Resistance Artery Creatine Kinase mRNA and Blood Pressure in Humans

Fares A. Karamat, Inge Oudman, Carrie Ris-Stalpers, Gijs B. Afink, Remco Keijser, Joseph F. Clark, Gert A. van Montfrans, Lizzy M. Brewster

Abstract—Hypertension remains the main risk factor for cardiovascular death. Environmental and biological factors are known to contribute to the condition, and circulating creatine kinase was reported to be the main predictor of blood pressure in the general population. This was proposed to be because of high resistance artery creatine kinase-BB rapidly regenerating ATP for vascular contractility. Therefore, we assessed whether creatine kinase isoform mRNA levels in human resistance arteries are associated with blood pressure. We isolated resistance-sized arteries from omental fat donated by consecutive women undergoing uterine fibroid surgery. Blood pressure was measured in the sitting position. Vessels of 13 women were included, 6 normotensive and 7 hypertensive, mean age 42.9 years (SE, 1.6) and mean systolic/diastolic blood pressure, 144.8 (8.0)/86.5 (4.3) mm Hg. Arteriolar creatine kinase isoform mRNA was assessed using quantitative real-time polymerase chain reaction. Normalized creatine kinase B mRNA copy numbers, ranging from 5.2 to 24.4 (mean, 15.0; SE, 1.9), showed a near-perfect correlation with diastolic blood pressure (correlation coefficient, 0.9; 95% confidence interval, 0.6–1.0) and were well correlated with systolic blood pressure, with a 90% relative increase in resistance artery creatine kinase B mRNA in hypertensives compared with normotensives, normalized copy numbers were, respectively, 19.3 (SE, 2.0) versus 10.1 (SE, 2.1), P=0.0045. To our knowledge, this is the first direct evidence suggesting that resistance artery creatine kinase mRNA expression levels concur with blood pressure levels, almost doubling with hypertension. These findings add to the evidence that creatine kinase might be involved in the vasculature’s pressor responses. (Hypertension. 2014;63:00-00.)

Key Words: creatine kinase ■ genetics, human ■ hypertension ■ microcirculation

Hypertension is an important worldwide public-health challenge. It is a common disease, affecting >25% of the adult population, around a billion people worldwide. Hypertension is identified as the leading risk factor for cardiovascular mortality and is ranked third as a cause of disability-adjusted reduction in life-years. The pathogenesis of hypertension is multifactorial, and environmental and biological circumstances contribute to the occurrence of the disease. We proposed that creatine kinase (CK), the central regulatory enzyme of energy metabolism, is the final common pathway leading to pressor responses. The enzyme regenerates and distributes ATP to subcellular locations of energy demands, catalyzing the reaction:

\[
\text{MgADP+Pcreatine+H}^+ \leftrightarrow \text{MgATP+creatine}\]

CK, tightly bound in the immediate proximity of A TP-utilizing enzymes, such as Na+/K+-ATPase and Ca2+-ATPase at membranes, and myosin light chain kinase and myosin ATPase at the contractile proteins, where it rapidly provides ATP to these enzymes. CK is thus thought to fuel highly energy-demanding processes, such as sodium retention, cardiovascular contractility, and remodeling of arteries. In accord with this, serum CK was found to be the main predictor of blood pressure in the general population. This was proposed to be because of high tissue CK, primarily the resistance artery CK-BB isoform rapidly regenerating ATP for vascular contractility. However, hitherto, there were no data to substantiate this proposal. The main objective of this study was to assess whether resistance artery CK mRNA levels are associated with blood pressure.

