Creatine kinase and blood pressure: Clinical and therapeutic implications

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Chapter 3

Overexpression of microvascular CK mRNA in human hypertension

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Abstract

Rationale Hypertension remains the main risk factor for cardiovascular death. Environmental and biological factors are known to contribute to the condition, and recently serum creatine kinase was reported to be the main predictor of blood pressure in the general population. This was proposed to be related to high resistance artery creatine kinase rapidly regenerating ATP for microvascular contractility, but hitherto there were no data to substantiate this.

Objective To assess whether creatine kinase mRNA levels in human resistance arteries are associated with blood pressure across the clinical spectrum of normotension, and stage 1 and 2 hypertension.

Methods and Results We isolated resistance-sized arteries from omental fat donated by consecutive women during uterine fibroid surgery. Blood pressure was measured in the sitting position. Vessels of 13 women were included, 7 white and 6 African-Dutch, mean age 42.9 (SE 1.6) y; systolic/diastolic blood pressure 144.8 (8.0)/86.5 (4.3) mm Hg; heart rate 79.9 (3.3)/min; and body mass index 26.0 (1.5) kg/m². Microvascular creatine kinase mRNA was assessed using quantitative realtime PCR. Normalized creatine kinase B mRNA copy numbers, ranging between 5.18 and 24.43 (mean 15.0, SE 1.9), were strongly correlated with blood pressure, with correlation coefficients of 0.64 (95% CI, 0.13 to 0.88) for systolic, and 0.88 (0.64 to 0.96) for diastolic blood pressure.

Conclusion To our knowledge, this is the first direct evidence suggesting that creatine kinase gene mRNA expression levels in microvasculature progressively increase with blood pressure. This finding adds to the evidence that creatine kinase is involved in the vasculature’s pressor responses.
INTRODUCTION

Hypertension is an important worldwide public-health challenge. It is a common disease, affecting over 25% of the adult population, nearly 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:

\[ \text{MgADP} + \text{P}_{\text{creatine}} + \text{H}^+ \leftrightarrow \text{MgATP} + \text{creatine}. \]

CK is tightly bound in the immediate proximity of ATP utilizing enzymes such as Na\(^+\)/K\(^+\)-ATPase and Ca\(^{2+}\)-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 due to high tissue CK, primarily resistance artery CK-BB isoenzyme rapidly regenerating ATP for microvascular contractility. However, hitherto, there were no microvascular 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 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. Sitting blood pressure was measured at the outpatient clinic with the Datascoper Accutorr Plus (Tascope Corp., Paramus, New Jersey, USA). 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 two major cytosolic CK protein subunits are CK-brain (B) and CK-muscle (M) that are encoded by the CKB gene on human chromosome 14q32 and the CKM gene on 19q13.32 respectively. The enzymatic functional form can be either a homodimer (BB or MM) or a MB heterodimer, thus creating 3 cytosolic isoenzymes.9,11,12 CK is also present in the mitochrondrion where it facilitates the formation of creatine phosphate, which is transported by CK to subcellular locations of high-energy demands.12,13 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 5q13.3.9,11,12 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.12 Cytosolic CK-B and CK-M 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 less than 66% identity with the cytosolic CK.12

**Microvessel tissue preparation and RT-qPCR**

After omental biopsy, the omental fat pad sample was immediately placed into cold (4 degrees Celsius), oxygenated, physiologic 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 degrees Celsius. 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 clean up 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 (AMV) transcriptase kit 0.8 μl (20 units) and random hexamers (Roche Applied Science, Indianapolis, IN, USA), 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 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 creatine kinase. The transcription of CK genes was normalized to the reference gene PSMD4. Primers and corresponding probes were identified using the Roche Universal Probe Library (UPL) Assay Design Center (Table 1). Amplicons were cloned in pGEM-T easy (Promega Corp., Madison, WI, USA), sequenced to validate amplification of the intended transcript, and used to prepare amplicon specific calibration curves.

Table 1. Primer sequence and probe number.

