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:

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

CK is tightly bound in the immediate proximity of ATP-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 isoenzyme 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 the Datascope Accutorr Plus (Tascope Corp, Paramus, NJ). High blood pressure was defined as systolic blood pressure (SBP) ≥140 or diastolic blood pressure (DBP) ≥90 mmHg 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 1q43-2 and the CKM gene on 19q13.3. The enzymatic functional form can be either a homodimer (BB and MM) or a heterodimer (MB) thus creating 3 cytosolic isoenzymes.\(^2\)\(^,\)\(^3\)\(^,\)\(^5\)\(^,\)\(^12\) 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.\(^2\)\(^,\)\(^11\) 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.\(^5\)\(^,\)\(^12\) All CK isoenzymes contain a highly conserved catalytic cysteine domain of CK. The transcription of CK is encoded by the CKB, CKM, CKMT1, and CKMT2 transcripts that amplify mRNA of the highly homologous tissue CK isoenzymes is edge the first report showing that simultaneous assessment of CK mRN A and Blood Pressure

**Real-Time-Quantitative 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).

**Microvessel Tissue Preparation and Real-Time-Quantitative 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, NaHCO\(_3\) 24.8, KCl 4.6, KH\(_2\)PO\(_4\) 1.2, MgSO\(_4\) 1.2, CaCl\(_2\) 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.

**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 150 to 180 mmHg,\(^10\)\(^,\)\(^13\)\(^,\)\(^14\) we estimated to need 8 patients to assess a similar association 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 correlations of blood pressure with non-CKB 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, Spearman rank-order correlation coefficient (r), the unpaired t test, and 1-way ANOVA with the Bonferroni procedure as a post hoc analysis—and to reanalyze the data as a sensitivity analysis with nonparametric methods (ie, 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 knowl-

<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>TCTTCAGGATGAGAGCTGCT</td>
<td>AGGCCATGAGGTCGTGAT</td>
<td>77</td>
</tr>
<tr>
<td>CKM</td>
<td>CCCAACAAAGTTCAGGCTG</td>
<td>GGCATGTGTGTATGTTT</td>
<td>63</td>
</tr>
<tr>
<td>CKMT1</td>
<td>GGTAGATGAGGAGATTTGAAAG</td>
<td>CAGGCCAGTTGCTTGATAAGAT</td>
<td>39</td>
</tr>
<tr>
<td>CKMT2</td>
<td>TGACGCGCAGAAGCTG</td>
<td>CGCGAAGGTCGATGCTG</td>
<td>39</td>
</tr>
<tr>
<td>PSM04</td>
<td>GGCAAGTACCATCCTCTGCA</td>
<td>CTTCCACAAAAGGCAATG</td>
<td>21</td>
</tr>
</tbody>
</table>

CKB indicates cytoplasmic brain–type creatine kinase; CKM, cytoplasmic muscle–type creatine kinase; CKMT1 and CKMT2 are, respectively, ubiquitous and sarcomeric mitochondrial creatine kinase; PSM04, 26S proteasome non–ATPase regulatory subunit 4; and UPL, number of the Universal ProbeLibrary probe (Roche).

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

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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.

Mean CKB mRNA copy numbers were ≈90% higher in hypertensives compared with normotensives, respectively, 19.3 (SE, 2.0) versus 10.1 (2.1), \( P = 0.0045 \). For the other isoenzymes, mRNA copy numbers in hypertensives compared with normotensives were 0.07 (0.02) versus 0.02 (0.01) for CKM, \( P = 0.031 \); 0.26 (0.1) versus 0.16 (0.1), for CKMT1, \( P = 0.21 \); and 2.0 (0.2) versus 1.0 (0.3), for CKMT2, \( P = 0.01 \). The correlations between non-CKB cytoplasmic and mitochondrial isoenzyme mRNA and blood pressure are shown in Table 3.

Nonparametric 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 (\( P < 0.001 \)).