Methods

Participants

Protocols were in accord with institutional guidelines and were approved by the local institutional review board. All participants gave written informed consent. Consecutive, self-defined white and African-Dutch women undergoing an abdominal procedure for fibroid enucleation or hysterectomy for fibroids were eligible for inclusion. Patients with pre-existent vascular abnormalities, such as vasculitis and diabetes mellitus, HIV infection, infectious hepatitis, and bleeding disorders, were excluded. Blood pressure was measured in the sitting position at the outpatient clinic with theDatascope Accutorr Plus (Tascope Corp, Paramus, NJ). High blood pressure was defined as systolic blood pressure (SBP) ≥140 or diastolic blood pressure (DBP) ≥90 mm Hg or the use of antihypertensive drugs.
CK Isoenzyme cDNA

The 2 major cytosolic CK protein subunits are CK-brain (B) and CK-muscle (M), respectively, encoded by the CKB gene on human chromosome 1q43 and the CKM gene on 1q13. The enzymatic functional form can be either a homodimer (BB and MM) or a heterodimer (MB) thus creating 3 cytosolic isoenzymes. The mitochondrial CK is also present in the mitochondrion where it facilitates the formation of creatine phosphate that is transported by CK to subcellular locations of high-energy demands. Two mitochondrial CK isoenzymes, an ubiquitous and a sarcomeric form, are encoded by, respectively, the CKMT1 gene on chromosome 15q15 and the CKMT2 gene on chromosome 5q13. All CK isoenzymes contain a highly conserved catalytic cysteine domain. However, the triplet encoding for this catalytic cysteine domain is GCC for cytoplasmic CK and GTC for mitochondrial CK. Cytosolic CKB and CKM cDNA share a 78% nucleotide sequence identity and 79% predicted amino acid sequence identities. The human CKMT1 and CKMT2 cDNA share a 73% nucleotide and 80% predicted amino acid sequence identities but have <66% identity with the cytosolic CK.

Microvessel Tissue Preparation and Real-Time-Q uantitative Polymerase Chain Reaction

After omental biopsy, the omental fat pad sample was immediately placed into cold (4°C), oxygenated, physiological salt solution (PSS) consisting of (mmol/L) NaCl 118.2, NaHCO3 24.8, KCl 4.6, KH2PO4 1.2, MgSO4 1.2, CaCl2, 2 EDTA 0.26, and HEPES 50. Resistance-sized arteries (200–400 μm in diameter) were dissected under a microscope, cleaned of adherent adipose and connective tissue, and stored in Trizol Reagent at −80°C. Total RNA was isolated using the Trizol protocol and purified using the QIAGEN RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) with subsequent DNase treatment. RNA cleanup was done using the RNeasy Minute cleanup kit (Qiagen). To determine tissue-specific transcription, the Clontech total RNA human tissue panel was used to assess isoenzyme distribution in brain, striated and smooth muscle. First-strand cDNA synthesis was performed on 97.5-ng/μL RNA using the Avian Myeloblastosis Virus transcriptase kit 0.8 μL (20 U) and random hexamers (Roche Applied Science, Indianapolis, IN), which are short oligodeoxyribonucleotides of random sequence that anneal to random complementary sites on target RNA, to serve as primers for DNA synthesis by the reverse transcriptase.

Specific polymerase chain reaction (PCR) primers were designed for the CKB, CKM, CKMT1, and CKMT2 transcripts that amplify all alternatively spliced transcript variants that contain the highly conserved cysteine catalytic domain of CK. The transcription of CK genes was normalized to the reference gene 26S proteasome non-ATPase regulatory subunit 4. Primers and corresponding probes were identified using the Roche Universal Probe Library Assay Design Center (Table 1). Oligonucleotides were cloned in pGEM-T easy (Promega Corp, Madison, WI), sequenced to validate amplification of the intended transcript, and used to prepare ampiclon-specific amplification curves.