<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>TTCTCAGAGGGGAGCTGTGTT</td>
<td>AGGCATGAGGTCTCGAT</td>
<td>77</td>
</tr>
<tr>
<td>CKM</td>
<td>CCCAACAAACGTTCAAGCGTT</td>
<td>GGCCATGTTGTTAATGTT</td>
<td>63</td>
</tr>
<tr>
<td>CKMT1</td>
<td>GGTATAGTGGAGGAGGTGTTGAAAG</td>
<td>CAGCCACGTCTTTGGATAAGT</td>
<td>39</td>
</tr>
<tr>
<td>CKMT2</td>
<td>TGAACCGGCAGAAAGTTGTTG</td>
<td>CGCAGTCTGTTGGATTG</td>
<td>32</td>
</tr>
<tr>
<td>PSMD4</td>
<td>GGCAAGATCAAGCTGTGCAGT</td>
<td>CTCCCACAAAGGGAAATGAT</td>
<td>21</td>
</tr>
</tbody>
</table>

*UPL indicates the number of the Universal Probe Library Probe (Roche).

RT-qPCR
Quantitative real time PCR (qPCR) 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, USA), 100 nmol/L UPL probe (Roche), 2.5 μl water and 10 μl Absolute qPCR mix (Thermo Fisher Scientific, Asheville, NC, USA), 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. Based on animal studies showing a 1.5 to 4.0-fold increase in cardiac CK or CK-mRNA with SBP rising from 120 to 150-180 mm Hg,10,13,14 we estimated to need 8 patients to assess a similar association with an alpha of 0.05 and a 1-beta of 0.8. Other outcomes were correlations of blood pressure with non-CKB isoenzymes, and with total CK; and the
difference in CK mRNA expression between different stages of hypertension. Because of the expected small sample size, the distribution of the data could not be formally tested. Parametric analysis may not be accurate with small sample sizes, and non-parametric analysis may lack power to detect a significant difference. Therefore, we used parametric statistics as our primary analysis (i.e. arrhythmic mean with SEM; Pearson product-moment correlation coefficient (r), the unpaired t test, and 1-way ANOVA with the appropriate post-test with Bonferroni correction); and reanalysed the data as a sensitivity analysis with non-parametric methods (i.e. median with interquartile range; Spearman rank order correlation coefficient (rho); Mann-Whitney test, or Kruskal-Wallis test with a Dunn's post-test). We considered a one-sided probability value of <0.05 to be statistically significant. Data in brackets are 95% confidence intervals, unless stated otherwise. Data were analysed with IBM SPSS statistical software package for Windows, version 20.0 (SPSS Inc., Chicago, IL, USA).

Table 2. Clinical characteristics of the participants.

<table>
<thead>
<tr>
<th>Clinical Parameter</th>
<th>Normotensive (n=6)</th>
<th>Stage 1 HT (n=3)</th>
<th>Stage 2 HT (n=4)*</th>
<th>Total group (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>41.2 (2.7)</td>
<td>42.7 (2.8)</td>
<td>45.5 (2.3)</td>
<td>42.9 (1.6)</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>124.2 (4.5)</td>
<td>142.3 (5.5)</td>
<td>177.8 (13.7)</td>
<td>144.8 (8.0)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>72.7 (3.3)</td>
<td>91.7 (3.3)</td>
<td>103.5 (2.7)</td>
<td>86.5 (4.3)</td>
</tr>
<tr>
<td>Heart rate / min</td>
<td>70.8 (3.6)</td>
<td>81.3 (4.8)</td>
<td>90.3 (3.8)</td>
<td>79.9 (3.3)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>24.8 (1.0)</td>
<td>24.0 (2.6)</td>
<td>32.5 (6.5)</td>
<td>26.0 (1.5)</td>
</tr>
</tbody>
</table>

Values are expressed as mean (SE). Normotension, Stage 1, and 2 hypertension (HT) indicate SBP <140, 140 to 159, and ≥160; DBP <90, 90-99, and ≥100 mm Hg respectively. Stage 1, only 1 patient treated, stage 2, 3 patients treated. None of the patients reached control. *In the Dutch setting, according to national guidelines treatment of uncomplicated hypertension is only imperative at systolic pressure levels ≥180 mm Hg.15