**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). We think that this is consistent with other
Correlation Coefficient (by guest on April 15, 2017 http://hyper.ahajournals.org/ Downloaded from

This myosin regulatory light chain phosphorylation leads to calmodulin–myosin regulatory light chain kinase complex. A unmeasured confounder than serum CK.20,21 Therefore, microvascular resistance artery CK mRNA is less likely to be because of an association between blood pressure in normotensives and hypertensives (n=13) expressed in mm Hg. A, Systolic blood pressure (SBP); (B), diastolic blood pressure (DBP). Closed circles indicate treated hypertensives. Resistance artery creatine kinase (CK)-B mRNA is expressed in normal copy numbers. CKB is the dominant isoenzyme in vascular smooth muscle. Analysis of the correlation coefficient between total CK (isoenzyme CKB, cytoplasmic muscle–type creatine kinase [CKM], CKMT1, and CKMT2 [CKMT1 and CKMT2 are, respectively, ubiquitous and sarcomeric mitochondrial creatine kinase]) and blood pressure showed identical correlation coefficients and statistical significance as CKB (data not shown).

physiological observations about CK being bound to contractile proteins in vascular smooth muscle. That is, the CKB would increase proportionally to changes in contractile muscle protein and that ratio remains relatively constant. So, changes in cytosolic CK can be estimated on the basis of an assumption of a constant relationship of protein bound CK.

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 ATP.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 cross-bridge formation between the myosin heads and the actin filaments, and hence, vascular smooth muscle contraction.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 Thus, 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 physiological observations about CK being bound to contractile proteins in vascular smooth muscle. That is, the CKB would increase proportionally to changes in contractile muscle protein and that ratio remains relatively constant. So, changes in cytosolic CK can be estimated on the basis of an assumption of a constant relationship of protein bound CK.

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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; SBP, systolic blood pressure; and DBP, diastolic blood pressure.

*P<0.05, †P(0.00–0.84), signifies (0.001–0.844).
showed evidence of the presence of endothelin-1 mRNA. However, no correlation with blood pressure was reported. We retrieved no further articles that assessed the transcrip-
tion of genes involved in the intracellular pathways of pres-
sor 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,
and are also highly correlated with SBP, whereas CK mRNA
expression nearly doubles with hypertension.

This is in line with previous findings of CK as a main denomi-
ator of blood pressure and reports of significant vasodila-
tion of isolated resistance arteries after CK inhibition. Moreover, our data were collected in subjects of African
and European ancestry and among the clinical spectrum of
normotension to 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. Yet,
the small sample size, related to the nature of isolated vessels
and European ancestry and among the clinical spectrum of
hypertension, and are also highly correlated with SBP, whereas CK mRNA
expression nearly doubles with hypertension.

Perspectives
We found evidence that human resistance artery CK mRNA
levels progressively increase with blood pressure, nearly dou-
bling in hypertension. Together with previous findings that
circulating CK is the main predictor of blood pressure in the
general population, and that human resistance artery contrac-
tility is highly CK dependent, these new data strengthen the
evidence that the enzyme may be involved in human hyperten-
sion. 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 as part of its Innovational Research Incentives Scheme. L.M. Brewster is an inventor on NL patent WO/2012/138226 (titled).

References
8. We have also previously found that higher resistance artery CK activity is associated with enhanced contractility in iso-
lated human resistance arteries and in the myocardium and aorta of animal models of hypertension or acute pressure over-
load, CK mRNA was increased with concomitant increase in CK protein levels, as compared with controls. High myocardial CK activity was also reported to precede the devel-
opment of hypertension in animal models, to further increase with the development of hypertension, and to reduce after successful antihypertensive treatment. Similar findings, of a reduction in vascular CK activity, were reported in the spontaneously hypertensive rat after antihypertensive treat-
ment. 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 constitu-
tive and induced, is likely to be translated into CK protein to
meet the increased energy requirements of high blood pres-
sure. Further studies are needed to confirm this and to assess
the relative contribution of constitutive versus induced CK in
human hypertensive disease.


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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.
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 and Lizzy M. Brewster

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