Table 1. Primers Used in the Quantitative Real-Time Polymerase Chain Reaction

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Forward Primer (5′→3′)</th>
<th>Reverse Primer (5′→3′)</th>
<th>UPL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CKB</td>
<td>TTTCTAAGAGTGGAGCTGTTG</td>
<td>AGGCATGATGCTGCGAT</td>
<td>77</td>
</tr>
<tr>
<td>CKM</td>
<td>CCCACACAAAGTTCACAGCTG</td>
<td>GGCCATCTGTTGATGTTT</td>
<td>63</td>
</tr>
<tr>
<td>CKMT1</td>
<td>GGTAAATGAGAAGATGGTTGAAAG</td>
<td>CAGCCAGCTTCTGATAGATG</td>
<td>39</td>
</tr>
<tr>
<td>CKMT2</td>
<td>TGACCAGCCAAAGCTTGGT</td>
<td>GCAGCTCTGAGATATCGC</td>
<td>32</td>
</tr>
<tr>
<td>PSM4</td>
<td>GGCAAGATATCCCTTCGCA</td>
<td>CTTCCACAGAAGGCAATG</td>
<td>21</td>
</tr>
</tbody>
</table>

CK indicates cytoplasmic brain–type creatine kinase; CKM, cytoplasmic muscle–type creatine kinase; CKMT1 and CKMT2 are, respectively, ubiquitous and sarcomeric mitochondrial creatine kinase; PSM4, 26S proteasome non–ATPase regulatory subunit 4; and UPL, number of the Universal ProbeLibrary probe (Roche).

Real-Time-Q uantitative PCR

Real-time quantitative PCR was performed on a LightCycler 480 system (Roche), according to the manufacturer’s protocol. Reaction mixtures contained 2.5-μL cDNA, 0.4 μmol/L of each primer (Invitrogen, Carlsbad, CA), 100 mmol/L Universal Probe Library probe (Roche), 2.5-μL water, and 10-μL absolute quantitative PCR mix (Thermo Fisher Scientific, Asheville, NC) in a total volume of 20 μL. Reactions were run in duplicate. Data were analyzed and quantified, using the second derivative maximum for Cp determination, with the LightCycler 480 software 1.5.0 (Roche).

Statistical Analysis

The main outcome was the strength of the association between blood pressure and CKB mRNA as measured with the Pearson product-moment correlation coefficient. On the basis of animal studies showing a 1.5- to 4.0-fold increase in cardiac CK or CK mRNA with SBP rising from 120 to 180 mmHg, we estimated to need 8 patients with a sensitivity analysis with an α of 0.05 and a 1–β of 0.8. The secondary outcome was the difference in CKB mRNA expression between hypertensives and normotensives. Other outcomes were correlational analyses between blood pressure and non-CBK cytoplasmic and mitochondrial isoenzymes and with total CK. Because of the expected small sample size, assessment of the distribution of the data was not expected to yield relevant data. Because parametric analysis may not be accurate with small sample sizes, and nonparametric analysis may lack power to detect a significant difference, we prespecified to use parametric statistics as our primary analysis—that is, arithmetic mean with SE, Pearson product-moment correlation coefficient (r), the unpaired t test, and 1-way ANOVA with the Bonferroni procedure as a post hoc test—and to reanalyze the data as a sensitivity analysis with nonparametric methods (i.e., median with interquartile range, Spearman rank-order correlation coefficient (ρ), Mann–Whitney test, or Kruskal–Wallis test with a Dunn post hoc analysis). We considered a 1-sided P value of <0.05 to be statistically significant. Data in brackets are 95% confidence intervals, unless stated otherwise. Data were analyzed with IBM SPSS statistical software package for Windows version 20.0 (SPSS Inc, Chicago, IL) and with GraphPad Prism Software version 5 (GraphPad Software Inc, San Diego, CA).