RESULTS

Vessels of 13 women, 7 white and 6 African Dutch, mean age 42.9 (SE 1.6) y; SBP 144.8 (8.0); DBP 86.5 (4.3) mm Hg; heart rate 79.9 (3.3)/min; body mass index (BMI) 26.0 (1.5) kg/m², were included. The clinical characteristics of these normotensive, and stage 1 and 2 hypertensive participants are depicted in Table 2, indicating the poor treatment status of the included subjects.
Overexpression of Microvascular CK mRNA in Human Hypertension

Figure 1. CK isoenzyme mRNA in different human tissues.
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 four creatine kinase (CK) transcripts (CKB, CKM, CKMT1, and CKMT2) normalized to the PSMD4 copy number. The total transcript level per tissue is set to 100% total normalized CK copy numbers respectively 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 CK protein levels as previously reported. Individual transcript fractions were calculated and marked as indicated. The results show CKM/CKMT2 transcription mainly in striated muscle, and CKB/CKMT1 transcription mainly in smooth muscle and other tissue as expected.

Table 3. Correlation coefficient of microvascular CKmRNA copy numbers and blood pressure.

<table>
<thead>
<tr>
<th>CK isoenzyme</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>

CKB, cytoplasmic brain type creatine kinase; CKM, cytoplasmic muscle type creatine kinase CKMT1 and CKMT2 are respectively ubiquitous and sarcomeric mitochondrial creatine kinase. SBP, systolic blood pressure; DBP, diastolic blood pressure. Pearson product-moment correlation coefficient (r) with 95% CI in brackets. *p<0.05; †(0.00 to 0.84), signifies (0.001 to 0.844).
With the CK transcripts as described in the method section, we first assessed CK isoenzyme mRNA in different human tissues as shown in Figure 1. The results indicate that CKM mRNA is predominant in striated muscle and CKB mRNA in other tissue as expected. This confirmed the specificity of our CK isoenzyme transcripts.

Subsequently, we assessed the human microvessels with the validated transcripts (Table 3). Normalized CK mRNA copy numbers of the vascular tissue and the correlation with systolic and diastolic blood pressure are depicted in Figure 2, showing the strong correlation between CKB mRNA and blood pressure. The differences in CK mRNA in different blood pressure strata is shown in Figure 3, indicating a highly significant increment in microvascular CK mRNA with increasing systolic or diastolic blood pressure levels, from normotension, to stage 1, up to stage 2 hypertension. Non-parametric statistical methods did not significantly change the direction or the magnitude of the outcomes, with a Spearman rank order correlation coefficient for the association between CKB mRNA and respectively SBP and DBP of 0.70 ($p=0.002$) and 0.83 ($p<0.001$).
Figure 3. Microvascular CKB mRNA in different blood pressure strata.
NT, normotension, systolic/diastolic blood pressure up to 139 or 89 mm Hg respectively; (n=6); Stage 1 Hypertension (HT), systolic/diastolic blood pressure between 140 to 159 or 90 to 99 mm Hg respectively (n=3); Stage 2 HT, systolic/diastolic blood pressure ≥160 or 100 mm Hg respectively (n=4); p<0.01 for differences in CK mRNA copy number between groups assessed with 1-way ANOVA.

**DISCUSSION**

We found a strong association between human resistance arteries CK mRNA and systemic systolic and diastolic blood pressure, including normotension, stage 1 and stage 2 hypertension. We had shown previously that CK is a main predictor of blood pressure, with an adjusted blood pressure increase in a random sample of a multi-ethnic population of 7.98 (3.27 to 12.68) systolic and 4.69 (1.88 to 7.5) mmHg diastolic per log CK increase. This was replicated in a large Norwegian population study.

Furthermore, we had shown that resistance artery contractility highly depends on CK, and that specific creatine kinase inhibitors greatly attenuate human vascular contractility ex vivo. The explanation proposed for these findings, was that in the absence of organ damage, high serum CK activity reflected high tissue CK activity. High CKB activity in resistance arteries was thought to lead to greater vascular contractility and higher blood pressures.

