 and M2. Further, the isoelectric points for MM1 (pl 6.90) and MM2 (pl 6.62) from skeletal muscle and plasma were identical, which does not substantiate Gulsli and Jacobs' claim that there are two different
MM series, one present in muscle and one in plasma. Multiple plasma CK-MM variants were present in male runners after the race, including two forms previously not described. Designated MM1B and MM2B, these variants may reflect altered amino acid compositions (Table 1, Figure 1). These variants, present when plasma was focused, whether diluted to 500 to 800 U/L or undiluted, have isoelectric points intermediate (pI = 6.76 and 6.48, respectively) between MM1 and MM2 and between MM2 and MM3. Perryman et al. (11) have described a carboxypeptidase-catalyzed hydrolysis of C-terminal lysine residues for production of MM2 and MM3, but the mechanism by which the additional variants are formed is not yet known. Women's plasma after the race showed patterns similar to the men, with a serial conversion scheme of MM1 → MM2 → MM3 (Table 1, Figure 2), but the MM1B and MM2B variants were not observed.

Morelli et al. (6) have suggested that AMI patients whose ratio shows a ratio of MM1/MM3 ≥ 1 may be undergoing a continuing release of CK from heart muscle. This ratio might also be used to assess the duration of CK release from injured skeletal muscle in the absence of damage to heart muscle. In the women runners, the plasma ratio peaked 24 (ratio = 1.87) to 48 (ratio = 1.90) h after the race, implying continuing CK release from skeletal muscle. This would be in harmony with electron-microscopic evidence that skeletal-muscle necrosis is greatest one to three days after a marathon race (15). For the men, however, the MM1:MM3 ratio was <1.0 at 24 h, indicating that release of CK from skeletal muscle may have been complete by 24 h. However, the additional MM variants, MM1B and MM2B, may complicate this interpretation. Thus, serial CK-MM variant analysis of ongoing enzyme release from exercise-induced injury to skeletal muscle might help explain the delayed onset of muscle soreness one to three days after a marathon race (23).

The source of the increased CK-MM activities in plasma of runners after a marathon race is thought to be the CK-MM-enriched skeletal muscle (12, 24). The men completed the race significantly (p < 0.05) faster than the women (mean times, 162 and 202 min, respectively), so a larger muscle fiber recruitment might have resulted in their higher CK activities in plasma. As discussed earlier, the greater sex-related CK-MM activities in men's skeletal muscles might give rise to higher CK-MM activities in their plasma. In this study, the sex-related difference in CK-MM activities observed after the race (Tables 1 and 2) substantiates our earlier report (21).

These results are the first to show the disappearance rates from plasma and the half-lives of CK-MM variants after a marathon race. The former, with respect to total CK-MM activities for men and women runners (Table 2), were relatively consistent with prior observations reported previously for total CK (CK-MM plus CK-MB) in runners after a marathon (21). Our observations are consistent with the patterns of disappearance of MM variants from plasma reported by Morelli et al. (6) after AMI and by Hashimoto et al. (5) during in vivo experiments on dogs. However, our absolute clearance rates were substantially slower. The CK-MM activity clearance was a composite of the individual clearances of MM variant. There were no significant sex-related differences in clearances of MM1, MM2, and MM3 by the runners. CK-MM1 was cleared fastest, CK-MM3 the slowest (Table 2).

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