Results

Vessels of 13 normotensive and hypertensive women were included. The clinical characteristics of the participants are depicted in Table 2. With the CK transcripts as described in the Methods section, we first assessed CK isoenzyme mRNA in different human tissues (Figure 1). This is to our knowledge the first report showing that simultaneous assessment of mRNA of the highly homologous tissue CK isoenzymes is feasible. The data indicated that CKM mRNA was predominant in striated muscle and CKB mRNA in other tissue as expected. This confirmed the specificity of our RT-quantitative PCR to detect the 4 highly homologous CK transcripts.
Subsequently, we assessed the human resistance arteries with these validated isoenzyme transcripts. Normalized CK mRNA copy numbers of the vascular tissue and the correlation with SBP and DBP are depicted in Figure 2 showing the strong correlation between CKB mRNA and blood pressure. The total transcript level per tissue is set to 100% (total mean 26S proteasome non–ATPase regulatory subunit 4 copy number.

Table 2. Clinical Characteristics of the Participants

<table>
<thead>
<tr>
<th>Clinical Parameter</th>
<th>Total Group (N=13)</th>
<th>Normotensives (n=6)</th>
<th>Hypertensives (n=7)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>42.9 (1.6)</td>
<td>41.2 (2.7)</td>
<td>44.3 (1.7)</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>144.8 (8.0)</td>
<td>124.2 (4.5)</td>
<td>162.6 (10.4)</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>86.5 (4.3)</td>
<td>72.7 (3.3)</td>
<td>98.4 (3.1)</td>
</tr>
<tr>
<td>Heart rate, min⁻¹</td>
<td>79.2 (3.1)</td>
<td>70.8 (2.9)</td>
<td>86.4 (3.3)</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>25.9 (1.3)</td>
<td>24.8 (1.0)</td>
<td>26.9 (2.4)</td>
</tr>
</tbody>
</table>

Data are expressed as mean (SE).

*Hypertension was defined as systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg, or the use of antihypertensive drugs. Three patients were diagnosed with stage 1 hypertension (systolic blood pressure, 140–159 mm Hg or diastolic blood pressure, 90–99 mm Hg), and 4 with stage 2 hypertension (systolic blood pressure, ≥160 mm Hg or diastolic blood pressure, ≥100 mm Hg). According to Dutch national guidelines, treatment of uncomplicated hypertension is imperative only at systolic blood pressure ≥180 mm Hg. Four hypertensives were treated, with angiotensin-converting enzyme inhibitors, β-adrenergic blockers, calcium channel blockers, or thiazide diuretics, as monotherapy or as combination therapy. None reached control.

Discussion

We found a strong association between human resistance artery CK mRNA and systemic SBP and DBP across the clinical spectrum of normotension and hypertension. We also provide a detailed method to assess simultaneously mRNA of 4 highly homologous CK isoenzymes in tissue.

We had shown previously that circulating CK is the main predictor of blood pressure in a random sample of a multiethnic population, with an adjusted blood pressure increase of 7.98 [3.27–12.68] SBP and 4.69 [1.88–7.50] mm Hg DBP per log CK increase; this was replicated in case control and independent population studies. Furthermore, we reported that human isolated resistance artery contractility depends on CK and that specific CK inhibitors greatly attenuate human vascular contractility in vitro. The explanation proposed for these findings was that in the absence of organ damage, high serum CK activity reflected high tissue CK activity. In particular, high CKB activity in resistance arteries was thought to lead to greater vascular contractility and higher blood pressures. Now, we have provided the first direct evidence that resistance artery CK mRNA expression is strongly associated with blood pressure, with a 90% relative increase in CK mRNA in hypertension.

We have no exact data on the protein levels, but the mRNA levels suggest that CK increases in mitochondrial as well as at cytoplasmic locations, at least in vascular smooth muscle (Table 3).

Figure 1. Creatine kinase (CK) isoenzyme mRNA in different human tissues. Validation of the CK isoenzyme transcripts. Real-time quantitative polymerase chain reaction was performed on RNA isolated from different human tissues as indicated on the left. Values represent the average of duplicate quantitative polymerase chain reaction experiments measuring copy number of the 4 CK transcripts (cytoplasmic brain-type creatine kinase [CKB], cytoplasmic muscle-type creatine kinase [CKM], CKMT1, and CKMT2 [CKMT1 and CKMT2 are, respectively, ubiquitous and sarcomeric mitochondrial creatine kinase]) normalized to the 26S proteasome non–ATPase regulatory subunit 4 copy number.