In this study, we have provided the first direct evidence that microvascular CK mRNA expression levels are strongly associated with blood pressure. The correlation coefficient between resistance artery CK mRNA and blood pressure is considerably
higher than previously reported for serum CK and blood pressure (0.19 for serum CK and SBP, vs 0.64 for CKB mRNA and SBP). The stronger association may indicate that the association with CK mRNA is less likely due to an unmeasured confounder than serum CK, and that microvascular CK mRNA is a more direct estimate of hypertension risk than serum CK.

Even though mRNA expression levels are commonly used as a proxy for estimating functional differences that occur at the protein level, the relation between mRNA and protein expression is not well established. However, in this study, we found an association with a potential functional characteristic of the protein, blood pressure. Therefore, it is likely that the CKmRNA is translated into functional protein. Because of the small size of the microvessels, CK activity could not directly be assessed with the gold standard of spectrophotometric enzyme assays, but using a bioassay with enzyme inhibition, we have previously found evidence that higher microvascular CK activity is associated with enhanced contractility in isolated human resistance arteries. In addition, the tissue isoenzyme mRNA data we present, including renal artery (Figure 1), correspond with the previously reported distribution of CK isoenzyme activity. Finally, in the myocardium and aorta of hypertensive animals or acute pressure overload, CK mRNA was increased with concomitant increase in CK protein levels, as compared to controls. High myocardial CK activity was also reported to precede the development of hypertension in animal models, and to further increase with the development of hypertension, to reduce after successful treatment. Finally, we found evidence in our population study, that otherwise healthy subjects with controlled hypertension have lower CK than those with uncontrolled hypertension. Thus, the existing data indicate that CK mRNA, both constitutive and induced, are likely to be translated into CK protein to meet the increased energy requirements of high blood pressure. Further studies are needed to confirm this, and to assess the relative contribution of constitutive versus induced CK in human hypertensive disease.

As previously reported by us and others, on a protein level, microvascular CK acts as an energy transducer at the smooth muscle contractile proteins, supplying ATP for the contractile process. Calcium dependent, RhoA/Rho kinase and nitric oxide (NO)–guanosine 3’, 5’-cyclic monophosphate pathways, the main intracellular effectors of blood pressure-regulating systems in vascular smooth muscle, are thought to converge on contractility responses fueled by CK. ATP is required for each actin-myosin complex formed. 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\textsuperscript{5,6,7,9,18,23,25}. If, because of greater creatine kinase 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\textsuperscript{5,6}. 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\textsuperscript{5,6}.

Although the resistance artery is central to the generation of blood pressure, to our knowledge, microvascular gene transcription and hypertension in humans have not been widely studied. Schiffrin et al.,\textsuperscript{26} then using in-situ hybridization, found that small arteries from untreated hypertensive patients with moderate-to-severe hypertension, but not with normotension, or mild hypertension, showed evidence of the presence of endothelin-1 messenger RNA. However, no correlation with blood pressure was reported.\textsuperscript{26} We retrieved no further papers that assessed the transcription of genes involved in the intracellular pathways of pressor responses in peripheral, non-coronary resistance arteries in humans, in relation to systemic blood pressure.

The main strength of this study is that we found, for the first time, that mRNA expression levels of the central regulatory enzyme of energy metabolism creatine kinase, shows an almost perfect correlation with diastolic blood pressure, and also with systolic blood pressure. This is in line with previous findings of CK as a main denominator of blood pressure,\textsuperscript{6,16,17} and reports of significant vasodilation of isolated resistance arteries after CK inhibition.\textsuperscript{18} Furthermore, our data were collected in subjects of African and white self-defined ethnicities, and among the wide clinical spectrum of normotension and stage 1 and 2 hypertension. A limitation of the study is the small sample size, related to the nature of isolated vessels studies, which require an invasive harvest procedure.\textsuperscript{26} However, the sample size was calculated to be sufficient for the primary outcome.

In summary, we now found evidence that human microvascular CK mRNA levels progressively increase with blood pressure. The association found precludes causal inferences, but together with previous findings that human resistance artery contractility is highly CK-dependent,\textsuperscript{18} these new data strengthen the evidence that CK is involved in pressor responses. Hyperexpression of this ATP regenerating enzyme may serve to meet the increased metabolic demands of enhanced microvascular contractility as implicated in hypertension.
Chapter 3

References