The total transcript level per tissue is set to 100% (total mean normalized CK copy numbers, respectively, are 331.3 for skeletal muscle, 85.9 for heart, 10.1 for renal artery, 4.8 for small intestine, 14.8 for colon, and 12.5 for brain tissue, in accord with tissue differences in total CK protein levels as previously reported).

Individual transcript fractions were calculated and marked as indicated. The results show CKB/CKMT2 transcription mainly in striated muscle, and CKB/CKMT1 transcription mainly in smooth muscle and other tissue. Importantly, this distribution pattern of tissue CK isoenzyme mRNA accords with previous reports on the distribution of tissue CK isoenzyme protein.
The correlation coefficient between resistance artery CK mRNA and blood pressure was considerably higher than previously reported for serum CK and blood pressure (0.19 for serum CK and SBP versus 0.64 for CKB mRNA and SBP).6 This may indicate that the association of blood pressure with resistance artery CK mRNA is less likely to be because of an unmeasured confounder than serum CK.20,21 Therefore, microvascular CK mRNA may be a more direct estimate of hypertension risk than serum CK.

As previously reported by us and others, on a protein level, vascular CK acts as an energy transducer at the smooth muscle contractile proteins, supplying ATP for the contractile process (Figure 3). Calcium-dependent, RhoA/Rho kinase, and NO-cGMP pathways, the main intracellular effectors of blood pressure–regulating systems in vascular smooth muscle, are thought to converge on contractility responses fueled by CK.5–7,19,22,23 The contraction is triggered by a rise in cytosolic Ca2+ and initiated by phosphorylation of the serine 19 residue of the myosin regulatory light chain by a specific Ca2+/calmodulin–myosin regulatory light chain kinase complex. This myosin regulatory light chain phosphorylation leads to cross-bridge formation between the myosin heads and the actin filaments, and hence, vascular smooth muscle contraction.5–7,23 ATP is required for each actin–myosin complex formed.5–11 Vascular smooth muscle contraction is thought to consist of a fast, force-generating component at relatively high-energy costs, and a slow, tonic maintenance of tension, for which ADP is required.5–7,19,22,23 If, because of greater CK activity, ADP levels at the contractile proteins do not achieve the level required for tonic maintenance of tension, then the smooth muscle tension response could be altered, leading to excessive contractility.5,6,19 High CK activity is thought to be associated with reduced NO biosynthesis, through reducing bioavailability of l-arginine. Creatine and NO are both synthesized from l-arginine, but creatine synthesis demands nearly 10× the flux of plasma l-arginine compared with NO synthesis and may inhibit NO-dependent functions.6,19 As expressed in the Poiseuille–Hagen formula, even a small increase in contractility and reduction in vascular diameter could have profound effect on resistance to flow and hence arterial pressure. Thus, even a small increase in CK activity might have a potentially large impact on blood pressure levels.5,6,19

Although the resistance artery is central to the generation of blood pressure, to our knowledge, resistance artery gene transcription in human hypertension has not been widely studied. Schiffrin et al,24 then using in situ hybridization, found that small arteries from untreated patients with moderate-to-severe hypertension, but not with normotension or mild hypertension, showed evidence of the presence of endothelin-1 mRNA. However, no correlation with blood pressure was reported.24 We retrieved no further articles that assessed the transcription of genes involved in the intracellular pathways of pressor

**Table 3. Correlation Coefficient of Microvascular CK mRNA Copy Numbers and Blood Pressure**

<table>
<thead>
<tr>
<th>CK mRNA</th>
<th>Normalized Copy Number</th>
<th>Correlation Coefficient ($r$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>CKB</td>
<td>15.00</td>
<td>1.91</td>
</tr>
<tr>
<td>CKM</td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td>CKMT1</td>
<td>0.19</td>
<td>0.08</td>
</tr>
<tr>
<td>CKMT2</td>
<td>1.53</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Pearson product-moment correlation coefficient ($r$) with 95% confidence interval in brackets. CKB indicates cytoplasmic brain-type creatine kinase; the predominant CK isoenzyme in smooth muscle; CKM, cytoplasmic muscle-type creatine kinase; CKMT1 and CKMT2 are, respectively, ubiquitous and sarcomeric mitochondrial creatine kinase; DBP, diastolic blood pressure; and SBP, systolic blood pressure.

*P=0.05, †(0.00–0.84), signifies (0.001–0.844).
responses in peripheral, noncoronary resistance arteries in humans, in relation to systemic blood pressure.

The main strength of this study is that we found, to our knowledge for the first time, that mRNA expression levels of the cytosolic form of the central regulatory enzyme of energy metabolism CK show an almost perfect correlation with DBP, whereas CK mRNA expression levels, use the results as a proxy for functional differences that occur at the protein level although mRNA levels probably may not adequately reflect protein levels.25 Notably, the expression of CKB was reported to be mediated at the level of mRNA,10 and the tissue isoenzyme mRNA data we present, including of the renal artery (Figure 1), correspond with the previously reported distribution of CK isoenzyme activity.5,7,9,10,12 We have also previously found that higher resistance artery CK activity is associated with enhanced contractility in isolated human resistance arteries;19 and in the myocardium and aorta of animal models of hypertension or acute pressure overload. CK mRNA was increased with concomitant increase in CK protein levels, as compared with controls.10 High myocardial CK activity was also reported to precede the development of hypertension in animal models, to further increase with the development of hypertension, and to reduce after successful antihypertensive treatment.10,13,14 Similar findings, of a reduction in vascular CK activity, were reported in the spontaneously hypertensive rat after antihypertensive treatment.22 Finally, we found evidence in our population study that otherwise healthy subjects with controlled hypertension have lower CK than those with uncontrolled hypertension.26 Thus, the existing data indicate that CK mRNA, both constitutive and induced, is likely to be translated into CK protein to meet the increased energy requirements of high blood pressure. Further studies need to confirm this and to assess the relative contribution of constitutive versus induced CK in human hypertensive disease.

Perspectives

We found evidence that human resistance artery CK mRNA levels progressively increase with blood pressure, nearly doubling in hypertension. Together with previous findings that circulating CK is the main predictor of blood pressure in the general population,6 and that human resistance artery contractility is highly CK dependent,19 these new data strengthen the evidence that the enzyme may be involved in human hypertension. Hyperexpression of resistance artery CK may serve to meet the increased metabolic demands of enhanced peripheral resistance, as implicated in hypertension. Future studies need to confirm these inferences, and establish whether inhibition of CK may lower blood pressure.

Disclosures

L.M. Brewster is a recipient of a VENI fellowship (grant number 916.10.156) awarded by the Netherlands Organisation for Scientific Research, and aorta of animal models of hypertension or acute pressure


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**Novelty and Significance**

**What is New?**

- It is unknown, why individuals with high circulating creatine kinase have higher blood pressure.
- We assessed mRNA expression levels of mitochondrial and cytoplasmic creatine kinase in isolated resistance arteries of individuals with normotension and hypertension.

**What is Relevant?**

- There is evidence that hypertension in individuals with high creatine kinase is more severe and more resistant to treatment.

**Summary**

Human resistance artery creatine kinase mRNA expression levels are strongly associated with blood pressure levels and are almost twice as high in hypertensives compared with normotensives. This indicates that creatine kinase may be involved in pressor responses. Further research should address whether creatine kinase inhibition lowers blood pressure.
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